Experimental

4.1. Sclerotial blight disease occurrence under natural conditions

Sclerotial blight disease is caused by *Sclerotium rolfsii* Sacc. (telemorph: *Athelia rolfsii* (Curzi) Tu & Kimbrough *Corticium rolfsii* Curzi). A survey was conducted to record the occurrence of sclerotial blight in various tea gardens of Dooars, the foot hills (terai) of Jalpaiguri district and hills of Darjeeling district. Sclerotial blight disease incidence was recorded for three consecutive years from five tea gardens such as Matigara Tea Estate, Washabarie Tea Estate, Tiriannah Tea Estate, Diana Tea Estate and Simulbarie Tea Estate. Highest sclerorial blight disease incidence (65%) was observed in Matigara Tea Estate (Plate 3) and Tiriannah Tea Estate. First appearance of the disease was during early April and continued up to August. Maximum disease was recorded during mid July (rainy season). Disease was always noticed in the plains but rarely in the hills. Generally it attacks nursery grown tea seedlings. The disease persisted in the same areas for years, causing gradual deterioration in the health of the tea seedlings in the nursery and loss of crop.

The fungus has been isolated from the tea nurseries and after completion of Koch's postulates the organism has been identified from Plant Diagnostic Laboratory, U.K. The fungus was identified as *Sclerotium rolfsii* and designated as isolate – Sr1. Similarly two more isolates (Sr-2 and Sr-3) were also isolated from naturally infected tea roots. Sclerotial blight disease became rampant in the nursery grown tea plants in all the Tea Estates mentioned above. The first visible symptom of sclerotial blight disease was observed as yellowing and wilting of lower leaves. The fungal mycelium first appeared at the base of tea seedlings near the soil line. The pathogen then grew upwards covering the stem with a cottony-white mass of mycelia. Later on, water-soaked and grey lesions appeared on the tea seedlings, which turned brown, resulting in death of the whole plant. A large number of small, light brown, mustard like sclerotia developed in the collar zone. After the pathogen established itself, its subsequent advancement and production of mycelia and sclerotia was quite rapid. The infected tea seedlings ultimately toppled down and died. *S. rolfsii* is able to survive within a wide range of environmental conditions. Growth is possible within a broad pH range, though best on acidic soils. The optimum temperature range for mycelial growth occurs between 25 and 35^0C with

Plate 3. Naturally infected tea seedling showing sclerotia of *Sclerotium rolfsii* on collar region. (Inset) *S. rolfsii* grown on PDA medium.

little or none at below 10^0C or over 40^0C. Sclerotial formation is also greatest at or near the optimum temperature for mycelial growth. Mycelium is killed at 0^0C, but sclerotia can survive at temperatures as low as – 10^0C. High moisture is required for optimal growth of the fungus. Sclerotia fail to germinate when the relative humidity is much below saturation. Mycelial growth and sclerotial germination occur rapidly in continuous light, though they may occur in darkness if other conditions are favourable.

4.2. Factors influencing mycelial growth of *S. rolfsii*

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

4.2.1. Media

Sclerotium rolfsii was grown in six different media *i.e.*, potato dextrose agar (PDA), potato sucrose agar (PSA), Richard's agar (RA), carrot juice agar (CJA), yeast dextrose agar (YDA) and Czapek-Dox agar (CDA). Results revealed that the fungus grew well in all media (Plates 4 and 5), except in CDA (Plate 5, fig. A) where the mycelial growth was very poor. Maximum growth was recorded in PDA (Plate 5, fig. F) followed by PSA and YDA but minimum growth was recorded in CDA where hyaline and submerged hyphal growth spread very loosely with no compact mycelial structure was observed. In most other media, white or hyaline advancing zones were observed and mycelial colour changed from white to mild white. Tan to brown mustard like sclerotia formation was observed in all media *i.e.*, PDA, PSA, RA, CDA, CJA and YDA.

4.2.2. Incubation period

S. rolfsii was grown in Richard's medium (RM) for a period of 20 days. Mycelial growth of the fungus was recorded after 4, 8, 12, 16 and 20 days of

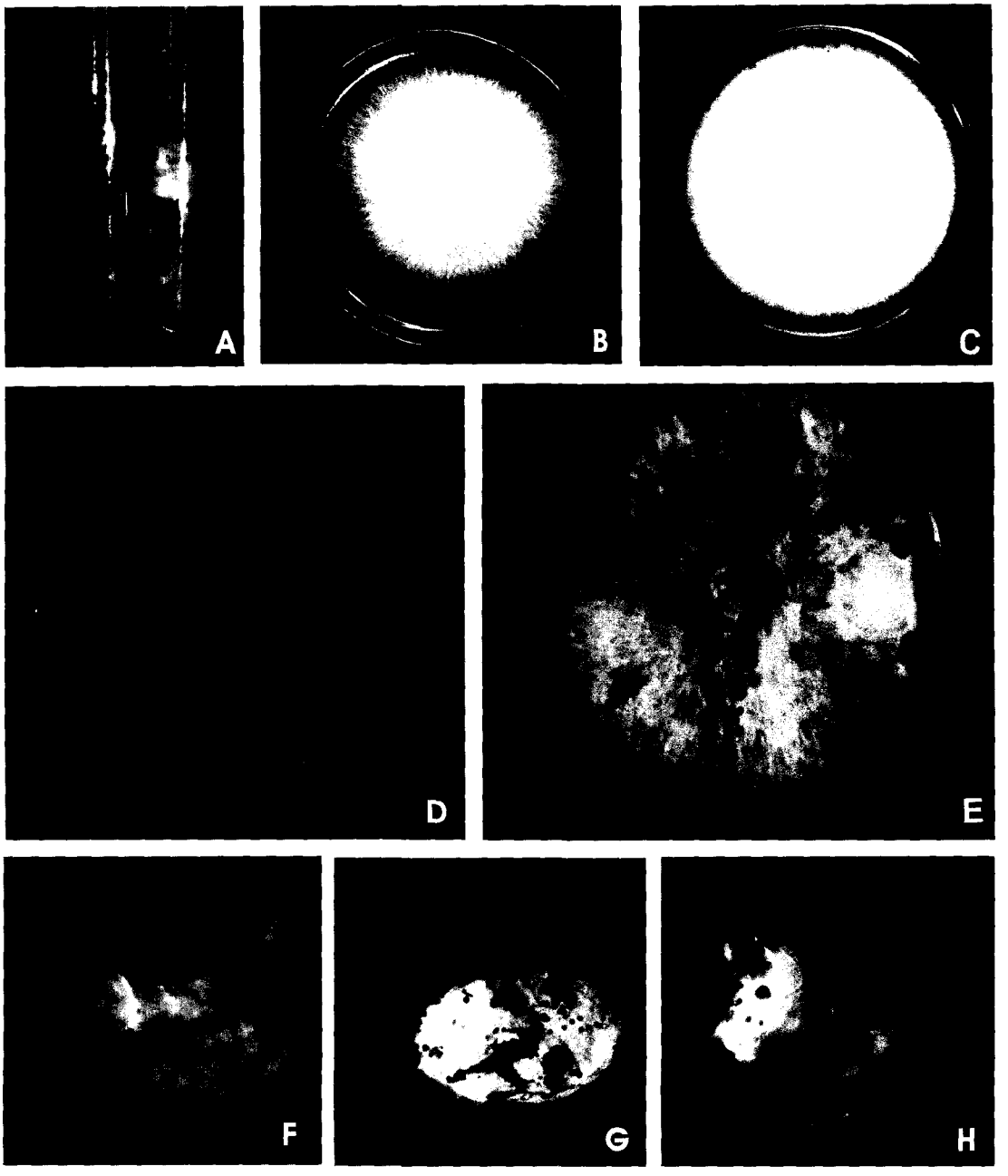

Plate 4 (figs. A-H). Growth of *S. rolfsii* isolates on potato dextrose agar (A-C,E) and potato dextrose broth (D, F-H). Isolate Sr-1(A-F), isolate Sr-2 (G) and isolate Sr-3 (H).

incubation at 28^{0}C. Results have been presented in Table 1 and Fig. 1 A. It revealed that maximum growth occurred after 8 days of incubation after which it declined.

Table 1: Effect of incubation period on mycelial growth of *Sclerotium rolfsii*

Incubation period in days	Mean mycelial dry wt (mg)[a]
4	258.0 ± 1.4
8	331.0 ± 2.8
12	272.0 ± 0.8
16	233.0 ± 2.4
20	191.0 ± 0.8

[a] Results are an average of 3 replicates.
± Standard error

4.2.3. pH of medium

The pH of the medium usually plays an important role in the growth of all micro-organisms and has to be used to stabilize the pH. In the present investigation buffer solutions with pH values ranging from 3.0 to 8.0 were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of M/30. The pH was finally adjusted using N/10 HCl or N/10 NaOH in each case. The medium and the buffer were sterilized separately by autoclaving for 15 min at 15 lb/in^2 pressure. Equal parts of the buffer solution and the medium (RM) were mixed before use. Each flask containing 50 ml of the medium was then inoculated with a mycelial block of *S. rolfsii* and incubated for 8 days at 28^{0}C. Results (Table 2 and Fig. 1 B) revealed that *S. rolfsii* grew to a lesser or greater extent in all the pH tested. Maximum growth was recorded at pH 6.0, while minimum growth occurred at pH 3.0. Sclerotial germination was very less in a pH as high as 8.0; however, pH 3.0 and pH 4.0 supported good sclerotial germination (Plate 6, figs. A-C).

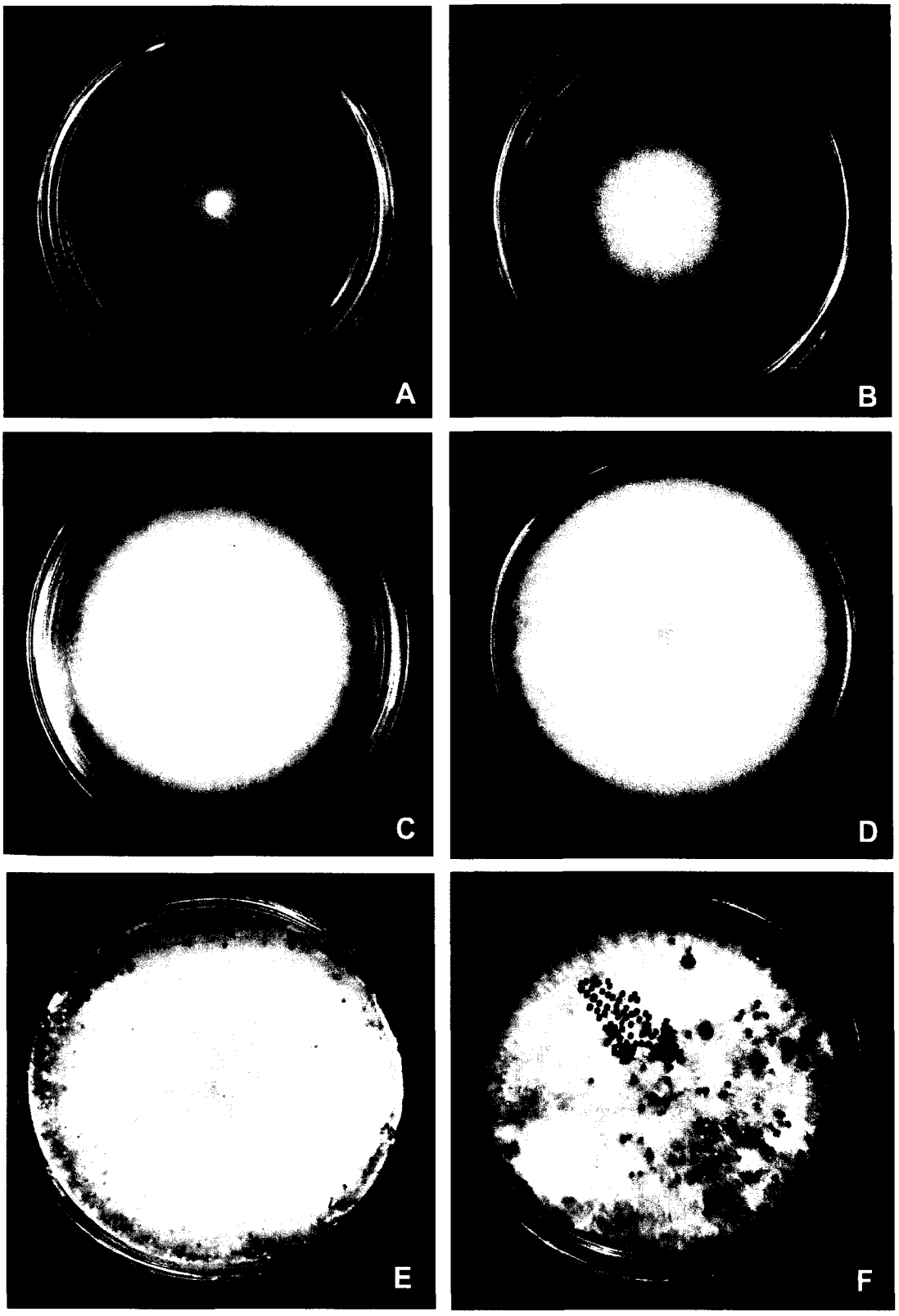

Plate 5 (figs. A-F). Mycelial growth of *S. rolfsii* on different media. (A) Richard's Agar, (B) Carrot juice Agar (C) Potato Sucrose Agar (D) Yeast Extract Dextrose Agar (E&F) Potato Dextrose Agar.

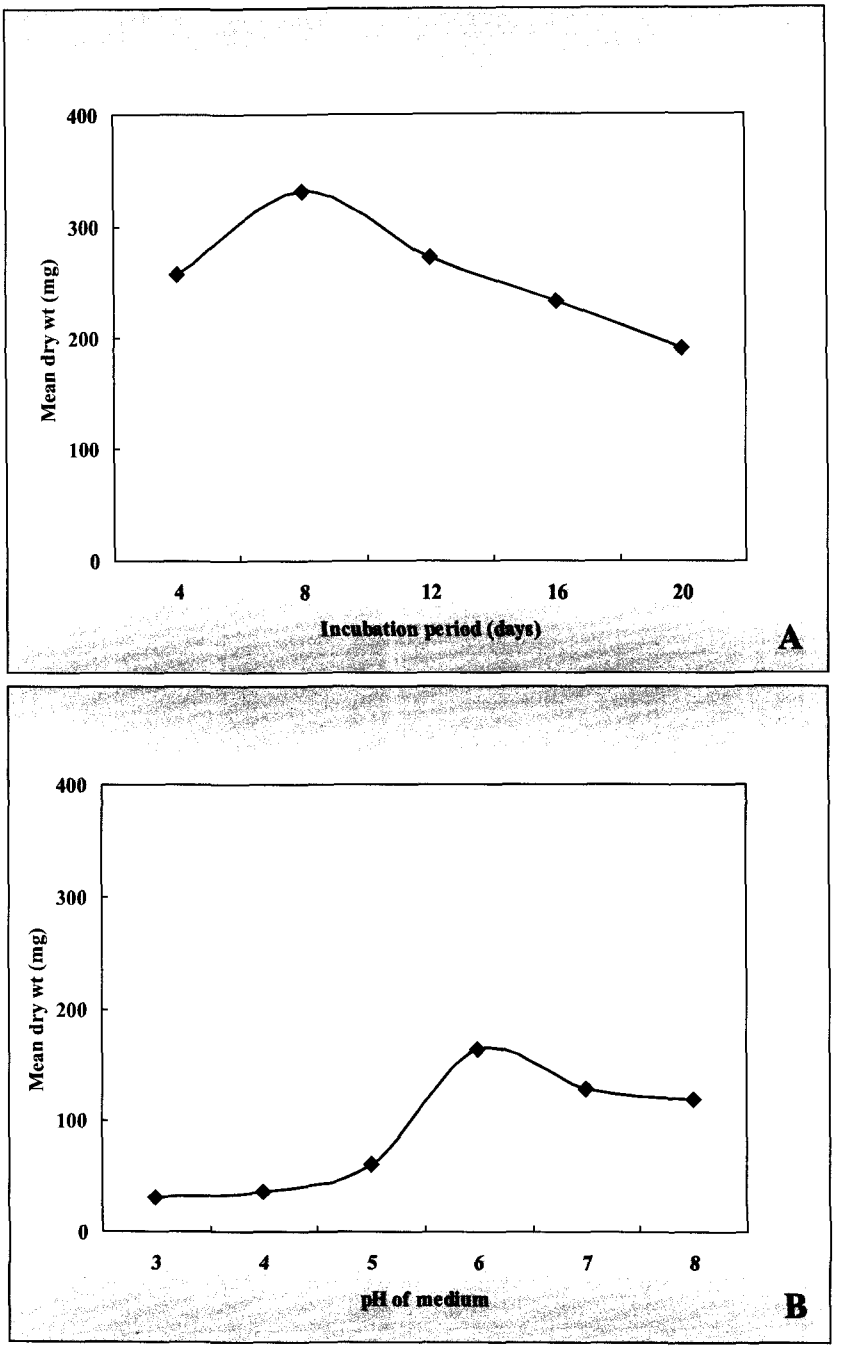

Fig. 1(A&B). Effect of incubation period (A) and pH (B) on mycelial growth of *S. rolfsii.*

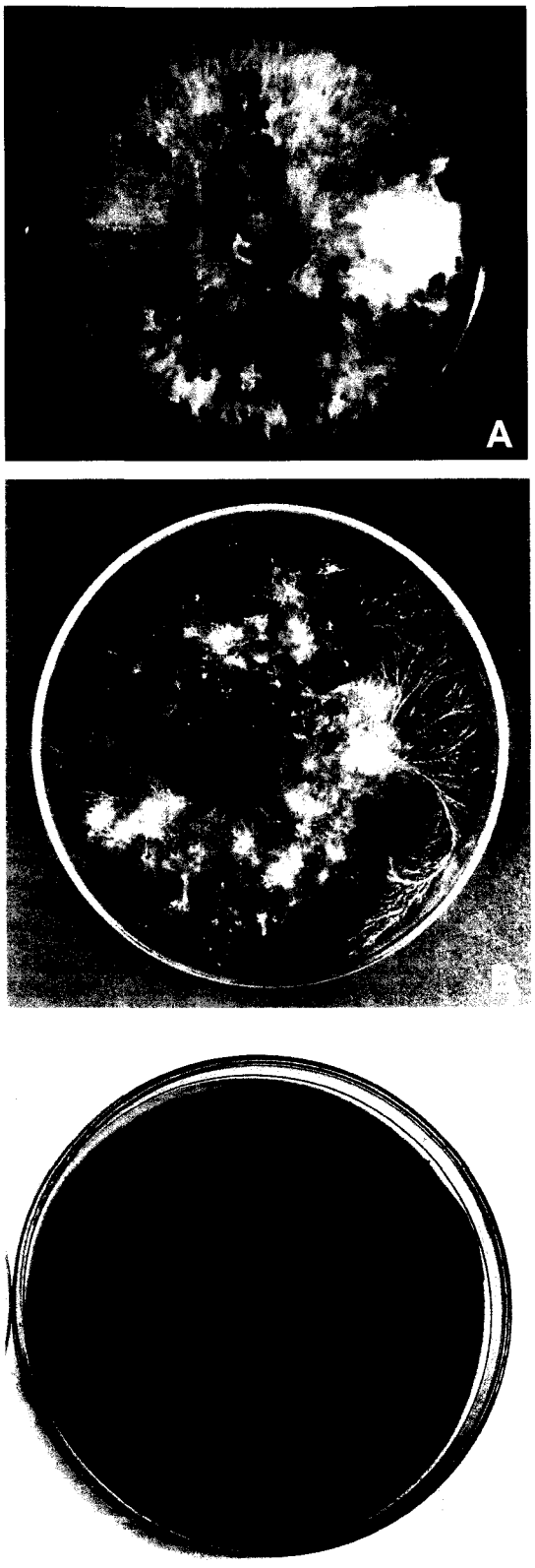

Plate 6 (figs. A-C). Germinated sclerotia of *S. rolfsii*

Table 2: Effect of pH on mycelial growth of *Sclerotium rolfsii*

pH of medium	Mean mycelial dry weight (mg)[a, b, c]
3.0	31.0 ± 1.6
4.0	36.0 ± 0.8
5.0	61.0 ± 0.7
6.0	164.0 ± 1.4
6.5	138.0 ± 1.6
7.0	129.0 ± 0.8
8.0	120.0 ± 2.1

[a] Results are an average of 3 replicates
[b] Incubation temperature 28^0C
[c] Incubation period – 8 days
± Standard error.

4.2.4. Carbon sources

The ability of fungi to grow in different media depends on their capacity to utilize the available nutrients, of which carbohydrates are the major ones. It was observed that the growth rate varies with different carbon sources. In this investigation, six different carbon sources (fructose, mannitol, sucrose, starch, maltose and dextrose) were tested for their effects on the growth of *S. rolfsii*. Richard's medium without sugar was used as the basal medium. The equivalent amount of carbon present in 1 percent glucose was used as standard and added separately to the basal medium.

Data were recorded after 8 days of incubation. A control set without any carbohydrate was also set up. Mycelial dry weight was recorded. Results given in Table 3 and Fig. 2 revealed maximum growth of *S. rolfsii* using dextrose as the carbon source. Maltose and starch also supported comparatively good growth. There was little growth in absence of any carbohydrate.

Table 3: Effect of different carbon sources on mycelial growth of *Sclerotium rolfsii*

Carbon sources	Mycelial dry weight (mg)[a, b, c]
D-Fructose	65.3 ± 0.5
Mannitol	19.0 ± 0.7
Sucrose	80.3 ± 0.9
Starch	103.0 ± 1.4
Maltose	128.0 ± 1.4
Dextrose	236.3 ± 1.8
Control (Without carbon)	16.3 ± 1.6

[a] Results are an average of 3 replicates
[b] Incubation temperature 28^0C
[c] Incubation period – 8 days
± Standard error.

4.2.5. Nitrogen sources

Nitrogen is the most important nutrient necessary for the growth of any organism. The availability of nitrogen depends to a great degree on the form in which it is supplied. Hence, the most suitable nitrogen source for any particular microorganism can only be determined by testing a number of sources including both inorganic and organic. The effects of complex organic sources (peptone, urea, yeast extract and beef extract) as well as inorganic nitrogen sources (calcium nitrate, sodium nitrate, ammonium sulphate and potassium nitrate) on the mycelial growth of *S. rolfsii* were tested. Richard's medium without nitrogen sources was used as the basal medium. A control set without any nitrogen source was considered as control. Data was recorded after 8 days of incubation. Results (Table 4) revealed that among the organic sources maximum growth was found in yeast extract. In inorganic nitrogen sources maximum growth was obtained in calcium nitrate followed by sodium nitrate (Fig. 3).

