EXPERIMENTAL

4.1. Charcoal stump rot incidence in tea of Terai and Dooars

Charcoal stump rot disease caused by *Ustulina zonata* occurs widely in Terai and Dooars region. In order to determine the incidence of this disease in different Tea Estates, a survey of ten tea gardens was carried out. The survey of the tea gardens revealed the occurrence of charcoal rot most commonly in four gardens i.e. Hansqua Tea Estate, Matigara Tea Estate (Plate 5, fig.A), Bijoynagar Tea Estate (Plate 5, fig.B) and Trihana Tea Estate (Plate 5, fig.C) . In severe cases, plants in specific areas had died off. Diseased plant root samples submitted by the planters to Nagrakata Tea Research Station were collected (Plate 6,fig.A-E) and fungal pathogen was isolated (Plate 6, fig.F&G) for further experiment.

4.2 Cultural conditions affecting growth of *U. zonata*

The study of the growth of *U. zonata in vitro* showed variation depending on different factors like medium, pH, temperature and seasonal changes. The young mycelia of *U. zonata* were white or hyaline initially, which turned into grayish black brittle crust gradually. The mycelial growth was generally superficial with fan shaped dull silky white mycelia or small black or white cushion like growth was found depending on the medium

4.2.1. Media

U. zonata was grown in eight different media i.e. potato dextrose agar (PDA), potato sucrose agar (PSA), Richard's Agar (RA), carrot juice agar (CJA) Czapek-Dox agar (CDA), Flentze's soil extract agar (FSEA), malt extract peptone dextrose agar (MPDA), yeast extract-dextrose agar (YDA). Results revealed that the maximum growth was recorded in PDA followed by PSA and RA but minimum growth was recoded in CDA. The fungus shows submerged, translucent mycelia to surfaced thick white growth which gets shriveled and turn dark, with black grey sporulation in oval patches.

4.2.2. Incubation period

U. zonata was grown in PDA medium for a period of 30 days, Mycelial growth was recorded after 5,10,15, 20, 25 and 30 days of growth and incubated at 25 $\pm$ 1°C. Maximum growth was recorded after 20 days of incubation (Table 1) after which it declined. After 5 days of incubation the growth was negligible.

Plate 5 (Fig. A-C): Naturally infected tea plants showing above ground symptoms of charcoal stump rot. (A) Hansqua Tea Estate (B) Matigara Tea Estate. (C) Bijoynagar Tea Estate.

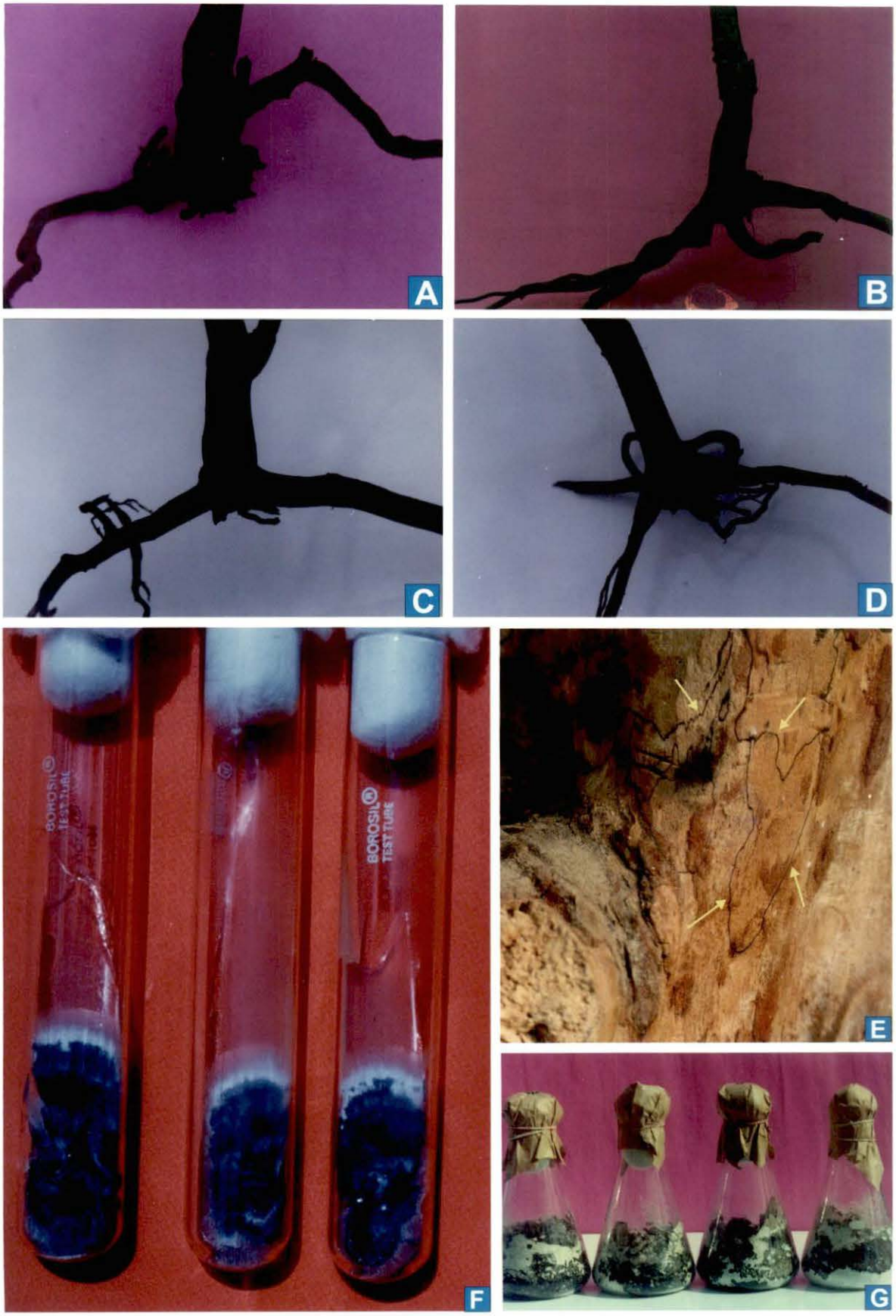


Plate 6 (Fig. A-G): Uprooted tea plants showing symptoms of charcoal stump rot disease (A-E), *Ustulina zonata* (Causal organism) grown in PDA (F) and sand maize meal medium (G).

Table 1 : Effect of incubation period on mycelial growth of *U. zonata*

Incubation Period (days)	Mycelial dry wt (mg)			
	Expt. 1	Expt.2	Expt.3	Mean
5	07.0	07.3	06.0	06.76±0.39
10	21.8	20.5	23.4	24.15±0.83
15	42.6	42.0	41.6	42.00±0.29
20	79.7	78.2	78.9	78.93±0.43
25	53.7	52.8	52.5	53.00±0.36
30	47.0	49.5	48.1	48.20±0.72

± Standard error; Temperature 25±1°C

4.2.3. pH

pH of the medium plays an important role in the growth of all microorganisms. To determine the effect of pH, buffer systems have to be used to stabilize the pH, Initially buffer solution with pH values ranging from 3.0 to 8.0 (3.0,3.5, 4.0, 4.5,5.0, 5.5, 6.0, 6.5, 7.0, 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of 0.03M. The pH finally adjusted using N/10 HCl or N/10 NaOH in each case. Potato dextrose medium and phosphate buffer was sterilized separately by autoclaving for 15min. at 15 lb p.s.i pressure and equal parts of the buffer solution and medium were mixes before use in Laminar Flow Bench. After mixing flasks were inoculated and incubated for 20 days after which dry wt. was taken as described previously. Results (Table 2) revealed that *U. zonata* grew to a lesser or greater extent over a wide range of pH (3.0-8.0), maximum growth was observed at pH 3.5 - 4.0 and then growth gradually declined.

4.2.4. Carbon source

Like the pH of the surrounding medium the growth of fungus is greatly influenced by available nutrients. The ability of fungi to grow in different media depends on their capacity to utilized by the available nutrients, of which carbohydrates are the major ones. All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. In the present investigations, eight different carbon sources (dextrose, fructose, lactose, mannitol, maltose, sorbose, starch and sucrose) were

tested for their effect on the growth of *U. zonata*. These were added separately to the basal medium. PDA medium without sugar was used as the basal medium which served as control set. Data were recorded after 20 days of incubation. Results (Table 3 , Fig.1) revealed maximum growth using lactose as the carbon source while no growth was observed in sorbose which was similar to control set. Fructose and sucrose also supported comparatively good growth.

Table 2 : Effect of different pH on mycelial growth of *U. zonata* Mycelial dry wt (mg)

pH of Medium[a]	Expt. 1	Expt.2	Expt.3	Mean
3.0	41.6	41.0	39.9	30.83± 0.49
3.5	58.5	58.1	58.9	58.50±0.23
4.0	54.8	53.8	54.5	54.36±0.29
4.5	51.3	51.0	51.6	51.30±0.17
5.0	44.5	43.7	43.9	44.03± 0.24
5.5	38.9	38.2	38.1	38.40±0.25
6.0	35.1	35.5	35.6	35.40±0.15
6.5	28.9	27.4	26.9	27.73±0.60
7.0	24.8	24.6	24.0	24.46±0.24
8.0	19.2	19.7	18.9	19.26±0.23

[a] PDA ; ± Standard error; Temperature 25 ±1°C ; Incubation period 20 days

Table 3 : Effect of different carbon sources on mycelial growth of *U. zonata*

Carbon Sources	Mycelial dry wt (mg)			
	Expt. 1	Expt.2	Expt.3	Mean
Fructose	56.8	56.0	48.2	53.66±2.746
Sorbose	01.7	01.5	01.3	01.50±0.057
Dextrose	47.4	46.9	47.9	47.40±0.289
Mannitol	32.0	31.0	27.6	30.20±1.333
Sucrose	41.1	43.2	38.4	40.90±1.390
Starch	26.0	24.8	21.6	24.13± 1.314
Maltose	30.4	27.3	29.6	29.10±0.930
Lactose	60.1	62.5	64.4	62.33±1.245
Control	01.8	01.5	1.8	01.70±0.173

± Standard error; Temperature 25 ±1°C; Incubation period 20 days

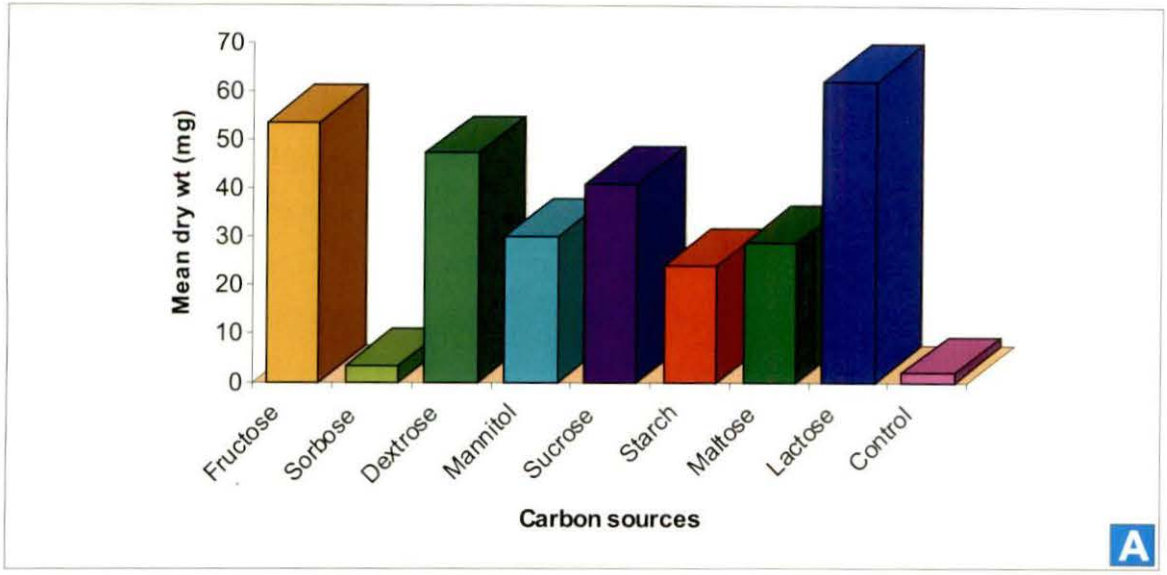
4.2.5. Nitrogen source

The availability of nitrogen for growth of the organism depends on the form in which it is supplied. Hence the most suitable medium for any particular microorganism can only be determined by testing a number of sources including both organic and inorganic. The effect of inorganic nitrogen sources (ammonium nitrate, ammonium sulphate, calcium nitrate, potassium nitrate and sodium nitrate) as well as complex organic sources (casein hydrolysate, beef extract, peptone, urea and yeast extract) on the mycelial growth of *U. zonata* was tested. A basal medium without any nitrogen source was considered as control. Results (Table 4, Fig.1) revealed maximum growth in beef extract followed by yeast extract and then in peptone. Among the inorganic sources calcium nitrate supported maximum growth. Other inorganic sources supported lower growth than organic sources, though no growth was observed in urea and insignificant growth was noted in basal medium without nitrogen.

Table 4 : Effect of different Nitrogen sources on mycelial growth of *U.zonata*

	Dry weight of fungal mass (mg)			
Nitrogen sources	Expt. 1	Expt.2	Expt.3	Mean
Inorganic				
Potassium nitrate	29.6	35.8	25.6	30.3±2.97
Sodium nitrate	34.5	30.8	28.6	31.3±1.72
Ammonium sulphate	32.6	31.7	32.0	32.1±0.26
Ammonium nitrate	43.2	46.0	48.7	45.9±1.58
Calcium nitrate	127.5	112.9	118.6	119.6±4.25
Organic				
Urea	-	-	-	-
Peptone	201.5	210.6	205.4	205.8±2.63
Casein hydrolysate	190.5	192.0	186.7	189.7±1.57
Yeast extract	230.0	215.0	213.9	219.6±5.19
Beef extract	256.0	290.0	310.6	285.5±5.19
Control (without nitrogen)	3.2	4.1	2.9	3.4±0.36

± Standard error; Temperature 30 ±1°C ; Incubation period 20 days



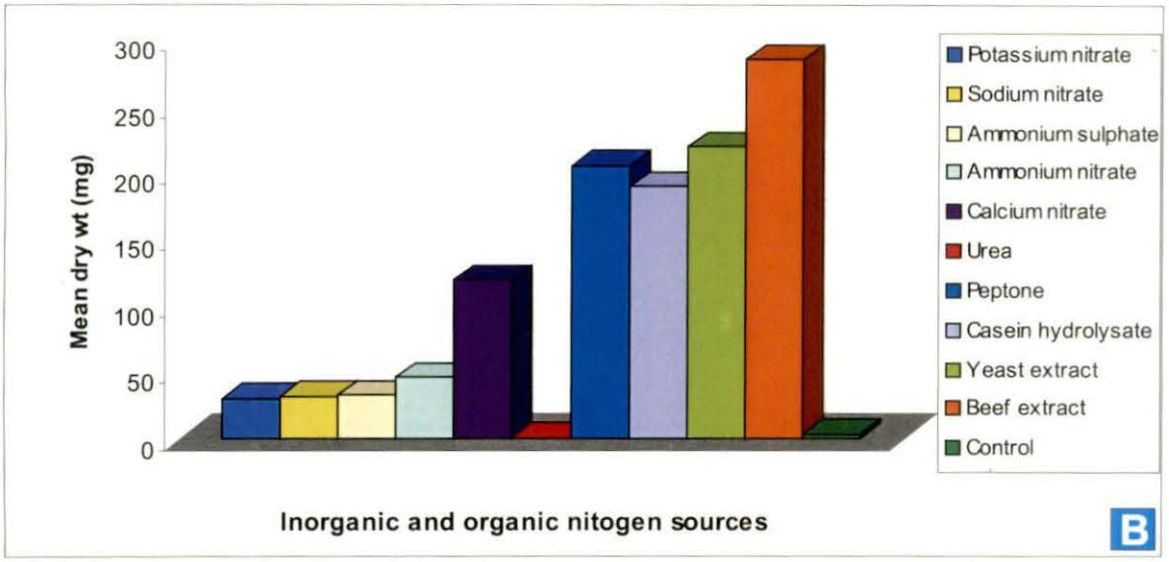


Figure 1: Effect of different carbon sources (A), inorganic and organic sources on mycelial growth of *U. zonata.*

4.3. Varietal resistance test of tea varieties against *U. zonata*

Naturally infected tea roots, showing symptoms of charcoal stump rot (Plate 2) was collected and the pathogen (*U. zonata*) was isolated from the naturally infected roots and then compared with the fungal culture received from Tocklai Experimental Station, Jorhat and was used for further studies involving different tea varieties. Varietal resistance test of tea against *U. zonata* was carried out with twenty four tea varieties. Three year old plant roots were inoculated with *U. zonata* and disease assessment was done on the basis of visual observation of symptoms and disease index (ranging from 1-6) was calculated after 20, 40 and 60 days of inoculation. Among the tested tea varieties BSS-2, BS/74/76 and AV-2 were found to be highly susceptible, while UPASI-9, UPASI-2, UPASI-3 were most resistant (Table 5; Fig. 2).

Table 5 : Varietal resistance test of tea varieties against *U. zonata*

	Disease Index		
Tea varieties [a]	20 days	40 days	60 days
TV-9	1.0	2.0	2.0
TV-18	1.1	1.9	2.0
TV-22	0.8	1.3	2.4
TV-23	0.5	2.0	2.8
TV-25	0.8	2.7	3.0
TV-26	0.5	2.0	2.9
TV-27	0.6	2.4	3.1
TV-28	1.0	2.1	2.6
TV-30	0.5	2.2	3.5
Teen Ali 17/1/54	0.9	1.6	1.9
BS/74/76	0.9	2.8	4.5
CP-1	0.4	1.5	2.6
AV-2	0.8	2.9	4.3
HV-39	0.8	0.9	2.5
T-135	0.4	1.3	2.2
S-449	1.1	2.0	2.2
P-1258	0.2	1.4	1.9
K1/1	0.4	1.6	2.3
UPASI-2	-	0.3	1.1
UPASI-3	-	0.2	1.3
UPASI-9	-	0.2	0.8
UPASI-26	0.9	2.1	3.6
BSS-2	1.9	3.7	4.6

Average of 30 separate inoculated plants of each variety ; ± Standard error

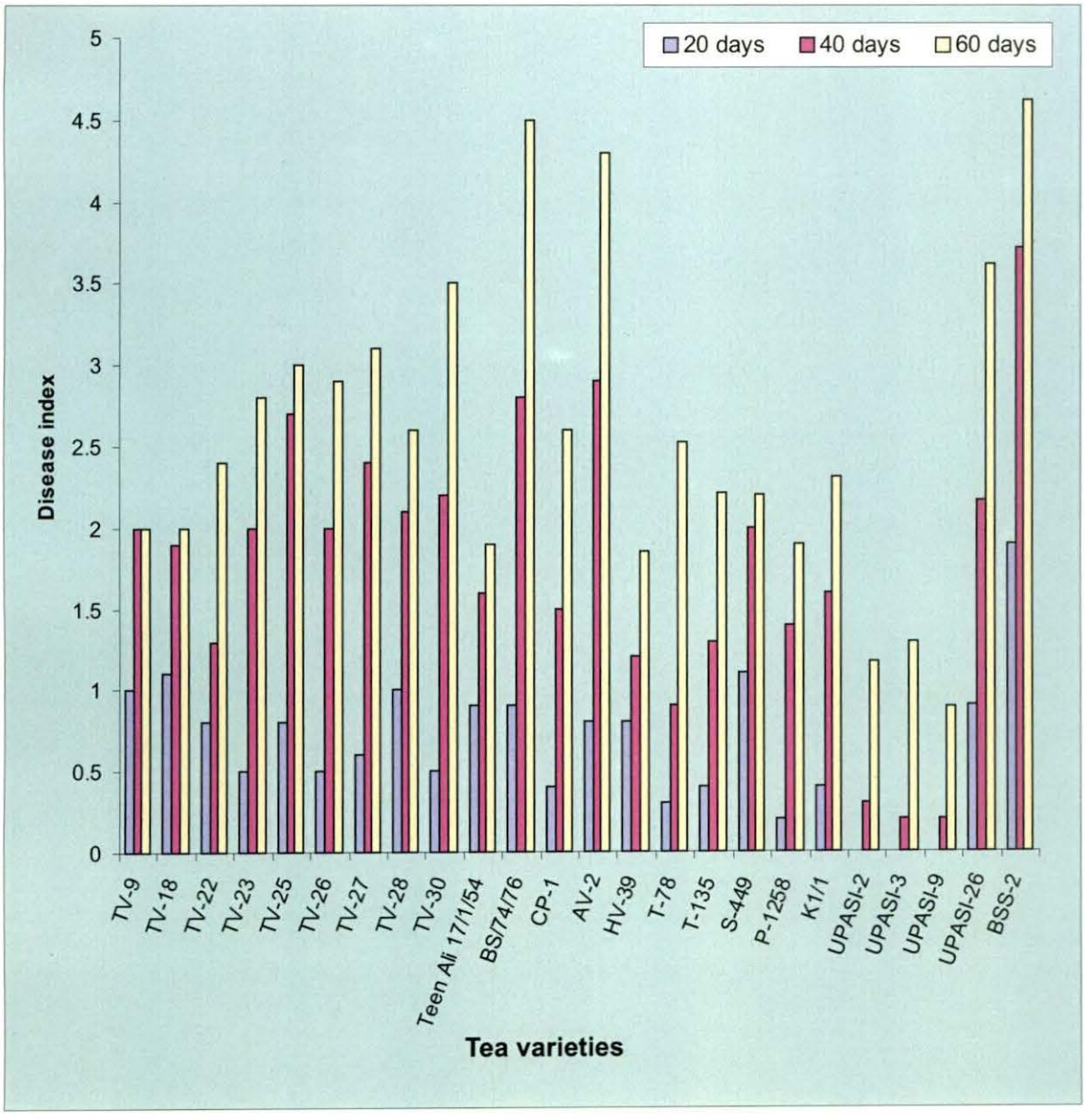


Figure 2: Varietal resistance test of tea against *U. zonata.*

4.4 Analysis of proteins in mycelia and tea roots

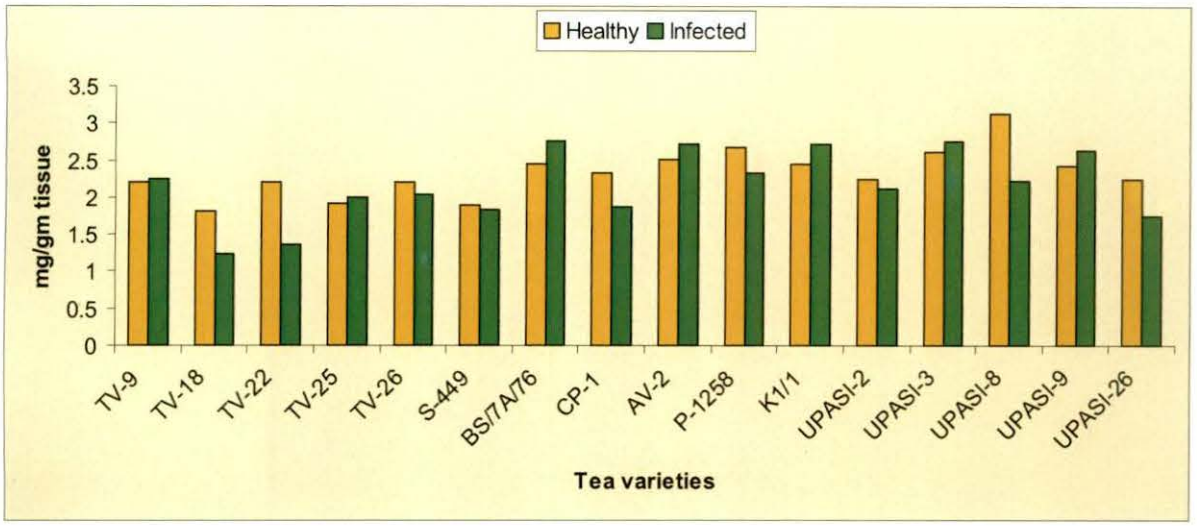
Soluble proteins of mycelia and cell wall of the pathogen as well as tea roots before and after inoculation with *U. zonata* was analysed . For this purpose estimation of protein content as well as SDS-PAGE was carried out with fungal proteins as well as proteins of healthy and infected tea roots.