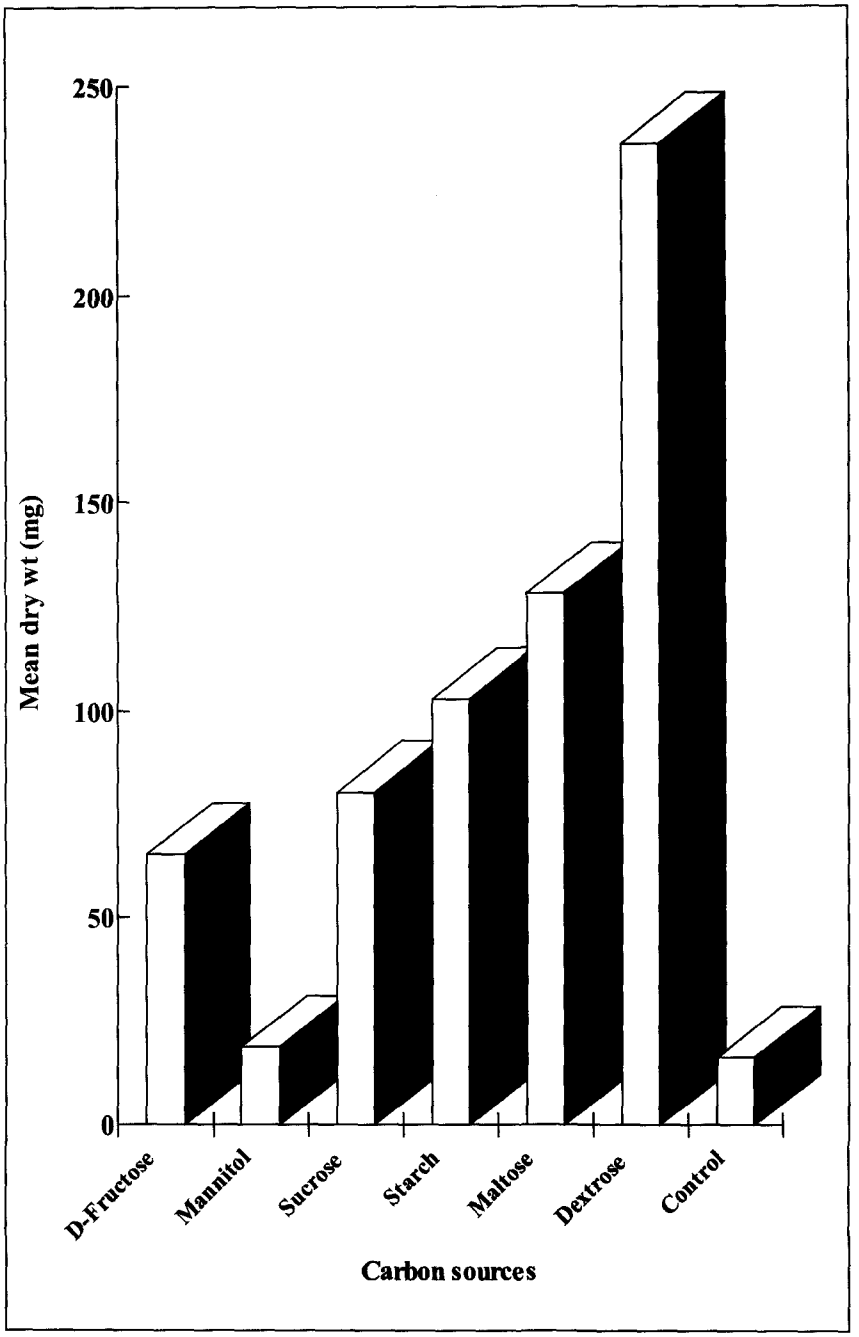

Fig. 2. Effect of different carbon sources on mycelial growth of *S. rolfsii*

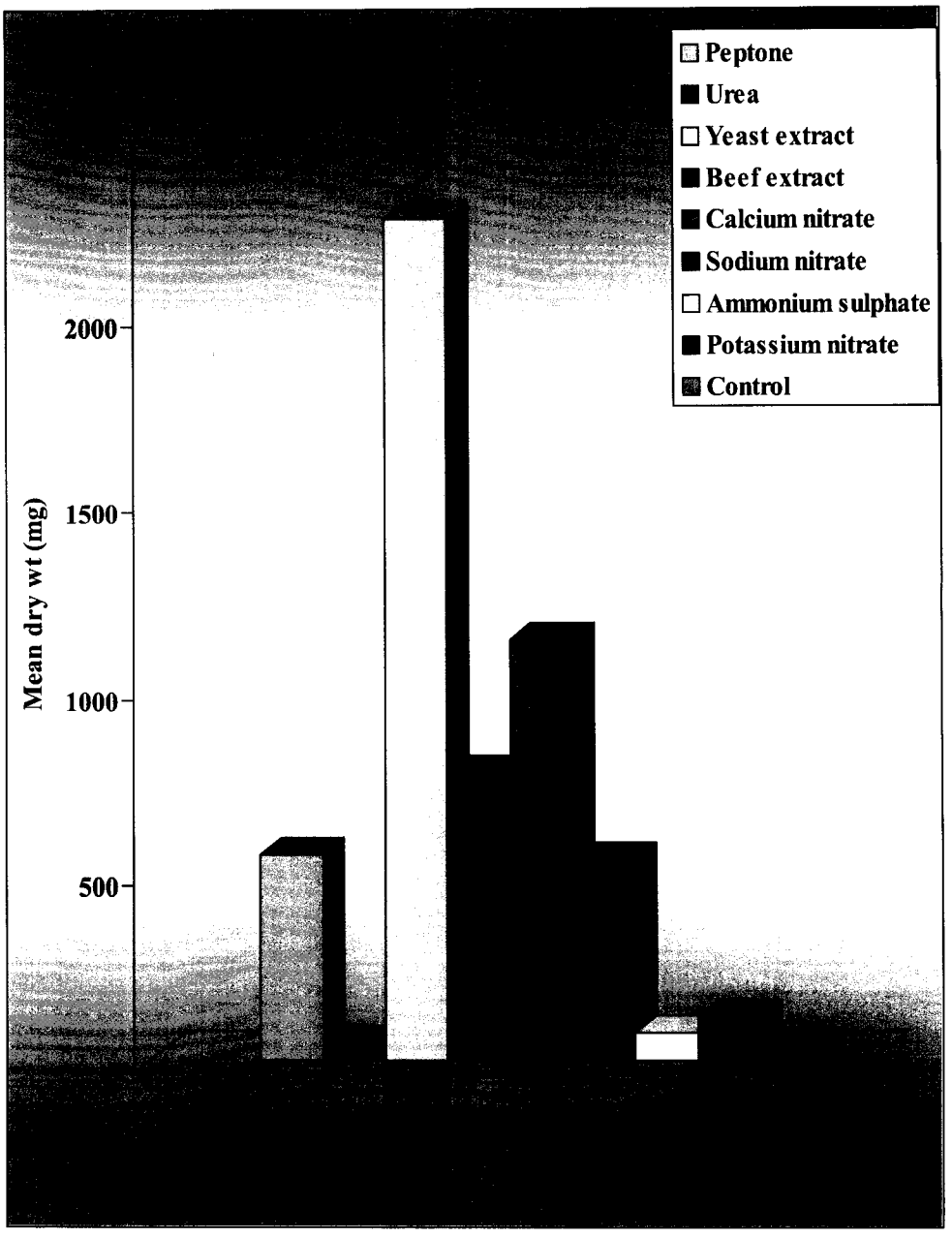

Fig. 3. Effect of different organic and inorganic nitrogen sources on mycelial growth of *S. rolfsii.*

Table 4: Effect of different nitrogen sources on mycelial growth of *Sclerotium rolfsii*

Nitrogen sources	Mycelial dry weight (mg) [a,b,c]
Organic	
Peptone	551.3 ± 0.5
Urea	47.3 ± 0.9
Yeast extract	2252.7 ± 1.8
Beef extract	776.0 ± 1.4
Inorganic	
Calcium nitrate	1130.0 ± 1.4
Sodium nitrate	538.3 ± 0.9
Ammonium sulphate	75.6 ± 1.2
Potassium nitrate	120.0 ± 0.8
Control (Richard's Agar without nitrogen)	43.3 ± 0.9

[a] Results are an average of 3 replicates; [b] Incubation temperature 28^{0}C
[c] Incubation period – 8 days; ± Standard error.

4.3. Varietal resistance test of tea against *Sclerotium rolfsii*

Pathogen (*Sclerotium rolfsii*) isolated from the naturally infected tea roots after completion of Koch's postulate was used for artificial inoculation of tea plants grown in earthen pots. Rhizosphere region of 1-year-old potted plants of 18 different tea varieties were inoculated with *S. rolfsii* which has already been described in materials and methods. Twenty plants of each tea variety were used. Among 18 varieties 5 were Tocklai varieties, 7 were from Darjeeling and 6 were UPASI varieties. Disease assessment was done on the basis of visual observation of symptoms and disease index was calculated as described earlier. Disease index ranged from 0-6 and was calculated after 15, 30 and 45 days of inoculation. Results are presented in Table 5 and Fig. 4. It revealed that among the Tocklai varieties TV-30 was the most susceptible while TV-26 was the most resistant. Among the Darjeeling varieties Teen Ali – 17, B-157, T-78, T-135 and AV-2 showed maximum susceptibility while HV-39 and K1/1 were the most resistant varieties. In case of UPASI varieties UP-8 and UP-26 were most susceptible where as UP-2 and BSS-2 resistant respectively. Among all 18 varieties UP-8 and Teen Ali-17 were most susceptible (Plate 7) whereas K1/1 and HV-39 were most resistant (Table 5).

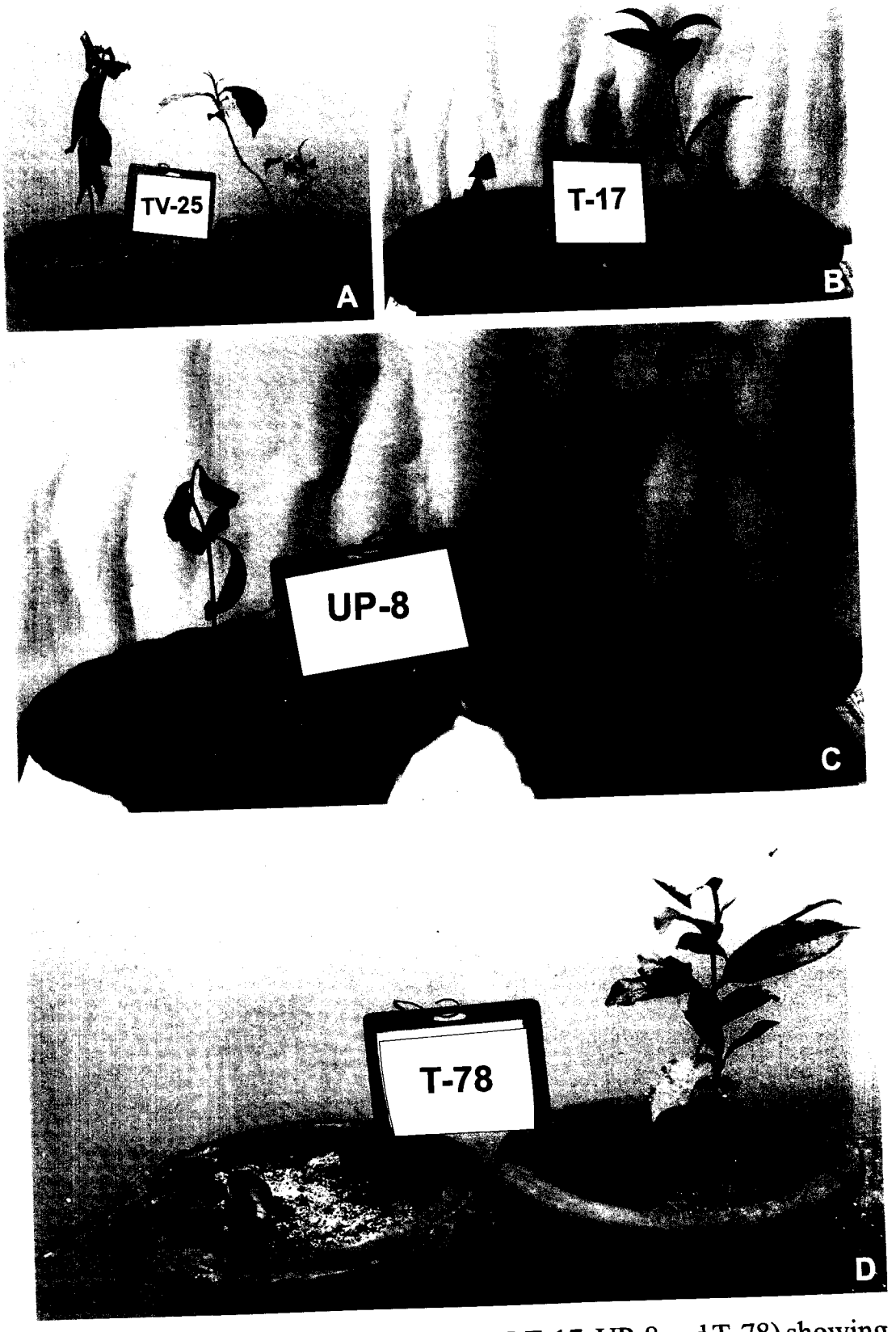

Plate 7 (figs. A-D). Tea varieties (TV-25,T-17, UP-8 and T-78) showing symptoms following inoculation with *S. rolfsii.*

Table 5: Varietal resistance test of *Sclerotium rolfsii* on different tea root varieties

Tea Varieties	Disease index [a, b]		
	15	30	45
TV-18	1.67 ± 0.03	3.53 ± 0.09	4.06 ± 0.08
TV-22	1.65 ± 0.02	3.46 ± 0.04	4.09 ± 0.08
TV-25	0.22 ± 0.01	4.63 ± 0.09	5.71 ± 0.06
TV-26	2.64 ± 0.01	3.02 ± 0.03	3.6 ± 0.04
TV-30	2.52 ± 0.03	4.91 ± 0.02	5.41 ± 0.01
UP-2	0.53 ± 0.02	2.08 ± 0.05	3.48 ± 0.06
UP-3	0.66 ± 0.01	3.65 ± 0.04	4.08 ± 0.061
UP-8	4.33 ± 0.01	5.1 ± 0.04	5.95 ± 0.03
UP-9	4.02 ± 0.02	5.14 ± 0.04	5.91 ± 0.03
UP-26	3.9 ± 0.08	4.94 ± 0.04	5.43 ± 0.01
BSS-2	1.64 ± 0.02	3.11 ± 0.06	3.48 ± 0.05
T-17	4.35 ± 0.03	5.07 ± 0.04	5.92 ± 0.01
T-78	1.37 ± 0.04	3.08 ± 0.08	4.02 ± 0.02
AV-2	0.66 ± 0.02	1.5 ± 0.03	3.54 ± 0.04
T-135	2.32 ± 0.02	4.25 ± 0.04	5.05 ± 0.03
B-157	2.66 ± 0.02	4.60 ± 0.01	5.67 ± 0.05
HV-39	0.22 ± 0.02	1.03 ± 0.05	1.44 ± 0.07
K1/1	0.49 ± 0.01	0.53 ± 0.04	0.67 ± 0.02

[a] Results are an average of 20 inoculated plants
[b] Days after inoculation
± Standard error

Key to disease index:

0 – No symptoms; 1 – Small roots turn rotten, lesions appeared at the collar region; 2 – Middle leaves start wilting and 10-20% of the roots turn brown; 3 – Leaves wilted and 20-40% roots become dry with browning of shoot; 4 – Extensive rotting at the collar region of root, 60-70% of the roots and leaves wilted, browning of shoot over 60%; 5 – 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80% and 6 – Whole plants die, since 100% roots were wilted.

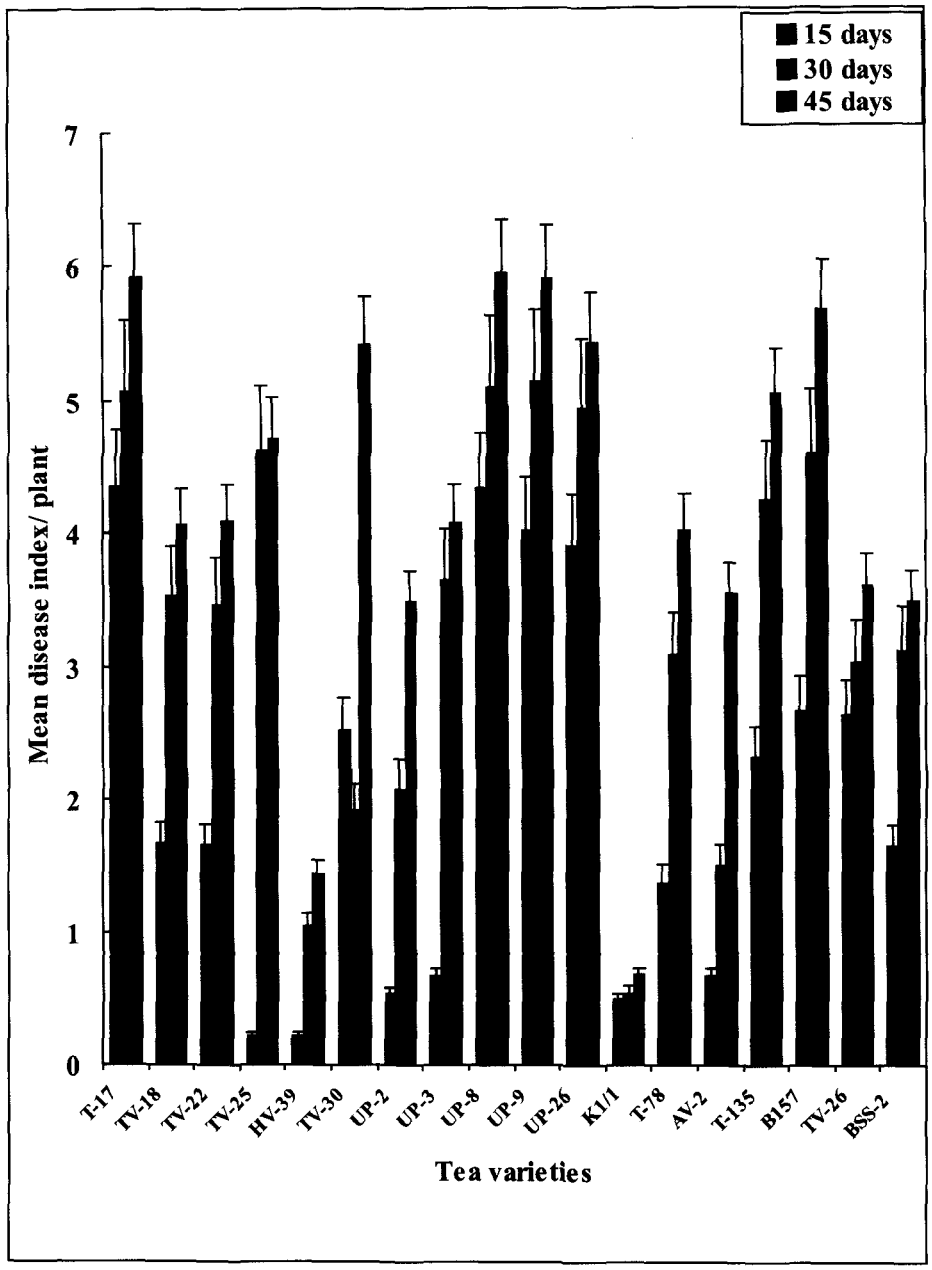

Fig. 4. Screening of tea varieties for resistance against *S. rolfsii*.

4.4. Estimation and analysis of proteins in fungal mycelia and tea roots following infection

Proteins are major biochemical components in all plants. Proteins are generally known to either increase or decrease due to infection by pathogen and more importantly their pattern may also change. A number of experiments were designed to investigate the biochemical changes in the protein patterns in fungal mycelia and tea roots following infection.

4.4.1. Protein content in tea roots following infection

Protein was extracted from tea roots and contents were estimated. Detailed procedures for extraction and estimation have already been presented under material and methods. In case of protein contents of roots, no significant differences were noted between the different tea varieties of neither healthy nor inoculated roots. Protein contents of healthy and infected tea roots are presented in Table 6 and Fig. 5.

4.4.2. Protein content in fungal mycelia

Soluble proteins of *S. rolfsii* were extracted from mycelia and the content was estimated. Estimation of mycelial protein revealed that *S. rolfsii* had a protein content of 7.0 mg/g fresh weight of tissue. Protein content of fungal mycelia of *S. rolfsii* was recorded after 3, 6, 9 and 12 days of incubation at 28^0C. Results are presented in Table 7. Maximum protein content of fungal mycelia of *S. rolfsii* occurred after 9 days of incubation after which it declined. The proteins were further analysed by SDS-PAGE.

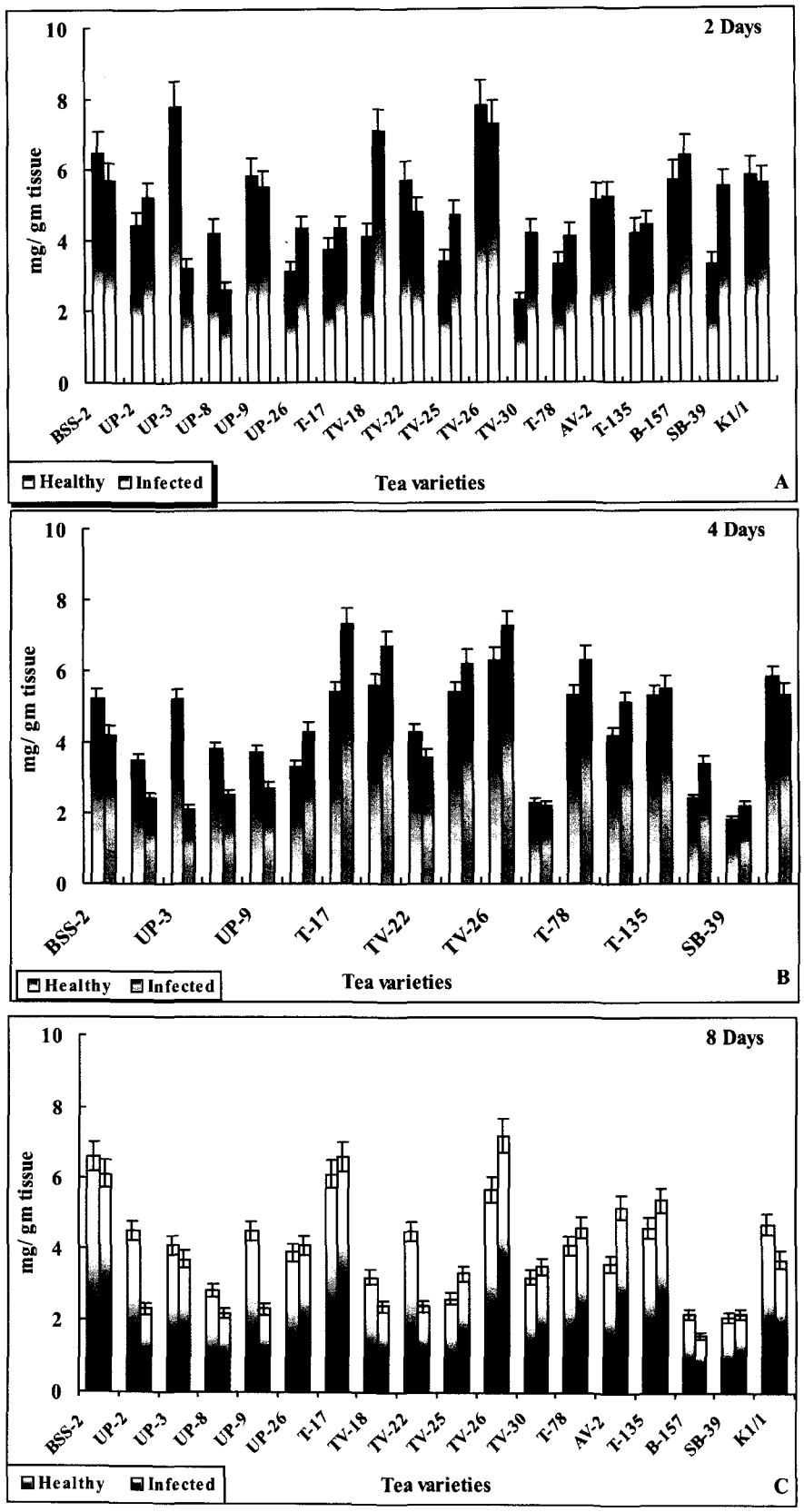

Fig. 5 (A-C). Protein content in healthy and *S. rolfsii* inoculated root of tea varieties.