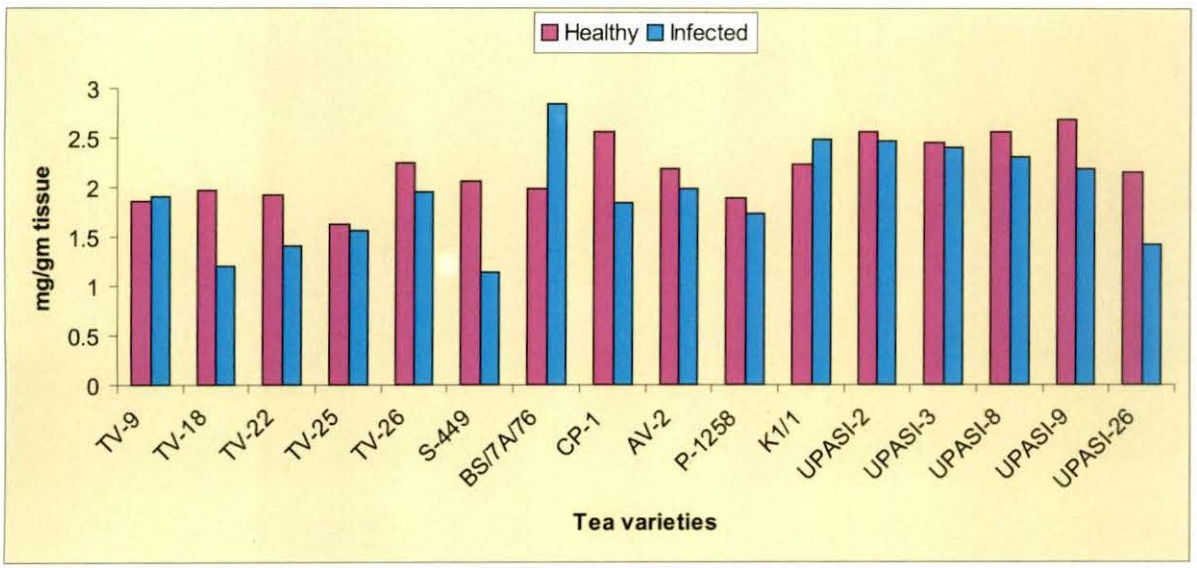
4.4.1. Protein content

Mycelial protein content of *U. zonata* was around 6.0mg/gm fresh weight tissue. Protein content of cell wall preparations was 5.8mg/g fresh weight of mycelial wall. Protein content of tea roots of tested varieties following artificial inoculation with *U. zonata* was estimated after 10,20 and 30days. Results (Table 6) revealed that protein content decreased following inoculation in most of the varieties tested (Fig 3). There was no relationship with susceptibility or resistance. In older plants (5yr old) protein content of susceptible varieties, which were greatly affected, showed a significant decrease in relation to control.

Table 6 : Protein content of healthy and infected tea root tissues.

	Protein content (mg/gm)					
	Days after inoculation					
Tea variety	10		20		30	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	2.20	2.24	1.86	1.90	1.52	1.55
TV-18	1.82	1.24	1.97	1.21	2.42	1.12
TV-22	2.21	1.35	1.92	1.41	1.96	1.36
TV-25	1.92	1.99	1.62	1.57	1.73	1.71
TV-26	2.21	2.03	2.25	1.95	2.08	1.70
S-449	1.90	1.84	2.07	1.14	2.12	1.23
BS/7A/76	2.46	2.76	1.98	2.84	2.54	2.63
CP-1	2.32	1.88	2.56	1.84	2.57	1.52
AV-2	2.51	2.72	2.19	1.99	1.92	1.64
P-1258	2.68	2.32	1.89	1.74	2.25	1.44
K1/1	2.46	2.72	2.23	2.48	2.32	2.47
UPASI-2	2.24	2.12	2.56	2.47	2.63	1.69
UPASI-3	2.62	2.75	2.46	2.41	2.24	2.02
UPASI-8	3.12	2.23	2.57	2.32	1.95	1.97
UPASI-9	2.42	2.64	2.69	2.18	2.89	2.99
UPASI-26	2.24	1.74	2.16	1.42	2.21	1.32





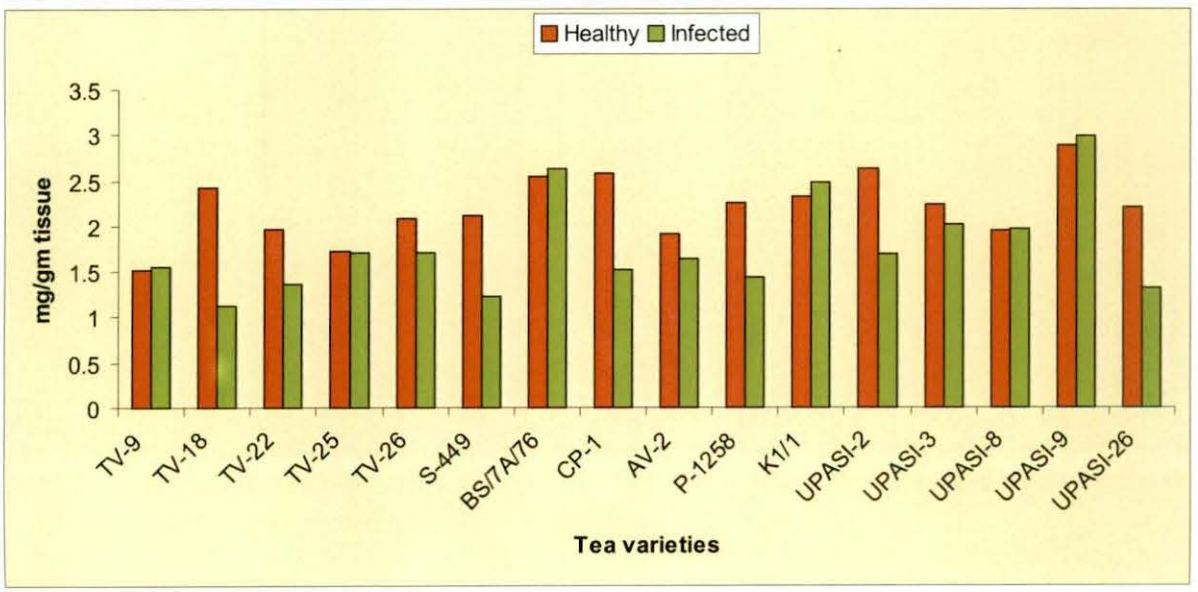


Figure 3: Soluble protein content of healthy and *U. zonata* inoculated tea roots.

4.4.2. Protein pattern

Proteins extracted from different sources were further analysed by SDS-PAGE. The molecular weight of protein bands visualized after staining with coomassie blue were determined from the known molecular weight marker.

4.4.2.1 Mycelial protein of *U. zonata*

Mycelial protein exhibited 19 bands in SDS-PAGE ranging in molecular weight (Ca.97.4 kDa to 12.7 kDa) and bands were of varying intensities and more proteins of lower molecular were present (Plate 7,fig.A). Maximum number of bands were found in 10 and 15 days of incubation following which a decline in number of bands was found. High intensity of bands were found in extracts from 10-15 days. In case of 5 days on incubation one deep and thick band (ca. 22 kDa) was found which gradually became and less intense in following days.

4.4.2.2. Cell wall protein of *U. zonata*

Cell wall preparation of *U. zonata* were resolved in SDS-PAGE as described earlier, fixed in fixer solution and stained with coomassie blue. Gel exhibited 15 protein bands ranging from ca.97.4 to 15 kDa of which 7 bands were of higher molecular weight and 8 of low molecular weight (Plate 7, fig.B).

4.4.2.3. Tea root protein

Since protein content of tea roots had decreased following infection, it was decided to analyse the changes in protein pattern by SDS PAGE. In the healthy roots of the tea varieties number of bands ranged from 10-18 of molecular wts. 70-13 kDa. Following inoculation number of bands decreased. In infected roots of Tocklai varieties number of bands ranged from 9-11, whereas in infected roots of UPASI varieties number of bands ranged from 10-12.

4.4.3. Con A-FITC binding

Mycelia and isolated cell wall of *U. zonata* were treated with FITC labeled Con A. Strong apple green fluorescence were evident in both mycelia and cell wall which confirmed glycoprotein nature of the cell wall.

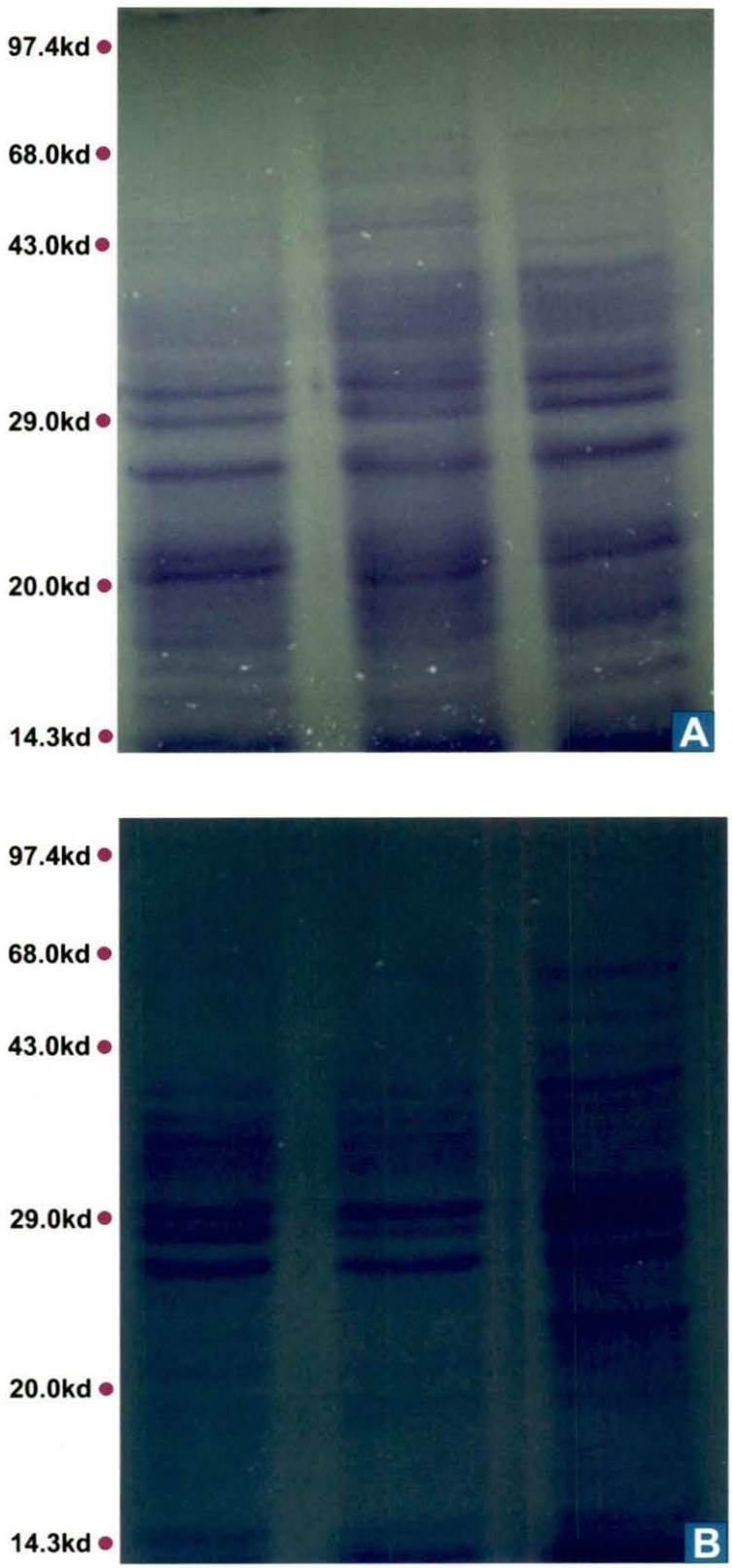


Plate 7 (Figs. A&B): SDS-PAGE analysis of soluble proteins of mycelia (A) and cell wall (B) of *Ustulina zonata*

4.5. Determination of levels of phenolics in tea varieties following inoculation with *U. zonata*

As polyphenols are the major constituents of tea roots it was decided to compare the level of total phenol and ortho-dihydroxy phenol in tea roots of resistant and susceptible varieties following inoculation with *U. zonata.* Sixteen tea varieties (TV-9, TV-18, TV-22, TV-25, TV-26, S-449, BS/7A/76, CP-1, AV-2, P-1258, K1/1, UPASI-2, UPASI-3, UPASI-8, UPASI-9 and UPASI-26) were selected for this experiment.

4.5.1 Total phenols

Total phenols from healthy and *U. zonata* inoculated tea roots of five tocklai varieties, six Darjeeling varieties and five UPASI varieties were extracted after 7 days of inoculation and estimated. Results (Table-7) revealed that total phenol decreased following inoculation with *U. zonata* in the susceptible varieties. However there is an increase in the phenol content of resistant varieties following inoculation. Among all the varieties tested UPASI-varieties showed maximum increase in total phenol following inoculation with the pathogen.

4.5.2 Ortho-dihydroxy phenols

Ortho-dihydroxy phenols were also extracted from healthy and *U.zonata* inoculated tea roots of five each of Tocklai and UPASI varieties and six Darjeeling varieties after 7 days of inoculation with the pathogen and estimated. Results (Table -8) revealed that ortho-dihydroxy content decreased in susceptible varieties and increased in resistant varieties following inoculation with *U. zonata* . Response of UPASI varieties against the pathogen were towards increasing the level of orthodihydroxy phenol.

4.5.3. Analysis of antifungal compound in tea roots following inoculation with *U.zonata*

Further experiments were carried out to detect the antifungal phenolics from relatively large samples of freshly harvested tea roots following artificial inoculation with *U.zonata* using facilitated diffusion technique. Antifungal compounds were extracted separately from healthy and *U. zonata* inoculated tea roots of two resistant varieties (UPASI-9 and UPASI-3) and two susceptible varieties (BS/74/6 and BSS-2) after 96 h of inoculation.

Table-7: Total phenol content in healthy and *U. zonata* inoculated tea varieties

Tea varieties	Total phenol (mg/g Tissue)[a]	
	Healthy	**Inoculated [b]**
TV-9	5.3	4.8
TV-18	4.5	4.1
TV-22	4.9	3.7
TV-25	4.6	4.0
TV-26	6.2	5.7
S-449	6.5	3.1
BS/7A/76	5.9	2.8
CP-1	5.7	2.8
AV-2	4.9	3.0
P-1258	5.3	3.4
K1/1	5.8	2.6
UPASI-2	4.7	5.2
UPASI-3	4.9	5.0
UPASI-8	5.1	5.6
UPASI-9	5.4	5.9
UPASI-26	4.3	4.8

a = average of three replicates

b =7 days following inoculation with *U. zonata*

4.5.3.1. Bioassay

Ethyl acetate fractions of both healthy and *U. zonata* inoculated tea root extracts were loaded on TLC plates, developed in chloroform : methanol (9:1, v/v) and sprayed with Folin-Ciocalteau's reagent. Colour reaction was noted at Rf 0.56. Crude extract (ethyl acetate fraction dissolved in methanol) prepared from healthy and *U. zonata* inoculated roots of four varieties were bioassayed following radial growth inhibition assay. Results (Table -9) revealed that mycelial growth of *U. zonata* was inhibited markedly in the medium supplemented with the extracts of inoculated roots of resistant varieties (UPASI-9 and UPASI-3) than those of susceptible varieties (BS/74/6 and BSS-2) tested in relation to their respective control (media supplemented with healthy root extract). Mycelial growth was measured in each treatment, when *U. zonata* covered full Petridish grown in PDA without any supplementation.

Table-8: Level of Ortho-dihydroxy phenol content in healthy and *U. zonata* inoculated tea varieties

Tea varieties	Ortho-dihydroxy phenol(mg/g Tissue)[a]	
	Healthy	Inoculated [b]
TV-9	2.1	1.8
TV-18	2.7	2.5
TV-22	3.7	3.2
TV-25	4.1	3.8
TV-26	4.3	3.6
S-449	2.3	1.6
BS/7A/76	1.8	0.7
CP-1	1.7	0.9
AV-2	2.3	1.2
P-1258	2.4	1.1
K1/1	1.7	0.6
UPASI-2	1.3	2.4
UPASI-3	1.8	2.0
UPASI-8	0.9	2.1
UPASI-9	1.4	1.9
UPASI-26	1.2	1.8

a = average of three replicates

b =7 days following inoculation with *U.zonata*

Table 9 : Effect of antifungal compounds extracted from healthy and inoculated tea root extracts on radial growth of *U. zonata*

Variety	Diameter of mycelial growth (mm) [a]	
	Healthy	Inoculated [b]
Resistant		
UPASI-9	15.1	4.6
UPASI-3	13.2	5.3
Susceptible		
BS/74/76	19.6	10.4
BSS-2	18.4	12.8
Distilled water control	30 mm	

[a] Average of three experimental sets ; [b] Inoculated with *U. zonata*

4.5.3.2 UV -spectrophotometric analysis

Results of the bioassay revealed the presence of antifungal compounds in inoculated tea roots. Partially purified compound (Rf 0.56) from extracts of healthy and inoculated tea roots of UPASI-9 were examined in a UV-spectrophotometer. It is interesting to note that extracts from *U. zonata* inoculated root tissues gave a peak at 272nm. Maximum absorption peak measured at 272 nm was identical to an authentic sample of pyrocatechol. Hence quantification of pyrocatechol was done from UV-spectrophotometric curve by considering molar extinction coefficient of authentic pyrocatechol 6000 at 272 nm. Pyrocatechol accumulation in two resistant and two susceptible varieties of tea after 96 h of inoculation was estimated and compared with healthy controls. It appears from results that in inoculated roots, greater amount (428-512 µg/g fresh wt). Concentration of this compound in healthy root tissues were very low (54-73 µg/g fresh wt).

4.6. Determination of enzyme activity in healthy and *U. zonata* inoculated tea roots

4.6.1. Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL) is the first enzyme of phenyl propanoid metabolism in higher plants and it has been suggested to play a significant role in regulating the accumulation of phenolics, and phytoalexins as well as lignins, three key factors responsible for disease resistance. In the present study, PAL activity of was assayed in sixteen tea root varieties following inoculation with *U. zonata*. PAL activity was assayed in each case after 2, 4 and 8 days after inoculation. Results have been presented in Table 10 and Figure 4. It showed that PAL activity increased after 4 days of inoculation markedly in all the varieties except TV-9 and UPASI-26. However, after 8 days of inoculation PAL activity decreased .

4.6.2. Peroxidase (PO)

PO activity was assayed as increase in absorbance when o-dianisidine was oxidized by the oxygen released from H_2O_2 which was oxidised by the enzyme. Peroxidase was extracted from healthy and *U. zonata* inoculated tea roots of sixteen varieties and their activity was also assayed after 2, 4 and 8 days of inoculation. Results

have been presented in Table 11. Peroxidase activity also increased in all the varieties tested following inoculation. Time course accumulation of peroxidase was highest after 4 days of inoculation and after this period peroxidase activity started declining. However, increased POX activity was noticed in TV-26, BS/74/76, CP-1, AV-2, K1/1 and UPASI-8 even after 8 days of inoculation with the pathogen (Fig.5).

Table 10 : Changes in phenylalanine ammonia lyase activity in tea roots following inoculation with *U.zonata*

	PAL activity in tea roots (µg cinnamic acid $g^{-1}m^{-1}$) [a]					
	Days after inoculation					
	2		4		8	
Tea Varieties	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	160.1	35.9	163.4	89.5	170.4	101.8
TV-18	73.9	79.2	105.3	127.1	119.3	87.6
TV-22	74.8	23.9	76.0	141.9	93.2	68.7
TV-25	89.0	149.2	81.0	158.6	79.5	132.5
TV-26	69.5	57.0	75.4	173.8	81.6	98.3
S-449	71.8	89.3	73.1	129.9	76.3	101.2
BS/7A/76	138.0	153.7	143.2	247.0	136.8	59.4
CP-1	106.3	67.5	189.2	292.7	89.9	104.5
AV-2	33.2	77.4	41.6	120.9	41.8	101.1
P-1258	102.5	140.1	123.0	201.9	126.6	63.0
K1/1	135.7	170.6	133.8	231.9	139.4	53.9
UPASI-2	105.7	73.8	134.2	241.9	103.1	39.1
UPASI-3	107.5	69.8	137.3	240.8	101.7	39.2
UPASI-8	83.03	69.1	87.3	142.6	90.1	54.5
UPASI-9	56.7	70.3	68.2	89.7	49.7	28.9
UPASI-26	160.5	71.2	150.4	142.3	189.7	48.8

[a] Average of 3 replicates.

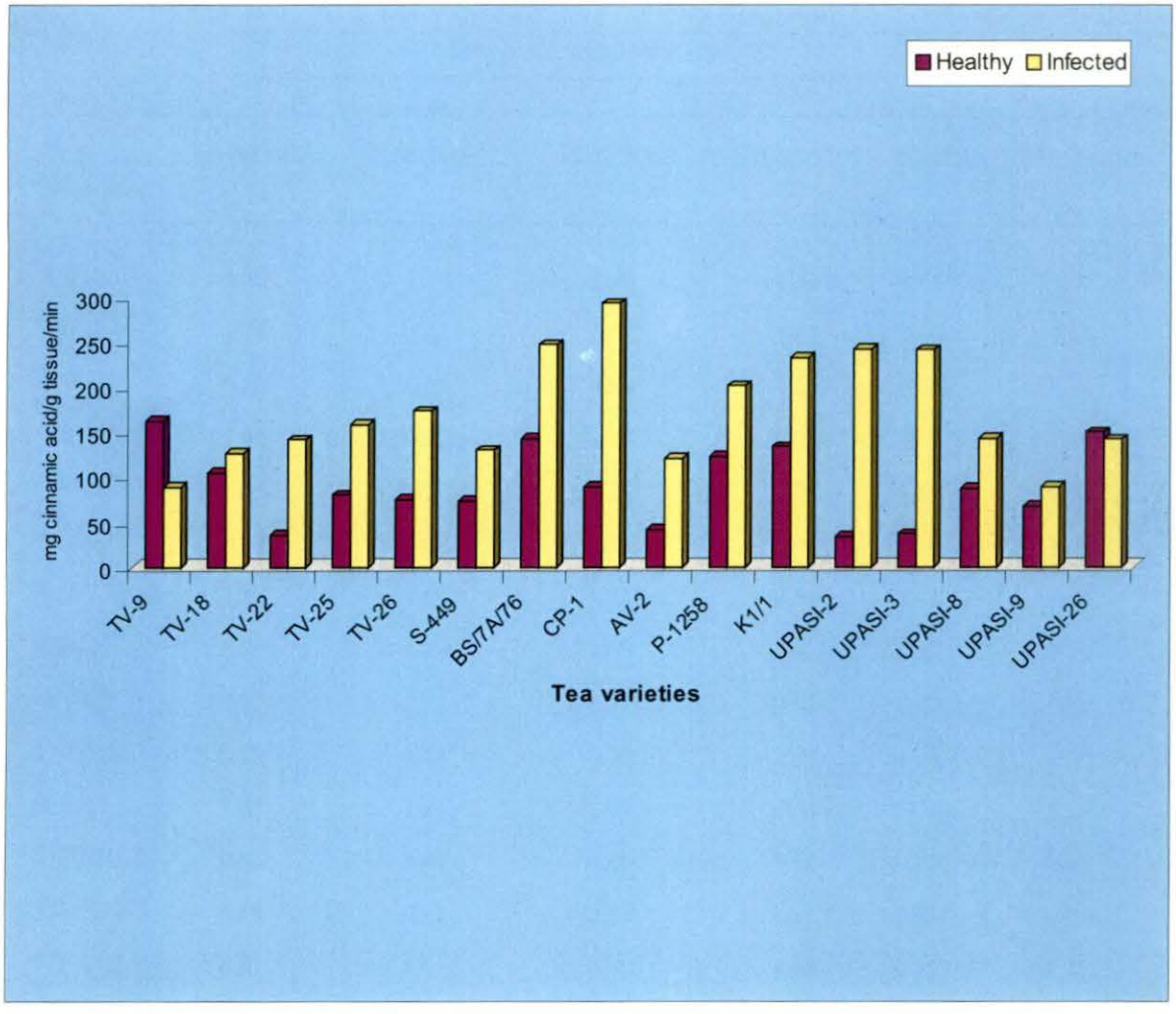


Figure 4: Phenylalanine ammonia lyase (PAL) activity in tea varieties following inoculation with *U.zonata.*

Table 11 : Changes in peroxidase activity in tea roots following inoculation with *U.zonata*

	PO activity in tea roots (ΔOD/g tissue/min) [a]					
	Days after inoculation[b]					
	2		4		8	
Tea Varieties	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	1.40	1.13	1.46	1.57	1.63	1.37
TV-18	1.48	1.02	1.58	1.63	1.49	1.17
TV-22	0.65	0.16	0.64	1.80	0.65	1.14
TV-25	1.03	1.10	1.11	1.22	1.17	1.19
TV-26	1.45	1.53	1.29	1.77	0.89	1.74
S-449	0.91	0.98	1.23	1.06	1.14	1.26
BS/7A/76	1.23	1.09	1.11	2.51	1.21	1.56
CP-1	0.79	1.23	1.03	1.62	1.23	1.50
AV-2	0.23	0.81	0.52	0.91	0.22	0.81
P-1258	0.59	1.18	0.72	1.09	1.33	1.00
K1/1	1.05	1.21	1.02	2.34	1.06	1.38
UPASI-2	0.82	0.69	0.88	1.41	0.99	0.93
UPASI-3	0.68	0.87	0.52	1.21	0.65	0.83
UPASI-8	0.61	1.4	0.68	1.43	0.71	1.22
UPASI-9	0.89	1.13	1.24	1.32	1.36	1.20
UPASI-26	0.69	0.88	0.89	1.76	0.97	1.01

[a] Average of 3 replicates ; [b] Days after inoculation

4.6.3. Polyphenol oxidase (PPO)

Sixteen tea varieties were selected and enzyme activity following inoculation with the pathogen was assessed. PPO activity in tea roots increased markedly after 4 days of inoculation with *U. zonata* in all the varieties tested. Results have been presented in Table 12 and Fig.6

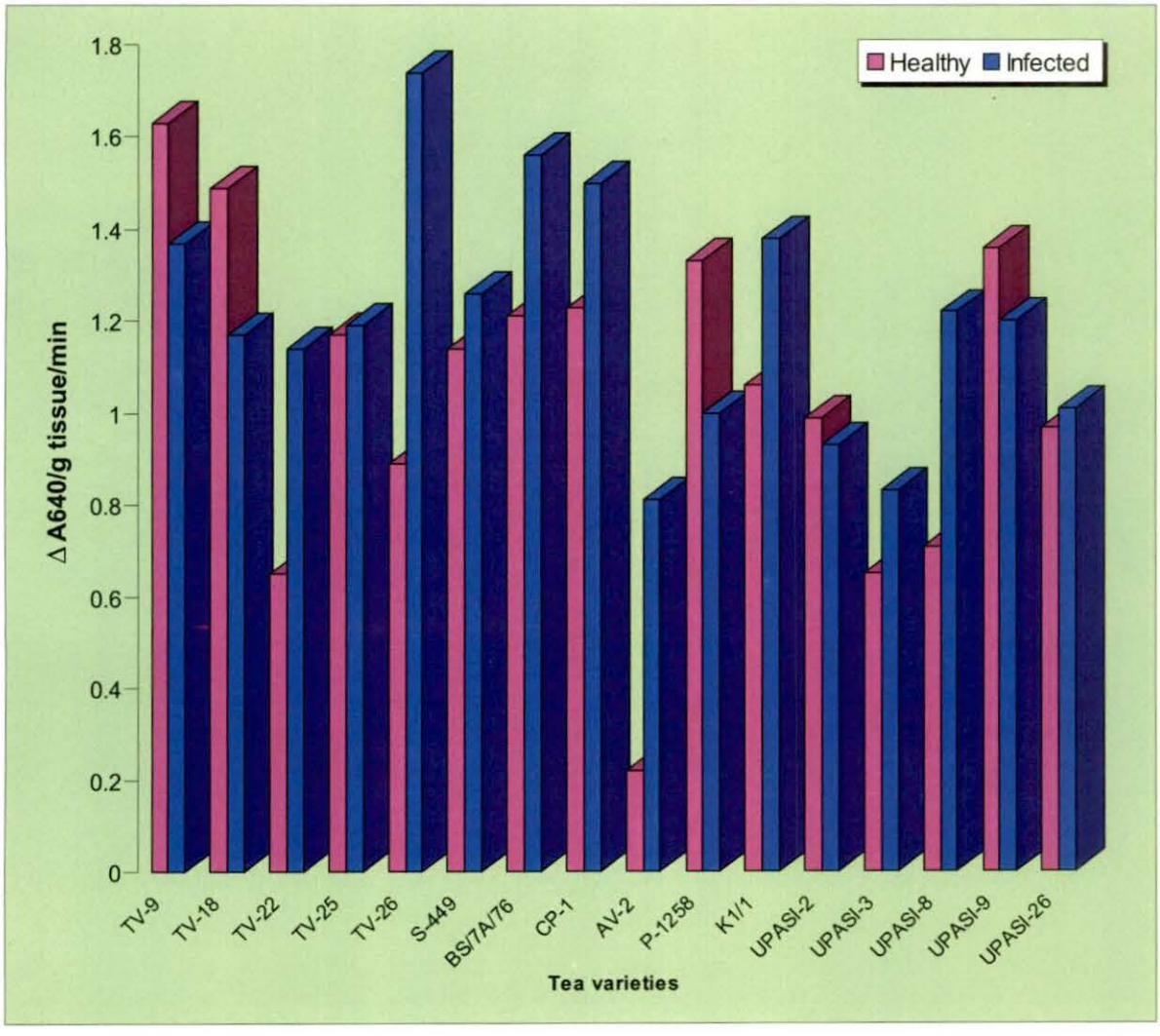


Figure 5: Peroxidase (POX) activity in tea varieties following inoculation with *U. zonata.*

Table 12 : Changes in polyphenol oxidase activity in tea roots following inoculation with *U.zonata*

	PPO activity in tea roots (ΔOD/g tissue/min) [a]					
	Days after inoculation[b]					
	2		4		8	
Tea Varieties	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	0.13	0.2	0.14	0.19	0.14	0.20
TV-18	0.15	0.16	0.15	0.19	0.17	0.21
TV-22	0.12	0.18	0.11	0.21	0.12	0.13
TV-25	0.15	0.14	0.16	0.21	0.16	0.19
TV-26	0.25	0.17	0.25	0.29	0.26	0.29
S-449	0.04	0.12	0.04	0.16	0.05	0.14
BS/7A/76	0.24	0.29	0.26	0.31	0.37	0.41
CP-1	0.11	0.08	0.13	0.28	0.15	0.21
AV-2	0.13	0.14	0.12	0.16	0.17	0.19
P-1258	0.04	0.11	0.08	0.28	0.09	0.21
K1/1	0.21	0.29	0.25	0.37	0.27	0.29
UPASI-2	0.23	0.21	0.19	0.26	0.14	0.19
UPASI-3	0.09	0.10	0.04	0.21	0.09	0.14
UPASI-8	0.24	0.25	0.18	0.22	0.25	0.09
UPASI-9	0.21	0.29	0.24	0.19	0.25	0.17
UPASI-26	0.13	0.12	0.15	0.16	0.13	0.08

[a] Average of 3 replicates ; [b] Days after inoculation

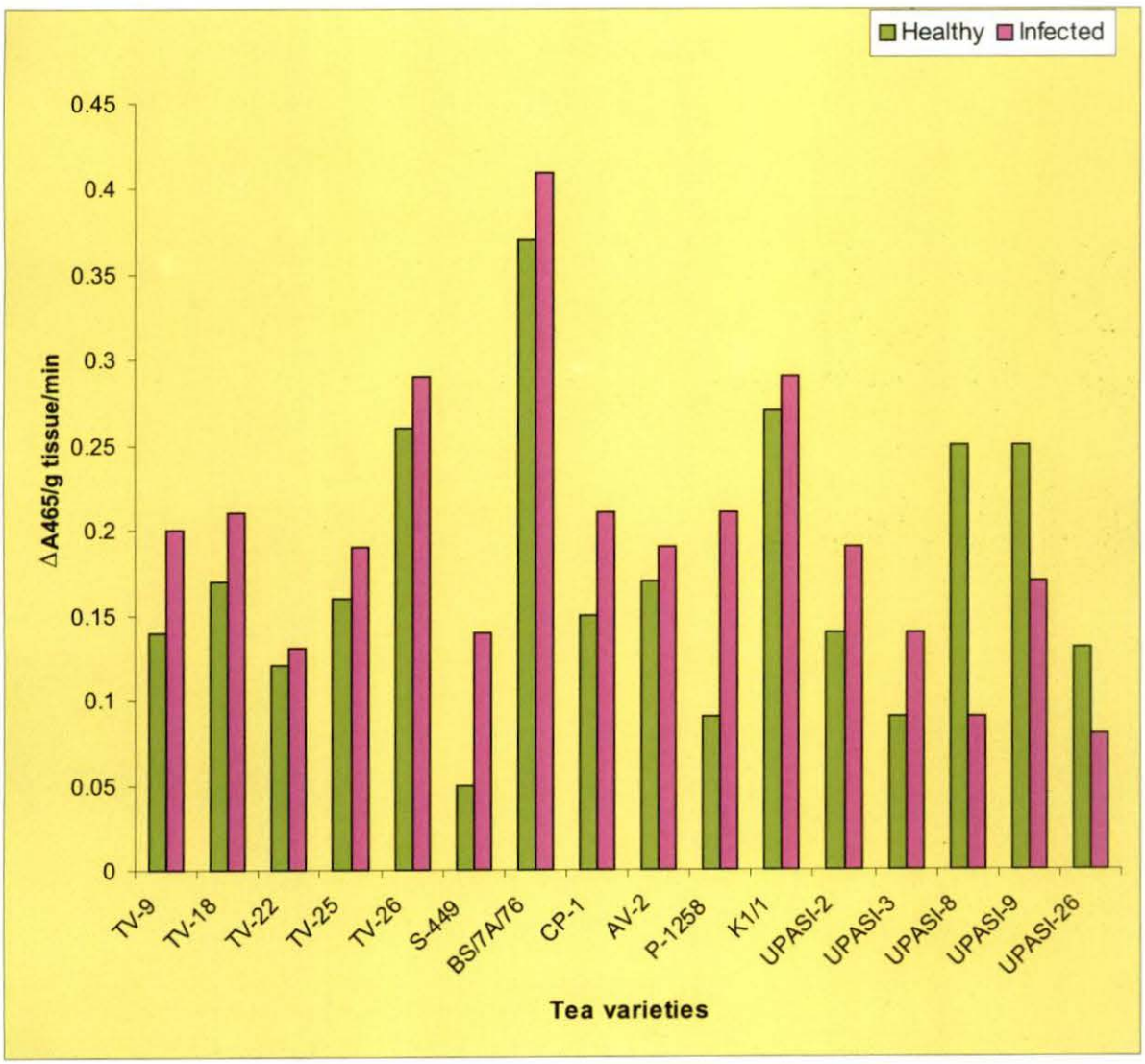


Figure 6: Polyphenol oxidase (PPO) activity in tea varieties following inoculation with *U. zonata.*

4.7. Agar gel double diffusion tests with PAbs of *U. zonata* and tea roots

Polyclonal antibodies (PAbs) were raised in rabbits against mycelial (100% SAS) and cell wall antigens of pathogen (*U. zonata*), mycelial antigens of two mycoparasites selected as biocontrol agents (*T. harzianum*, *T. viride*), as well as tea root antigens (UPASI-26) and these were used for development of immunodiagnostic kits using various serological assays. The PAbs obtained from different bleedings were initially checked for the effectiveness of each antigen preparation in raising PAbs by homologous cross reaction using agar gel double diffusion tests. Control sets involving normal sera and antigen of pathogen and tea roots were all negatives. Strong precipitin reaction occurred when PAb of *U. zonata* raised against mycelia and cell wall antigens were reacted with its own antigen (Plate 8 figs. A&B). Besides, strong positive reaction was also noticed when PAb raised against tea root antigen was checked in immumodiffusion test.

4.8. Optimization of PAb and antigen concentrations by ELISA

Enzyme linked immunosorbent (ELISA) assay is one of the most sensitive serological techniques for detection of cross reactive antigens between host and the pathogen as well as for detection of pathogen in diseased tissue and surrounding soil. Optimization using plate trapped antigen coated (PTA)- ELISA technique followed by primary antibody and finally secondary antibody labels with enzyme were performed separately with PAbs raised against mycelial and cell wall antigens of *U. zonata* and tea root antigens. PAbs in each case were collected by different bleedings at definite time intervals.

4.8.1. PAb raised against mycelia of *U. zonata*

Optimization of ELISA was done by considering two variables i.e. concentration of the antigen and concentration of PAb. Reactions were done with PAb obtained after different bleedings. Enzyme concentration was 1:10.000 while substrate was used at a concentration of 1mg/ml.

4.8.1.1. IgG concentration

Different concentrations of IgG (ranging from 0.312-40 µg/ml) from PAb were tested against homologous antigens at a concentration of 10µg/ml Absorbance values in PTA-ELISA increased with increase in concentration of IgG with a maximum value of 2.8 in 40 µg/ml (Table-13). This concentration of IgG was selected for further experiments.

Table 13: ELISA reaction with different concentration of PAb of *U. zonata* (mycelia) and homologous antigen

Antisera (IgG) concentration (µg/ml)	Absorbance at 405nm			
	Expt. 1	Expt.2	Expt.3	Mean
0.312	0.524	0.550	0.540	0.538± 0.007
0.625	0.721	0.743	0.738	0.734±0.006
1.25	0.892	0.889	0.868	0.883±0.007
2.5	1.064	1.067	1.021	1.050±0.014
5	1.341	1.456	1.401	1.399±0.033
10	1.567	1.621	1.501	1.563±0.034
20	2.078	2.154	2.240	2.157±0.046
40	2.871	2.909	2.91	2.896±0.012

Antigen concentration - 10 µg/ml
± Standard error

4.8.1.2. Antigen concentration

Antigen concentration ranging from 0.156-20 µg/ml were tested against IgG from 1st to 5th bleeding at a concentration of 40 µg/ml. Results (Table 14, Fig.7) revealed that ELISA values decreased with the decrease of antigen concentration. However concentration as low as 0.156 µg/ml could also be well detected by ELISA and maximum ELISA value was obtained in IgG 3rd followed by 4th bleeding.

Table 14 : ELISA reaction of mycelial PAb of *U. zonata* obtained from different bleedings with different concentration of homologous antigen.

Antigen concentration (µg/ml)	Absorbance at 405nm				
	1st bleed	2nd bleed	3rd bleed	4th bleed	5th bleed
0.156	0.501	0.592	0.639	0.649	0.656
0.312	0.723	0.732	0.759	0.768	0.789
0.625	0.811	0.871	0.889	0.892	0.898
1.25	0.841	0.862	0.878	0.901	0.912
2.5	1.064	1.167	1.191	1.201	1.223
5	1.341	1.456	1.471	1.488	1.562
10	1.507	1.600	1.621	1.678	1.897
20	1.634	1.756	1.811	1.892	1.909
40	1.758	1.987	2.010	2.210	2.340

IgG concentration 40 µg/ml

4.8.2. PAb raised against cell wall of *U. zonata*

PAb raised against antigens prepared from cell wall of *U. zonata* were used to for optimization, considering two variables, antiserum concentration and antigen concentration on ELISA reactivity.

4.8.2.1. IgG concentration

A series of IgG concentration tested ranging from 0.312-40 µg/ml, prepared from PAb of cell wall preparation of the pathogen. Cell wall antigens were used at a concentration of 10 µg/ml. Absorbance values increased with increase in concentration of IgG with a maximum value 2.58 in 40 µg/ml (Table 15)

4.8.2.2 Antigen concentration

ELISA reaction with different concentration of cell wall antigen from 0.312-40 µg/ml were determined at an IgG concentration 40 µg/ml in respect of different bleedings. Absorbance values increased with increasing concentration (Table 16 & Fig.7) Result also revealed maximum ELISA values in IgG- 4th bleed followed by IgG-5th bleed raised against cell wall .

Table 15 : ELISA reaction of cell wall antigen of *U. zonata* with different concentration of homologous PAb.

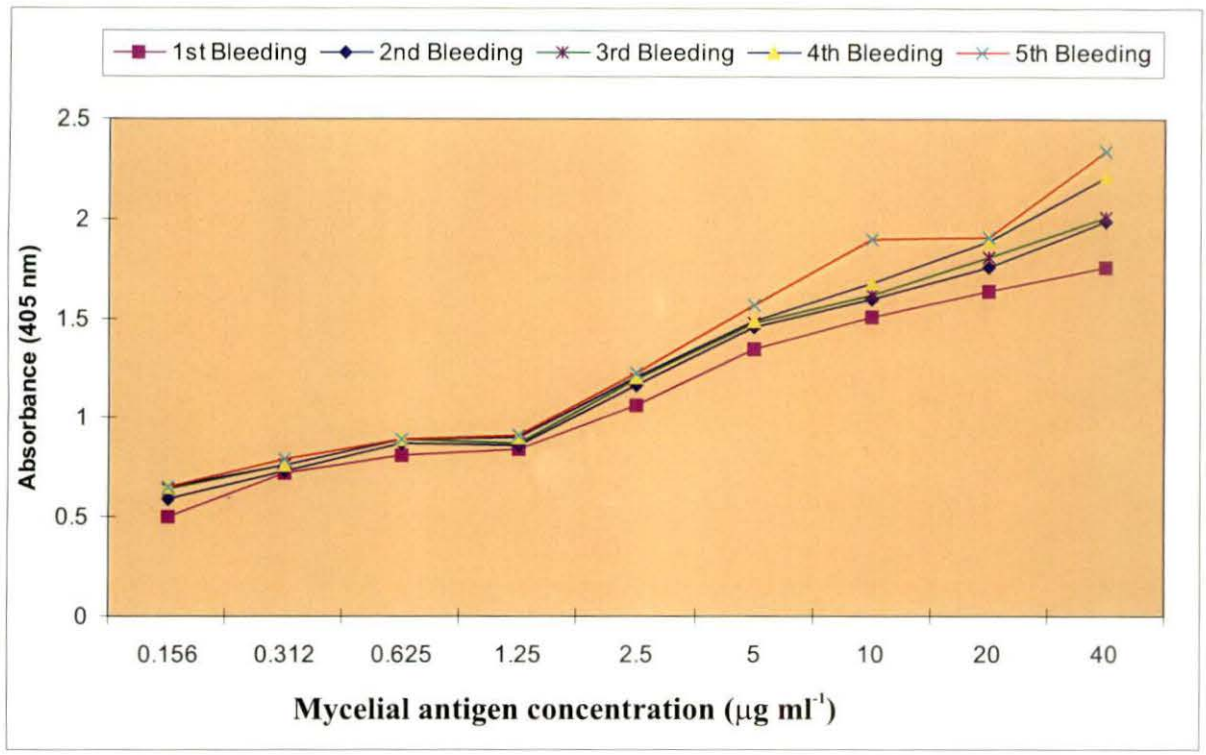
IgG concentration (µg/ml)	Absorbance at 405nm			
	Expt.1	Expt.2	Expt.3	Mean
0.312	0.841	0.839	0.840	0.858± 0.0005
0.625	0.894	0.896	0.897	0.895±0.0008
1.25	0.923	0.926	0.924	0.924±0.0008
2.5	0.923	0.926	0.924	0.981±0.0008
5	1.23	1.40	1.52	1.380±0.0842
10	1.87	1.89	1.92	1.890±0.0145
20	2.23	2.34	2.25	2.270±0.0338
40	2.56	2.61	2.59	2.580±0.0145

Antigen concentration 10 µg/ml; ±Standard error.