Table 6: Protein content in tea roots following inoculation with *Sclerotium rolfsii*

Tea varieties	Protein content (mg/g of tissue) [a]					
	2[b]		4		8	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
BSS-2	6.5 ± 0.08	5.7 ± 0.04	5.2 ± 0.12	4.2 ± 0.14	6.6 ± 0.16	6.1 ± 0.09
UP-2	4.4 ± 0.21	5.2 ± 0.18	3.5 ± 0.14	2.4 ± 0.21	4.5 ± 0.24	2.3 ± 0.12
UP-3	7.8 ± 0.02	3.2 ± 0.14	5.2 ± 0.04	2.1 ± 0.09	4.1 ± 0.12	3.7 ± 0.08
UP-8	4.2. ±0.04	2.6 ± 0.21	3.8 ± 0.09	2.5. ±0.08	2.8 ± 0.04	2.2. ± 0.08
UP-9	5.8 ± 0.02	5.5 ± 0.12	3.7 ± 0.02	2.7 ± 0.09	4.5. ± 0.08	2.3. ± 0.14
UP-26	3.1 ± 0.08	4.3 ± 0.04	3.3 ± 0.08	4.3 ± 0.14	3.9 ± 0.02	4.1 ± 0.09
TV-18	4.1 ± 0.08	7.1 ± 0.04	5.6 ± 0.12	6.7 ± 0.14	3.2 ± 0.09	2.4 ± 0.04
TV-22	5.7 ± 0.13	4.8 ± 0.11	4.3 ± 0.12	3.6 ± 0.02	4.5 ± 0.11	2.4 ± 0.09
TV-25	3.4 ± 0.11	4.7 ± 0.19	5.4 ± 0.05	6.2 ± 0.06	2.6 ± 0.07	3.3 ± 0.02
TV-26	7.8 ± 0.07	7.3 ± 0.19	6.3 ± 0.10	7.2 ±0.09	5.7 ± 0.16	7.2 ± 0.08
TV-30	2.3 ± 0.37	4.2 ± 0.10	2.3 ± 0.24	2.2 ± 0.05	3.2 ± 0.12	3.5 ± 0.08
T-17	3.7 ± 0.28	4.3 ± 0.16	5.4 ± 0.12	7.3 ± 0.09	6.1 ± 0.08	6.6 ± 0.14
T-78	3.3 ± 0.32	4.1 ± 0.03	5.3 ± 0.31	6.3 ± 0.20	4.1 ± 0.11	4.6 ± 0.19
AV-2	5.1 ± 0.02	5.2 ± 0.15	4.2 ± 0.05	5.1 ± 0.04	3.6 ± 0.17	5.2 ± 0.03
T-135	4.2 ± 0.02	4.4 ± 0.04	5.3 ± 0.08	5.5 ± 0.05	4.6 ± 0.12	5.4 ± 0.10
B157	5.7 ± 0.13	6.4 ± 0.20	2.4 ± 0.21	3.4 ± 0.08	2.2 ± 0.07	1.6 ± 0.09
HV-39	3.3 ± 0.15	5.5 ± 0.12	1.8 ± 0.13	2.2 ± 0.08	2.1 ± 0.09	2.2 ± 0.04
K1/1	5.8 ± 0.15	5.6 ± 0.11	5.8 ± 0.04	5.3 ± 0.12	4.7 ± 0.10	3.7 ± 0.11

[a] Results are an average of 3 replicates; [b] Days after inoculation; ± Standard error

Table 7: Variation of protein content of fungal mycelia of *Sclerotium rolfsii* with age

Incubation period (days) [a]	Protein content of fungal mycelia of *S. rolfsii* (mg/g) [b]
3	1.9
6	6.8
9	7.3
12	3.6

[a] Incubation temperature – 28°C

[b] Results are an average of 3 replicates

4.4.3. SDS-PAGE analysis of fungal protein

Mycelial proteins of *S. rolfsii* were analyzed by SDS-PAGE. Twenty four protein bands ranging in molecular weight from 6.5 to 205 kDa were detected (Table 8). The molecular weights were determined by comparison with standard molecular weight markers as described previously. Protein profile of soluble proteins extracted from 9-day-old mycelia of *S. rolfsii* and resolved on SDS-PAGE has been presented in Plate 8, fig. A.

Table 8: SDS-PAGE analysis of mycelial protein of *Sclerotium rolfsii*

Protein source	No. of bands	Molecular weight (kDa)
Mycelia	24	205, 161.9, 97.4, 90.1, 66, 61.4, 56.8, 52.2, 43, 37.4, 34, 29, 25.4, 23, 20, 18.6, 17.8, 15.2, 14.6, 12.5, 11, 10.5, 8.8, 6.5

4.5. Detection of cross reactive antigens between *Sclerotium rolfsii* and tea varieties

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

immunodiffusion, ELISA and immunofluorescence. A number of experiments were performed and the results obtained have been presented below.

4.5.1. Immunodiffusion tests

The effectiveness of antigen preparations from *S. rolfsii* (isolate Sr-1) and tea roots (B-157 and AV-2) for raising polyclonal antibodies were checked by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and antigens of pathogen (*S. rolfsii* isolates; Sr-1, Sr-2 and Sr-3) and tea roots (B-157 and AV-2) were all negative. When the polyclonal antibody (PAb) raised against mycelial antigens of *S. rolfsii* (Sr-1) was reacted with its own antigen and antigen of the other two isolates (Sr-1 and Sr-2), strong precipitin reaction occurred (Plate 8, figs. B-E). When PAb of *S. rolfsii* was cross reacted with root antigens prepared from 18 tea varieties (5 Tocklai, 6 UPASI and 7 Darjeeling), 8 varieties (T-17, TV-30, UP-3, UP-8, UP-9, UP-26, T-135 and B-157) exhibited strong precipitin bands in agar gel double diffusion tests (Table 9). However, weak precipitin reactions occurred with antigens of other 8 varieties (TV-18, TV-22, TV-25, TV-26, UP-2, T-78, AV-2 and BSS-2). No such precipitin bands were observed in case of root antigens prepared from 2 specific varieties (HV-39 and K1/1) as well as root antigen prepared from *Oryza sativa* (non host plant) and *Fusarium graminearum* (non pathogen of tea).

Reciprocal cross reaction using PAb raised against AV-2 and antigens prepared from tea roots of 18 varieties, one non host and one non pathogen species and three isolates of *S. rolfsii* were also carried out. Results (Table 10) revealed that none of the isolates of *S. rolfsii* could develop any precipitin reaction with anti-AV-2 antiserum. Non-host species and non pathogen also failed to develop any precipitin bands. Serological cross reactivity among UPASI, Tocklai and Darjeeling varieties was revealed by the appearance of precipitin bands in diffusion tests. However, strong bands were observed in case of UP-8, UP-9, UP-26, T-17, TV-25, TV-30, UP-3, T-135 and B-157 while weak precipitin reactions were observed in case of UP-2, TV-26, BSS-2, TV-18, TV-22, AV-2, HV-39 and K 1/1.

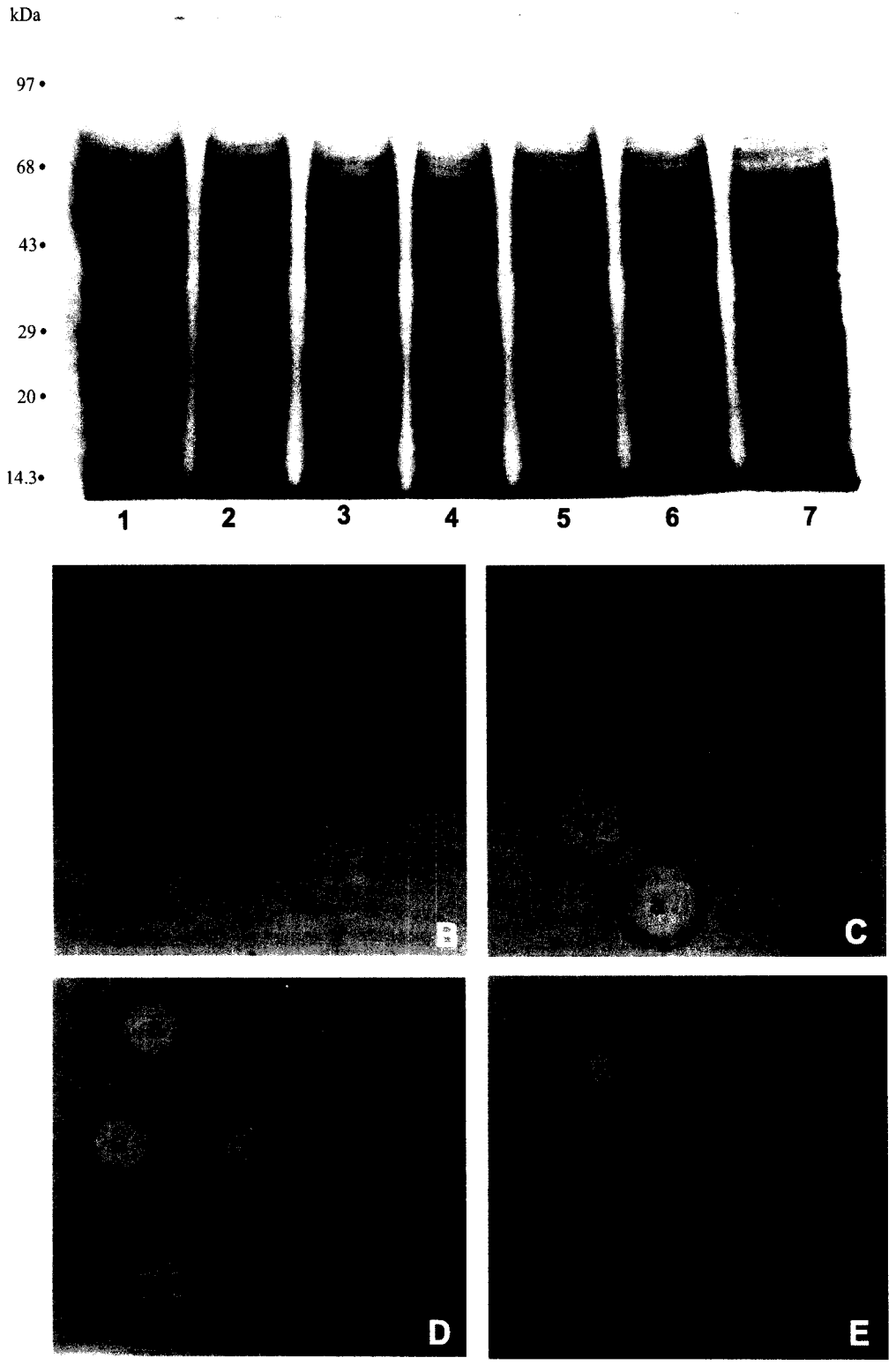

Plate 8 (figs. A-E). Mycelial antigens of *Sclerotium rolfsii.* (A) SDS-PAGE analysis (B-E) Agar gel double diffusion test with PAb of *S. rolfsii* (central wells) and homologous antigen (peripheral wells).

To confirm the presence of common antigens between *S. rolfsii* (isolate Sr-1) and tea varieties reciprocal cross reaction with PAb raised against B-157 was also carried out with root antigens of host and non host as well as with mycelial antigens of pathogen isolates and non pathogen. Results are presented in Table 10. Strong precipitin reactions were observed in homologous reactions with most of the Tocklai varieties (TV-18, TV-22, TV-25 and TV-30), four UPASI varieties (UP-3, UP-8, UP-9 and UP-26) and five Darjeeling varieties (T-135, T-17, T-78, AV-2 and B-157). However weak precipitin reactions were noticed in cross reactions between PAb of B-157 and root antigens of TV-26, UP-2, BSS-2, HV-39 and K1/1.

It is interesting to note that reciprocal cross reactions using PAb of B-157 and antigen of isolate Sr-1 gave strong precipitin bands, while, weak precipitin bands were noticed in reactions with mycelial antigens of isolate Sr-2 and Sr-3. No precipitin reactions were observed when non host and non pathogen antigens were reacted with PAb of B-157 in immunodiffusion test.

Table 9: Detection of cross reactive antigens among tea varieties and *Sclerotium rolfsii* following agar gel double diffusion tests

Antigens of host parasite	PAb of *Sclerotium rolfsii*
UPASI varieties	
UPASI-2	±
UPASI-3	+
UPASI-8	+
UPASI-9	+
UPASI-26	+
BSS-2	±
Tocklai varieties	
TV-18	±
TV-22	±
TV-25	±
TV-26	±
TV-30	+
Darjeeling varieties	
T-17	+
T-78	±
AV-2	±
T-135	+
B-157	+
HV-39	-
K1/1	-
Pathogen	
S. rolfsii isolate - Sr1	+
S. rolfsii isolate - Sr2	+
S. rolfsii isolate - Sr3	+
Non pathogen	
Fusarium graminearum	-
Non host	
Oryza sativa	-

+ Common precipitin band present
± Week precipitin band present
- Common precipitin band absent.

Table 10: Serological cross reactivity among different tea varieties and *Sclerotium rolfsii* isolates following immunodiffusion test

Tea root antigens	Polyclonal antibody raised against tea root antigens	
UPASI varieties	**AV-2**	**B-157**
UPASI-2	±	±
UPASI-3	+	+
UPASI-8	+	+
UPASI-9	+	+
UPASI-26	+	+
BSS-2	±	±
Tocklai varieties		
TV-18	±	±
TV-22	±	+
TV-25	+	+
TV-26	±	±
TV-30	+	+
Darjeeling varieties		
T-17	+	+
T-78	+	+
AV-2	+	+
T-135	+	±
B-157	±	+
HV-39	±	±
K1/1	±	±
Pathogen		
S. rolfsii isolate – Sr1	–	+
S. rolfsii isolate – Sr2	–	±
S. rolfsii isolate – Sr3	–	±
Non pathogen		
Fusarium graminearum	–	–
Non host		
Oryza sativa	–	–

+ Common precipitin band present
± Week precipitin band present
- Common precipitin band absent

4.5.2. Plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA)

In plate trapped antigens-enzyme linked immunosorbent assay (PTA-ELISA) antigens are linked to a solid carrier, after which the antibody is allowed to bind to the antigen. To this antigen-antibody complex, the conjugate (an antibody conjugated to an enzyme) is added. Finally a non-coloured substrate is added which is converted to a coloured end product, which is generally detected by a reader. In the present study, PTA-ELISA formats have been used for serological assays. Since ELISA depends on a number of factors and these vary from system to system, it was considered essential to optimize the conditions in this particular host (tea) and pathogen (*S. rolfsii*) system.

4.5.2.1. Optimization of ELISA

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

4.5.2.1.1 Enzyme dilution

In this experiment, keeping the antigen (10 µg/ml) and antiserum dilution (1:125) constant, different dilutions of alkaline phosphatase was used. Dilution ranged from 1:10,000 to 1:40,000. On the basis of results, enzyme concentration 1:10,000 was used while substrate was standardized at a concentration of 10 mg/ml.

4.5.2.1.2 Antiserum dilution

PAb raised against *S. rolfsii* (isolate Sr-1) were pooled in two batches. First and second bleedings were separately purified for IgG. These two batches of IgG were diluted ranging from 1:125 to 1:16,000 and then tested against homologous antigen (mycelial antigen of Sr-1 isolate) at a concentration of 10 µg/ml. Results are given in Table 11. Absorbance values in ELISA decreased from the dilution of 1:125 to 1:16,000 prepared for IgG of first and second bleeding (Fig. 6A). Highest

absorbance value 2.143 was obtained with second bleeding whereas 1.782 was obtained with first bleeding at 1:125 dilutions. In all serological assays IgG prepared from second bleeding was considered.

4.5.2.1.3 Antigen dilution

Antigen dilutions of *S. rolfsii* ranging from 10,000 ng/ml to 78 ng/ml were tested against two antisera dilutions (1:125 and 1:250). ELISA values increased with the concomitant increase of antigen concentration (Table 12 and Fig. 6B). Mycelial antigen concentrations as low as 78 ng/ml could be easily detected by ELISA at both antisera dilutions.

Table 11: ELISA reaction with various dilution of anti *Sclerotium rolfsii* antiserum and homologous antigen

Antiserum dilution	Absorbance at 405 nm	
	First bleeding	Second bleeding
1:125	1.782 ± 0.091	2.143 ± 0.023
1:250	1.647 ± 0.032	1.950 ± 0.034
1:500	1.572 ± 0.055	1.812 ± 0.065
1:1000	1.393 ± 0.052	1.781 ± 0.044
1:2000	1.165 ± 0.044	1.563 ± 0.058
1:4000	0.921 ± 0.009	1.493 ± 0.013
1:8000	0.869 ± 0.006	1.036 ± 0.003
1:16000	0.756 ± 0.012	0.962 ± 0.017

± Standard error

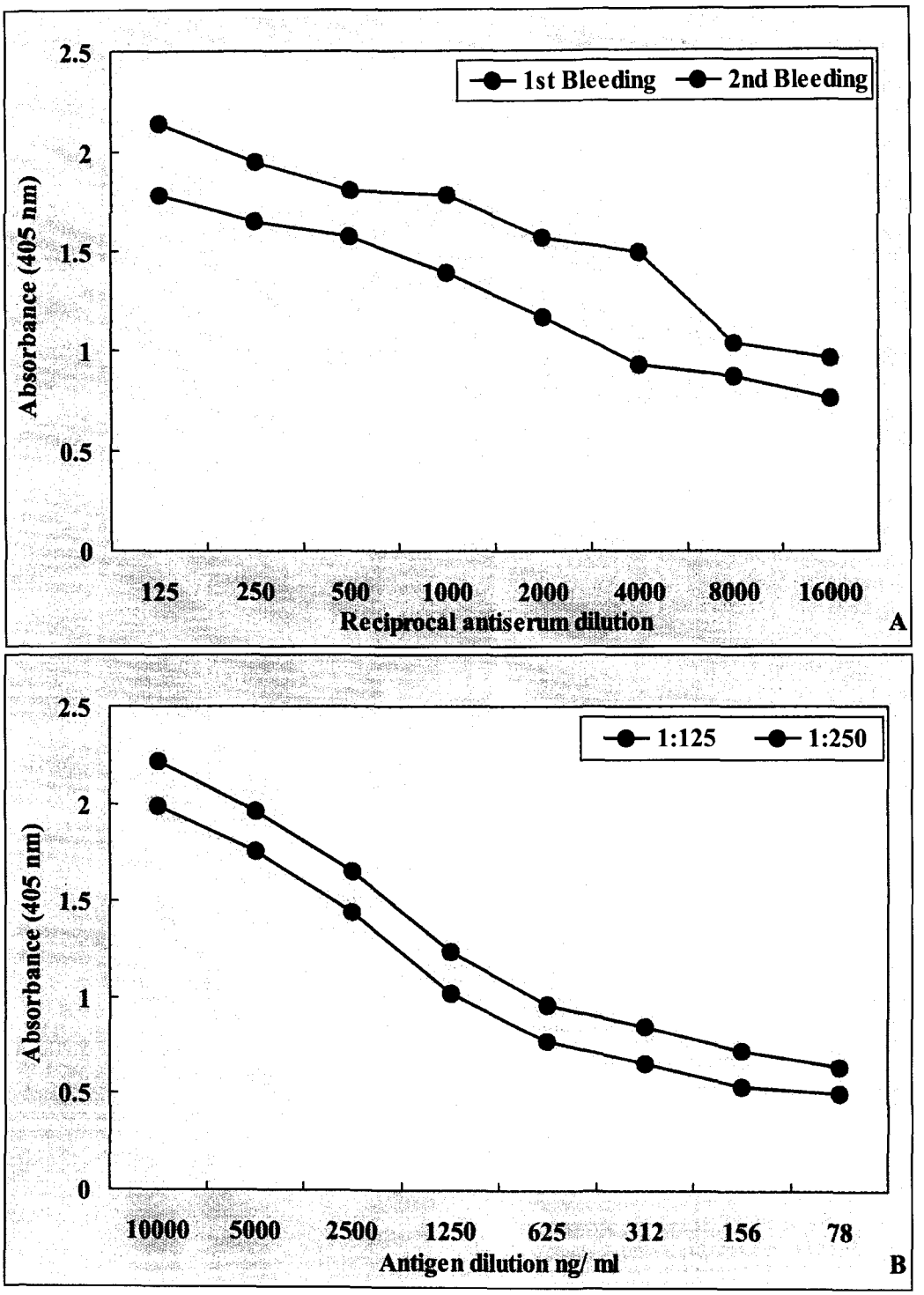

Fig. 6 (A&B). Effect of dilution of antiserum (A) and antigen (B) of *S. rolfsii* on homologous reaction using PTA-ELISA format.

4.5.3. Comparison of ELISA reactivity among antigens of different tea varieties against antiserum of *S. rolfsii*

Among 18 tea varieties tested for varietal resistance towards *S. rolfsii*, differential responses were obtained. Certain varieties were found to be highly susceptible, while others were moderately susceptible or moderately resistant. Disease development to some extent was visible in all the varieties tested. Conventional techniques for determination of host resistance or susceptibility are being replaced by more rapid, sensitive and reproducible modern serological techniques. Detection of pathogen in plant tissue in soil even before appearance of the disease symptom has become routine practice now a days in different agricultural stations. It was therefore considered promising to find out serological cross reactivity of all the tea varieties against the pathogen (*S. rolfsii*) using PTA-ELISA formats.