Table 16 : ELISA reaction of cell wall PAb of *U. zonata* obtained from different bleedings with different concentration of homologous antigen.

Antigen concentration (µg/ml)	Absorbance at 405nm				
	1st bleed	2nd bleed	3rd bleed	4th bleed	5 th bleed
0.312	0.687	0.691	0.721	0.798	0.813
0.625	0.691	0.711	0.856	0.872	0.976
1.25	0.823	0.837	0.924	0.956	0.965
2.5	0.928	0.977	0.982	0.993	1.102
5	1.223	1.488	1.512	1.545	1.684
10	1.687	1.869	1.882	1.889	1.909
20	1.923	1.934	1.995	2.102	2.198
40	2.209	2.451	2.287	2.897	2.567

IgG concentration 40 µg/ml; ± Standard error.



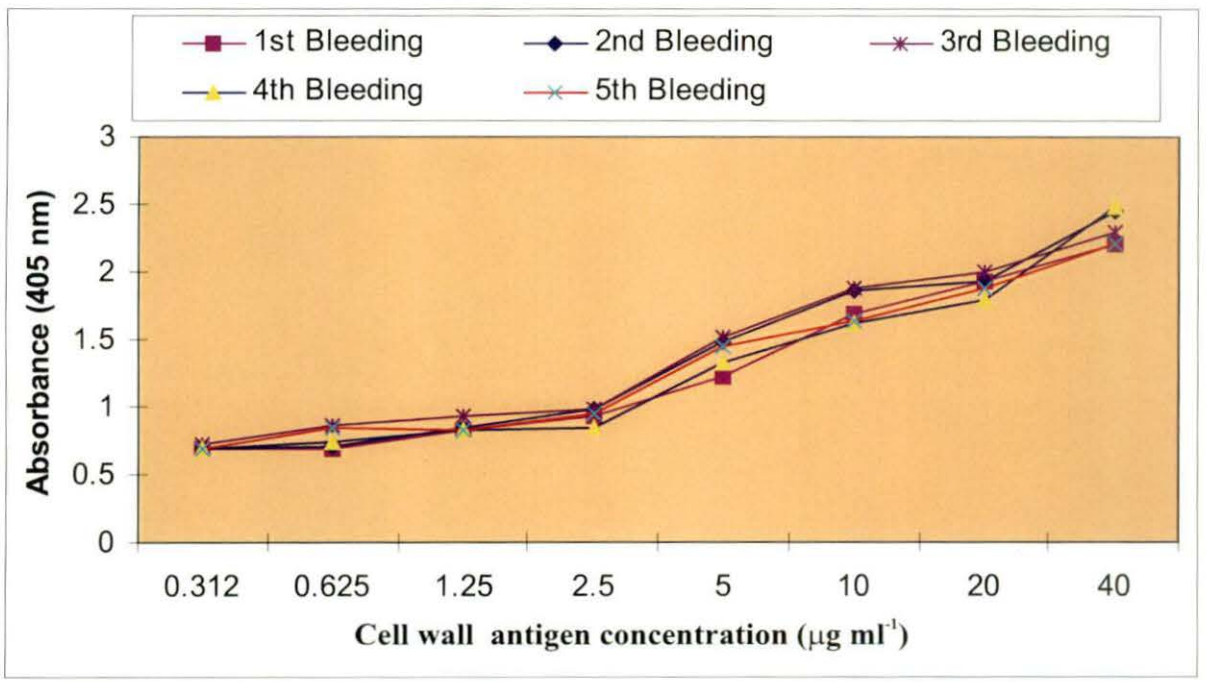


Figure 7: Optimization of mycelial and Cell wall antigen concentration of *U. zonata* using homologous PAb of the pathogen.

4.8.3. PAb raised against tea root

Optimization of ELISA was also done using PAb against root antigens of UP-26. The effect of two variables, antiserum concentration and antigen concentration on ELISA reactivity, were determined in this case also.

4.8.3.1. IgG concentration

Homologous reactions were carried out using different concentration of Pab raised against UP-26 root tissue ranging from 0.312 - 40μg/ml. A405 values decreased with decrease in concentration as revealed in Table 17.

Table 17 : ELISA reaction of tea root (UP-26) antigen with different concentration of homologous PAb.

IgG concentration (μg/ml)	Absorbance at 405nm			
	Expt.1	Expt.2	Expt.3	Mean
0.312	0.543	0.560	0.547	0.550± 0.005
0.626	0.587	0.590	0.579	0.585±0.003
1.25	0.629	0.634	0.633	0.632±0.001
2.5	0.691	0.692	0.689	0.691±0.000
5	0.710	0.715	0.712	0.712±0.001
10	0.921	0.923	0.920	0.921±0.000
20	1.112	1.128	1.121	1.120±0.004
40	1.234	1.245	1.240	1.239±0.003

Antigen concentration 10 μg/ml
± Standard error.

4.8.3.2. Antigen concentration

To determine the effect of antigen concentration on ELISA reactivity, various concentrations of root antigen ranging from 0.312 – 40 μg/ml were used. IgG was used at a concentration of 40μg/ml. Absorbance value decreased with decrease in concentration (Table 18). In both mycelial and cell wall PAb, high A405 values were obtained even with 312ng/ml, indicating that the PAb could detect even lower concentrations.

Table 18: ELISA reaction of PAb of tea root (UP-26) with different concentration of root antigen

Antigen concentration (μg/ml)	Absorbance at 405nm			
	Expt.1	Expt.2	Expt. 3	Mean
0.312	0.467	0.560	0.434	0.487± 0.037
0.626	0.553	0.498	0.545	0.532±0.017
1.25	0.612	0.620	0.625	0.619±0.003
2.5	0.662	0.649	0.660	0.657±0.004
5	0.731	0.741	0.742	0.738±0.003
10	0.879	0.881	0.889	0.883±0.003
20	1.113	1.201	1.118	1.144±0.028
40	1.321	1.320	1.228	1.289±0.030

IgG concentration = 40 μg/ml
± Standard error.

4.9. Detection of cross reactive antigens between *U. zonata* and tea roots

4.9.1 Indirect ELISA

Indirect ELISA could readily detect cross reactivity between pathogen antisera and host tissues. Cross reactive antigens have been shown to be the determinants of compatible reaction in several host pathogen combinations. In the present study sixteen tea varieties revealed variable responses ranging from high susceptibility to high resistance. In order to determine whether resistance or susceptibility could be correlated with involvement of CRA, PTA-ELISA formats were designed with antigens of all tested tea varieties using PAbs raised against mycelia and cell wall. Results were compared with those of the varietal resistance test.

4.9.1.1. PAb of *U. zonata*

4.9.1.1.1. Mycelia

Antigens were prepared from tea roots of sixteen varieties, one non-host and one non-pathogen as well as mycelia of *U. zonata.* ELISA reaction were carried out with these antigens against purified mycelial PAb of *U. zonata.* Maximum absorbance values were recorded in reactions with antigens of susceptible varieties however, in

general, absorbance values were rather low (Table 19). PTA- ELISA reactivity of tea root antigens with PAb of *U. zonata* increased with age of plant.

Table 19 : Indirect ELISA values (A405) of antigens of tea root, *U. zonata*, non-pathogen and non- host reacted with mycelial PAb of *U. zonata*.

	Absorbance at 405nm			
Antigens	Expt.1	Expt.2	Expt.3	Mean
TV-9	0.567	0.561	0.559	0.562± 0.002
TV-18	0.617	0.612	0.600	0.609±0.005
TV-22	0.523	0.512	0.520	0.518±0.003
TV-25	0.462	0.437	0.431	0.443± 0.009
TV-26	0.456	0.451	0.449	0.452±0.002
S-449	0.597	0.589	0.592	0.592±0.002
BS/7A/76	0.472	0.469	0.461	0.467±0.003
CP-1	0.648	0.642	0.641	0.643±0.002
AV-2	0.478	0.471	0.456	0.468±0.006
P-1258	0.612	0.621	0.611	0.615±0.003
K1/1	0.532	0.554	0.530	0.539±0.007
UPASI-2	0.451	0.443	0.441	0.445±0.001
UPASI-3	0.475	0.471	0.477	0.474±0.000
UPASI-8	0.459	0.461	0.460	0.460±0.005
UPASI-9	0.461	0.443	0.450	0.451±0.011
UPASI-26	0.651	0.611	0.640	0.634±0.002
Mycelia of *U.zonata*	2.870	2.879	2.875	2.875±0.001
Non-pathogen				
Fusarium oxysporum	0.444	0.440	0.439	0.441±0.001
Non-host				
Oryza sativa	0.371	0.366	0.379	0.372±0.003

Antigen concentration = 20µg/ml
PAb Concentration of *U. zonata* (Mycelia) = 40µg/ml

4.9.1.1.2. Cell wall

ELISA reactions were carried out with antigens prepared from root of sixteen tea varieties against cell wall PAb of *U. zonata*. Results (Table 20) also revealed higher A405 values in case of susceptible varieties and slightly lower in case of resistant ones.

Table 20 : Indirect ELISA values (A405) of tea root antigens, cell wall antigen of*U. zonata*, non-pathogen and non host reacted with PAb of *U. zonata* (cell wall)

	Absorbance at 405nm			
Antigens	**Expt.1**	**Expt.2**	**Expt.3**	**Mean**
Tea Varieties				
TV-9	0.554	0.549	0.552	0.552± 0.001
TV-18	0.692	0.681	0.696	0.689±0.004
TV-22	0.579	0.581	0.572	0.577±0.002
TV-25	0.527	0.531	0.535	0.531±0.002
TV-26	0.515	0.520	0.511	0.515±0.002
S-449	0.601	0.592	0.605	0.599±0.003
BS/7A/76	0.519	0.522	0.525	0.522±0.001
CP-1	0.610	0.618	0.607	0.612±0.003
AV-2	0.535	0.526	0.530	0.530±0.002
P-1258	0.666	0.660	0.653	0.560±0.003
K1/1	0.523	0.519	0.528	0.523±0.002
UPASI-2	0.462	0.469	0.460	0.464±0.002
UPASI-3	0.543	0.549	0.557	0.549±0.004
UPASI-8	0.568	0.564	0.562	0.565±0.001
UPASI-9	0.512	0.509	0.511	0.511±0.000
UPASI-26	0.682	0.687	0.627	0.665±0.019
Cell wall	2.827	2.870	2.770	2.822±0.028
U. zonata				
Non-pathogens				
Beauveria bassiana	0.346	0.339	0.350	0.345±0.003
Non-host				
Leucaena leucocephala	0.356	0.370	0.377	0.368±0.006

Antigen concentration = 20µg/ml; IgG concentration of cell wall = 40µg/ml

4.9.1.2. PAb of tea root

PAb raised against healthy tea root antigen (UP-26) was tested to detect the cross reactive antigens of the root tissues with other tea varieties as well as root pathogens. The PAb of UP-26 reacted with the antigens of all other varieties and A405 values were quite high. Absorbance values were even higher where this PAb was treated with antigens of tea root pathogens. Strong reaction in ELISA reaction was noticed when PAb of the tea root reacted with pathogen antigens (Table 21).

Table 21 : Indirect ELISA values (A405) of tea root antigens, and mycelial antigens of root pathogens with PAb raised against tea (UP-26) root immunogens.

	Absorbance at 405nm			
Antigens	Expt.1	Expt.2	Expt.3	Mean
_Tea Varieties				
TV-9	0.829	0.828	0.831	0.829± 0.000
TV-18	0.820	0.819	0.809	0.816±0.003
TV-22	0.846	0.847	0.841	0.845±0.001
TV-25	0.782	0.780	0.778	0.780±0.001
TV-26	0.811	0.806	0.815	0.811±0.002
S-449	0.723	0.732	0.730	0.728±0.002
BS/7A/76	0.729	0.713	0.733	0.725±0.006
CP-1	0.760	0.769	0.777	0.769±0.004
AV-2	0.701	0.709	0.699	0.703±0.003
P-1258	1.010	1.021	1.001	1.038±0.005
K1/1	0.728	0.786	0.791	0.768±0.020
UPASI-2	0.919	0.921	0.925	0.922±0.001
UPASI-3	0.927	0.905	0.933	0.922±0.008
UPASI-8	0.821	0.825	0.819	0.822±0.001
UPASI-9	0.809	0.804	0.803	0.805±0.001
UPASI-26	1.200	1.211	1.190	1.200±0.006
Pathogen				
U. zonata	1.085	1.062	1.073	1.073±0.006

Antigen concentration 20µg/ml

PAb concentration (Mycelia)=40µg/ml

4.9.2. Western blot

Cross reactivity between *U. zonata* and tea root antigens were also determined by Western Blot analysis. It was observed that when healthy tea root extracts were probed with PAb of *U. zonata*, in the susceptible varieties 2-3 bands out of 4 bands (ca.79, 50, 21 and 19 kDA) were visible while in the others no bands or sometimes only 1 (ca.68kDa) was evident. In case of homologous reactions of mycelial and cellwall PAb showed 12bands (ranging from ca.80 to 16 kDa) and 7 (ranging from ca.68 to 18 kDa) respectively (Table 22 and Plate 8, fig. E & F) were observed.

Table 22 : Western blot analysis of mycelial and cell wall proteins of *U. zonata*.

Mycelia	12	79.4,68.9,50.1,43.0, 38.6, 29.2, 27.1, 24.5,20.0,18.4,17.4,16.3
Cell wall	7	68.9,43.0, 29.2,27.1,24.5,20.0,18.4

Western blotting using PAb of tea root (UP-26) revealed that the homologous antigens showed 15 bands ranging from 97.4 to 18.5kDa. Antigens of seven healthy tea roots (BSS-2, UP-9, T-17, TV-18, TV-26, CP-1 ant T-78) were probed with PAb to determine the cross reactivity among different tea varieties. The cross reactive antigens were found in case of BSS-2 (i.e. ca.79 and 50 kDa bands) while UP-9 showed 8 bands ranging from 97.4 to 18.5 kDa. The band patterns were more or less same in case of Tocklai varieties with appearance of 7-8 bands, most of which were of higher molecular weight and a single thick, very prominent band (ca. 70 kDa). An additional band of about 24 kDa was found in TV-18 and a band of about 97 kDa was absent in TV-26. The two Darjeeling varieties (CP-1 and T-78) tested showed 5-6 bands, of which 3 bands, (ca.97, 59 and 24kDa) were common .

4.10. Detection of *U.zonata* in infected tea root tissues

To detect the pathogen in host tissues a number of immunodetection assays have proved effective where antisera raised against the pathogen reacted with antigens of infected material to give high absorbance values. These include ELISA, Western Blot

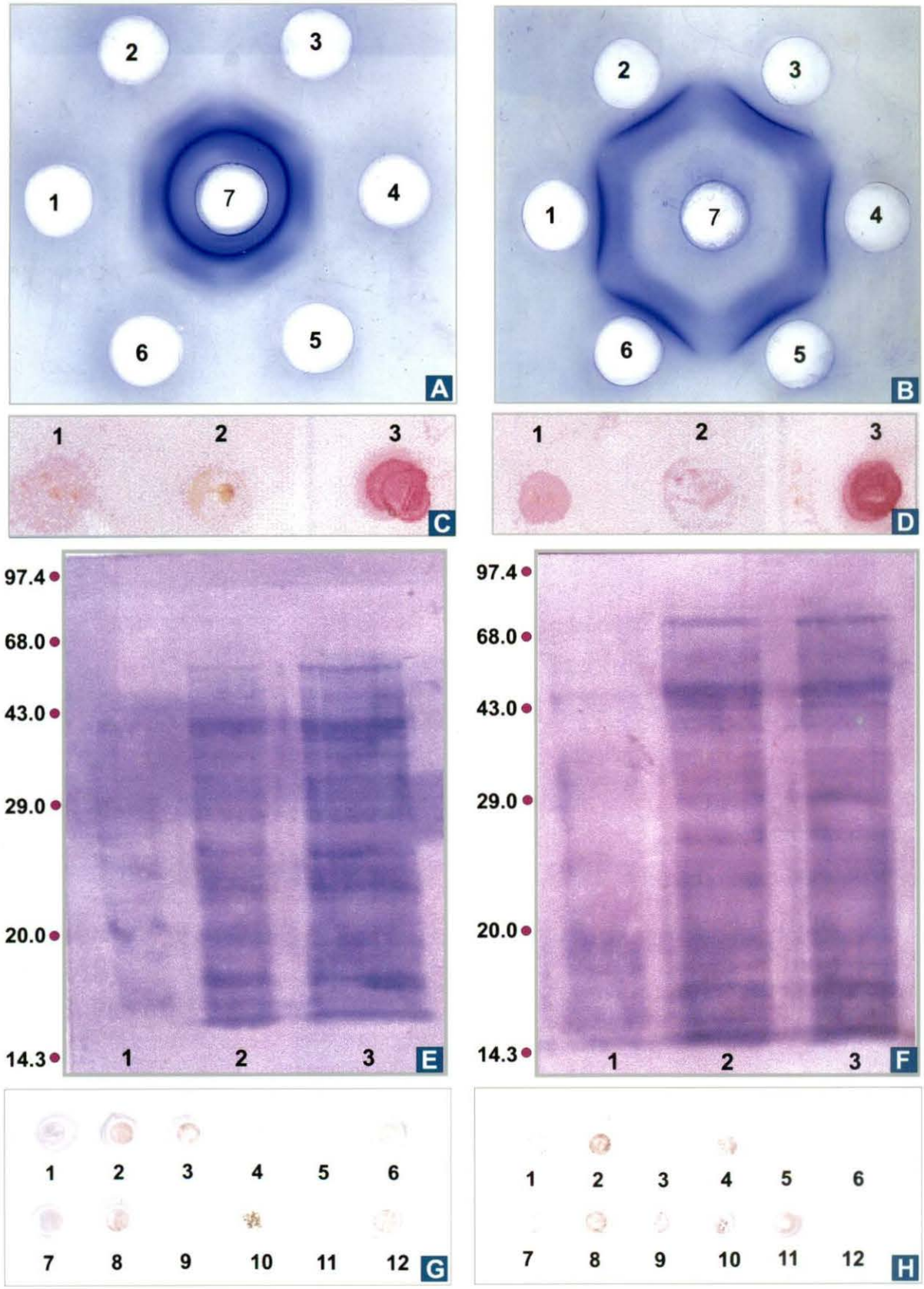


Plate 8 (Figs A-H): Serological assay of *Ustulina zonata* using(A,C,E&G) PAb of mycelia and (B,D,F&H) PAb of cell wall. (A&B) Agar gel double diffusion tests-antigens (1-6) and PAb of *U. zonata* (7). (C&D) Dot immunobinding assay-soil antigens(1&2) fungal antigen (3). (E&F) Western blot analysis- soil antigen (lane 1) fungal antigen (2&3). (G&H) Dot immunobinding assay of infested soil (1-12) collected from tea gardens.

and Dot Blot analyses. In the present study, following varietal resistance test and determination of cross reactive antigens in the different varieties attempts were made to detect *U. zonata* in infected tea root tissues by ELISA and Dot immunobinding assay.

4.10.1. ELISA

ELISA is the most routinely used detection assay as the reactivity can be measured quantitatively by difference in A405 values between healthy and infected host antigens.Two commonly used ELISA formats are PTA-ELISA and DAS-ELISA which differ in the time of PAb coating.

4.10.1.1. PTA– ELISA

4.10.1.1.1. PAb of mycelia

4.10.1.1.1.1. Artificially infected root tissues

PAb raised against mycelia of *U. zonata* was reacted in ELISA against the root antigens prepared from healthy and inoculated roots of sixteen varieties (Tocklai, UPASI and Darjeeling) of 2 year old tea plants. Antigens were prepared from healthy and inoculated root tissues at 15, 30 and 45 day intervals following the date of inoculation. Results revealed that in all tested varieties infected extracts showed higher ELISA values than the healthy extracts but the difference was not significance except in susceptible varieties. Results have been presented in Tables 23, 24 & 25. In Tocklai varieties maximum ELISA values were obtained in TV-18. Among the UPASI varieties the infected extracts and healthy extracts was obtained in UP-26 followed by UP-9. Significantly higher ELISA values in infected root extracts was obtained in the susceptible varieties (Fig 8).

Subsequently, tea root antigens from healthy and infected extracts of seven selected varieties (TV-9, TV-26, BSS-2, BS/7A/76, UPASI-2, UPASI-3 and UPASI-9) were tested with PAb of *U. zonata* mycelia, obtained from 4 bleeds . Significant differences between healthy and infected tea root antigens were obtained in all varieties and against PAb from all bleedings. Maximum A405 values were obtained in 3rd and 4th bleed (Table 26). However, it was observed that the detection could be done even with PAb of 1st bleed.

Table 23 : ELISA values showing reaction PAb of *U. zonata* with antigens of healthy and inoculated tea roots of Tocklai varieties.

	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
Tea Varieties	**Healthy**	**Infected**	**Healthy**	**Infected**	**Healthy**	**Infected**
TV-9	0.441	0.565	0.506	0.687	0.509	0.690
TV-18	0.568	0.733	0.582	0.811	0.599	0.798
TV-22	0.546	0.650	0.548	0.662	0.545	0.682
TV-25	0.462	0.571	0.473	0.592	0.491	0.673
TV-26	0.449	0.489	0.461	0.620	0.493	0.670

Age of plant 2 yr.;Antigen concentration = 20µg/ml;
PAb concentration (Mycelia) = 40µg/ml

Table 24 : ELISA values showing reaction of PAb of *U. zonata* with antigens of healthy and inoculated tea roots of UPASI varieties.

	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
Tea Varieties	**Healthy**	**Infected**	**Healthy**	**Infected**	**Healthy**	**Infected**
BSS-2	0.431	0.532	0.436	0.540	0.429	0.612
UPASI-2	0.562	0.599	0.561	0.750	0.558	0.781
UPASI-3	0.471	0.515	0.469	0.588	0.473	0.681
UPASI-8	0.440	0.511	0.453	0.559	0.480	0.576
UPASI-9	0.439	0.684	0.592	0.800	0.594	0.870
UPASI-26	0.581	0.682	0.581	0.803	0.590	0.871

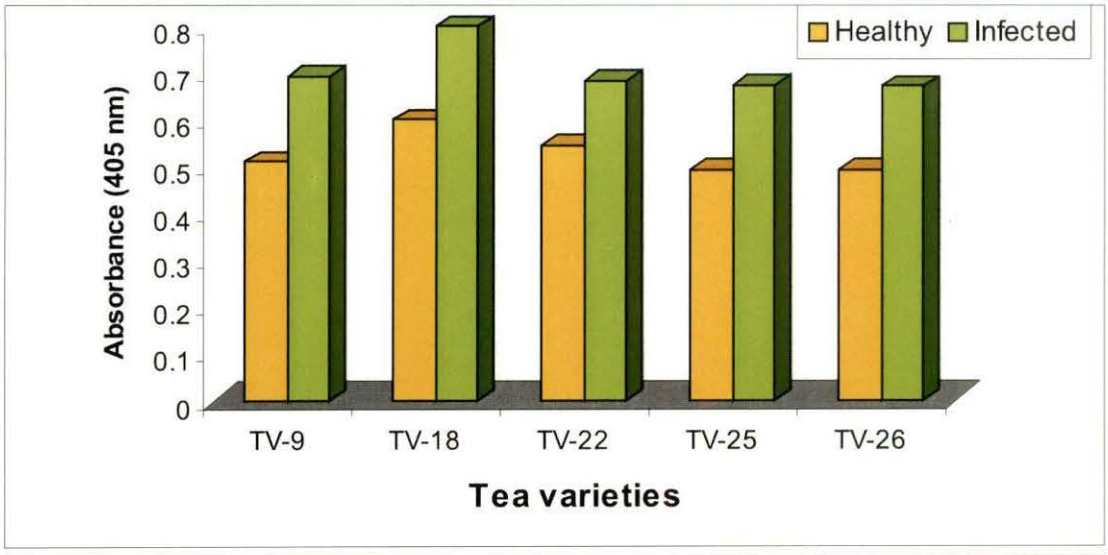
Antigen concentration 20µg/ml; PAb concentration (Mycelia)= 40µg/ml

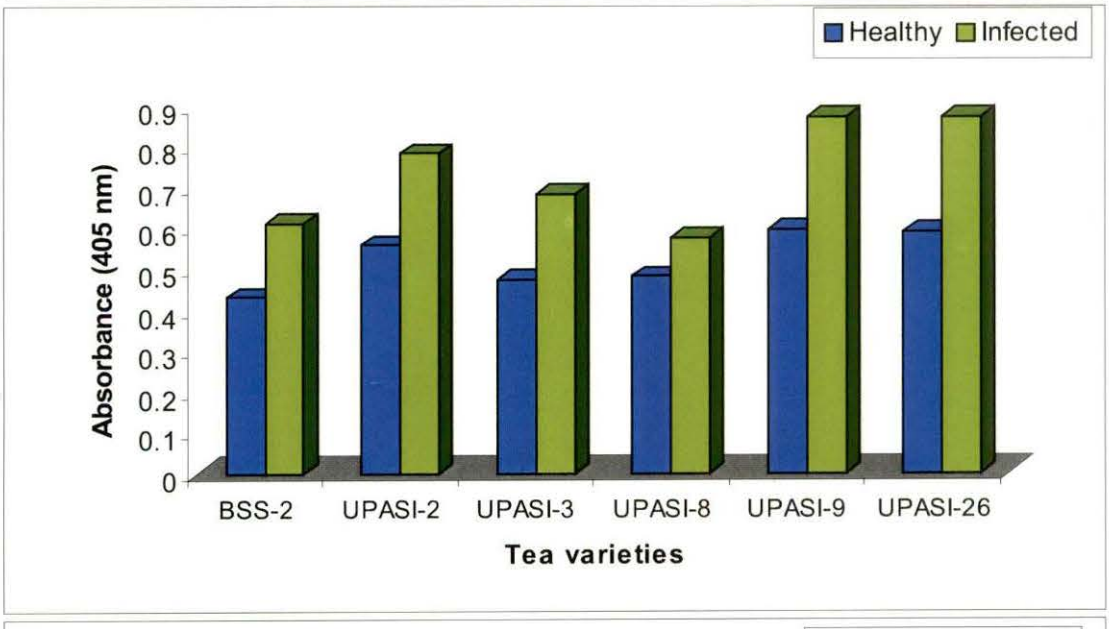
Table 25 : ELISA values showing reaction of PAb of *U. zonata* with antigens of healthy and inoculated tea roots of Darjeeling varieties.

	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
Tea Varieties	Healthy	Infected	Healthy	Infected	Healthy	Infected
BS/7A/76	0.392	0.469	0.400	0.559	0.410	0.569
CP-1	0.548	0.562	0.552	0.670	0.554	0.722
AV-2	0.440	0.483	0.453	0.571	0.463	0.623
P-1258	0.552	0.581	0.558	0.570	0.587	0.713
K1/1	0.468	0.487	0.475	0.589	0.492	0.612

Antigen concentration 20µg/ml; IgG concentration = 40µg/ml

In a further experiment, it was decided to determine ELISA responses of healthy and inoculated tea root extracts obtained from plants of ages varying from 1 to 4 years. Absorbance values of healthy and infected extracts were found to increase with the age of the plants (Table 27). In susceptible varieties the A405 of extracts from infected plants 3 to 5 years old were very high, whereas in resistant varieties, they did not increase with age (Table 27). A good correlation was therefore obtained with disease symptoms rated visually and ELISA responses. It seems probable that the entry of the mycelia in resistant varieties is rather restricted, whereas in susceptible varieties, the mycelia had spread into the tissues of the older plants.





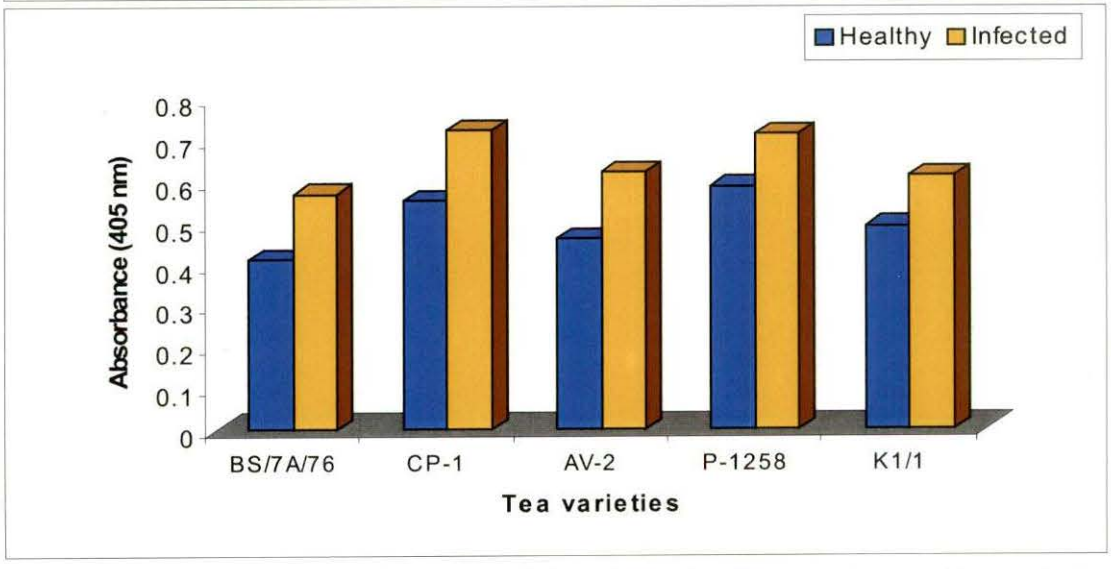


Figure 8: PTA-ELISA of healthy and artificially inoculated root antigens of tea varieties using PAb of *U. zonata*.

Table 26 : PTA-ELISA values of healthy and infected tea root antigens with PAb of *U. zonata* raised against mycelial antigen

	Absorbance at 405 nm							
	Bleeding No.							
	1st		2nd		3rd		4th	
Tea Varieties	**H**	**I**	**H**	**I**	**H**	**I**	**H**	**I**
TV-9	0.353	0.499	0.387	0.770	0.511	0.801	0.534	0.901
TV-26	0.372	0.599	0.463	0.711	0.589	0.734	0.631	0.897
BS/7A/76	0.532	0.736	0.569	0.792	0.634	0.989	0.672	1.897
BSS-2	0.469	0.660	0.475	0.675	0.482	0.892	0.521	1.439
UPASI-2	0.333	0.436	0.376	0.457	0.387	0.592	0.431	0.735
UPASI-3	0.347	0.503	0.436	0.557	0.476	0.612	0.472	0.618
UPASI-9	0.372	0.639	0.475	0.701	0.589	0.802	0.577	0.813

Antigen concentration 20µg/ml; IgG concentration = 40µg/ml

Table 27 : PTA- ELISA values of healthy and inoculated roots of a few selected tea varieties of different ages reacted with PAb of *U. zonata.*

	Absorbance at 405 nm					
	Age of the plants (years)					
	2 Yr		3 Yr		5 Yr	
Tea Varieties	**H**	**I**	**H**	**I**	**H**	**I**
BS/7A/76	0.432	0.679	0.713	0.990	0.817	1.542
BSS-2	0.469	0.660	0.570	0.871	0.938	1.835
UPASI-2	0.313	0.431	0.372	0.561	0.730	0.789
UPASI-3	0.347	0.392	0.438	0.493	0.634	0.710
UPASI-9	0.372	0.639	0.475	0.701	0.537	0.601

Antigen concentration 20µg/ml; IgG concentration = 40µg/ml

4.10.1.1.1.2. Naturally infected root tissues

It was decided to test whether natural charcoal stump rot infection could be detected with the PAb of *U. zonata* that was used to detect infection in artificially inoculated root tissues. For the purpose, infected tea roots were collected from different tea gardens showing symptoms of charcoal stump rot infection and antigens were prepared from these roots. Using antigen extracts from healthy and naturally infected tea roots PTA-ELISA was performed against mycelial PAb of *U.zonata*. Results presented in Table 28 revealed that the infected extract of Hansqua Tea Estate and Bijaynagar Tea Estate had significantly higher absorbance values in comparison to the healthy extracts.

Table 28 : PTA-ELISA values of healthy and naturally infected tea root antigens with PAb of *U. zonata*

		Absorbance at 405 nm	
Tea gardens		Healthy	Infected
Hansqua Tea Estate	Plot-1	0.582	0.793
	Plot-2	0.600	1.022
	Plot-3	0.530	0.702
Matigara Tea Estate	Plot-1	0.588	0.813
	Plot-2	0.600	0.913
	Plot-3	0.537	0.736
Trihana Tea Estate	Plot-1	0.606	0.938
	Plot-2	0.437	0.719
Bijaynagar Tea Estate	Plot-1	0.569	1.900
	Plot-2	0.598	1.002
	Plot-3	0.496	0.692

Antigen concentration =20µg/ml ; PAb concentration = 40µg/ml;

4.10.1.1.2. PAb of cell wall

In this experiment, healthy and inoculated root antigens were prepared from nine tea varieties and tested against the PAb raised against cell wall *U.zonata* by DAS-ELISA. ELISA values in general were higher in this case than those obtained using mycelial PAb. Infected root antigens gave higher reactivity than healthy ones (Table 29) with results similar as in case of mycelial PAb.

4.10.1.2. DAS-ELISA with PAb of mycelia

DAS-ELISA involves coating of ELISA plates first with the primary antibody, followed by antigens, which again is followed by secondary antibody labeled with enzyme. Detection of *U.zonata* in the infected roots of selected varieties using the DAS-ELISA format was also carried.

Table 29 : DAS-ELISA values of healthy and infected tea root antigens with PAb of *U. zonata* raised against cell wall in respect of different bleedings.

	Absorbance at 405 nm							
	Bleeding No.							
	1st		2nd		3rd		4th	
Tea Varieties	H	I	H	I	H	I	H	I
TV-9	0.430	0.489	0.382	0.779	0.516	0.841	0.54	0.910
TV-26	0.372	0.590	0.453	0.769	0.469	0.800	0.531	0.832
BS/7A/76	0.532	0.736	0.531	0.735	0.631	0.892	0.734	1.932
K 1/1	0.537	0.639	0.586	0.734	0.539	0.937	0.633	1.703
UPASI-2	0.411	0.521	0.403	0.532	0.422	0.600	0.531	0.819
UPASI-3	0.322	0.636	0.434	0.735	0.532	0.799	0.568	0.670
UPASI-9	0.431	0.536	0.451	0.707	0.492	0.790	0.613	0.745

Antigen concentration= 20µg/ml
PAb concentration =40µg/ml

Results presented in Table 29, revealed significantly higher ELISA values with infected extracts than healthy ones. In comparison to PTA- ELISA A405 values in both healthy and infected antigens were higher in this case.

Table 30 : DAS-ELISA values of healthy and infected tea root antigens tested against PAb of *U.zonata*.

	Absorbance at 405 nm	
Tea varieties	Healthy	Infected
BS/7A/76	0.870	1.201
K-1/1	0.713	1.436
UPASI-2	0.791	0.932
UPASI-3	0.643	0.790
UPASI-9	0.716	0.801

Age of plants 3year
Antigen concentration = 20µg/ml
IgG concentration = 40µg/ml

4.10.2. Dot - Blot

Root antigens of different tea varieties were reacted with mycelial and cell wall, PAbs of *U. zonata* in dot-blot as described earlier. Results (Table 31) revealed that the antigens from infected roots of BS/7A/76 and K1/1 showed pink coloured (Fast Red-substrate) dots while UP-2, UP-3 and UP-9 had very light coloured dot, indicating weak reaction. These varieties had shown resistance in previous tests including pathogenicity and ELISA. When antigens of healthy and infected tea roots of plants of different ages (1-5yr) were similarly reacted in dot-blot, it was observed that in healthy extracts of 1 yr old plants, there was no reaction, while weak reactions were observed in root extracts from 2 and 3yr. old plants. Colour intensity of dots in reactions of root antigens from 5 yr old plants were higher. In reactions with antigens from infected tea roots, positive reactions were obtained in all cases, though intensity increased with age (Table 32).

Table 31 : Dot – blot analysis of healthy and inoculated tea plants using PAb of *U.zonata*.

	Colour intensity[a]			
	Mycelial PAb (3rd bleed)		Cell wall PAb (3rd bleed)	
Tea varieties	Healthy	Infected	Healthy	Infected
BS/74/76	-	+	-	+
K-1/1	-	+	-	+
UPASI-2	-	±	-	±
UPASI-3	-	±	-	±
UPASI-9	-	±	-	±

[a] Fast red colour intensity : Pinkish red ; + + + + Bright, + + + High, + + Medium, + Low, ± Faint, - no reaction ; Age of plants 3year; IgG concentration 40µg/ml.

Table 32 : Dot-blot analysis of healthy and inoculated antigens of different ages of tea plants with PAbs of *U.zonata*.

		Colour intensity [a]			
Antigen source		Mycelial PAb (3rd bleed)		Cell wall PAb (3rd bleed)	
Tea varieties	Plant age (yr)	Healthy	Infected	Healthy	Infected
BS/74/76	2	-	+	-	+
	3	-	+ +	-	+ +
	4	±	+ +	-	+ + +
BSS-2	2	-	+	±	+
	3	-	+	-	+ +
	4	-	+ +	±	+ + +

[a] Fast red colour intensity : Pinkish red ; + + + + Bright, + + + High, + + Medium, + Low, ± Faint, - no reaction ; Age of plants 2 year; IgG concentration 40µg/ml.

Healthy and infected root antigens of selected tea varieties (5yrs old) were further tested in Dot-Blot with PAbs of *U.zonata* (mycelia and cell wall). As well as PAbs reised against root antigens of 2 tea varieties, one susceptible (UP-26) ad other resistant (TV-26). Among the 4 selected varieties 3 were most susceptible (TV-18, UP-26, T-78) and one was resistant (TV-26). Reactions with PAbs of tea roots showed dots of high intensity in general. Besides homologous reaction, PAb of TV-26 reacted very strongly with antigens from healthy roots of TV-26 & T-78, and PAb of UP-26 reacted with antigens of UP-26 & T-78 strongly, where deep violet coloured dots appeared. In cases of reactions with PAbs of roots, infected root antigens showed dots of lesser intensity then healthy ones while with PAbs of *U.zonata* (mycelia and cell wall) infected extracts showed dots of higher intensity (Table 33).

Table 33 : Comparison of dot-blot reaction of PAbs from different sources with healthy and incoculated root antigens of 5yr old tea varieties.

Tea Varieties	Plant Condition	Colour intensity [a] PAbs raised against		
		U.zonata Cell wall	BSS2	BS/7A/76
TV-18	Healthy	-	-	-
	Infected	+	±	±
BSS-2	Healthy	-	-	-
	Infected	+ +	+	+
UP-2	Healthy	±	-	-
	Infected	±	+	±
UP-9	Healthy	-	-	-
	Infected	+	±	±

[a] NBT / BCIP colour intensity : + + + Deep violet; + + violet; + light violet ; ± Faint ; - no reaction ; IgG concentration 40µg/ml.

4.10.3 Western blotting

In Western blot analysis, it was observed that when healthy root extracts were probed with PAb of *U. zonata*, in susceptible varieties 2-3 bands out of 4 bands (ca.79, 50, 21 and 19 kDa) were visible and infected root extracts from 2yr old plants. More bands (ca.101, 97, 79, 71, 61, 54, 53, 37, 32, 28, 25 & 21) were found in case of plants from 3 yr onwards.

4.11. Detection of *U. zonata* in soil

4.11.1. PTA - ELISA

In order to determine whether PAb of *U. zonata* could detect the pathogen in soil, samples were collected from various locations including several tea estates. Antigens were also prepared from amended soil infested with the propagules of *U.zonata* either in field condition or in potted condition. In this investigation, antigens were prepared from 38 samples from root rhizosphere soil and tested against the PAb of *U.zonata*. Result (Table 34) revealed only low A405 values in the range of 0.3 – 0.5 in most soil samples collected, except few samples which gave high absorbance valüe (ranging from 0.7 – 0.9) in ELISA. This indicated the presence of propagules of *U. zonata* only in these soils. In case of amended soils, high values were obtained. Both positive and negative controls were in expected ranges.

4.11.2. Dot blot

Identification of *U. zonata* propagules in artificially infested and non infested root rhizosphere soil was carried out through dot immunobinding reaction also. Soil antigens were prepared from soil samples from *U.zonata* amended soil and different locations including tea gardens and reacted with PAb of *U.zonata*. Among 38 Collected soil samples only 4 sample showed positive reactions, though dots were of low intensity ; in all other samples, either not dots could be detected or the reactions were very weak. was (Table 35).

Table 34 : ELISA responses of different soil antigens with PAb of *U. zonata* (mycelia).

Soil sample	Absorbance at 405nm	Soil sample	Absorbance at 405nm [a]
S – 1	0.489	S - 21	0.693
S - 2	0.362	S – 22	0.465
S – 3	0.552	S – 23	0.593
S – 4	0.443	S – 24	0.502
S – 5	0.456	S – 25	0.487
S – 6	0.584	S – 26	0.499
S – 7	0.792	S – 27	0.419
S – 8	0.596	S – 28	0.343
S – 9	0.498	S – 29	0.373
S – 10	0.462	S – 30	0.741
S – 11	0.479	S – 31	0.398
S – 12	0.468	S – 32	0.356
S – 13	0.472	S – 33	0.354
S – 14	0.422	S – 34	0.378
S – 15	0.856	S – 35	0.374
S – 16	0.763	S – 36	0.999
S – 17	0.560	S – 37	1.582
S – 18	0.460	S – 38	2.446
S – 19	0.532	Homologous	
S – 20	0.482	mycelia	2.567

PAb concentration= 40µg/ml.

Soil antigen – S- 1 = Control soil; S-2 Sterile soil. 3-34 = collected from different tea growing field; [Hansqua T.E. – Section B : Plot 7 (S-3), 8 (S-4), 9 (S-5); Trihana T.E. Section C : Plot 1 (S-6,7), 4 (S-8,9), 5(S-30); Cooch Behar T.E. Plot 2(S-10),3(S-11,12), 4(S-13); Bijoynagar T.E. – Section A : Plot 1 (S-14,15), 2(S-16); Section B : Plot 1 (S-17,18), 3(S-19,20), 4(S-21); Matigara T.E.-Section A: Plot 1(S-22), 2(S-23) 5 (S-24), 6 (S-25); Section D : Plot 4(S-26,27), 5(S-28,29); Chandmoni T.E. – Section A : Plot 2(S-31,32), 3(S-33,34), S-35-38 = Amended soil of *U. zonata* (60 days after amendment).

Table 35 : Dot-blot of different soil antigens with PAb of *U.zonata*.