Table 12: ELISA reaction with various concentration of mycelial antigen of *Sclerotium rolfsii* and homologous antiserum

Antigen dilution (ng/ml)	Absorbance at 405 nm	
	1: 125 [a]	1:250
10000	2.215 ± 0.011	1.984 ± 0.012
5000	1.959 ± 0.015	1.754 ± 0.026
2500	1.657 ± 0.030	1.446 ± 0.036
1250	1.238 ± 0.019	1.026 ± 0.009
625	0.968 ± 0.017	0.883 ± 0.018
312	0.854 ± 0.030	0.656 ± 0.027
156	0.723 ± 0.021	0.54 ± 0.016
78	0.638 ± 0.014	0.506 ± 0.046

[a] Anti-*S. rolfsii* antiserum (dilutions 1:125 and 1:250)

Enzyme dilution: 1:10,000

± Standard error

Table 13: Indirect ELISA values (A 405 nm) of root antigens (host and non host) and mycelial antigens (pathogen and non pathogen) reacted with PAb raised *Sclerotium rolfsii* (isolate Sr-1).

Antigen of host and parasite (40 µg/ml)	**Polyclonal antibody of *S. rolfsii* dilutions**	
Tea varieties	**1:125**	**1:250**
UPASI-2	0.960 ± 0.028	0.822 ± 0.022
UPASI-3	1.039 ± 0.017	0.952 ± 0.017
UPASI-8	1.648 ± 0.026	1.464 ± 0.029
UPASI –9	1.448 ± 0.035	1.349 ± 0.034
UPASI-26	1.128 ± 0.016	1.026 ± 0.023
BSS-2	0.970 ± 0.014	0.896 ± 0.063
TV-18	1.181 ± 0.055	0.953 ± 0.026
TV-22	1.033 ± 0.012	0.899 ± 0.082
TV-25	1.459 ± 0.029	1.260 ± 0.030
TV-26	0.955 ± 0.034	0.778 ± 0.056
TV-30	1.463 ± 0.018	1.276 ± 0.030
T-17	1.745 ± 0.030	1.565 ± 0.030
T-78	1.180 ± 0.023	1.050 ± 0.043
AV-2	1.125 ± 0.024	0.929 ± 0.095
T-135	1.406 ± 0.076	1.096 ± 0.025
B-157	0.942 ± 0.033	1.155 ± 0.011
HV-39	0.862 ± 0.033	0.953 ± 0.026
K1/1	0.844 ± 0.022	0.717 ± 0.012
Pathogen		
S. rolfsii (isolate Sr-1)	2.811 ± 0.016	2.145 ± 0.037
S. rolfsii (isolate Sr-2)	2.059 ± 0.027	1.740 ± 0.039
S. rolfsii (isolate Sr-3)	2.058 ± 0.028	1.734 ± 0.043
Non pathogen		
F. graminearum	0.457 ± 0.033	0.261 ± 0.013
Non host		
O. sativa	0.348 ± 0.032	0.216 ± 0.020

± Standard error.

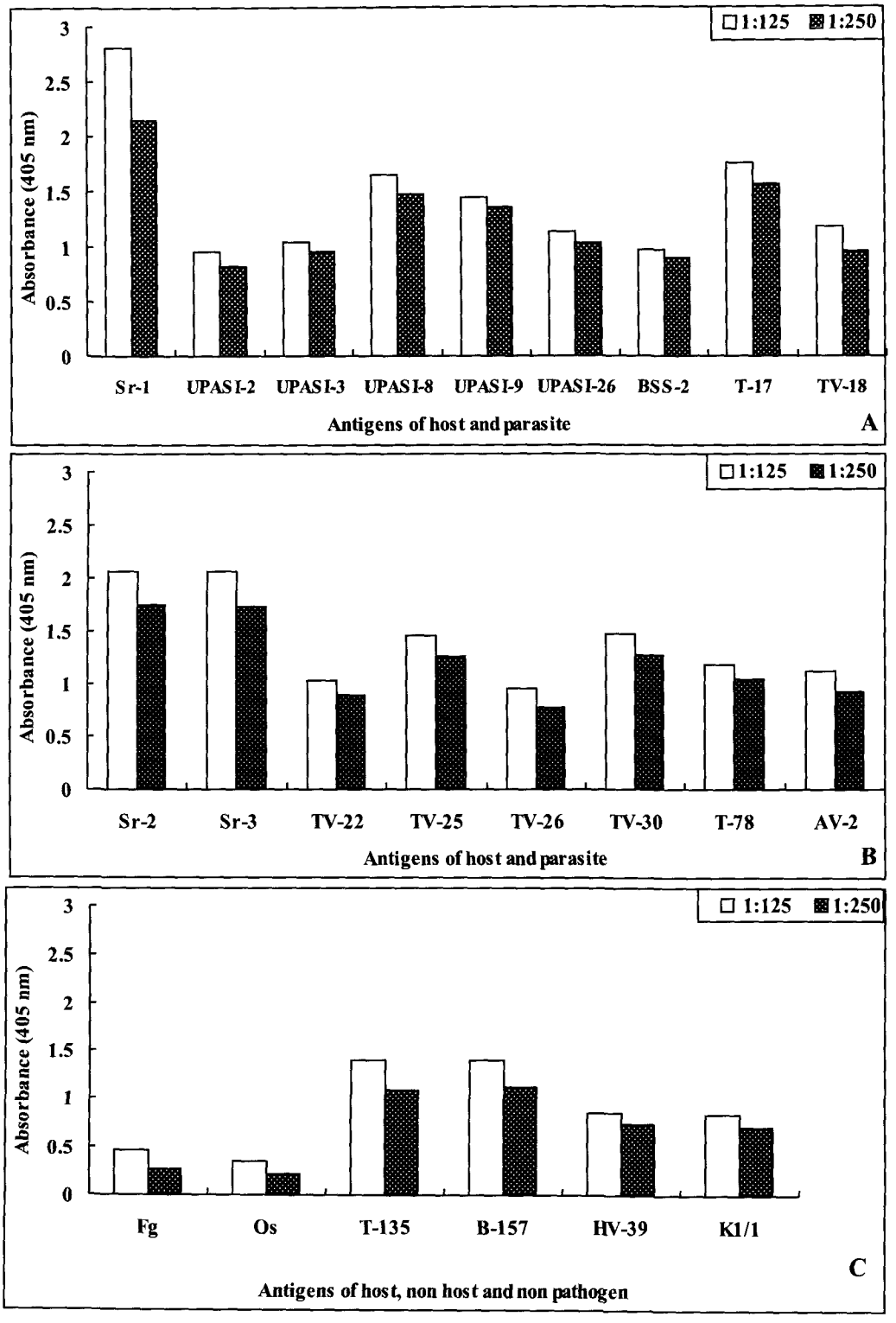

Fig. 7. PTA-ELISA responses of healthy root antigens of different tea varieties against PAb of *S. rolfsii.*

Tea root antigens were prepared from 6 UPASI varieties (UP-2, UP-3, UP-8, UP-9, UP-26 and BSS-2), 5 Tocklai varieties (TV-18, TV-22, TV-25, TV-26 and TV-30) and 7 Darjeeling varieties (T-17, T-78, AV-2, T-135, B-157, HV-39 and K1/1) root antigens of non host (*O. sativa*), mycelial antigens of three isolates of *S. rolfsii* and non pathogen (*F. graminearum*). All of these antigens at a concentration of 40 µg /ml were tested against 1:250 dilution of PAb raised against *S. rolfsii* (isolate Sr-1) by using PTA-ELISA formats. Experiments were repeated thrice keeping same concentrations of antigens and antisera under same incubation conditions. Results have been presented in (Table 13 and Fig. 7).

Reciprocal cross reaction involving antisera B-157 and AV-2 and antigen preparations from all eighteen tea varieties, three isolates of *S. rolfsii*, non host and non pathogen were studied using PTA-ELISA formats. Results have been presented in Table 14 and Figs. 8 & 9. Susceptible varieties showed positive reaction and highest absorbance values were detected in these cases. Whereas resistant varieties exhibited lower absorbance values than susceptible varieties. Absorbance for non host and non pathogen antigen preparation with these antisera were always found to be low.

4.5.4. Cellular location of CRA using immunofluorescence

Fluorescent antibody labeling with FITC is known to be one of the powerful techniques to determine the cell or tissue location of major CRA shared by host and parasite. In the present study following immunodiffusion and PTA-ELISA the presence of CRA shared by *Camellia sinensis* and *S. rolfsii* has been detected. It was decided to determine the tissue and cellular location of CRA in tea root tissue.

Cross sections of healthy tea roots of three susceptible varieties (UPASI-3, Teen Ali-17 and B-157) were treated separately with normal serum, homologous and pathogen antiserum, then reacted with FITC. Root sections exhibited a natural autofluorescence under UV light on the cuticle. Same observation was noted when the root sections were treated with normal serum and FITC. Root sections treated with antiserum of *S. rolfsii* and then reacted with FITC developed bright fluorescence which was distributed throughout the root tissue, mainly in the epidermis, cortex and endodermal cells (Plate 9, figs. A - D and Plate 10, figs A - D).

Table 14: Indirect ELISA values (A 405nm) of root antigens (host and non-host) and mycelial antigens (pathogen and non pathogen) reacted with PAb raised against tea root antigens (AV-2 and B-157).

Antigen of host and parasite (40µg/ml)	**Polyclonal antibody of *S. rolfsii* dilutions**	
Tea varieties	**AV-2**	**B-157**
UPASI-2	0.934 ± 0.032	0.890 ± 0.024
UPASI-3	1.171 ± 0.16	1.775 ± 0.038
UPASI-8	1.623 ± 0.025	1.781 ± 0.081
UPASI –9	1.145 ± 0.014	1.227 ± 0.023
UPASI-26	1.160 ± 0.020	1.086 ± 0.033
BSS-2	0.890 ± 0.073	0.839 ± 0.016
TV-18	1.157 ± 0.017	1.172 ± 0.020
TV-22	1.220 ± 0.027	0.856 ± 0.034
TV-25	1.476 ± 0.052	0.953 ± 0.059
TV-26	0.925 ± 0.020	0.955 ± 0.034
TV-30	1.473 ± 0.052	1.902 ± 0.057
T-17	1.791 ± 0.074	1.056 ± 0.031
T-78	1.186 ± 0.016	1.040 ± 0.013
AV-2	1.194 ± 0.028	1.065 ± 0.031
T-135	1.565 ± 0.033	1.903 ± 0.067
B-157	1.155 ± 0.011	1.942 ± 0.041
HV-39	0.862 ± 0.033	0.935 ± 0.043
K1/1	0.551 ± 0.042	0.935 ± 0.043
Pathogen		
S. rolfsii (isolate Sr-1)	0.577 ± 0.021	0.921 ± 0.029
S. rolfsii (isolate Sr-2)	0.453 ± 0.034	0.855 ± 0.043
S. rolfsii (isolate Sr-3)	0.446 ± 0.036	0.833 ± 0.063
Non pathogen		
F. graminearum	0.268 ± 0.025	0.237 ± 0.022
Non host		
O. sativa	0.364 ± 0.020	0.253 ± 0.029

± Standard error.

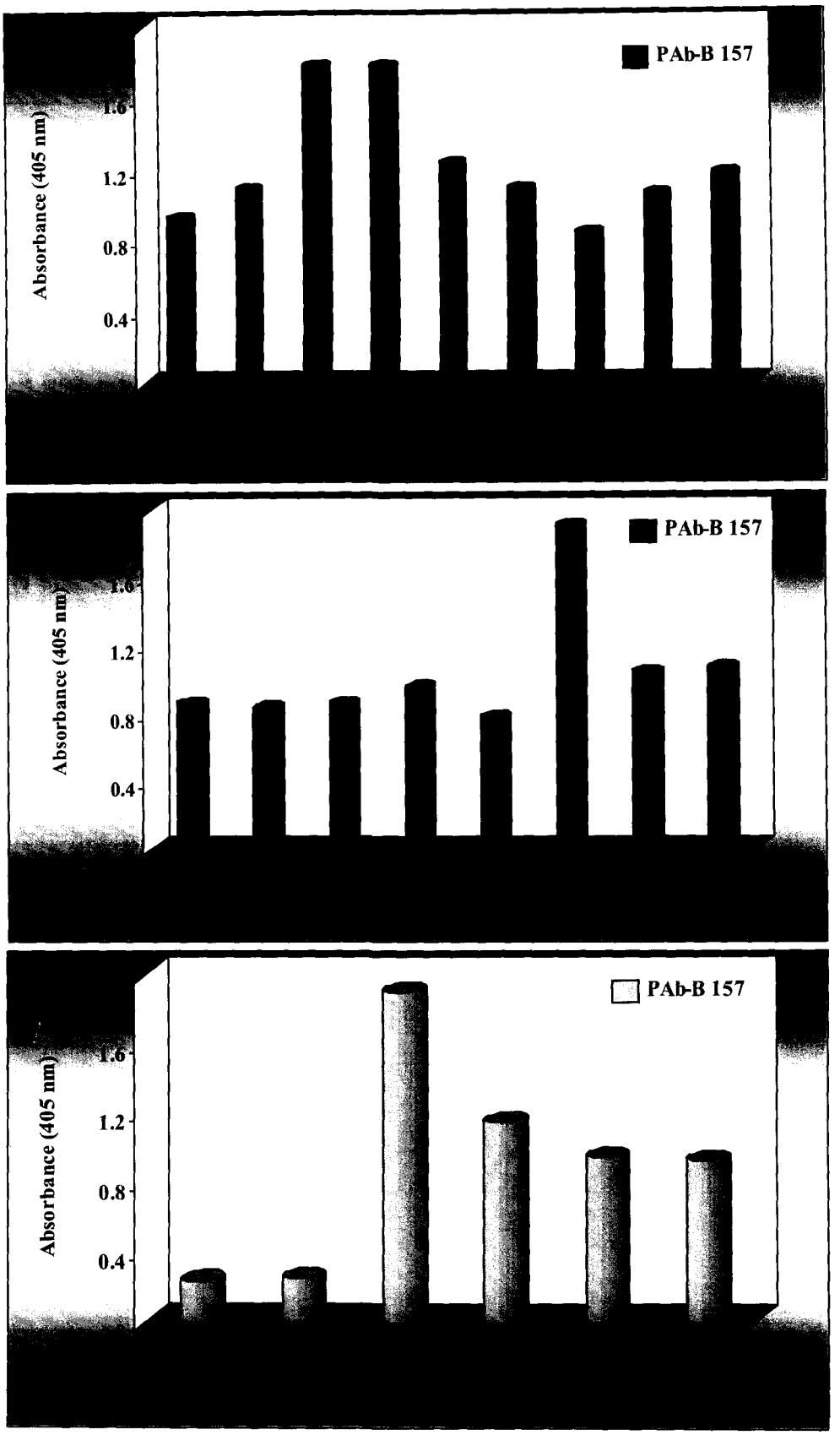

Fig. 8. Reciprocal cross reaction of root antigens of tea varieties (host), non host, mycelial antigen of pathogen and non pathogen with PAb of tea variety (B-157) using PTA-ELISA format.

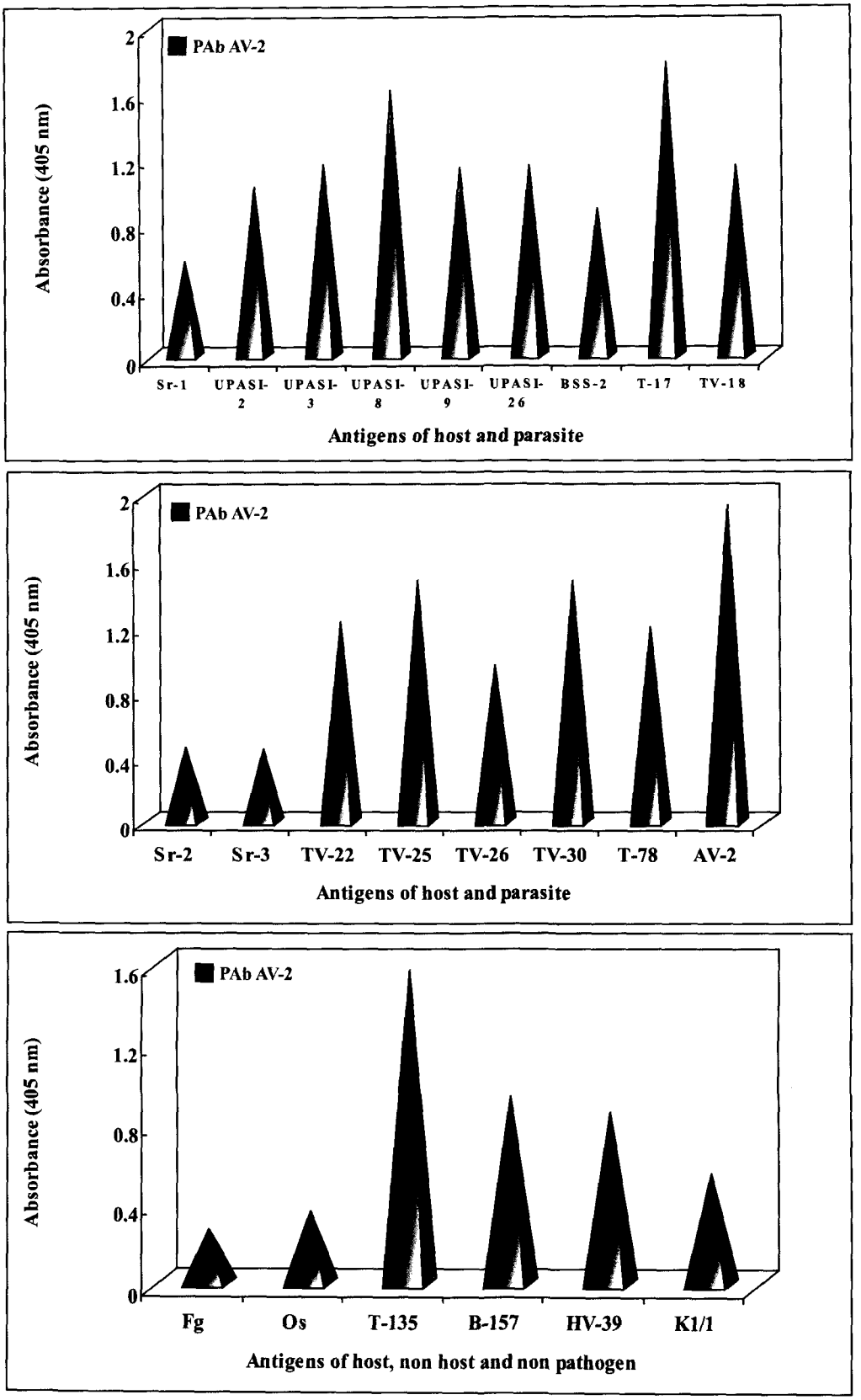

Fig. 9. Reciprocal cross reaction of root antigens of tea varieties (host), non host, mycelial antigen of pathogen and non pathogen with PAb of tea (AV-2) using PTA-ELISA format.

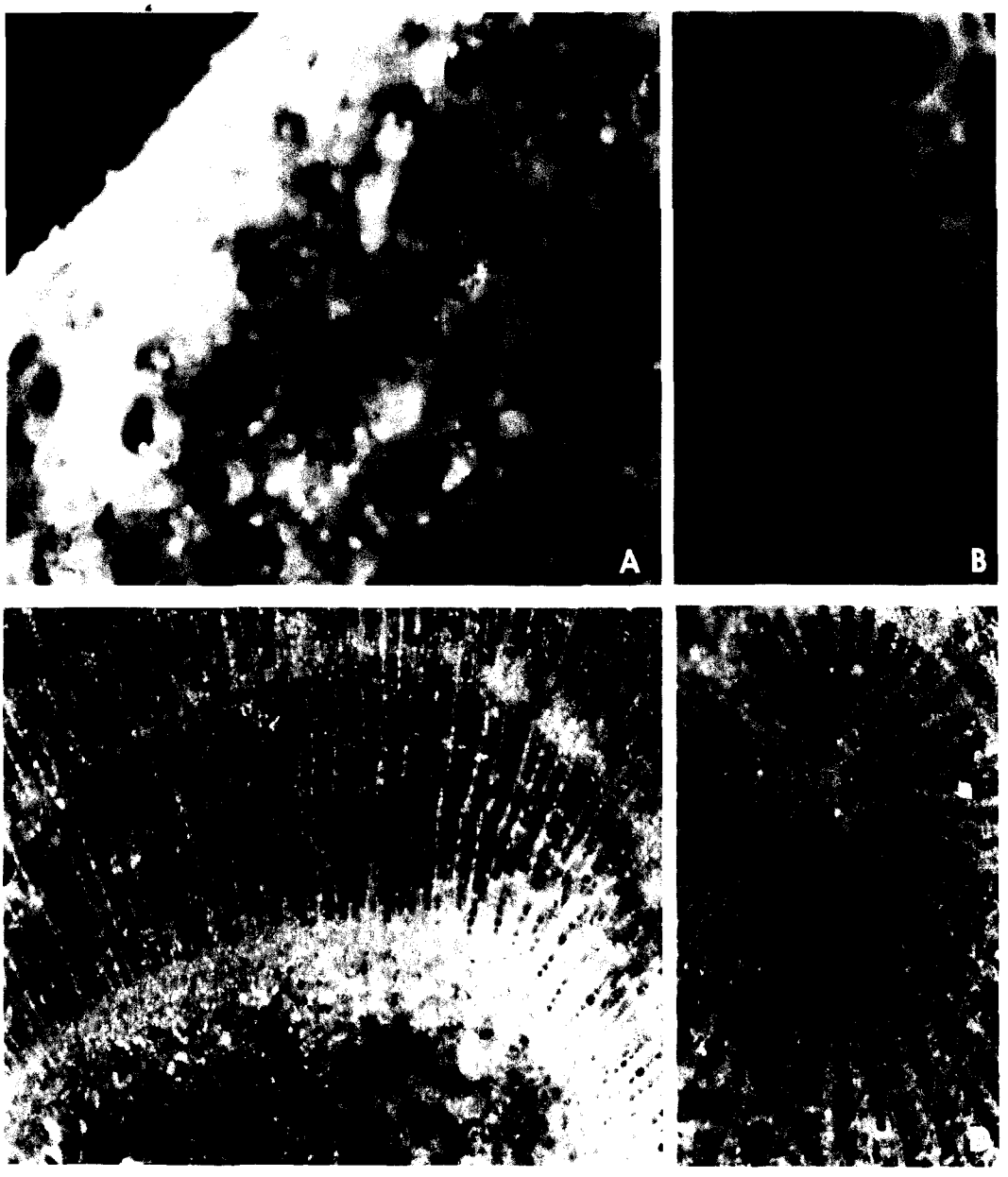

Plate 9 (figs. A-D). Fluorescent antibody staining of tea root tissues for cross reactive antigens shared with *S. rolfsii*. Healthy root tissues treated with PAb of *S. rolfsii* and labeled with FITC antibodies of goat specific for rabbit globulin (A&B) UP-3; (C&D) T-17.

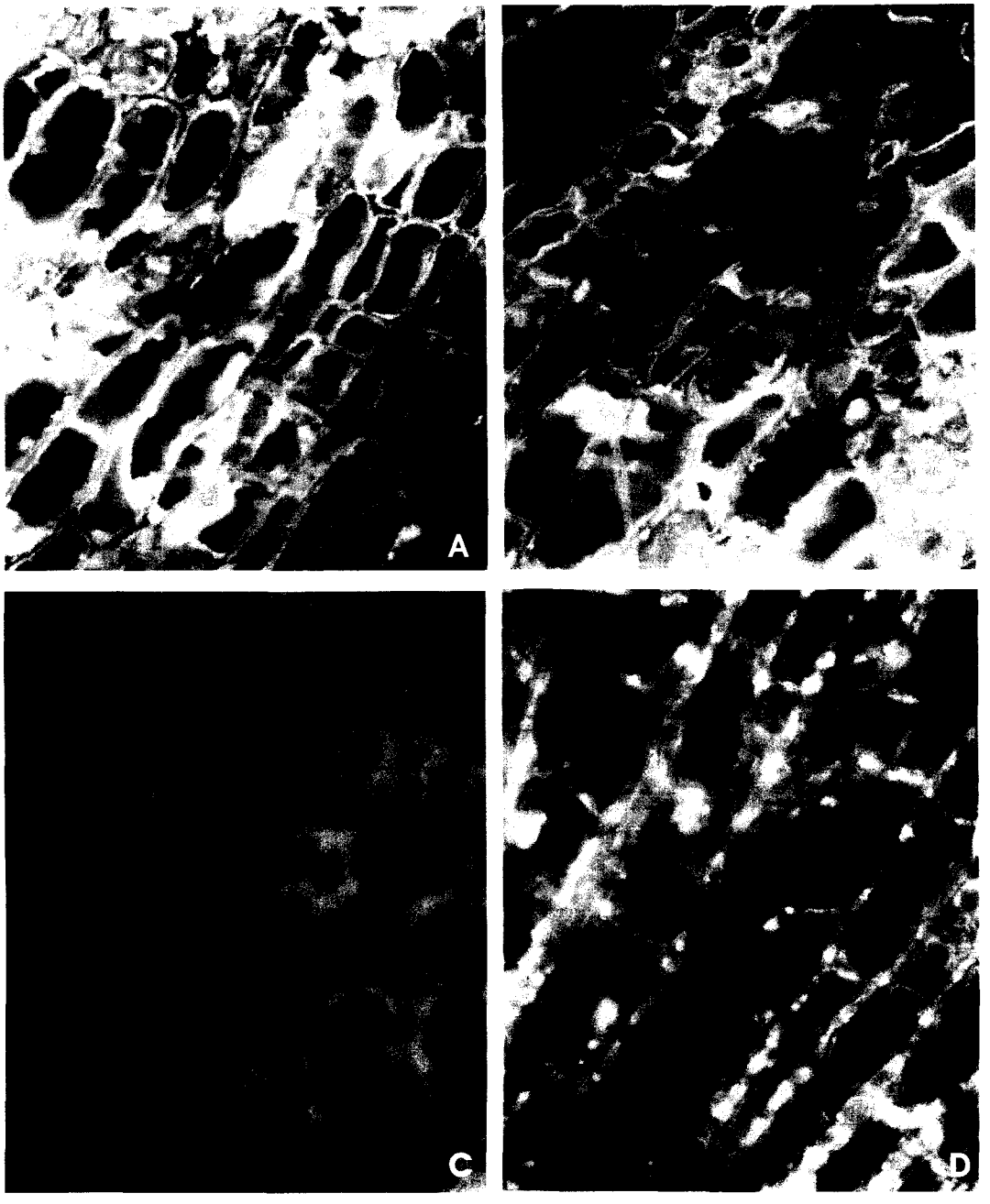

Plate 10 (figs. A-D). Fluorescent antibody staining of tea root tissues (B-157) for cross reactive antigens shared with *S. rolfsii.* Healthy root tissue treated with PAb of *S. rolfsii* and labeled with FITC antibodies of goat specific for rabbit globulin

4.6. Detection of *Sclerotium rolfsii* in artificially inoculated tea root tissue

4.6.1. PTA-ELISA

The efficacy of the antiserum raised against *S. rolfsii* was tested for its ability to detect the pathogen in infected root tissue by PTA-ELISA. For this experiment the roots were artificially inoculated with *S. rolfsii* for 15 days. After that antigens were prepared from healthy as well as infected tea roots of five varieties (TV-30, AV-2, Teen Ali-17, UPASI-8 and UPASI-9) and tested by using PTA-ELISA formats. Results have been presented in Table 15. Higher absorbance values were recorded in all the tested inoculated varieties (after 15 days of inoculation) in comparison to healthy root antigens.

Table 15: Absorbance value in PTA-ELISA reactions with healthy and inoculated tea root antigens

Antigens (40 µg/ml)	Absorbance values at 405 nm [a]	
	Healthy	Inoculated
AV-2	1.434 ± 0.02	1.986 ± 0.04
T-17	1.493 ± 0.01	1.781 ± 0.04
UP-8	1.393 ± 0.05	1.781 ± 0.04
UP-9	1.103 ± 0.02	1.433 ± 0.03
TV-30	1.755 ± 0.02	2.058 ± 0.02

[a] PAb of *S. rolfsii*

± Standard error

4.6.2 Dot immunobinding assay

Dot immunobinding technique is a rapid and sensitive method for detection of pathogen in the soil and the root tissue. In the present study, dot blot was used to detect pathogen in healthy and *S. rolfsii* inoculated tea root tissue as well as amended soil.

The antigen preparations from healthy and artificially inoculated tea root tissues were spotted on nitro-cellulose paper carefully and tagged with antiserum of *S. rolfsii*. This was finally probed with the conjugates. Results have been presented in Plate 11 fig. A. Clear and intense colour reactions were observed in case of mycelial antigens prepared from three isolates (Sr-1, Sr-2 and Sr-3) of the pathogen (Plate 11, fig. B) Cross-reactivity between other soil pathogens (*Fomes lamaoensis*, *Fusarium oxysporum* f.sp. *lycopersici*, *Aspergillus niger* and *Poria hypobrumea*) were also examined. Those fungi when reacted with PAb of *S. rolfsii*, showed various intensities of colour in cross reactivities (Plate 11, fig B and Table 16).

Table 16: Dot-blot reaction of antigens of different pathogens with PAb of *Sclerotium rolfsii*

Antigen source	**Colour intensity** [a]
Fusarium oxysporum f. sp. *lycopersici*	+
S. rolfsii (Sr-1)	++++
Poria hypobrumea	+
S. rolfsii (Sr-2)	++++
Aspergillus niger	++

[a] Colour intensity of dots: + very light violet ; ++ light violet; +++ violet; ++++ deep violet.
PAb concentration: 40 µg / ml; NBT/BCIP used as substrate.

4.6.3. Western blot

Molecular probing of mycelial antigens of three isolates of *S. rolfsii* (Sr-1, Sr-2 and Sr-3) was also performed with PAb of *S. rolfsii* using western blotting technique. It revealed two bands for all three isolates, whereas the isolate Sr-1, for which polyclonal antibodies were prepared yielded more profuse bands (Plate 11, figs C&D).

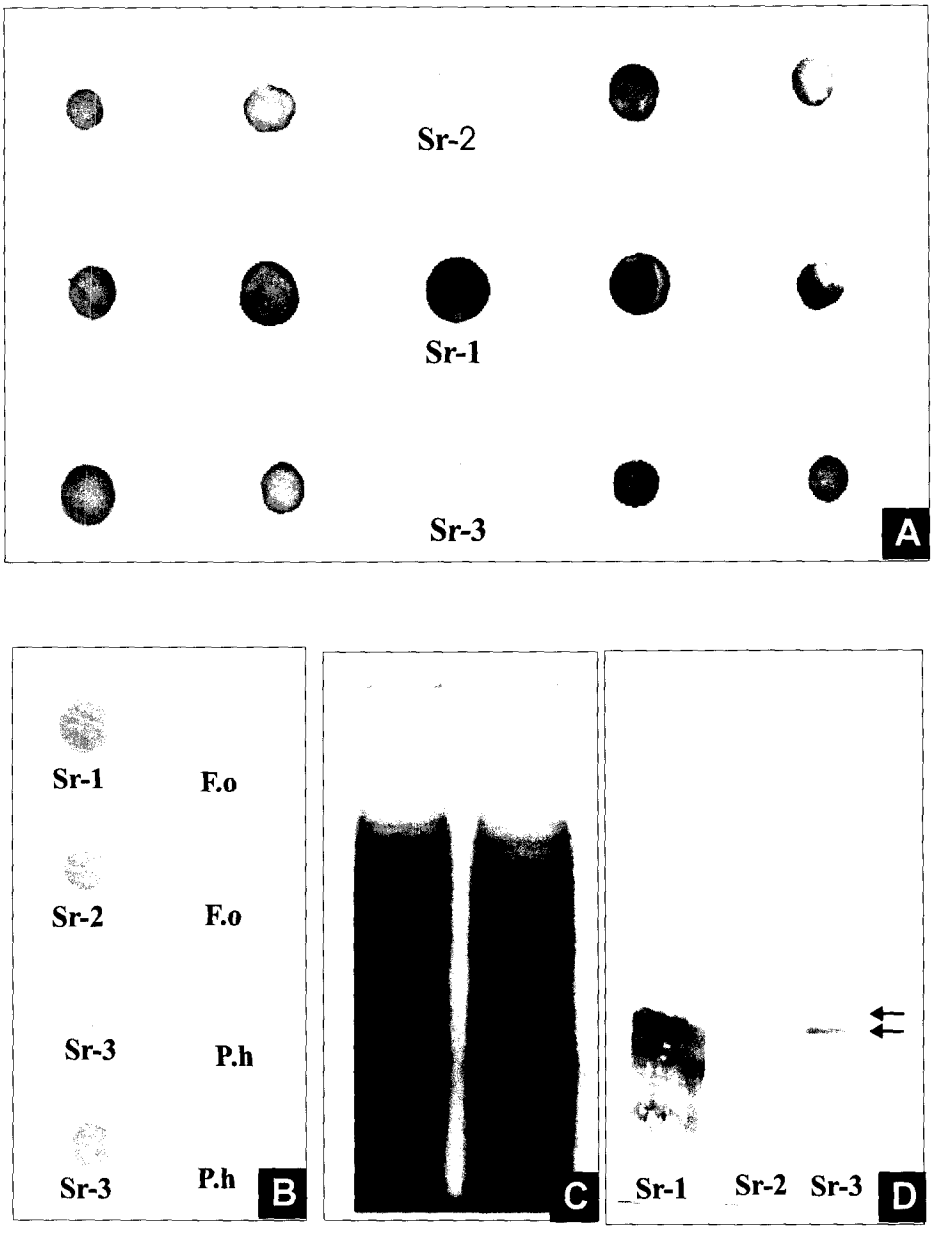

Plate 11 (figs A-D): Dot immunobinding assay and Western blot analysis of mycelial antigens with PAb of *S. rolfsii*. (A&B) Antigens of *S.rolfsii* (isolates Sr-1, Sr-2 & Sr-3), *F.oxysporum* f.sp. *lycopersici, Poria hypobrumea* reacted with PAb of *S. rolfsii*. (C) SDS-PAGE analysis of mycelial antigens of *S. rolfsii* (isolate Sr-1), (D) Western blot analysis of mycelial antigens of *S. rolfsii* (isolates Sr-1, Sr-2 & Sr-3)

4.6.4. Indirect immunofluorescence

4.6.4.1. Mycelia

Mycelia of three isolates of *S. rolfsii* were not autofluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of mycelia of *S. rolfsii* with homologous antiserum and FITC showed a general fluorescence that was more intense on young hyphal tips (Plate 12, figs. A-D).

4.6.4.2. Sclerotia

Sclerotia of three isolates were also not autofluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of young germinated sclerotia of *S. rolfsii* with homologous antiserum and FITC showed more intense fluorescence (Plate 12 figs E&F).

4.6.4.3. Tea root tissue

Three varieties of tea plants (UPASI-3, Teen Ali-17 and B-157) were artificially inoculated with *S. rolfsii*. After 20 days of inoculation, cross sections of healthy as well as inoculated tea roots of the three varieties were treated separately with normal serum as well as pathogen antiserum and then reacted with FITC. Root sections exhibited a bright apple green fluorescence under UV light on the epidermis, cortical tissue and endodermal layers. Fungal hyphal penetration within the tissue elements was visible in all three varieties tested (Plate 13, figs A-F).

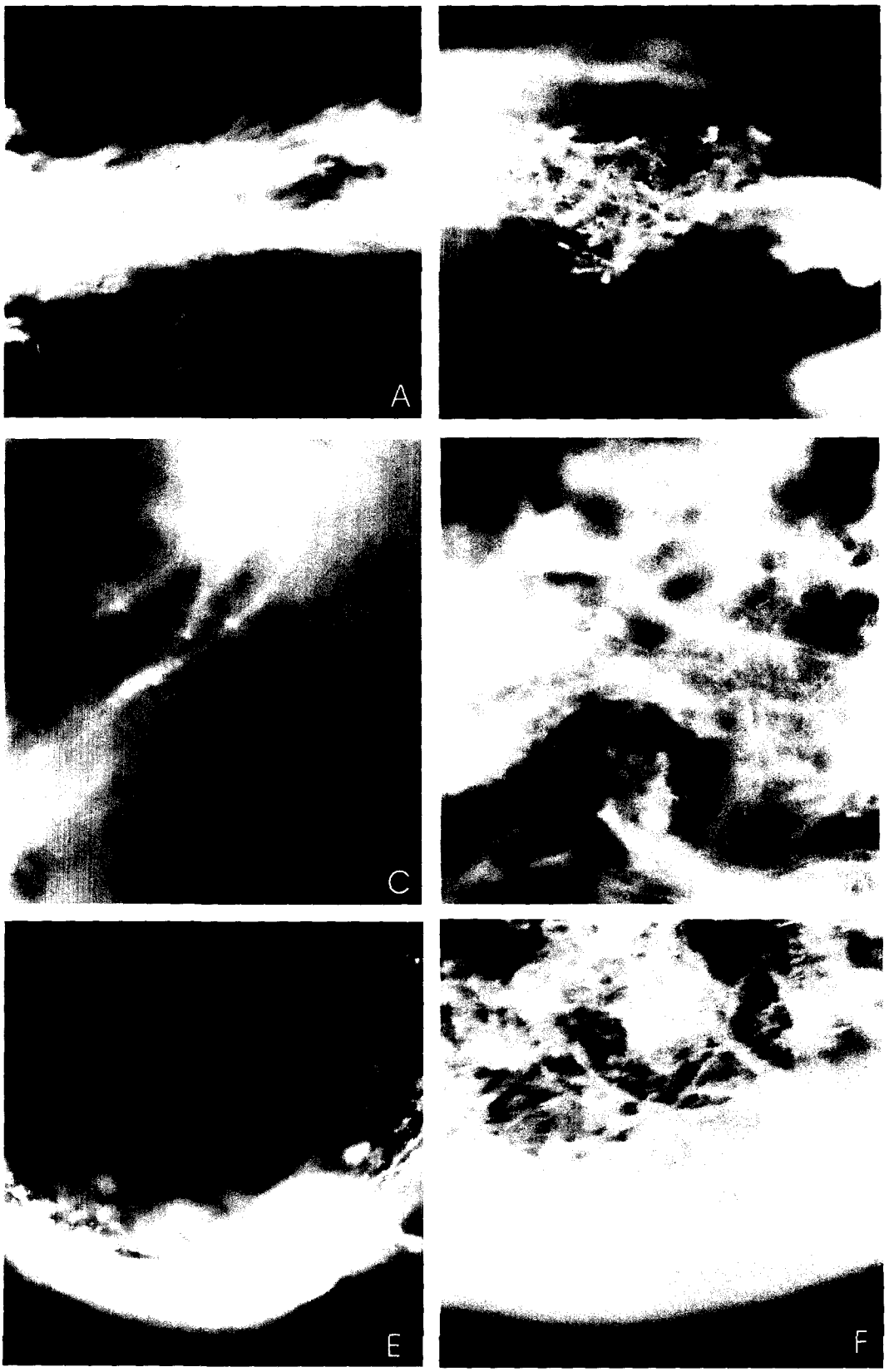

Plate 12 (figs. A-F). Indirect immunofluorescence of hyphae (A-D) and Sclerotia (E-F) of *S. rolfsii* treated with PAb of *S. rolfsii* and FITC labeled antibodies of goat specific for rabbit globulin.

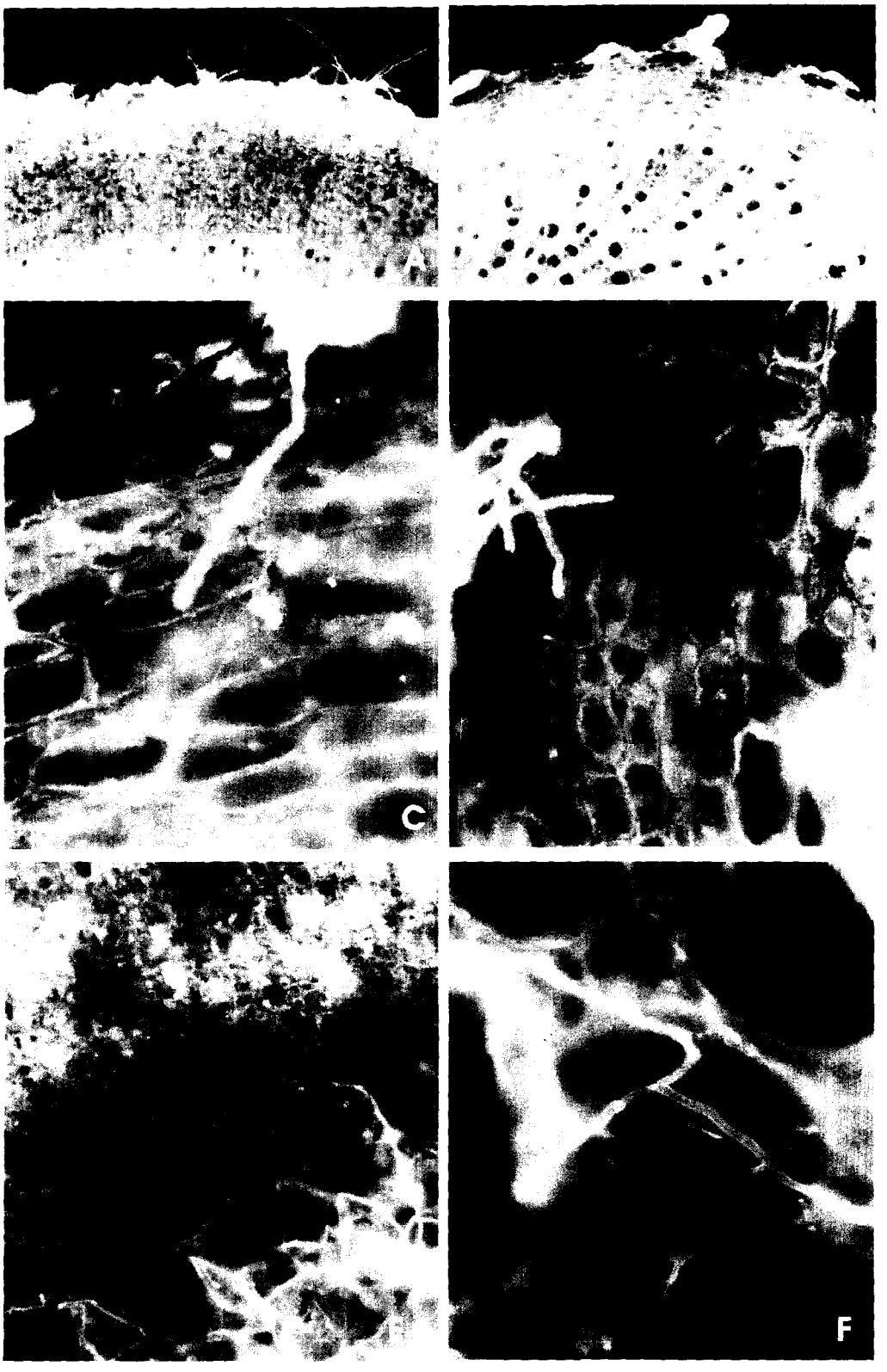

Plate 13 (figs. A-F). Fluorescent antibody staining of infected tea root tissues (A&B: UP-3; C&D: T-17 and E&F: B-157) treated with PAb of *S. rolfsii* and labeled with FITC antibodies of goat specific for rabbit globulin.

4.7. Determination of levels of phenolics in tea roots of resistant and susceptible varieties following inoculation with *S. rolfsii*

In many cases there is a greater increase in phenolics biosynthesis in resistant host species than susceptible ones (Mahadevan, 1991). As polyphenols are the major constituents of tea roots it was decided to compare quantitative changes in the phenolics of resistant and susceptible varieties. At the onset, the simple phenolics present in the healthy roots were characterized following which quantitative estimation of total phenol and ortho-dihydroxy phenol was done.

4.7.1. Total phenols

Total phenols from healthy and *S. rolfsii* inoculated tea roots of 17 tea varieties (T-17, TV-18, TV-22, TV-25, TV-26,TV-30, UP-2, UP-3, UP-8, UP-26, BSS-2, B-157, AV-2, T-78, T-135, K-1/1 and HV-39) were extracted after 7 days of inoculation and estimated. Results are given in Table 17 and Fig. 10. Total phenol content decreased following inoculation with *S. rolfsii* in the susceptible varieties. However, there was an increase in the phenol content of resistant varieties following inoculation. Among all the varieties tested, K1/1 showed maximum increase in total phenol following inoculation with *S. rolfsii* .