Soil antigens	Colour intensity[a]	Soil antigens	Colour intensity[a]
S – 1	-	S - 21	±
S - 2	-	S – 22	-
S – 3	±	S – 23	±
S – 4	±	S – 24	+
S – 5	-	S – 25	-
S – 6	-	S – 26	-
S – 7	-	S – 27	±
S – 8	+	S – 28	±
S – 9	-	S – 29	-
S – 10	±	S – 30	-
S – 11	±	S – 31	-
S – 12	-	S – 32	-
S – 13	±	S – 33	±
S – 14	-	S – 34	±
S – 15	-	S – 35	-
S – 16	+	S – 36	-
S – 17	±	S – 37	+
S – 18	-	S – 38	++
S – 19	±	Homologous	
S – 20	+	mycelia	++++

[a] Fast red colour intensity : Pinkish red ; + + + + Bright, + + + High, + + Medium, + Low, ± Faint, - no colour ;

PAb concentration= 40µg/ml.

Soil antigen – S- 1 = Control soil; S-2 Sterile soil. 3-34 = collected from different tea growing field; [Hansqua T.E. – Section B : Plot 7 (S-3), 8 (S-4), 9 (S-5); Trihana T.E. Section C : Plot 1 (S-6,7), 4 (S-8,9), 5(S-30); Cooch Behar T.E. Plot 2(S-10),3(S-11,12), 4(S-13); Bijoynagar T.E. – Section A : Plot 1 (S-14,15), 2(S-16); Section B : Plot 1 (S-17,18), 3(S-19,20), 4(S-21); Matigara T.E.-Section A: Plot 1(S-22), 2(S-23) 5 (S-24), 6 (S-25); Section D : Plot 4(S-26,27), 5(S-28,29); Chandmoni T.E. – Section A : Plot 2(S-31,32), 3(S-33,34), S-35-38 = Amended soil of *U. zonata* (60 days after amendment).

4.12. Determination of cross reactivity of PAb of *U.zonata*

4.12.1. PTA- ELISA

4.12.1.1. Fungal mycelial antigens of different soil fungi

Cross reactivity of the PAb raised against *U. zonata* was tested against a number of soil fungi of which some were pathogenic to tea (*Poria hypobrumea, Ustilina zonata, Sphaerostilbe repens, Rosellinia arcuata*) and other were non-pathogenic (*Fusarium oxysporum, Metarhizium anisopliae, Beauveria bassiana, Trichoderma viride, Trichoderma. harzianum, Sclerotium rolfsii*). Antigens prepared from the mycelia of all the above were tested against PAb of *U. zonata* by PTA-ELISA. Results presented in Table 36 and Fig. 9 revealed that among all the fungi tested PAb of *U. zonata* reacted to some extent with antigens of *Rosellinia arcuata* in relation to its homologous reaction.

Table 36 : Indirect ELISA values (A405) of PAb of *U.zonata* reacted with antigens of soil fungi

Antigen Source	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Sphaerostilbe repens	0.562	0.559	0.423	0.515± 0.045
Poria hypobrumea	0.572	0.592	0.621	0.595± 0.014
Fomes lamaoensis	0.496	0.635	0.546	0.559± 0.040
Rosellinia arcuata	0.726	0.643	0.721	0.697± 0.026
Fusarium oxysporum	0.477	0.544	0.328	0.449± 0.063
Metarhizium anisoplia	0.392	0.342	0.401	0.378± 0.018
Beauveria bassiana	0.469	0.367	0.450	0.429± 0.031
Trichoderma viride	0.481	0.492	0.490	0.488± 0.003
Trichoderma harzianum	0.320	0.347	0.458	0.375± 0.0422
Sclerotium rolfsii	0.423	0.489	0.420	0.444± 0.0225
Ustilina zonata	2.523	2.420	2.726	2.556± 0.0899

Antigen concentration 100µg/ml

IgG source - U.zonata mycelial 4 bleed PAb; concentration 40µg/ml

± Standard error.

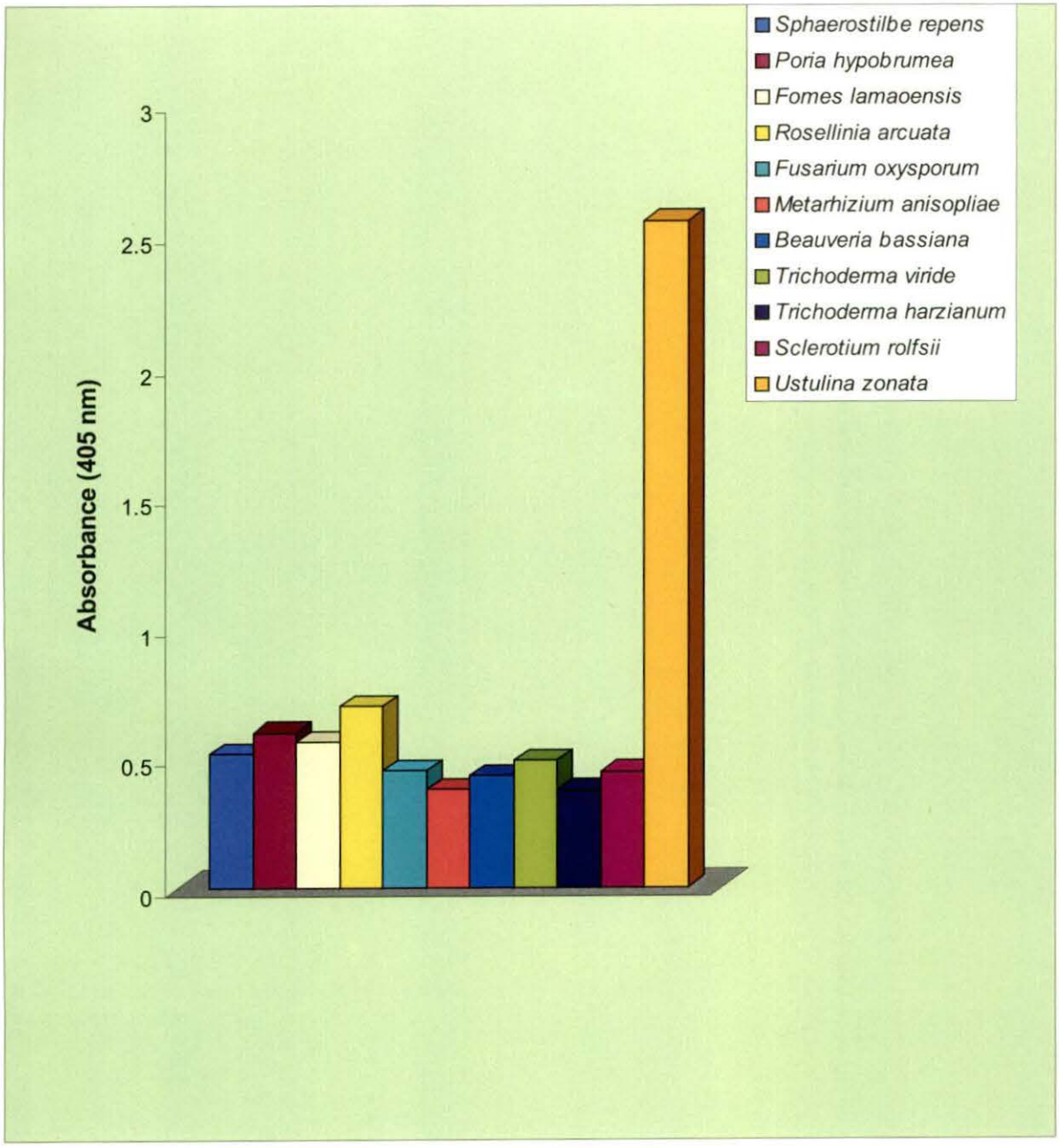


Figure 9: PTA-ELISA of mycelial antigens prepared from soil fungi using Pab of *U. zonata*.

4.12.1.2 Fungi in tea roots infected with other root pathogens

A number of pathogens can infect tea roots causing different types of root rot diseases of tea. So, it was considered worthwhile to investigate whether PAb of *U.zonata* could also react with the antigens from tea roots infected with other root pathogens. Hence, the PAb of *U.zonata* was reacted by PTA-ELISA with antigens prepared from tea roots (BSS -2, BS/7A/76, UPASI-2, UPASI 3,and UPASI-9) infected with *Spaerostilbe repens* (violet root rot). Results (Table 37) revealed that reactivity of these antigens was lesser when compared with *U.zonata* infected root antigens though here also infected root extracts showed higher ELISA values than the healthy ones. Thus a certain degree of cross reactivity was observed through this investigation.

Table 37 : PTA-ELISA values of healthy and infected (with different root pathogen) tea root antigens reacted with PAb of *U.zonata.*

Tea Varieties	Absorbance at 405 nm				
	Healthy	Infected			
		F. lamaoensis	*R.arcuata*	*S.repens*	*U.zonata*
UPASI-2	0.791	0.315	0.401	0.424	0.523
UPASI-3	0.643	0.348	0.309	0.444	0.816
UPASI-9	0.716	0.459	0.422	0.369	0.849
BSS-2	0.713	0.460	0.364	0.458	1.550
BS/7A/76	0.870	0.279	0.412	0.581	1.872
U. zonata (mycelia)					2.053

Antigen concentration = 20µg/ml
PAb concentration of *U. zonata* (mycelia) = 40µg/ml

4.12.2. Dot Blot

Cross reactivity of *U. zonata* PAb (mycelia and cell wall) with antigens of other fungi were tested by dot-blot also using antigens of other root pathogens. Antigens of *S. repens, R. arcuata* and *T. viride* showed slightly positive reaction, though the dots were of much lesser intensity than the homologous reaction (Table 38).

Table 38 : Dot – blot reaction of antigens of different soil fungi with *U. zonata* PAbs.

Antigen Source	Colour intensity[a]	
	Mycelia- PAb	Cell wall -PAb
S. repens	±	+
R. arcuata	+	+
P. hypobrumia	±	±
A. mellea	±	±
T. harzianum	±	±
T. viride	+	+
U.zonata		
Mycelia	+ + +	+ ++
Cell wall	+ ++	+ + +

Fast red colour intensity : Pinkish red ; (+ + + +) Bright, (+ + +) High, (+ +) Medium, (+) Low, (±)Faint, (-) no colour

4.13. Purification of antigen by ammonium sulphate precipitation

The crude antigens preparations were purified by ammonium sulphate precipitation, as described under materials and methods. In order to determine the fractions, which contained the antigenic proteins, immunodiffusion, PTA-ELISA and Western blot were performed with PAb raised against mycelia of *U. zonata* the results of which are given below. The precipitin reaction of fractionated proteins (0-20%, 20-40%, 40-60%,60-80% and 80 – 100% SAS) with PAb raised against mycelial extracts was tested in immundiffusion. Result (Table 39) revealed 3 separated, strong precipitin bands in 40-60% in 60 – 80% fraction, 4 bands in 100% SAS while one strong band was observed in 80 – 100 % fraction and no bands in 0 - 20% and one weak band in 20 – 40%. The reactions of the different fractions could not be differentiated by PTA-ELISA. SDS-PAGE of the proteins from different fractions were stained by coomassie blue, and another set was used for Western Blot. In SDS-PAGE, a number of bands of different molecular weights were found in the different fractions, with maximum bands in the fraction 40 – 60% SAS . In case of Western blot, however, maximum bands due to

antigen-antibody reaction was evident in 60-80% SAS. Based on the results of immunodiffusion and Western blot, 60 – 80% SAS fraction was selected as the fraction containing the maximum antigenic proteins and was used as immunogen.

Table 39 : Western Blot analysis of fractionated (SAS) mycelial proteins of *U.zonata* with PAb of *U.zonata*.

	Molecular weight (kDa)						
Antigen Source							
S.No.	**Crude**	**100% SAS**	**0-20% SAS**	**20-40% SAS**	**40-60% SAS**	**60-80% SAS**	**80-100% SAS**
1.	102.2	102.1	102.1	-	102.1	102.1	-
2.	95.2	95.2	-	-	-	95.2	95.2
3.	75.3	75.3	-	75.3	75.3	75.3	-
4.	-	59.8	-	-	-	-	-
5.	52.0	52.0	-	-	-	52.0	-
6.	45.6	45.6	-	-	45.6	45.6	45.6
7.	39.3	39.3	39.3	39.3	39.3	-	-
8.	24.5	24.5	24.5	-	24.5	24.5	24.5
9.	-	20.8	20.8	20.8	-	20.8	20.8
10.	18.2	18.2	-	-	-	-	-
11.	17.6	17.6	-	17.6	17.6	17.6	17.6
12.	16.0	16.0	-	-	16.0	16.0	-
13.	14.9	14.9	-	-	-	14.9	-
14.	-	13.7	-	-	-	13.7	-
15.	-	12.2	-	-	-	12.2	-

IgG source – 3rd bleed PAb concentration = 40µg/ml.

4.14. Evaluation of PAb raised against purified mycelial antigen

4.14.1. Immunodiffusion

The precipitin reaction was also done with PAb raised against 60-80% fractionated protein and results shows four separated, sharp bands in 60-80% and 100% SAS, two strong band in 40-60%, one in 80-100%, no bands in 0-20% and 20-40% SAS. IgG fractions were purified and experiments were done with purified IgG fraction of this PAb.

4.14.2. Cross reactive antigens

PAbs raised against 60-80% SAS fraction of mycelial antigen of *U.zonata* were also reacted with tea root antigens of all sixteen varieties tested. ELISA responses obtained were similar trends to that obtained with PAbs raised against mycelia and cell wall of the pathogen (Table 40).

4.14.3. Detection of *U. zonata* in tea root tissues

4.14.3.1. PTA- ELISA

Ability of PAb raised against 60-80% SAS fraction of *U.zonata* to detect the pathogen in root tissues was tested by PTA-ELISA, Dot-Blot and Western Blot analysis. In PTA-ELISA differences in A405 values between healthy and infected roots were highly significant in the susceptible varieties (Table 41).

4.14.3.2. Dot Blot

In dot-blot, where the root antigens were reacted with the above PAb, healthy root antigenic extracts showed only faint dots, while infected showed dots which were either light violet or violet. In no case, deep coloured dots were visible (Table 42).

4.14.3.3. Western Blot

Western Blot analysis was also carried out with PAb and root antigens. No bands were visible when healthy root extracts were reacted but four bands of ca. 23.2, 27.4, 37.6 and 40.8 kDa were visible .When compared to the reaction with PAb raised against mycelial extract, these bands were lesser in number, but these may be more specific.

Table 40 : PTA-ELISA values (A405) of tea root antigens, 60-80% SAS mycelial antigen of *U. zonata*, non-pathogen and non host reacted with anti-60-80% SAS PAb of *U.zonata.*

	Absorbance at 405 nm			
Antigens	**Expt.1**	**Expt.2**	**Expt.3**	**Mean**
Tea varieties				
TV-9	0.420	0.392	0.425	0.412± 0.010
TV-18	0.391	0.412	0.418	0.407± 0.008
TV-22	0.452	0.444	0.450	0.448± 0.002
TV-25	0.467	0.470	0.453	0.463± 0.005
TV-26	0.381	0.411	0.424	0.405± 0.012
S-449	0.756	0.882	0.869	0.835± 0.040
BS/74/76	0.943	0.890	0.949	0.927± 0.018
CP-1	0.791	0.693	0.787	0.757± 0.032
AV-2	0.821	0.820	0.825	0.822± 0.001
P-1258	0.711	0.723	0.709	0.714± 0.004
K1/1	0.801	0.793	0.805	0.799± 0.003
UPASI-2	0.550	0.559	0.553	0.554± 0.002
UPASI-3	0.573	0.579	0.600	0.584± 0.008
UPASI-8	0.582	0.573	0.585	0.580± 0.003
UPASI-9	0.566	0.549	0.554	0.556± 0.005
UPASI-26	0.590	0.587	0.580	0.586± 0.002
60-80% SAS of *U. zonata*	2.770	2.862	2.860	2.831± 0.030
Non-pathogen				
Beauveria bassiana	0.344	0.337	0.351	0.344± 0.004
Non-host				
Leucaena leucocephala	0.356	0.378	0.376	0.370± 0.007

Antigen concentration = 20µg/ml; PAb concentration= 40µg/ml

± Standard error.

Table 41: PTA-ELISA values of healthy and inoculated tea root antigens tea varieties with anti-60-80% mycelial SAS PAb of *U. zonata*

Root antigens		**Absorbance at 405nm**			
Tea varieties	**Condition**	**Expt.1**	**Expt.2**	**Expt.3**	**Mean**
K-1/1	Healthy	0.719	0.815	0.810	0.781± 0.031
	Infected	1.539	1.630	1.535	1.568± 0.031
BS/7A/76	Healthy	0.964	0.955	0.960	0.960± 0.002
	Infected	1.119	1.303	1.124	1.182± 0.060
UP-2	Healthy	0.709	0.721	0.716	0.715± 0.003
	Infected	0.915	0.922	0.932	0.923± 0.004
UP-3	Healthy	0.648	0.642	0.640	0.643± 0.002
	Infected	0.793	0.786	0.790	0.789± 0.002

Antigen concentration 20µg/ml; PAb concentration of *U. zonata* (mycelia) = 40µg/ml
± Standard error.

Table 42 : Comparison of dot-blot reaction of PAbs from mycelial 100% and 60-80% SAS of *U. zonata* with healthy and inoculated root antigens tea varieties.

		Colour intensity [a]	
		PAbs raised against	
Tea Varieties	**Plant Condition**	**100% SAS**	**60 – 80% SAS**
BS/7A/76	Healthy	-	-
	Infected	+ +	+
K-1/1	Healthy	-	+
	Infected	+ +	+
UP-2	Healthy	±	-
	Infected	±	±
UP-3	Healthy	-	-
	Infected	+	±

[a] NBT / BCIP colour intensity : + + + Deep violet; + + violet; + light violet ; ± Faint ; - no reaction ; PAb concentration = 40µg/ml.

4.15. Immunofluoresence

Fluorescent antibody labeling with fluorescein isothiocyanide (FITC) is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens (CRA) shared by host and parasite as well as for detection of pathogen in plant tissue. Present study reports the use of indirect immunofluorescent test using polyclonal antibodies to determine the tissue and cellular location of the pathogen in root tissue of tea varieties, tea rhizosphere soil as well as mycelia of *U. zonata*. Mycelia, cross section of root tissue as well as soil preparations were photographed under UV- fluoresecence and the intensity of bright apple-green fluorescence indicated the positive reaction.

4.15.1. Mycelia

Pre-immune sera did not show reactivity with the mycelia of *U.zonata* followed by FITC and mycelia was not auto-fluorescent. Examination of mycelia treated with homologous PAbs of mycelia, cell wall and 60-80% fractionated mycelial protein and stained indirectly with FITC indicated strong fluorescence throughout the mycelia, specially in young hyphal tips (Plate 9 Fig. E).

4.15.2. Root tissue

Cross section of tea roots were treated separately with normal and PAb of *U. zonata* and then reacted with FITC. Root sections exhibited a natural autofluoresence under UV-light. When the cross section of infected tea root tissue of susceptible varieties incubated with PAb of *U. zonata* and stained with FITC, strong fluorescence was observed in the infected root tissues. Present of fungal mycelium was evident with strong fluorescence in infected root tissue. Fluorescence was evident throughout the sections, extending upto the vascular tissues as well as outer surface (Plate 9 Figs.A-E). Healthy sections exhibited no fluorescence.

4.15.3. Soil sample

Amended soil preparation was done for immunoflurescece study as described under materials and methods. Microscopic observation under UV-fluorescence revealed that presence of strongly fluorescing mycelia. Thus immunofluorescence could be used to detect the pathogen in soil.

4.16. Immunocytochemical staining

Another approach adopted to study the interaction of *U. zonata* with root tissue was by direct observation, immunocytochemical staining, based on specific antibodies produced anainst *U. zonata*, provided a means of visualizing hyphae within root tissues of infected tea plants. Production of a specific immunocytochemical stain involves preparation of suitable antigen, appropriate methods for evaluating specificity of the antibodies, and development of the immynocytochemical staining procedure. In the present study three susceptible tea varieties were artificially inoculated with *U. zonata* in the experimental field. After 40 days of inoculation the affected plants were uprooted and washed properly. Cross-sections were made from the infected tea roots and fungal hyphae which penetrated the root tissues were probed with PAb raised against mycelial antigens of *U. zonata*. In root sections stained immunocytochemically, hyphae of *U. zonata* were growing along the epidermis and hyphal penetration throughout root tissues was evident (Plate 9 , Figs.F&G). Hyphae, which were growing horizontally in the cortical regions could be distinguished as strands, whereas those which were growing vertically either inter or intracellularly appeared as blue coloured masses (cross section of hyphae). Deep blue coloured thick layer was found on outer surface of root which was an evidence of deep brown and blackish sheet on root surface formed by fungal mycelia. This staining clearly showed the penetration of hyphae throughout the tissue.

4.17. Biological control of Charcoal stump rot disease with *Trichoderma harzianum and Trichoderma viride*

The present work was aimed at developing a management strategy to control Charcoal stump rot of tea by biological means. Antibiosis to *U. zonata* by biocontrol agents [*Trichoderma harziamum* and *T.viride*] were evaluated *in vitro* and *in vivo* condition.

4.17.1. *In vitro* test

Both the biocontrol agents tested *in vitro* were effective in causing significant suppression of growth of *U. zonata* (Plate 10). After 3-4 days of incubation *T. harzianum* and *T. viride* over grew the pathogen. But in control plate pathogen grew characteristically on PDA.