4.7.2. Ortho-dihydroxy phenols

Ortho-dihydroxy phenols from healthy and *S. rolfsii* inoculated tea roots of 18 varieties were also extracted after 7 days of inoculation and estimation. The method of extraction and estimation has been described in detail under materials and methods. Results (Table 18 and Fig. 11) revealed that ortho-dihydroxy phenol content decreased in susceptible varieties (T-17, TV-18, TV-22, TV-25, TV-30, UP-3, UP-8, UP-26, B-157, AV-2, T-78 and T-135) and increased in resistant varieties (UP-2, TV-26, BSS-2, K-1/1 and HV-39) following inoculation with *S. rolfsii.* Among 5 of the resistant varieties tested, tea roots of K 1/1, followed by TV-26 and BSS-2 showed maximum increase in orthodihydroxy phenol content after inoculation with *S. rolfsii.*

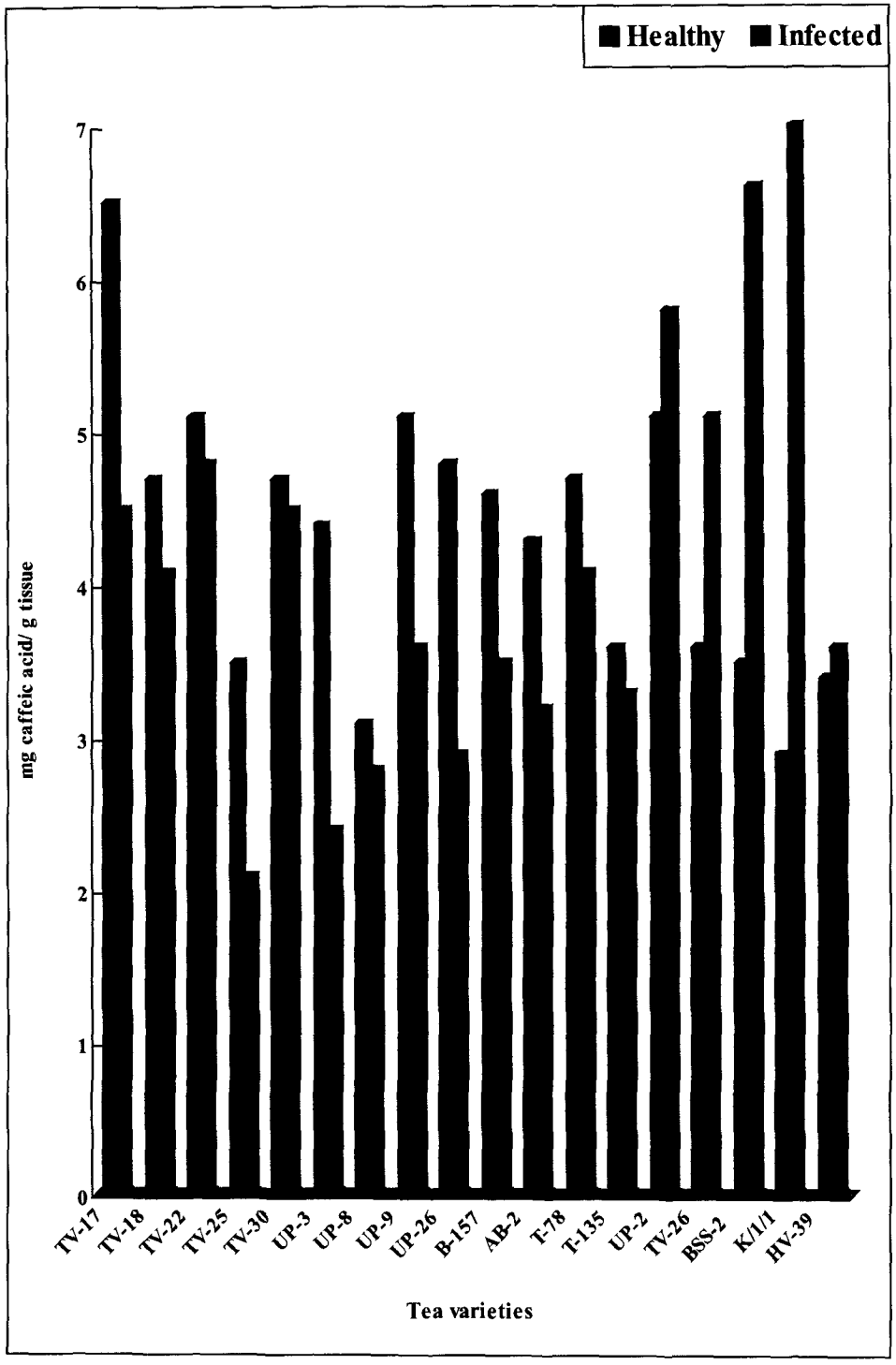

Fig. 10. Total phenol content in tea varieties following inoculation with *S. rolfsii*

Table 17: Total phenol content in healthy and *Sclerotium rolfsii* inoculated tea roots

Tea Varieties	Total phenol (mg/g tissue)[a]	
	Healthy	Inoculated [b]
Susceptible		
T-17	65	4.5
TV-18	4.7	4.1
TV-22	5.1	4.8
TV-25	3.5	2.1
TV-30	4.7	4.5
UP-3	4.4	2.4
UP-8	3.1	2.8
UP-9	5.1	3.6
UP-26	4.8	2.9
B-157	4.6	3.5
AV-2	4.3	3.2
T-78	4.7	4.1
T-135	3.6	3.3
Resistant		
UP-2	5.1	5.8
TV-26	3.6	5.1
BSS-2	3.5	6.6
K 1/1	2.9	7.0
HV-39	3.4	3.6

[a] Average of three replicates
[b] 7 days following inoculation with *S. rolfsii*

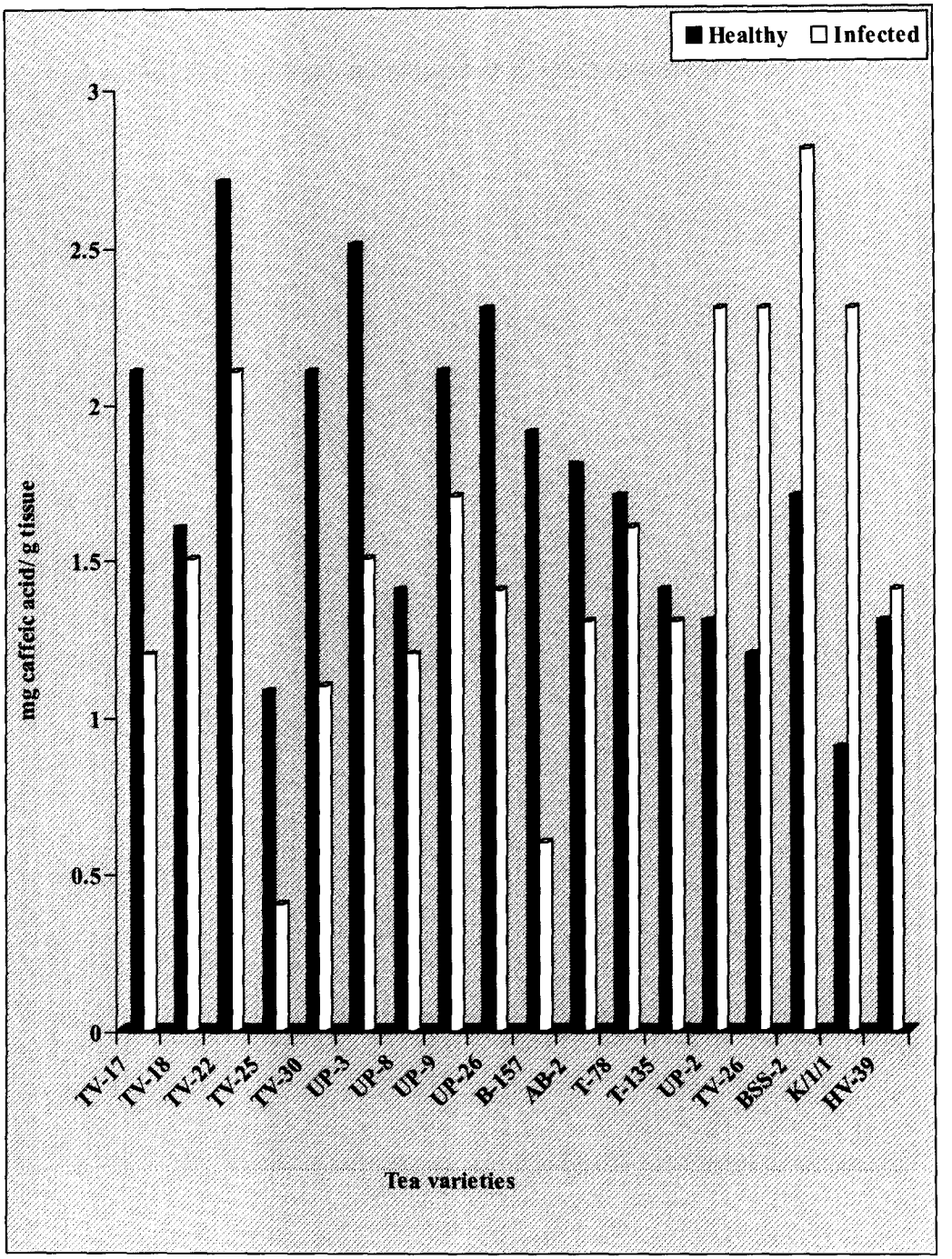

Fig. 11. Orthodihydroxy phenol content in healthy and *S. rolfsii* inoculated roots of tea varieties.

Table 18: Level of Ortho-dihydroxy phenol in healthy and *Sclerotium. rolfsii* inoculated tea roots

Tea Varieties	Ortho-dihydroxy phenol (mg / g root tissue)[a]	
	Healthy	Inoculated [b]
Susceptible		
T-17	2.1	1.2
TV-18	1.6	1.5
TV-22	2.7	2.1
TV-25	1.08	0.4
TV-30	2.1	1.1
UP-3	2.5	1.5
UP-8	1.4	1.2
UP-9	2.1	1.7
UP-26	2.3	1.4
B-157	1.9	0.6
AV-2	1.8	1.3
T-78	1.7	1.6
T-135	1.4	1.3
Resistant		
UP-2	1.3	2.3
TV-26	1.2	2.3
BSS-2	1.7	2.8
K 1/1	0.9	2.3
HV-39	1.3	1.4

[a] Average of three replicates
[b] 7 days following inoculation with *S. rolfsii*

4.7. 3. Analysis of antifungal compound in tea roots following inoculation with *S. rolfsii*

In the present investigation further experiments were carried out following facilitated diffusion technique for the detection of antifungal phenolics from

relatively large samples of freshly harvested healthy tea roots as well as *S. rolfsii* inoculated roots. Antifungal compounds were extracted separately from healthy and S.rolfsii inoculated tea roots of two resistant varieties (K 1/1 and TV-26) and two susceptible varieties (B-157 and UP-3) after 96 h of inoculation. Ethyl acetate fractions of both healthy and *S. rolfsii* 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 0.58.

4.7.3.1. Bioassay

Crude extract (ethyl acetate fraction dissolved in methanol) prepared from healthy and *S. rolfsii* inoculated roots of four varieties (K 1/1, TV-26, B-157 and UP-3) were bioassayed following radial growth inhibition assay. Results (Table 19) revealed that mycelial growth of *S. rolfsii* was inhibited markedly in the medium supplemented with the extracts of inoculated roots of resistant varieties (K1/1 and TV-26) than those of susceptible varieties (B-157 and UP-3) tested in relation to their respective control (media supplemented with healthy root extract). Mycelial growth was measured in each treatment, when *S. rolfsii* covered full petridish (3 mm dia) grown in PDA without any supplementation. It is interesting to note that sclerotial germination of *S. rolfsii* was completely inhibited, when tested directly with extract of inoculated roots of K 1/1 in glass slide bioassay in relation to distilled water control wherein full sclerotial germination was evident.

4.7.3.2. UV-spectrophotometric analysis

Results of the bioassay revealed the presence of antifungal compounds in inoculated tea roots. Partially purified compound (Rf 0.58) from extracts of healthy and inoculated tea roots (variety K 1/1) were examined in a UV-spectrophotometer (Figs. 12 A&B) . It is interesting to note that extracts from *S. rolfsii* inoculated root tissues gave a peak at 274 nm. Maximum absorption peak measured at 274 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 274 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 (525-678 µg/g fresh wt) of antifungal compound (pyrocatechol) accumulated in resistant varieties than in the susceptible varieties (212-290 µg/g fresh wt). Concentration of this compound in healthy root tissues were very low (60-93 µg/g fresh wt).

Table 19: Effect of antifungal compounds extracted from healthy and inoculated tea root extracts on radial growth of *Sclerotium rolfsii*

Variety	**Diameter of mycelial growth (mm)** [a]	
	Healthy	**Inoculated**[b]
Resistant		
K 1/1	15.5	6.3
TV-26	14.2	7.0
Susceptible		
B- 157	19.8	11.6
UP-3	20.0	13.5
Distilled water control	30	

a Average of three experimental sets

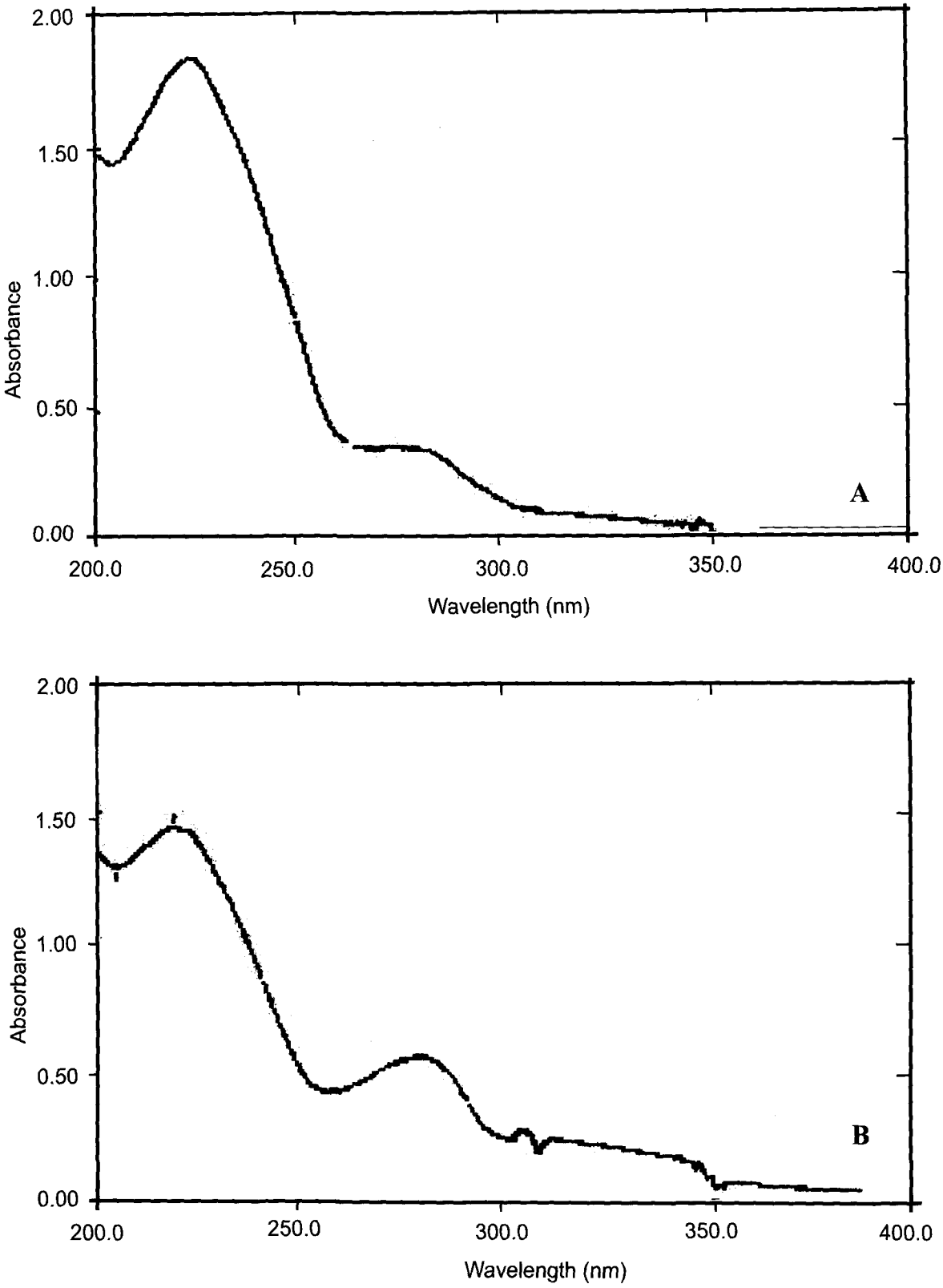

Fig. 12. (A&B): UV-spectrophotometric analysis of antifungal phenolics of tea plants(K1/1) [A] Healthy root, [B] *S. rolfsii* inoculated root.

4.7.3.3. HPLC analysis

Antifungal phenolics extracted from healthy and artificially inoculated (with *S. rolfsii*) tea root samples (variety K 1/1) were used for HPLC analysis. The elution pattern of the phenolic compounds is illustrated in Fig. 13 (A&B). In both cases noticeable peaks were resolved (Table 20), however, in the inoculated sample one new peak was evident.

Table 20: HPLC analysis of antifungal phenolics in tea root tissue (variety K 1/1)

Sample	Peak no.	Retention time (min)	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
Healthy	P1	2.9	97995.1	991.5	1.2	87.9	76.4
	P2	3.1	1385.3	63.5	0.3	1.2	4.9
Inoculated[a]	P1	2.8	31350.3	1007.2	0.4	20.4	22.1
	P2	3.1	2734.9	98.9	0.5	2.4	2.7
	P3	4.4	2090.8	29.1	0.7	0.8	0.6

a Inoculated with *S. rolfsii*

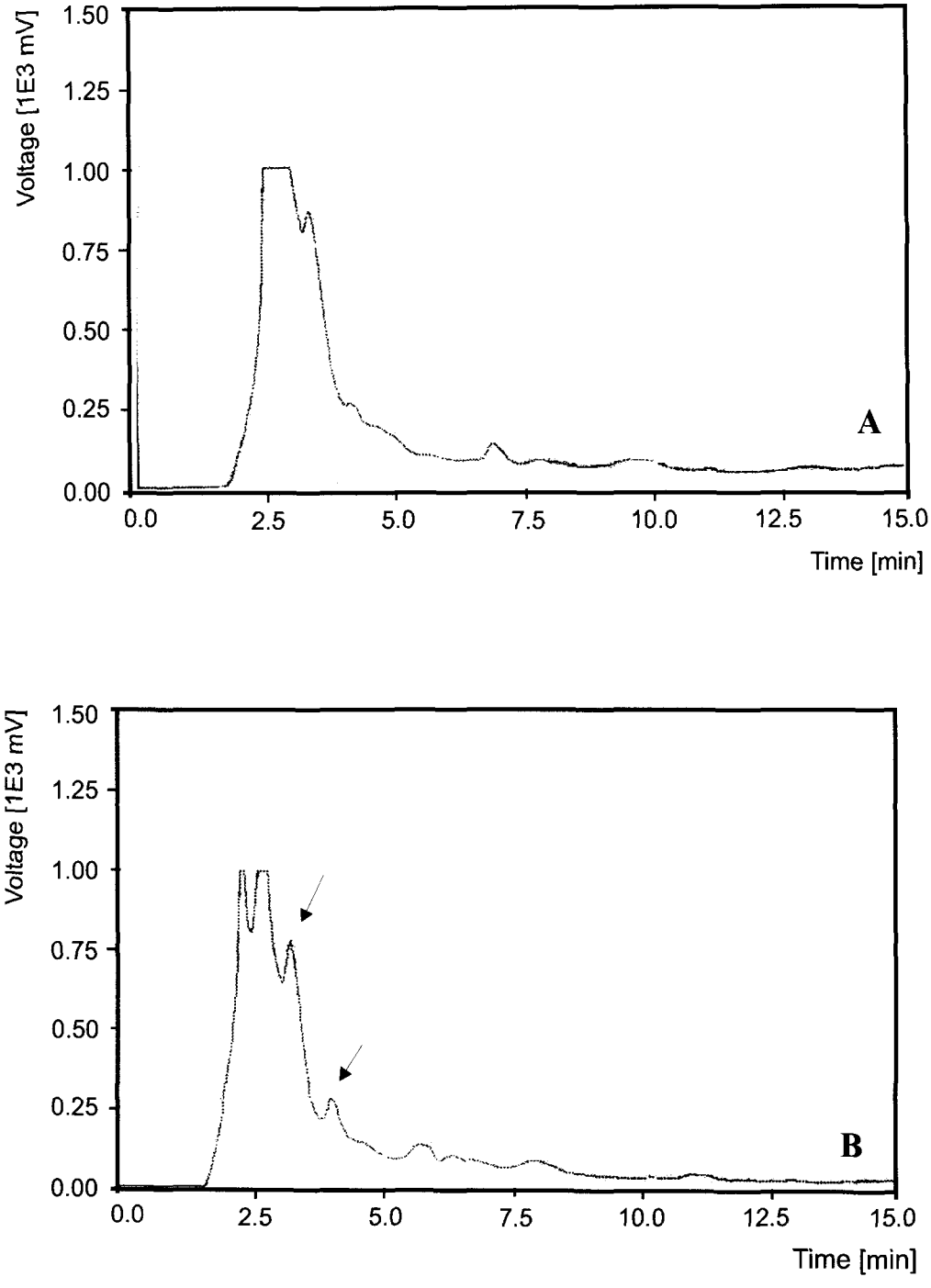

Fig. 13 (A & B). HPLC elution profiles of antifungal phenolics of tea plants (K1/1) (A) Healthy root (B) *S. rolfsii* inoculated root

4.8. Determination of enzyme activity in healthy and *S. rolfsii* inoculated tea roots

4.8.1. Phenylalanine ammonia lyase (PAL)

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, phytoalexins and lignins, three key factors responsible for disease resistance. In the present study, activity of phenylalanine ammonia lyase was assayed in 18 different tea root varieties following inoculation with *S. rolfsii*. PAL activity was assayed in each case after 2, 4 and 8 days after inoculation. Results have been presented in Table 21. It showed that PAL activity increased after 4 days of inoculation in TV-18, TV-25, TV-30, UP-26, AV-2, T-78, T-135, UP-2, BSS-2, K-1/1 and HV-39 markedly. However, highest increase in PAL activity was seen tea roots of TV-26 after inoculation with *S. rolfsii* (Fig.14).

4.8.2. 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 oxidized by the enzyme. Peroxidase was extracted from healthy and *S. rolfsii* inoculated tea roots of 18 varieties and their activity was assayed after 2, 4 and 8 days of inoculation. Results have been presented in Table 22. Peroxidase activity also increased in all the varieties tested, highest increase was noticed in HV-39 after 4 days of inoculation (Fig. 15).

4.8.3. Polyphenol oxidase (PPO)

PPO activity in tea roots increased markedly after 4 days of inoculation with *S. rolfsii* in all the varieties tested. Results have been presented in Table 23 and Fig. 16.

Table 21: Changes in phenylalanine ammonia lyase activity in tea roots following inoculation with *Sclerotium rolfsii*

Tea Varieties	PAL activity in tea roots (µg cinnamic acid $g^{-1}m^{-1}$)[a]					
	2		4		8	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
BSS-2	144.3 ± 1.54	90.8 ± 0.96	136.6 ± 0.63	151.5 ± 0.81	132.6 ± 0.49	42.1 ± 1.24
UP-2	107.9 ± 0.26	72.6 ± 0.66	31.6 ± 0.26	249.1 ± 2.13	105.9 ± 0.47	35.3 ± 0.84
UP-3	117.1 ± 0.53	101.7 ± 0.32	127.8 ± 1.17	111.94 ± 0.31	121.67 ± 1.04	55.6 ± 0.49
UP-8	84.08 ± 0.62	70.4 ± 0.52	89.5 ± 0.47	144.8 ± 0.59	92.2 ± 2.20	56.4 ± 0.37
UP-9	56.08 ± 0.42	30.7 ± 0.62	58.9 ± 0.32	92.1 ± 0.23	55.2 ± 0.85	29.5 ± 0.77
UP-26	165.3 ± 1.02	74.3 ± 1.31	155.06 ± 0.47	148.9 ± 0.49	159.9 ± 0.51	27.7 ± 0.9
TV-18	164.6 ± 1.17	37.01 ± 0.58	167.2 ± 0.70	175.06 ± 1.77	162.3 ± 0.47	140.5 ± 1.89
TV-22	89.2 ± 0.81	22.7 ± 0.39	32 ± 0.40	150.5 ± 0.40	94.9 ± 0.44	77.1 ± 0.79
TV-25	87 ± 0.53	156.03 ± 0.38	84.2 ± 0.75	165.4 ± 0.32	81.2 ± 0.86	130.07 ± 0.09
TV-26	72.7 ± 1.51	58 ± 0.40	76.1 ± 1.31	175.9 ± 0.53	81.9 ± 1.24	98.6 ± 0.98
T-17	64.9 ± 0.28	74.8 ± 0.47	116.8 ± 0.47	131.2 ± 0.95	121.8 ± 0.47	90.3 ± 0.81
TV-30	95 ± 1.98	28.0 ± 0.57	92.27 ± 0.65	135.8 ± 0.54	90.6 ± 0.42	52 ± 0.57
T-78	91.6 ± 0.48	49.6 ± 0.54	94.7 ± 1.03	165.2 ± 0.38	89.7 ± 0.32	143.2 ± 0.14
AV-2	33.7 ± 0.44	78.8 ± 0.5	43.8 ± 0.37	128.8 ± 0.59	45.9 ± 0.98	100.8 ± 0.29
T-135	108.8 ± 0.65	68.06 ± 0.75	91.7 ± 0.64	296.2 ± 0.53	96.2 ± 0.65	103.9 ± 0.37
B157	73.6 ± 0.59	91.4 ± 0.94	74.2 ± 1.14	136.6 ± 0.32	77.5 ± 0.56	111.4 ± 0.28
HV-39	132.6 ± 0.49	170.7 ± 0.62	84.3 ± 0.94	178.8 ± 0.51	135.1 ± 0.83	64.8 ± 0.12
K1/1	133.06 ± 0.47	165.2 ± 0.57	135.9 ± 0.53	234.06 ± 0.41	142.9 ± 0.94	65.9 ± 0.5

[a] Average of 3 replicates.
± Standard error

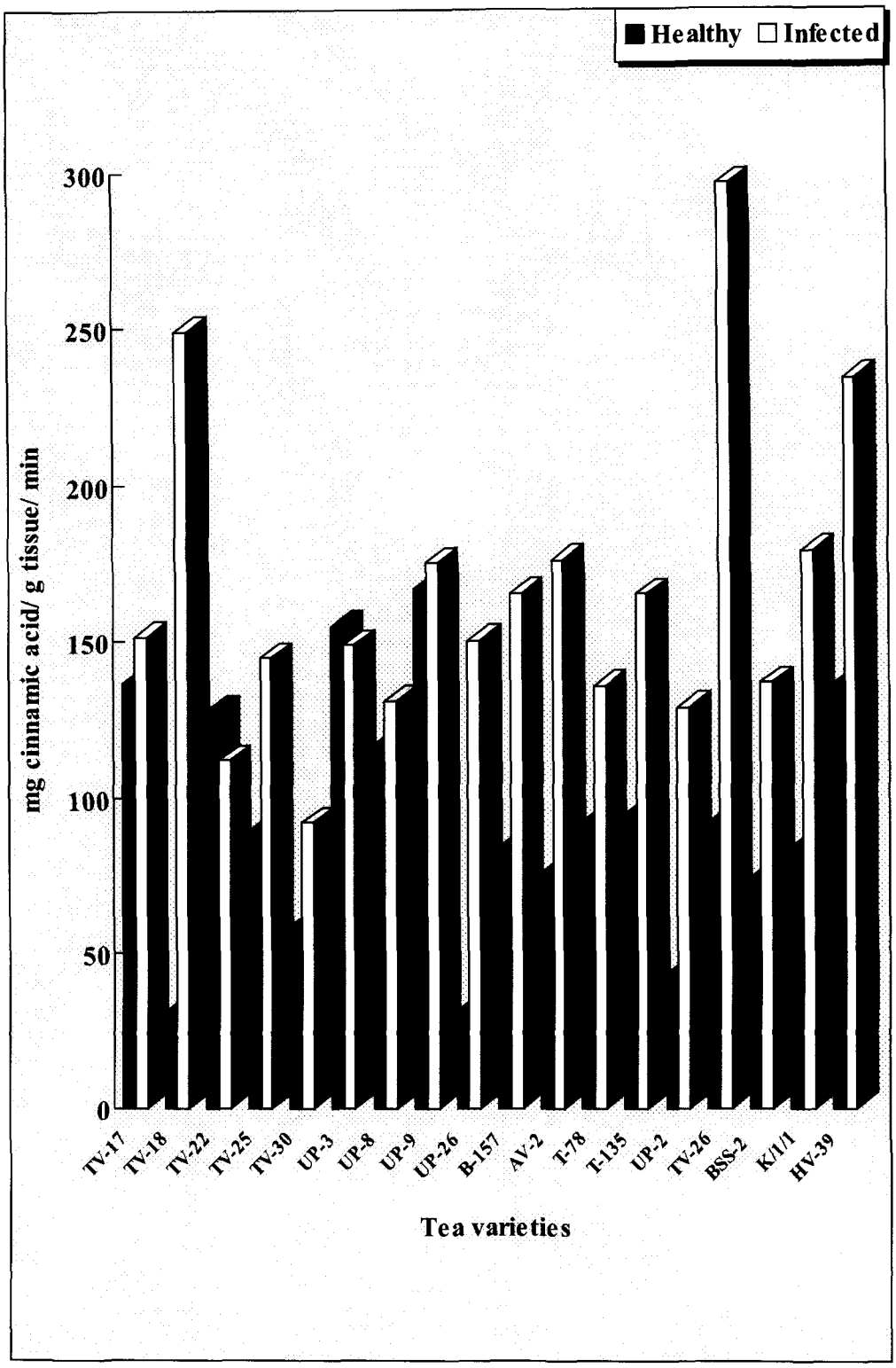

Fig. 14. Phenylalanine ammonia lyase (PAL) activity in tea varieties following inoculation with *S. rolfsii.*

Table22: Changes in peroxidase activity in tea roots following inoculation with *Sclerotium. rolfsii*

Tea Varieties	PO activity in tea roots (ΔOD/g tissue/min)[a]					
	2		4		8	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
BSS-2	0.93 ± 0.02	0.74 ± 0.02	0.87 ± 0.01	1.31 ± 0.01	0.94 ± 0.03	0.96 ± 0.02
UP-2	0.66 ± 0.03	0.89 ± 0.07	0.55 ± 0.12	1.23 ± 0.04	0.68 ± 0.02	0.92 ± 0.02
UP-3	0.54 ± 0.12	0.75 ± 0.11	0.655 ± 0.12	1.26 ± 0.06	0.55 ± 0.12	0.76 ± 0.03
UP-8	0.63 ± 0.04	1.2 ± 0.08	0.66 ± 0.12	1.66 ± 0.12	0.73 ± 0.13	1.27 ± 0.44
UP-9	0.9 ± 0.8	1.03 ± 0.12	1.03 ± 0.12	1.2 ± 0.07	1.16 ± 0.12	1.1 ± 0.16
UP-26	0.77 ± 0.13	0.87 ± 0.04	0.87 ± 0.12	1.6 ± 0.08	0.83 ± 0.07	0.87 ± 0.01
TV-18	1.57 ± 0.17	1.12 ± 0.05	1.67 ± 0.2	1.76 ± 0.12	1.5 ± 0.08	1.14 ± 0.02
TV-22	0.67 ± 0.12	0.17 ± 0.01	0.68 ± 0.16	1.82 ± 0.09	0.66 ± 0.12	1.17 ± 0.2
TV-25	1.13 ± 0.01	1.17 ± 0.01	1.12 ± 0.05	1.31 ± 0.08	1.16 ± 0.02	1.18 ± 0.02
TV-26	1.6 ± 0.12	1.73 ± 0.12	1.3 ± 0.07	1.89 ± 0.03	0.91 ± 0.08	1.76 ± 0.06
T-17	0.81 ± 0.06	1.11 ± 0.06	1.1±0.35	1.71 ± 0.08	1.31 ±0.08	1.4 ± 0.04
TV-30	1.03 ± 0.01	1.07 ± 0.02	1.07 ± 0.01	1.36 ± 0.12	1.04 ± 0.02	1.08 ± 0.01
T-78	1.23 ± 0.2	1.72 ± 0.17	1.08 ± 0.02	1.91 ± 0.02	1.06 ± 0.02	1.76 ± 0.03
AV-2	0.21 ± 0.08	0.83 ± 0.05	0.3 ± 0.16	0.93 ± 0.04	0.23 ± 0.12	0.84 ± 0.01
T-135	0.69 ± 0.16	1.29 ± 0.08	0.76 ± 0.12	1.1 ± 0.49	1.42 ± 0.09	1.09 ± 0.08
B157	1.16 ± 0.02	1.33 ± 0.16	1.11 ± 0.07	1.46 ± 0.3	1.14 ± 0.1	1.16 ± 0.01
HV-39	0.63 ± 0.12	1.63 ± 0.12	0.86 ± 0.04	3.06 ± 1.44	0.86 ± 0.04	01.85 ± 0.07
K1/1	1.06 ± 0.3	1.06 ± 0.3	1.03 ± 0.41	2.4 ± 0.08	1.04 ± 0.02	1.47 ± 0.02

[a] Average of 3 replicates.
[b] Days after inoculation
± Standard error

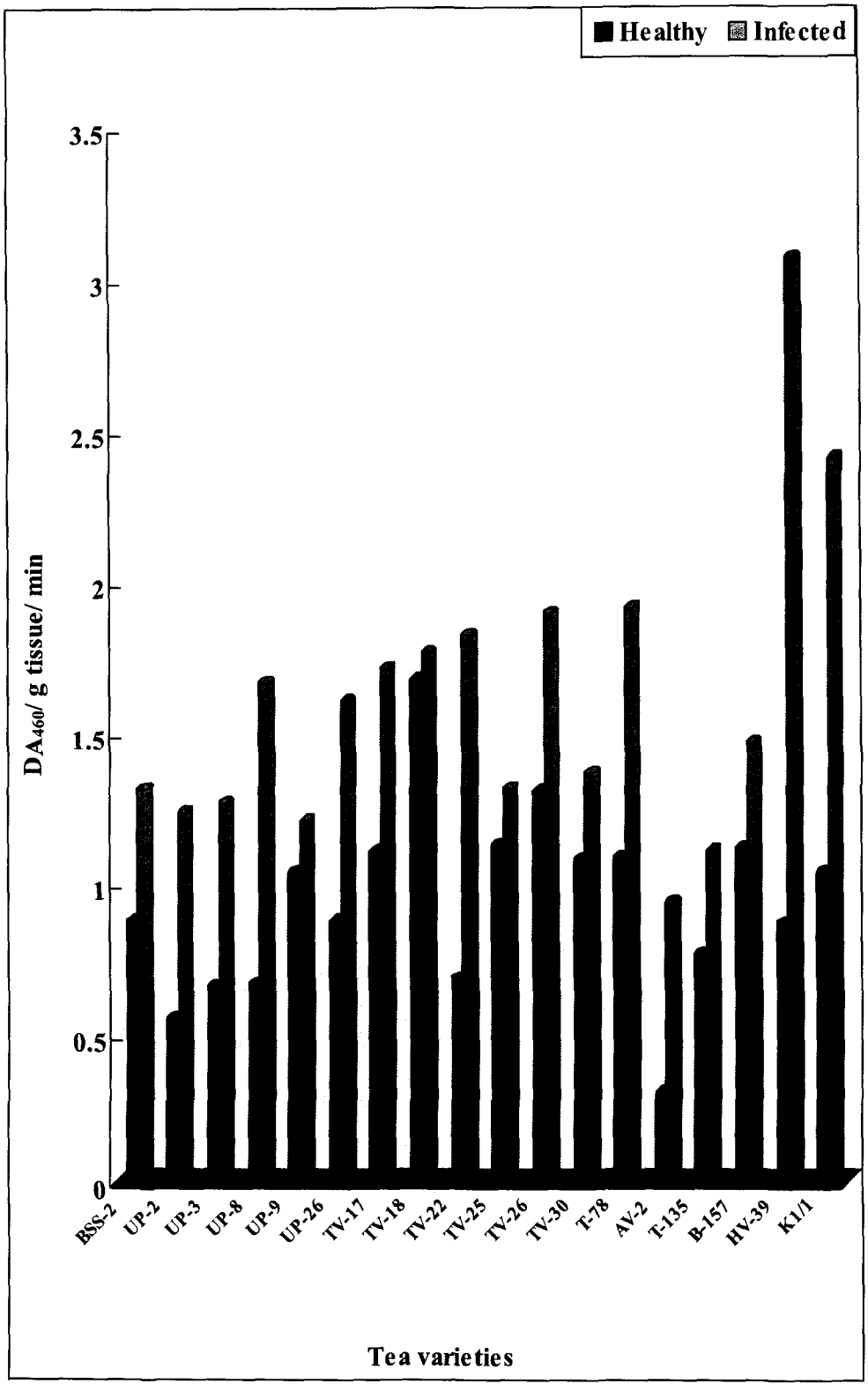

Fig. 15. Peroxidase (PO) activity in tea varieties following inoculation with *S. rolfsii.*

Table23: Changes in polyphenol oxidase activity in tea roots following inoculation with *Sclerotium rolfsii*

Tea Varieties	PPO activity in tea roots (ΔOD/g tissue/min)[a]					
	2		4		8	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
BSS-2	0.16±0.04	0.9±0.08	0.13±0.01	0.34±0.01	0.13±0.01	0.24±0.01
UP-2	0.27±0.12	0.22±0.09	0.2±0.08	0.28±0.02	0.18±0.02	0.2±0.05
UP-3	0.07±0.02	0.08±0.02	0.04±0.01	0.22±0.02	0.06±0.03	0.16±0.02
UP-8	0.26±0.02	0.25±0.02	0.2±0.08	0.26±0.04	0.27±0.01	0.08±0.02
UP-9	0.23±0.04	0.31±0.01	0.21±0.02	0.18±0.02	0.23±0.04	0.15±0.03
UP-26	0.14±0.04	0.13±0.01	0.15±0.03	0.17±0.02	0.14±0.04	0.07±0.04
T-17	0.27±0.05	0.27±0.06	0.2±0.04	0.28±0.01	0.2±0.04	0.3±0.01
TV-18	0.14±0.04	0.15±0.04	0.14±0.04	0.29±0.10	0.18±0.02	0.31±0.02
TV-22	0.12±0.01	0.19±0.01	0.12±0.02	0.22±0.01	0.12±0.01	0.13±0.04
TV-25	0.16±0.05	0.16±0.02	0.16±0.05	0.22±0.08	0.15±0.04	0.2±0.03
TV-26	0.27±0.08	0.19±0.01	0.27±0.07	0.3±0.04	0.25±0.05	0.22±0.07
TV-30	0.16±0.03	0.17±0.02	0.15±0.03	0.18±0.09	0.15±0.02	0.06±0.02
T-78	0.05±0.05	0.23±0.04	0.04±0.04	0.25±0.04	0.03±0.04	0.13±0.05
AV-2	0.14±0.03	0.15±0.03	0.13±0.02	0.17±0.01	0.15±0.02	0.16±0.02
T-135	0.06±0.04	0.21±0.04	0.07±0.02	0.27±0.06	0.07±0.02	0.23±0.08
B157	0.04±0.04	0.13±0.05	0.04±0.05	0.17±0.02	0.06±0.04	0.06±0.04
HV-39	0.12±0.06	0.06±0.04	0.12±0.06	0.30±0.01	0.14±0.04	0.22±0.03
K1/1	0.21±0.04	0.28±0.08	0.24±0.05	0.34±0.02	0.22±0.06	0.27±0.06

[a] Average of 3 replicates.
[b] Days after inoculation
± Standard error

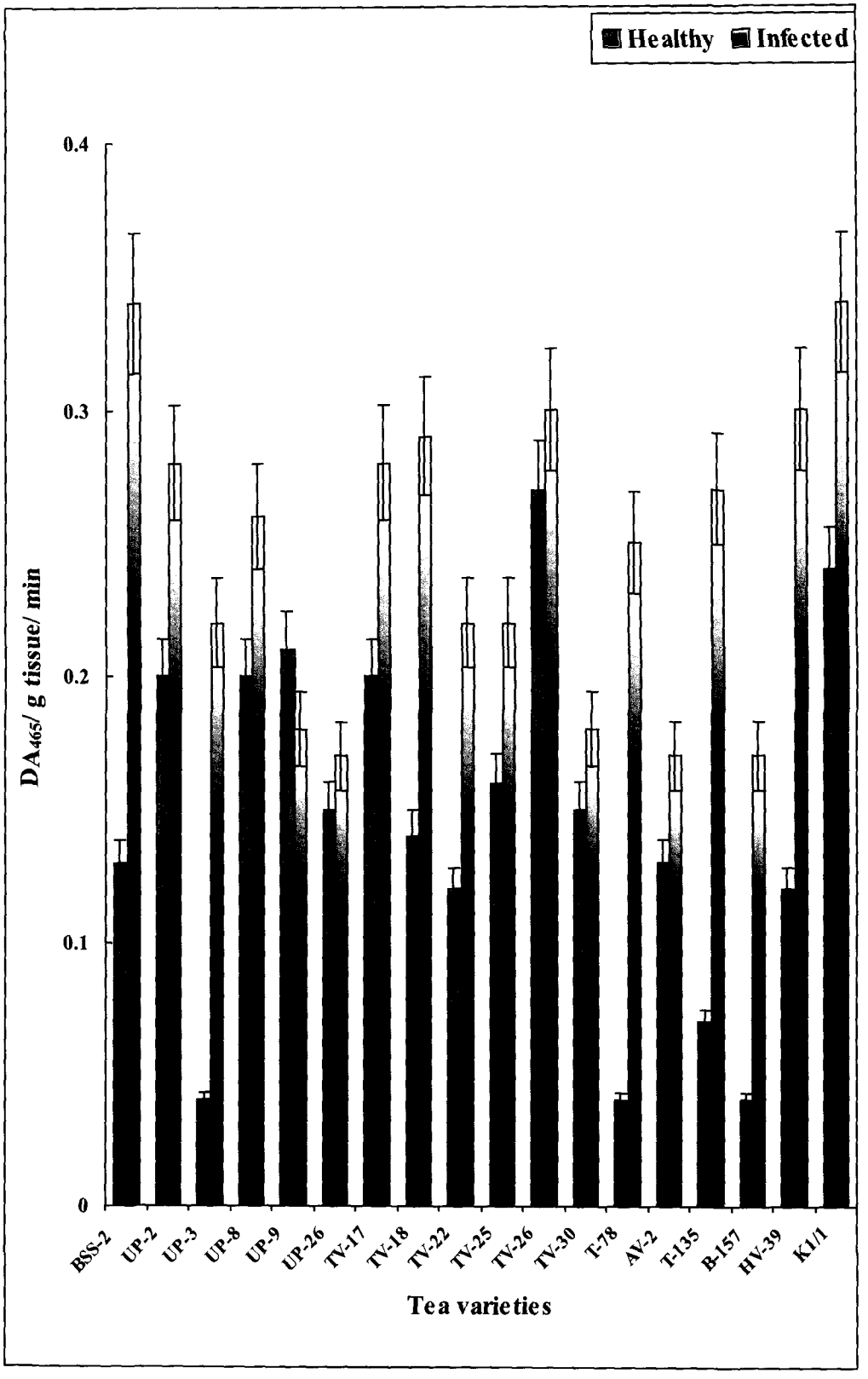

Fig. 16. Polyphenol oxidase (PPO) activity in tea varieties following inoculation with *S. rolfsii*

4.9. Management of seedling blight

In order to develop effective integrated management practices for seedling blight disease of tea using plant extracts, biocontrol agents, organic additives along with selected fungicides, initially *in vitro* tests were performed against *S. rolfsii*.

4.9.1. *In vitro* evaluation

4.9.1.1. Plant extract

Plant extracts prepared from *Azadirachta indica* and *Catharanthus roseus* were tested *in vitro* by two different ways. These extracts prepared as stock (100%) were diluted to 50% and 10% and accordingly mixed in PDA medium and mycelial growth of *S. rolfsii* was measured after 72 h and compared in relation to medium control. The results (Table 24) revealed that both the plant extracts were inhibitory to the mycelial growth of *S. rolfsii*. As the concentration of extracts increased in the medium the effectiveness of extracts also increased and maximum growth inhibition was recorded at 100% concentration in both plant extracts.

Table 24: Efficacy of antifungal effect of different plant extracts on *Sclerotium rolfsii*

Plant	Part	Diameter of fungal mycelia (cm)			
		Control	10%	50%	100%
Azadirachta indica	Leaf	9.2	7.3	7	3
Catharanthus roseus	Leaf	9.2	6.5	6	4

In another experimental set up sclerotial germination tests were performed by direct contact on sterile filter paper kept in sterile Petri plates. Filter papers were soaked with plant extracts separately and on each soaked filter paper a minimum of 20 sclerotia was placed with at least three replicates. These were allowed to germinate for 24-48 h and finally percentage germination and diameter of mycelial growth were measured. Greater inhibition in the germination of sclerotia was noticed with *C. roseus* than *A. indica* (Plate14, figs. A-G) in relation to distilled water control (Plate 14, figs H&I).