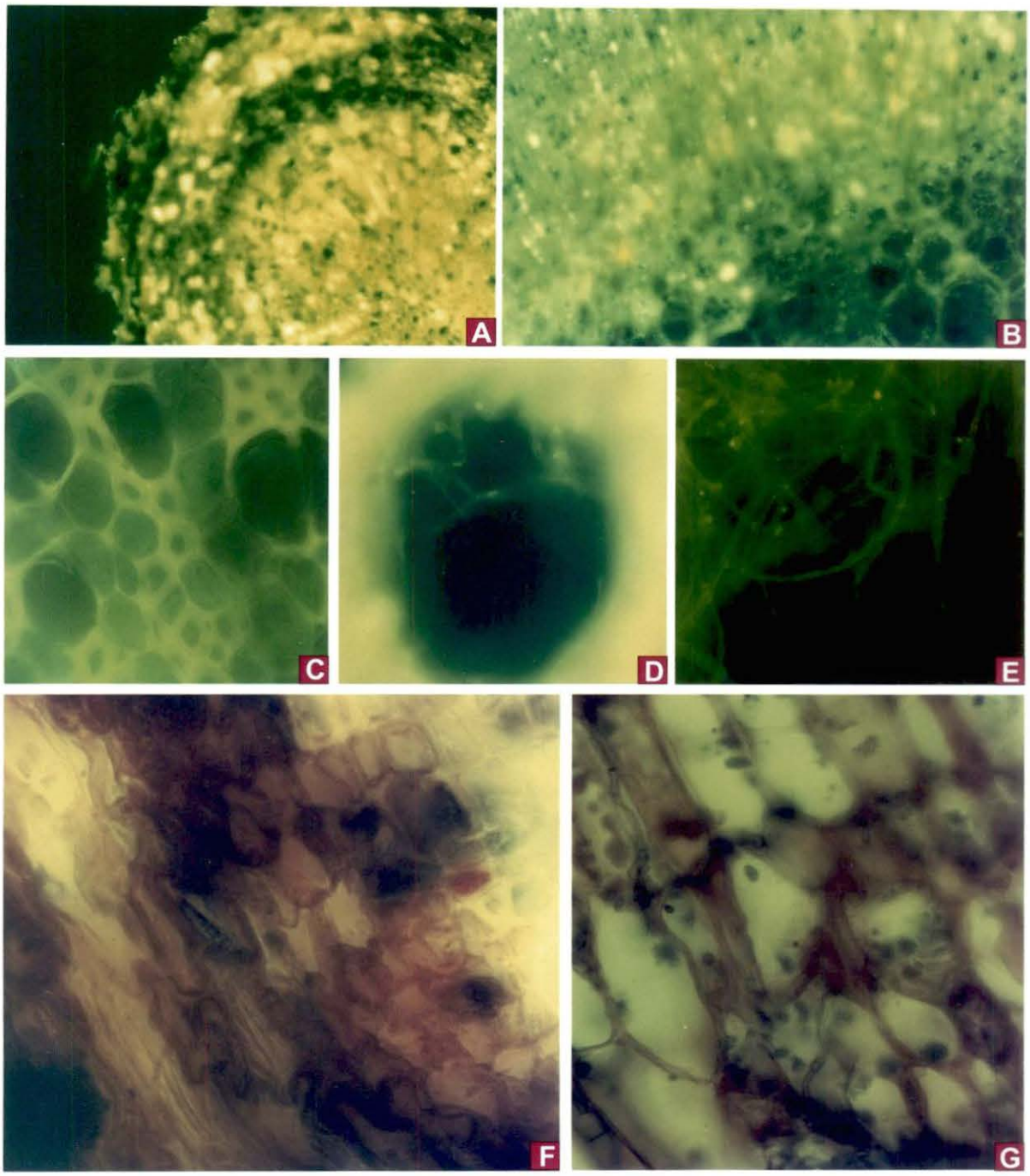


Plate 9 (Figs. A-G): Cross section of *Ustulina zonata* inoculated tea roots treated with PAb of *U. zonata* and labelled with FITC (A-D); stained with fast blue BB salt (F&G) and mycelia of *U. zonata* treated with homologous PAb and labelled with FITC (E).

4.17.2. *In vivo* test

To manage the Charcoal stump rot disease using biocontrol agents in potted and field conditions above two Trichoderma species were used. The experimental set up was carried out with following treatments : (a) pathogen (*U. zonata*), (b) *T. harzianum*, (c) *T.viride*, (d) *U. zonata* and *T. harzianum*, (e) *U.zonata* and *T.viride* and (f) control plants i.e. healthy. Inocula were prepared in different media for mass multiplication as described in materials and methods. Among them the most useful inculum was sand-maize meal and tea root for pathogen and tea waste/wheat bran medium for biocontrol agents. Biocontrol agents were infested in the rhizosphere 7 days before inoculation with pathogen.

4.17.2.1. Potted plants

Three years old tea plants (BSS-2, BS/7A/76) grown in potted condition were used for this purpose. Ten replicates of each treatment were taken and disease ratings was as described in materials and methods. Results revealed that treatment with either *T.harzianum* or *T.viride* reduced disease significantly (Table 43) and population of *U. zonata* also decreased significantly in rhizosphere soil.

Table 43 : Effect of *T. harzianum* and *T. viride* on Charcoal stump rot disease in potted condition.

	Disease Index [a]	
	Tea varieties [b]	
Treatments	**BSS-2**	**BS/7A/76**
U.zonata	3.58	3.87
U.zonata + *T.harzianum*	0.92	0.99
U.zonata + *T.viride*	0.87	0.76

No disease was observed in uninoculated control, or those inoculated with either *T.harzianum* or *T.viride* alone.

[a] 0 = No symptoms;
1 = Small roots turn brownish and start to rot;
2 = Leaves start withering and 20-40% of root turn brown;

3 = Leaves withered with 50% of roots affected ;
4 = Shoot tips start withering 60-70% of roots affected;
5 = Shoots withered with defoliation of lower withered leaves, 80% roots affected;
6 = Whole plants die, with upper withered leaves still remaining attached; roots fully Rooted.

[b] Age of plants 3 yr
Average of 10 separate inoculated plants
40 days after inoculation

4.17.2.2. Field grown plants

For field experiments 4 selected varieties of 5 year old plants were taken and among them 2 varieties were susceptible (BSS-2 and BS/7A/76) and one was resistant (UP-2) as proved in earlier pathogenicity test as well as different immunoenzymatic reactions. Different treatment with bio-control agents were done and disease incidences were recorded as described in earlier experiments. In this experiment also both *T. harzianum* and *T.viride* significantly reduced the disease intensity (Table 44, Fig. 9). In case of treatment with *U.zonata* alone all the plants BSS-2 and BS/7A/76 varieties died after 40 days of inoculation.

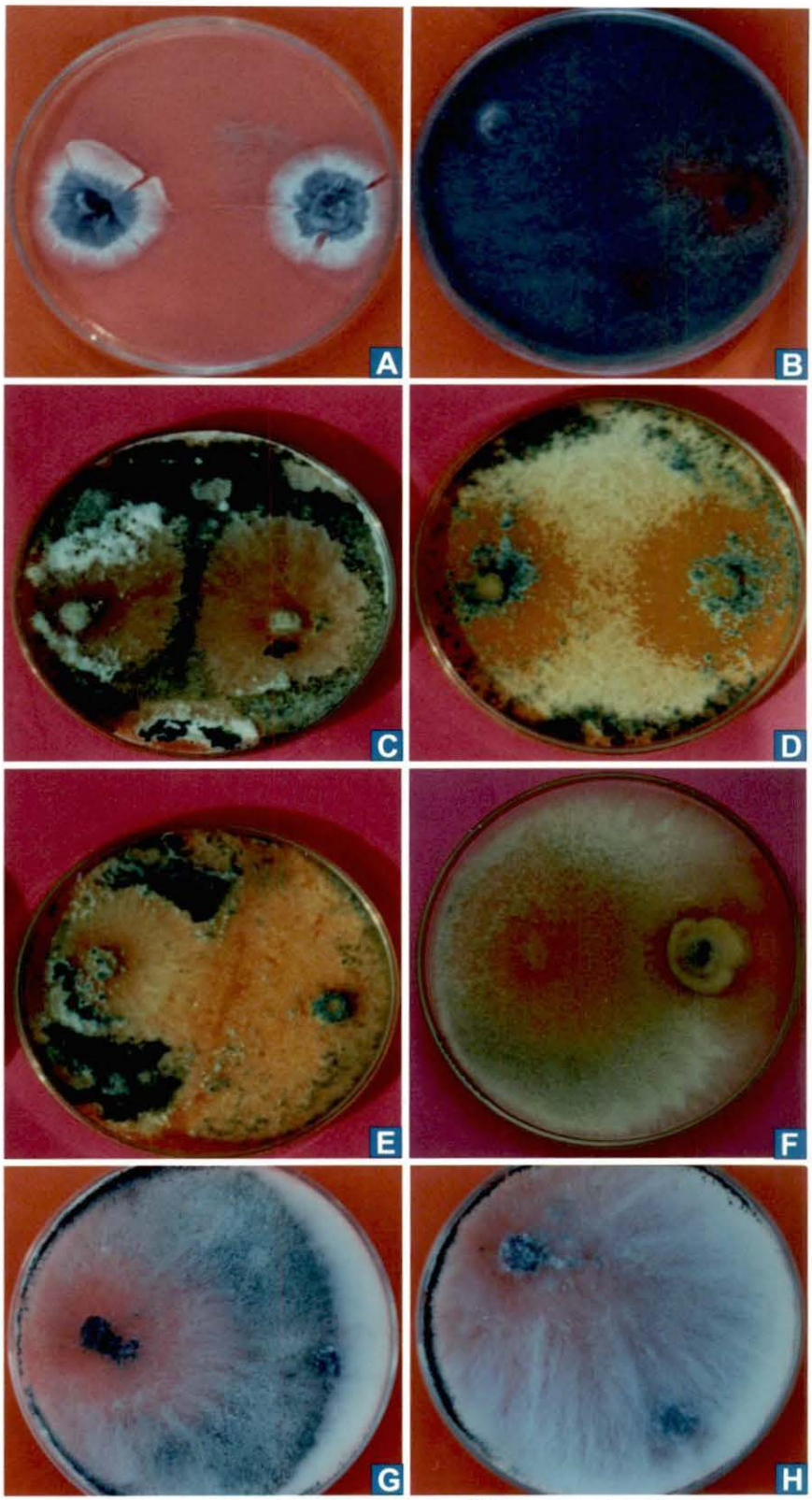


Plate 10 (Figs. A-H): *In vitro* pairing of *Ustulina zonata* with *Trichoderma harzianum* and *Trichoderma viride*. Homologous pairing of *U.zonata* (A&B); *T. harzianum* (C) *T. viride* (D). Pairing of *T. harzianum* with *T. viride* (E), *U. zonata* with *T. viride* (F) and *U. zonata* with *T. harzianum* (G&H).

Table 44 : Effect *of T. harzianum* and *T. viride* on development of Charcoal stump rot disease in field condition.

		Disease Index [a]		
		Days after inoculation		
Tea varieties [b]	**Treatments**	**10**	**20**	**30**
BS/7A/76	*U.z.*	2.78	3.62	3.95
	U.z.+ T.h	0.43	0.38	0.27
	U.z. + T.v	0.49	0.41	0.33
BSS-2	*U.z*	2.62	3.12	4.20
	U.z.+ T.h	0.45	0.32	0.25
	U.z. + T.v	0.52	0.41	0.29
UP-2	U.z.	0.72	0.81	0.99
	U.z.+ T.h	0.29	0.22	0.19
	U.z. + T.v	0.36	0.31	0.30
UP-3	U.z.	0.92	1.09	1.22
	U.z.+ T.h	0.33	0.27	0.18
	U.z. + T.v	0.47	0.38	0.32

No disease was observed in uninoculated control, or those inoculated with either *T.harz ianum* of *T. viride* alone.

[A] 0 = No symptoms;
1 = Small roots turn brownish and start to rot;
2 = Leaves start withering and 20-40% of root turn brown;
3 = Leaves withered with 50% of roots affected ;
4 = Shoot tips start withering 60-70% of roots affected;
5 = Shoots withered with defoliation of lower withered leaves, 80% roots affected;
6 = Whole plants die, with upper withered leaves still remaining attached; roots fully Rotted.

[b] Age of plants 3 yr. Average of 10 separate inoculated plants

U.z. = U. zonata,T.h.= T. harzianum, T.v. = T. viride

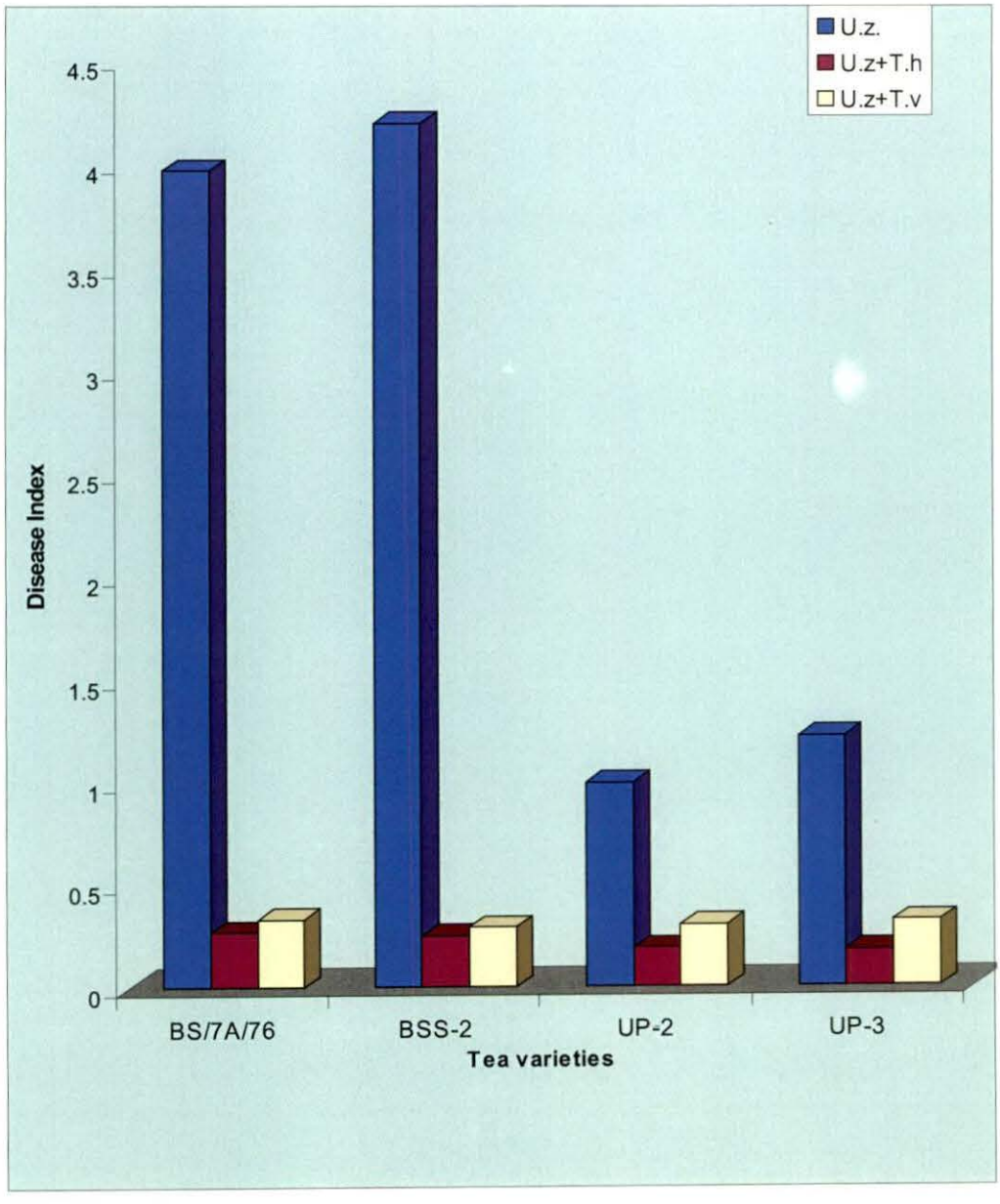


Figure 10: Charcoal stump rot disease development following application of biological control agents.

4.18. Detection of *U. zonata* in tea root and soil following treatment with biocontrol agents.

4.18.1. PTA-ELISA

Since the application of biocontrol agents in rhizosphere soil reduced intensity of charcoal stump rot disease, it was decided to investigate whether this reduction could also be determined immunologically in both root tissues and soil. For this purpose PTA-ELISA as well as competition ELISA were carried out. ELISA reactions were performed with root antigens from different treatments as well as soil antigens.

4.18.1.1. Root tissues

Root antigens were prepared from uprooted plants (2yr old) of different treatment after 40 days of pathogen inoculation for biocontrol experiment of potted plants. These antigens were reacted in PTA-ELISA using PAb of *U. zonata*. Results showed that ELISA values of roots treated with *T. harizianum* and *T. viride* were significantly lesser than with *U.zonata* alone (Table 45). In case of 5yr old field grown plants also similar results were observed (Table 46), though A405 values were higher than the potted plants.

Table 45 : ELISA of reactions of PAb *U.zonata* with root antigens of tea varieties following treatments with Biocontrol agents.

	Absorbance at 405nm	
Antigen Source	**BSS-2**	**BS/7A/76**
Healthy Plant	0.592	0.572
Treatments		
U.zonata + T.harzianum	0.378	0.266
U.zonata + T.viride	0.312	0.343
U.zonata	1.618	1.554
T. harzianum	0.216	0.230
T.viride	0.292	0.201

Antigen concentration 20µg/ml
PAb concentration of *U. zonata* (mycelia) = 40µg/ml

Table 46 : ELISA values of reaction of PAbs with root antigens of tea varieties following treatment with biocontrol agents.

Antigen Source	Absorbance at 405nm					
	U. zonata		*T. viride*		*T. harzianum*	
	K-1/1	BS/74A/76	K-1/1	BS/74A/76	K-1/1	BS/74A/76
Healthy Plant	0571	0.582	0.349	0.368	0.378	0.369
Treatments						
U. zonata + *T. viride*	0.483	0.400	0.388	0.367	0.584	0.673
U. zonata + *T. harzianum*	0.550	0.572	0.627	0.731	0.785	0.881
U. zonata	1.543	1.538	0.410	0.428	0.520	0.529
T. viride	0.574	0.591	1.333	1.322	0.996	0.987
T. harzianum	0.423	0.419	0.327	0.321	1.237	1.226

Antigen concentration = 20µg/ml
PAb of *U. zonata* (mycelia) concentration = 40µg/ml

4.18.1.2. Soil

Soil samples of the rhizosphere of different treatments were collected at a depth of 7-9 inches from soil surface. *U. zonata* was evaluated through PTA- ELISA and competition ELISA by reacting the antigens from collected soils after 30 days of Control set was prepared from uninfested soil of control plants.

In PTA-ELISA results from soil treated with *U. zonata* and *T. harzinum* or *U. zonata* and *T. viride* reacted with PAb of *U. zonata* showed significantly lower absorbance values than that of soil antigen treated with *U. zonata* alone. This indicated that population of *U. zonata* soil had been reduced by the biocontrol agents (Table 47). When soil samples treated either with *T. harzianum* or *T. viride* were reacted with their homologous antisera, absorbance values were comparable with the soil samples treated with biocontrol agent(s) and inoculated with pathogen.

Table 47 : ELISA values of soil antigens of different treatments with PAbs of *U. zonata*, *T. harzianum* and *T. viride*.

	Source of PAbs		
Antigen Source	***U. zonata***	***T. viride***	***T. harzianum***
Unifested soil	0.301	0.253	0.212
Treatments			
U.zonata + *T.harzianum*	0.480	0.807	0.806
U.zonata + *T.viride*	0.486	0.723	0.747
U.zonata	1.282	0.335	0.432
T. harzianum	0.423	0.809	1.402
T. viride	0.367	1.328	0.873

Soil samples collected 30 days after pathogen inoculation;

The same trend of result (Table 48) was also obtained in case of competition ELISA which is an inhibition ELISA. Reduction of population in soil treated with *T.harzianum / T.viride* and *G.viriens* was confirmed using this ELISA format and detailed procedure has been outlined in materials and methods. Antigens were prepared from soils under various treatments as mentioned earlier and were used under doubling dilutions of from 1:25 to 1:400. Since competition ELISA is a double binding assay, where PAb is allowed to react to the test antigen first and the residual PAb is once again reacted with homologous antigen in separate plates, higher ELISA values in this procedure would indicate lower reactivity to a test sample.

Table 48 : Competition ELISA of various dilution of treated soil antigens with PAbs of *U. zonata*, *T. viride* and *G. virens*.

Soil antigen		Absorbance at 405nm		
		Source of PAbs		
Treatment	**Dilution**	***U. zonata***	***T. viride***	***T.harzianum***
U. zonata	1:25	0.502	1.040	1.186
	1:50	0.719	1.241	1.356
	1:100	0.754	1.298	1.445
	1:200	0.853	1.388	1.536
	1:400	0.947	1.423	1.618
U. zonata +	1:25	1.652	0.465	1.283
T. harzianum	1:50	1.693	0.718	1.475
	1:100	1.758	0.842	1.504
	1:200	1.847	0.880	1.685
	1:400	1.930	0.986	1.694
U. zonata +	1:25	1.787	0.850	0.637
T. viride	1:50	1.843	1.022	0.830
	1:100	1.880	1.051	0.942
	1:200	1.921	1.148	1.016
	1:400	1.973	1.253	1.044
T. harzianum	1:25	1.623	0.438	0.936
	1:50	1.787	0.693	1.332
	1:100	1.800	0.758	1.389
	1:200	1.875	0.813	1.416
	1:400	1.967	0.930	1.472
T. viride	1:25	1.683	0.807	0.411
	1:50	1.738	0.921	0.710
	1:100	1.778	0.979	0.872
	1:200	1.902	1.091	0.901
	1:400	1.933	1.276	1.082
Control	1:25	1.875	1.443	1.485
	1:50	1.923	1.520	1.551
	1:100	1.967	1.542	1.673
	1:200	2.083	1.545	1.725
	1:400	2.129	1.588	1.750

Sample collected 30 days after pathogen inoculation;
IgG concentration = 40µg/ml

4.18.2. Dot - Blot

Results presented in Table 49 and Plate 11 revealed that when PAb of *U. zonata* (mycelia and cell wall) was treated in the antigens from soil subjected to different treatments (described earlier), positive reaction was obtained in case of treatment with *U. zonata* alone. Similarly when these were treated with PAbs of *T. harzianum* or *T.viride* positive reactions were shown in cases where soil was amended with the particular fungus. In cases where soil was treated with both *U.zonata* and *T. harzianum /T.viride* also, positive reaction were not evident.

Table 49 : Dot-blot of soil antigens of different treatment (with combinations of *Trichoderma* sp and *U. zonata*) collected from root rhizosphere of field.

	Colour intensity [a]			
	PAbs raised against			
Antigen Source	***U. zonata*** **Mycelia**	***U. zonata*** **Cell wall**	***T.harzianum*** **Mycelia**	***T.viride*** **Mycelia**
Sterile Soil	-	-	-	-
Control Soil	-	-	-	-
Soil treated with				
U. zonata	+ +	+ +	-	-
T.harzianum	-	±	+	+
T. viride	-	-	+	+
U. zonata +	-	-	+	-
T.harzianum	-	±	+	+
U. zonata +	-	-	+	±
T. viride				
Mycelia				
U. zonata	+ + + +	+ + +	-	-
T. viride	+	+	+ + +	+ + + + +
T.harzianum	±	+	+ + + + +	+ + +

[a] Fast Red colour intensity (Pinkish red) : (+ + + +) Bright; (+ + +) High; (+ +) Medium; (+) Low, (±) Faint ; (-) no colour.

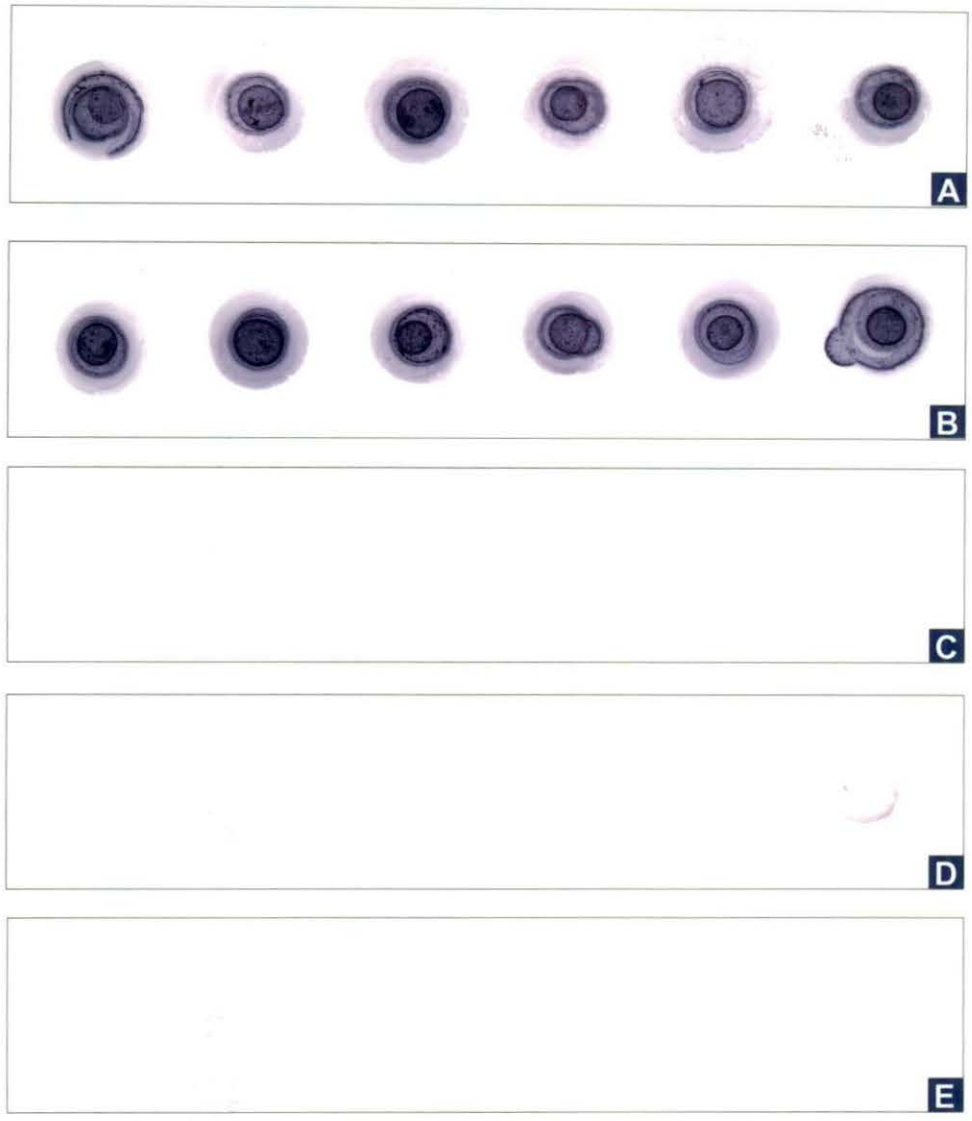


Plate 11 (Figs. A-E): Dot immunobinding assay of rhizosphere soil of tea plants following treatment with *Trichoderma harzianum* , *Trichoderma viride* and after inoculation with *U. zonata* and probed with PAb of *U. zonata.* (A) Mycelial antigen of *U. zonata*, (B) Plants inoculated with *U. zonata* (C&D) Treated with *T. harzianum* and inoculated with pathogen (E) Treated with *T. viride* and inoculated with pathogen.

4.18.3. Western Blot

Western Blot analysis of soil samples from different treatments set up using PAb of *U. zonata* revealed four bands with molecular weight of ca. 79,64,55 and 31 kDa. These four bands were also present in homologous reaction, with mycelial antigen though 18-19 bands were found in case of homologous reaction.

In case of soil from rhizosphere of plants inoculated with the biocontrol agents prior to inoculation with the pathogen, no bands were visible (Table 50). When these soil antigens were treated with PAb of *T. viride*, 4 bands of ca. mol. wt. 93, 50, 41 & 39 kDa were obtained with soil inoculated with *T. viride*, 3 bands (ca.93, 50 & 39 kDa mol. Wt.) with *U. zonata* + *T. viride* and 2 bands with *U. zonata* + *T. harzianum.*

Table 50 : Western blot analysis of different soil antigens from root rhizosphere infested with different combination of *Trichoderma* sp. and *U. zonata*

Antigen source	**IgG No.**			
	U. zonata		***T. viride***	
	No. of bands	**Mol.wt.kDa**	**No. of bands**	**Mol.wt.kDa**
Mycelia				
U. zonata	17	104.3, 97.4, 79.0, 60.9, 50.1, 43.0, 38.6, 32.2, 27.1, 24.5, 22.2, 19.0, 18.4, 17.4, 15.3, 14.3, 12.7,		NR
T.viride	NR		10	92.8, 83.6, 72.6, 68.0, 54.5, 50.0, 43.0, 40.8, 38.6, 29.8.
Soil treated with				
(a) *U.zonata*	4	79.0, 63.5, 54.5, 31.2		
(b) *T.harzianum*	Nil		Nil	
(c) *T.viride*	Nil		4	92.8, 50.0, 4.8 38.6
(d) *U.zonata* + *T. harzianum*	Nil		2	63.5, 54.5
(e) *U.zonata* + *T. viride*	Nil		3	92.8, 50.0, 38.6
(f) Uninfested soil	Nil		Nil	

IgG concentration = 40µg/ml.

4.19. Management of Charcoal stump rot disease using organic additives

In addition to biocontrol agents attempts were also made to develop effective integrated management practices for charcoal stump rot disease of tea using organic additives.

4.19.1. Growth promotion in tea plants

Two varieties of tea plants (BS/7A/76 and K 1/1) were grown in soil amended with neem cake and oil cake separately. Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded after one-month interval and up to two months following the treatment of neem cake and oil cake and after inoculation with *U. zonata*. Results (Table 51) revealed that the growth of tea plants increased following amendment with neem and oil cakes than those plants inoculated with *U.zonata* in relation to untreated uninoculated control as recorded after two months following treatment. It has been observed that the percentage increase in shoot length with neem cake and oil cake in treated inoculated with *U. zonata* tea plants was more than the treated uninoculated one (Table 52).

Similarly three tea varieties (UP-3 BS/7A/76 and K-1/1) were grown in soil amended separately with cowdung, rabbit manure and chicken manure. Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded after one month interval up to two months following the treatment of organic components and after inoculation with *U. zonata*. It has been observed that the growth of tea seedlings had been increased in treated uninoculated than treated inoculated tea seedlings (Table 53). Among the three treatments with organic components, rabbit manure gave very good and healthy growth the tea seedlings than chicken manure and cowdung.

Table 51 : Growth promotion in tea seedlings following soil amendment with neem cake oil cake

Tea Variety	One month				Two month			
	Healthy		Infected		Healthy		Infected	
	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves
BS/7A/76 Untreated	3	5	0	2	5	7	7.5	2
Treated Neem Cake	3	4	1	0	1.5	7	2	5
Oil cake	1	3	1.5	2	2	6	1.5	3
K 1/1 Untreated	1	2	1	0	3	1	1	3
Treated Neem Cake	2	3	2	0	1	2	0.5	2
Oil cake	1	4	1.5	0	2	3	1	1

Table 52 : Percentage increase in shoot length in tea seedlings following treatment with neem cake and oil cake

Tea Variety	Percentage increase in shoot length after two months treatment	
	Healthy	Infected
BS/7A/76 Untreated	5.5	2.3
Treated Neem cake	7.8	1.2
Oil cake	6.5	5.3
K 1/1 Untreated	8.1	4.8
Treated Neem cake	9	7
Oil cake	9.5	5.6

Table 53 : Growth promotion in tea seedlings by different organic components after inoculation with *U.zonata*

Tea Variety	One month				Two month			
	Healthy		Infected		Healthy		Infected	
	Increase in height (cm)	Increas No. Of leaves	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves
UP-3								
Untreated	2	0	1	0	3	2	1.5	1
Treated Cowdung	5	2	3	1	5	2	1.5	1
Rabbit Manure	9	0	6	1	5	1	4	0
Chicken Manure	4	3	2	1	3	1	2	0
BS/7A/76								
Untreated	1	0	0	0	1	2	1	0
Treated Cowdung	4	2	2	1	4	3	1.5	1
Rabbit Manure	7	4	5	1	5	3	2	2
Chicken Manure	4	0	2	1	4	3	2	1
K-1/1								
Untreated	2	0	1	0	2	1	0	0
Treated Cowdung	3	2	1	1	2.5	3	1	2
Rabbit Manure	8	5	5	2	8	2	4	3
Chicken Manure	6	3	4	0	3	3	2	1

4.19.2. Disease development

Under pot culture conditions *T. harzianum* alone and in combination with neem cake and oil cake provided best effective management practices of charcoal stump rot in

all the three modes of application viz., simultaneous, repeated and pot infection. Combination with neem cake and oil cake showed 66.2% disease incidence where as in oil cake, neem cake and *Trichoderma harzianum* in combination disease incidence were recorded 15.5% . But in combination with cowdung, neem cake, chicken manure and rabbit manure, results were insignificant as shown in (Table 54).

Table 54 : Effect of simultaneous treatments with biocontrol, fungicide, organic amendments and plant extract on development of seedling blight of tea following inoculation with *U.zonata*.

Treatment	Disease incidence (%	Disease control (%
Trichoderma harzianum	10.0	90.0
Oil Cake with Neem cake	66.2	33.8
T. harzianum, oil cake and neem cake	15.5	84.5
Cowdung, Neem cake and Oil cake	40.0	60.0
Chicken manure, Neem cake and Oil cake	44.5	54.5
Rabbit manure, Neem cake and Oil cake	42.4	55.6
Untreated Control	100	0

4.20. Biochemical changes associated with reduction of charcoal stump rot disease in tea plants

As polyphenols are the major constituents of tea plants, their role in the resistance mechanism was investigated. Changes in the levels of phenolic substances (total phenols and ortho-dihydroxy phenols) were determined in the untreated and treated varieties (K1/1 and BS/7A/76) after inoculation with pathogen (*U. zonata*). Results have been presented in Table 55 and Fig.11. It revealed that total phenol content increased in treated plants following inoculation than untreated inoculated plants. It has also been observed that total phenol levels increased in treated inoculated tea root varieties with *U.zonata* than treated uninoculated tea root varieties.

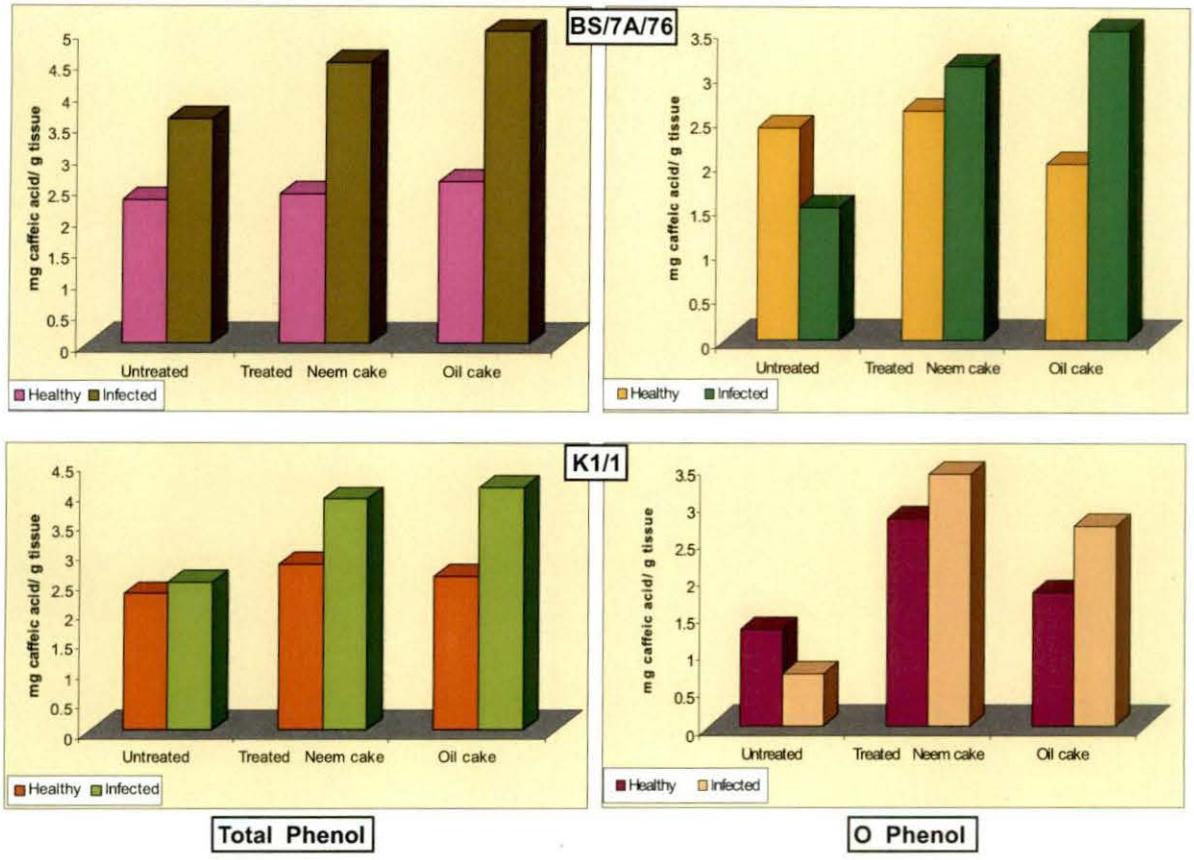


Figure 11: Total phenol and ortho-dihydroxy phenol content in tea varieties following application with organic additives and inoculation with *U. zonata*.

Level of ortho-dihydroxyphenol was also determined in these varieties (K1/1 and BS/7A/76) after treatment with neem cake and oil cake following inoculation with *U. zonata*. Results (Table 56, Fig. 11) revealed that ortho-dihydroxy phenol decreased in untreated inoculated tea root varieties in comparison to uninoculated healthy control. Ortho-dihydroxy phenol levels increased in treated roots following inoculation with the pathogen than treated healthy plants. Similar pattern was noted in case both the varieties tested. It is interesting to note that the plants grown in soil amended with neem cake and oil cake could resist the pathogen and changes in the level of total phenols as well as ortho-dihydroxy phenol can be correlated with the development of resistance in susceptible plants following such treatments.

Changes in the level of phenolics were also determined in two varieties of tea plants (UP-3, BS/7A/76 and K 1/1) grown separately in soil amended with cowdung, rabbit manure and chicken manure following inoculation with *U.zonata*. Results revealed that total phenol content decreased in untreated plants of two susceptible varieties (BS/7A/76 and K1/1) following inoculation with the pathogen in relation to healthy control, whereas the resistant variety (K 1/1) responded against inoculation with the pathogen. In this case total phenol and ortho-dihydroxy phenol content increased in comparison with untreated healthy control (Table 57 and 58; Fig. 12).

Table 55 : Total phenol content in tea varieties after treatment with Neem cake and oil cake following inoculation with *U. zonata*.

Tea variety	Phenol content (mg / g) [a]	
	Healthy	**Infected**
BS/7A/76		
Untreated	2.3	3.6
Treated		
Neem cake	2.4	4.5
Oil cake	2.6	5.0
K1/1		
Untreated	2.3	2.5
Treated		
Neem cake	2.8	3.9
Oil cake	2.6	4.1

Table 56 : Ortho-dihydroxy phenol content in tea varieties after treatment with Neem cake and oil cake following inoculation with *U.zonata* in treated tea root variety

Tea variety	Ortho-dihydroxy phenol content (mg / g) [a]	
	Healthy	**Infected**
BS/7A/76 Untreated	2.4	1.5
Treated Neem cake	2.6	3.1
Oil cake	2.0	3.5
K1/1 Untreated	1.3	0.7
Treated Neem cake	2.8	3.4
Oil cake	1.8	2.7

[a] Average of 3 replicates.

It has also been observed that total phenol levels increased in all the varieties tested following treatment with organic amendments. Rabbit manure responded markedly and in this case total phenol increased following inoculation with the pathogen in relation to treated healthy as well as untreated healthy control. Level of ortho-dihydroxy phenol increased markedly in soil amended with cowdung, whereas level of ortho-dihydroxyphenol increased in plants grown in soil amended with rabbit manure following inoculation with the pathogen.

Table 57 : Changes in the level of total phenol content in tea roots grown in soil amended with organic additives following inoculation with *U.zonata.*

Tea variety	Phenol content (mg / g) [a]	
	Healthy	Infected
UP-3 Untreated	2.8	2.3
Treated Cowdung	3.0	3.3
Rabbit Manure	3.7	5.8
Chicken manure	3.5	3.9
BS/7A/76 Untreated	3.6	2.9
Treated Cowdung	2.7	3.4
Rabbit Manure	4.0	4.9
Chicken manure	4.5	5.0
K-1/1 Untreated	2.9	6.5
Treated Cowdung	5.3	8.1
Rabbit manure	4.3	8.7
Chicken manure	4.4	5.3

[a] Average of 3 replicates.

Table 58 : Changes in the level of ortho-dihydroxy phenol content in tea roots grown in soil amended with organic additives following inoculation with *U. zonata.*

Tea variety	Ortho-dhydroxy content (mg / g) [a]	
	Healthy	**Infected**
UP-3 Untreated	2.0	1.4
Treated Cowdung	2.5	2.8
Rabbit Manure	2.3	2.5
Chicken manure	2.3	2.7
BS/7A/76 Untreated	1.0	0.7
Treated Cowdung	1.9	2.3
Rabbit Manure	1.7	2.1
Chicken manure	1.9	2.4
K-1/1 Untreated	0.9	2.1
Treated Cowdung	2.1	3.4
Rabbit manure	3	4.4
Chicken manure	1.5	2.5

[a] Average of 3 replicates.

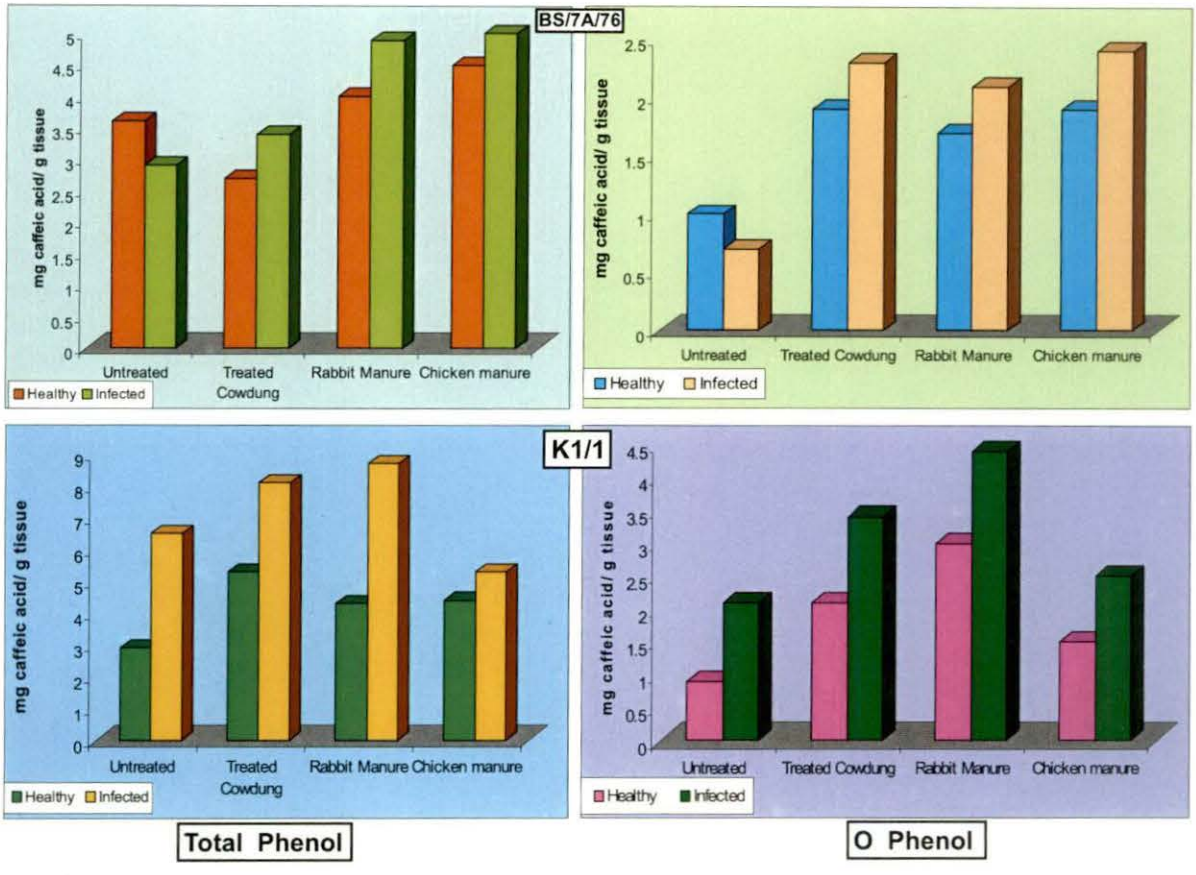


Figure 12: Total phenol and ortho-dihydroxy phenol content in treated tea varieties following application with organic manure and inoculation with *U. zonata.*