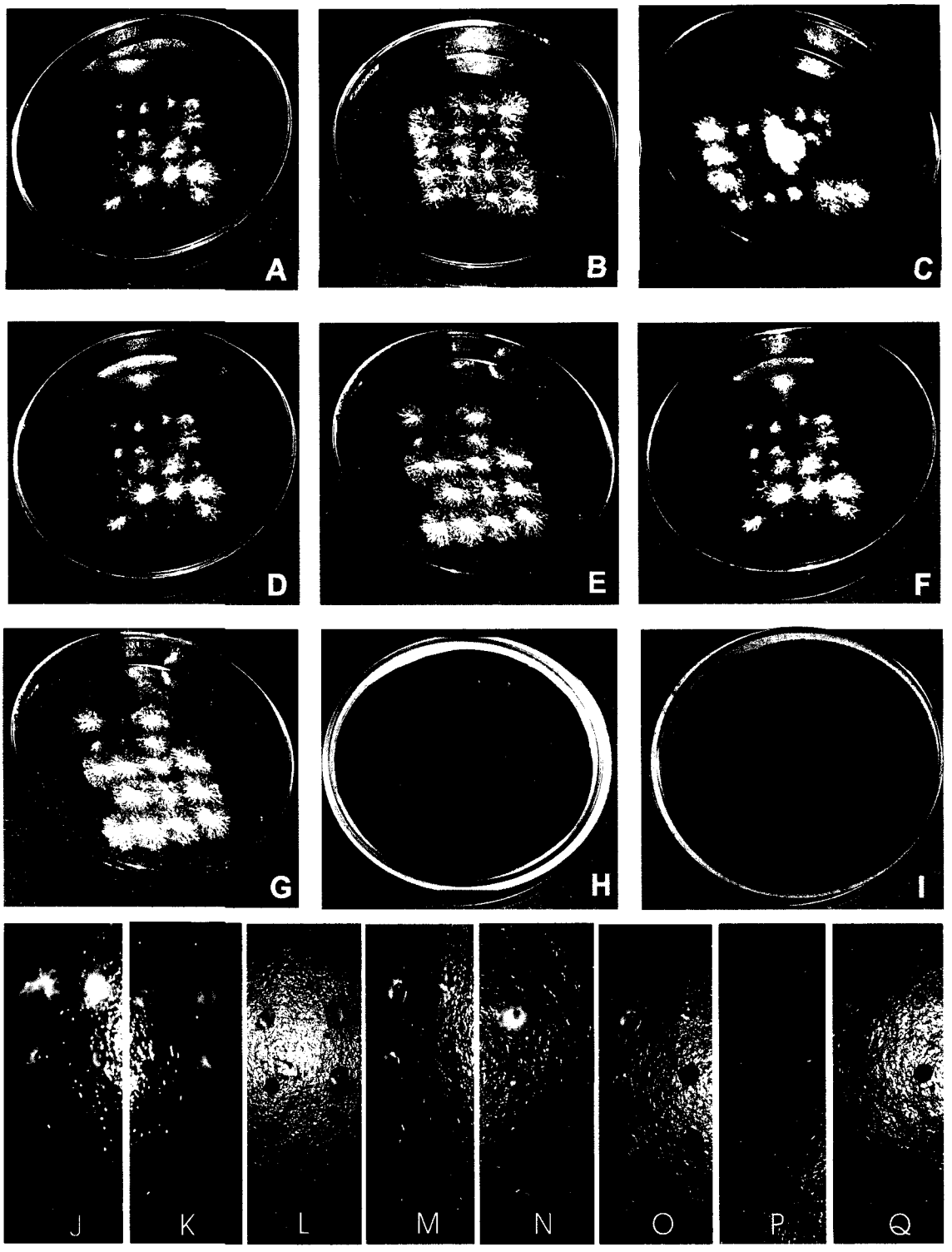

Plate 14 (figs. A-Q) Sclerotial germination bioassay of *S. rolfsii* against plant extracts and fungicides. (A-D) *Catharanthus roseus,* (E-G) *Azadirachta indica,* (H&I) Distilled water control, (J&K) Captan, (L&M) Carbendazim, (N&O) Thiodan, (P&Q) Calixin

4.9.1.2. Fungicides

All fungicides were significantly superior over control in checking the mycelial growth of *Sclerotium rolfsii* (Table 25). However Thiodan and Calixin, were completely arrested the growth of pathogen at a concentration as low as 0.0125% concentration. Carbendazim (Bavistin), captan and Indofil M-45 were less inhibitory at 0.1% concentration. Calixin also completely inhibited the germination of sclerotia of *S. rolfsii* (Plate 14, figs. P&Q) in comparison to captan, carbendazim and thiodan (Plate 14, figs. J-O). These were compared with sterile distilled water control (Plate 14, figs. H&I) where 100% sclerotial germination was evident.

4.9.1.3. Biocontrol agents

Antagonistic properties of *Trichoderma harzianum* and *Trichoderma viride* were studied through dual plate method. Mycelial discs of 6 mm dia cut from the margin of 5-day-old cultures of both test pathogen (*Sclerotium rolfsii*) and antagonists (*T. harzianum* and *T. viride*) were placed opposite to each other on PDA in Petri plates (9cm dia). The distance between inoculum blocks was 7cm. A set of plates was inoculated with *S. rolfsii* and after 24 h the same plates were inoculated with the antagonist. In the second set, the antagonists were inoculated first and after 24 h *S. rolfsii* was inoculated. In the third set, *S. rolfsii* and antagonists were inoculated simultaneously. Each treatment was replicated thrice and petriplates were incubated at 28^0C for 8 days and also appropriate control was maintained. Observations on colony diameter of *S. rolfsii* were recorded to calculate inhibition zone. The maximum inhibition of mycelial growth of *S. rolfsii* was recorded in *T. harzianum* when inoculated 24 h prior to inoculation of *S. rolfsii* The inhibition degree of mycelial growth of *S. rolfsii* decreased when antagonists were inoculated 24 h after inoculation with *S. rolfsii*. *T. harzianum* and *T. viride* were at par with one another in their ability to inhibit the pathogen. Simultaneously, *T. harzianum* and *T. viride* were equally effective in inhibiting the radial growth of *S. rolfsii* as illustrated in Plate 15, figs A-E.

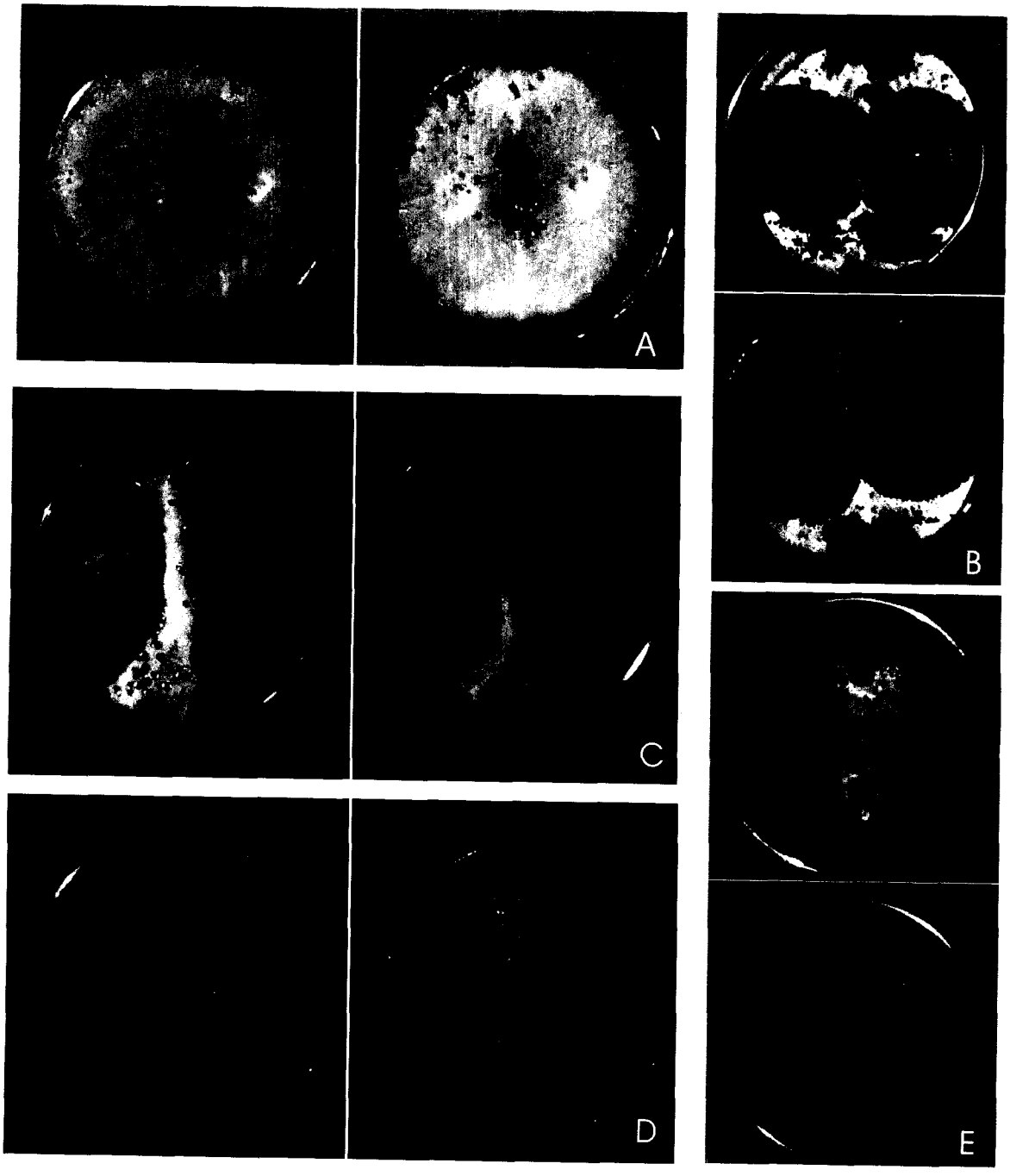

Plate 15 (figs. A-E): Pairing of *S. rolfsii* with *Trichoderma harzianum* and *Trichoderma viride.* Homologous pairing of *S. rolfsii* (A), *T. harzianum* (B) and *T. viride* (E) respectively. Pairing of *S. rolfsii* with *T. harzianum* (C) and with *T. viride* (D).

Table 25: *In vitro* efficacy of different concentration of fungicides against *Sclerotium rolfsii*

Fungicides	Concentration	Diameter of fungal mycelia (cm)
Distilled water control		9.2
Thiodan	0.1%	0
	0.05%	0
	0.025%	0
	0.0125%	0
Calixin	0.1%	0
	0.05%	0
	0.025%	0
	0.0125%	0
Captan	0.1%	3
	0.05%	4
	0.025%	5.2
	0.0125%	7.2
Carbendazim	0.1%	4
	0.05%	4.7
	0.025%	5.2
	0.0125%	7.2
Indofil M-45	0.1%	3.8
	0.05%	6
	0.025%	6.8
	0.0125%	7

After 8 days, *T. harzianum* and *T. viride* overgrew the pathogen and lysed it over a period of time. The pathogen formed an inhibition zone around it though pathogen also was not able to grow further *i.e.*, its growth was ceased. *T. harzianum* and *T. viride* inhibited the mycelial growth of the pathogen by 61.11% and 58.44% respectively on simultaneous inoculation with a least number of sclerotial production as in Table 26.

Table 26: *In vitro* antagonistic effect of biotic agent on mycelial growth of *Sclerotium rolfsii.*

Antagonists	24 h prior to the inoculation of *S. rolfsii*		24 h after inoculation of *S. rolfsii*		Simultaneous inoculation	
	Mycelial growth (cm)	Inhibition (%)	Mycelial growth (cm)	Inhibition (%)	Mycelial growth (cm)	Inhibition (%)
T.harzianum	1.8	80	4.3	52.22	3.5	61.11
T.viride	2.0	77.8	4.2	53.33	3.74	58.44
Control	9.0	0.0	9.0	0.0	9.0	0.0

Table 27: Effect of culture filtrates of bio agents on mycelial dry weight of *Sclerotium rolfsii*

Name of the culture	Dry weight (g)
Sclerotium rolfsii	0.68
S. rolfsii + 25% culture filtrate of *T. harzianum*	0.26
S. rolfsii + 25% culture filtrate of *T. viride*	0.27

Data from the Table 27 also indicated that 25% concentration of culture filtrates of *T. harzianum* and *T. viride* after 5 days reduced the mycelial weight of sclerotial blight pathogen by 55.56% and 50% respectively. This observation thus indicated that the volatile and non-volatile extracellular extracts of *Trichoderma* spp. may have some inhibitory growth retardation effect against this pathogen.

4.9.2. *In vivo* test

4.9.2.1. Growth promotion in tea seedlings

Tea seedlings of two varieties (B-157 and TeenAli-17/1/57) 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 *S. rolfsii*. Results (Table 28) revealed that the growth of tea seedlings had been increased following amendment with neem and oil cakes than those treated plants inoculated with *S. rolfsii* in relation to untreated uninoculated tea seedlings as recorded after two months following treatment.

Table 28: Growth promotion in tea seedlings following soil amendment with neem cake and oil cake

Tea variety	One month				Two months			
	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
T17/1/54 Untreated	2	4	0	2	5	6	1	2
Treated Neem cake	2	3	1	0	2	8	2	4
Oil cake	1	3	2.5	2	2	4	2	3
B-157 Untreated	1	3	1	0	4	6	1	2
Treated Neem cake	2	2	1	0	0	1	2	3
Oil cake	1	4	1.5	0	2	0	2	1

It has been observed that the percentage increase in shoot length after two months of treatment with neem cake and oil cake in treated inoculated with *S. rolfsii* tea seedlings was more than the treated uninoculated tea seedlings (Table 29).

Table 29: 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
T –17/1/54 Untreated	6.5	2.6
Treated Neem cake	8.6	1.0
Oil cake	7.8	5.9
B-157 Untreated	8.3	4.6
Treated Neem cake	8.3	7.6
Oil cake	9.5	5.5

Similarly seedlings of three tea varieties (UP-3, B-157 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 *S. rolfsii.* It has been observed that the growth of tea seedlings had been increased in treated uninoculated than treated inoculated tea seedlings (Table 30). Among the three treatments with organic components, rabbit manure gave very good and healthy growth of tea seedlings (Plate 16, fig. F) than chicken manure (Plate 16, figs.C-E) and cowdung.

Table 30: Growth promotion in tea seedlings by different organic components after inoculation with *Sclerotium rolfsii*

Tea variety	One month				Two months			
	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
UP-3 Untreated	2	0	1	0	3	0	1	0
Treated Cow dung	6	1	3	1	4	1	1.5	0
Rabbit manure	9	0	6	0	6	1	4	0
Chicken manure	4	1	2	0	3	1	2	0
B-157 Untreated	1	0	0	0	1	2	1	0
Treated Cow dung	3	1	3	1	4	1	1	1
Rabbit manure	8	1	5	0	5	1	2	1
Chicken manure	4	0	2	1	4	3	2	1
K – 1/1 Untreated	2	0	1	0	2	1	0	0
Treated Cow dung	3	0	1	0	2	0	0	0
Rabbit manure	9	4	7	0	8	0	2	0
Chicken manure	6	2	4	0	3	3	2	0

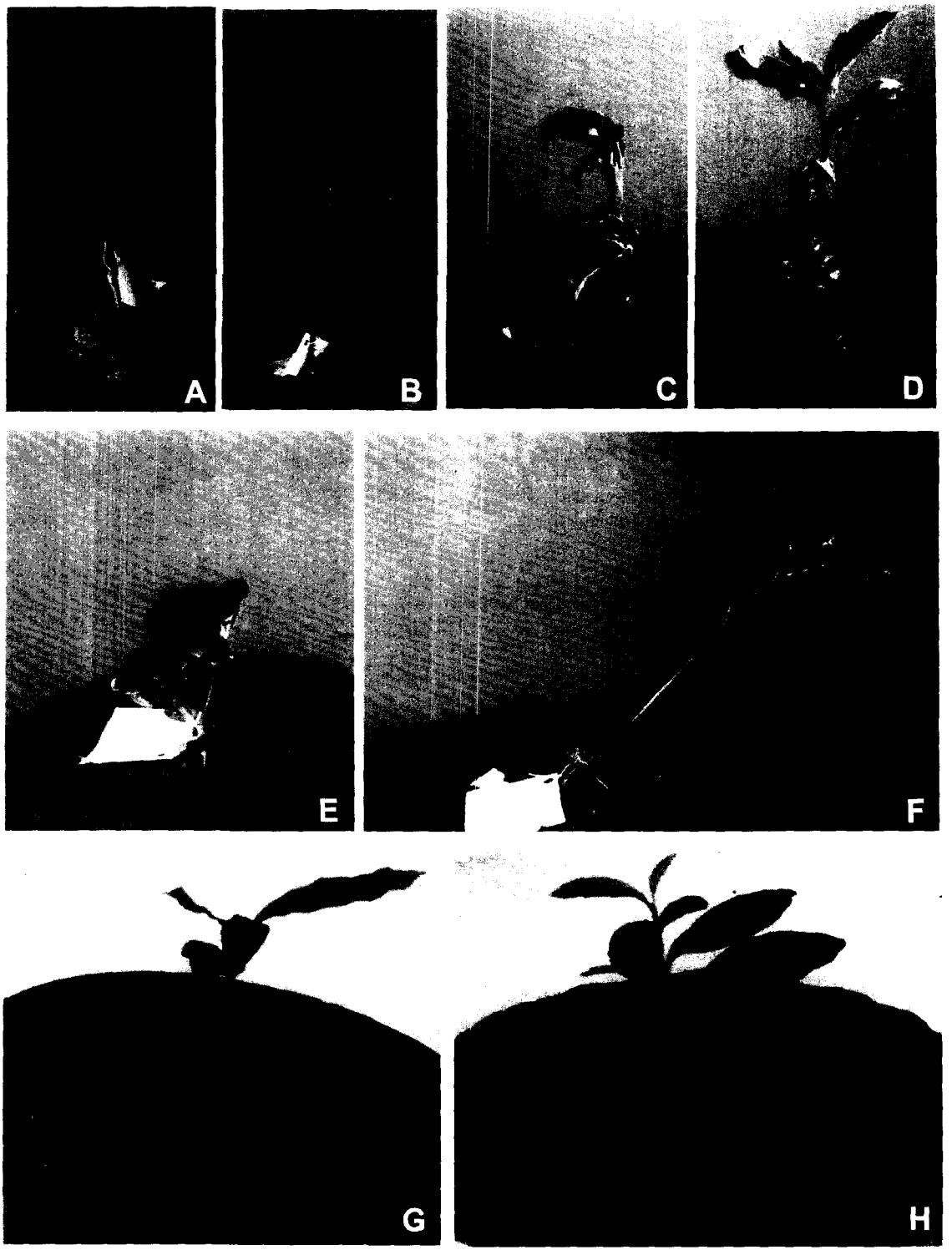

Plate 16 (figs. A-H). Tea plants following treatment with biocontrol agents and organic amendments. **(A)** Untreated inoculated with *S. rolfsii* **(B)** Untreated healthy **(C-E)** Ammended with chicken manure **(F)** Ammended with rabbit manure **(G)** *T. harzianum* inoculated **(H)** *T. viride* inoculated

4.9.2.2. Disease development

Under pot culture conditions *T. harzianum* alone and in combination with neem cake, oil cake and *Azadirachta indica* provided best effective management practices of seedlings blight in all the three modes of application *viz.*, simultaneous, repeated and pot infection. Combination with neem cake and oil cake showed 66.4% disease incidence where as in oil cake, neem cake and *Azadirachta indica* in combination disease incidence were recorded 11.1%. But in combination with cowdung, neem cake, oil cake, chicken manure and rabbit manure, results were insignificant as shown in (Tables 31 and 32).

Table 31: Effect of simultaneous treatments with biocontrol, fungicide, organic amendments and plant extract on development of seedling blight of tea following inoculation with *Sclerotium rolfsii*

Treatment	Disease incidence (%)	Disease control (%)
Trichoderma harzianum	0	100
Oil cake with Neem cake	66.4	33.6
Oil cake, Neem cake and *Azadirachta indica* (aqueous extract)	11.1	88.9
T. harzianum with *Azadirachta indica* (aqueous extract), oil cake and neem cake	0	100
Cowdung, Neem cake and Oil cake	44.6	55.4
Chicken manure, Neem cake and Oil cake	47.5	52.5
Rabbit manure, Neem cake and Oil cake	46.6	53.4
T. harzianum, Calixin (0.1%) and *Azadirachta indica* (aqueous extract)	0	100
Untreated Control	100	0

Table 32: Comparative efficacy of application of organic amendments and formulation against *Sclerotium rolfsii*

Treatment	Disease incidence (%)		
	Simultaneous	**Repetitive**	**Post infection**
Trichoderma harzianum	0	0	0
Oil cake, Neem cake and *Azadirachta indica* (aqueous extract)	15.8	0	44.6
T. harzianum, Azadirachta indica (aqueous extract) Oil Cake and Neem cake	0	0	0
Cowdung, Neem cake and Oil cake	40.6	30.5	77.7
Rabbit manure, Neem cake and Oil cake	46.3	33.0	85.8
Chicken manure, Neem cake and Oil cake	47.5	35.5	88.2
T. harzianum, Calixin (0.1%), *Azadirachta indica* (aqueous extract)	0	0	0
Untreated Control	100	100	100

4.10 Changes associated with induction of resistance in tea plants

4.10.1. Biochemical changes

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 (TeenAli-17/1/54 and B-157) after inoculation with pathogen (*S. rolfsii*). Results have been presented in Table 33 and Fig. 17 (A&C). 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 *S. rolfsii* than treated uninoculated tea root varieties. Level of total phenol increased by 3.12%, 4.69% in T-17/1/54 following treatments with neem cake and oil cake respectively, whereas, 2.86% and 5.71% increased in total phenol was noticed in neem cake and oil cake treated B-157 respectively after inoculation with *S. rolfsii* as compared to healthy untreated control.

Level of ortho-dihydroxyphenol was also determined in these varieties (TeenAli-17/1/54 and B-157) after treatment with neem cake and oil cake following inoculation with *S. rolfsii*. Results (Table-34) 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 of both the varieties tested (Figs. 17 B & D). 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.

Table 33: Total phenol content in tea varieties after treatment with Neem cake and oil cake following inoculation with *Sclerotium rolfsii*

Tea variety	Phenol content (mg /g)[a]	
	Healthy	Infected
T –17/1/54 Untreated	2.5	3.6
Treated		
Neem cake	2.8	4.8
Oil cake	2.7	5.2
B-157 Untreated	2.5	2.6
Treated Neem cake	2.7	3.6
Oil cake	2.8	3.7

[a] Average of 3 replicates.

Table 34: Ortho-dihydroxy phenol content in tea varieties after treatment with Neem cake and oil cake following inoculation with *Sclerotium rolfsii* in treated tea root variety

Tea variety	Ortho-dihydroxy phenol content (mg /g)[a]	
	Healthy	Infected
T –17/1/54 Untreated	2.1	1.2
Treated Neem cake	2.0	2.7
Oil cake	2.2	3.0
B-157 Untreated	1.1	0.6
Treated Neem cake	1.4	2.1
Oil cake	1.5	2.2

[a] Average of 3 replicates.

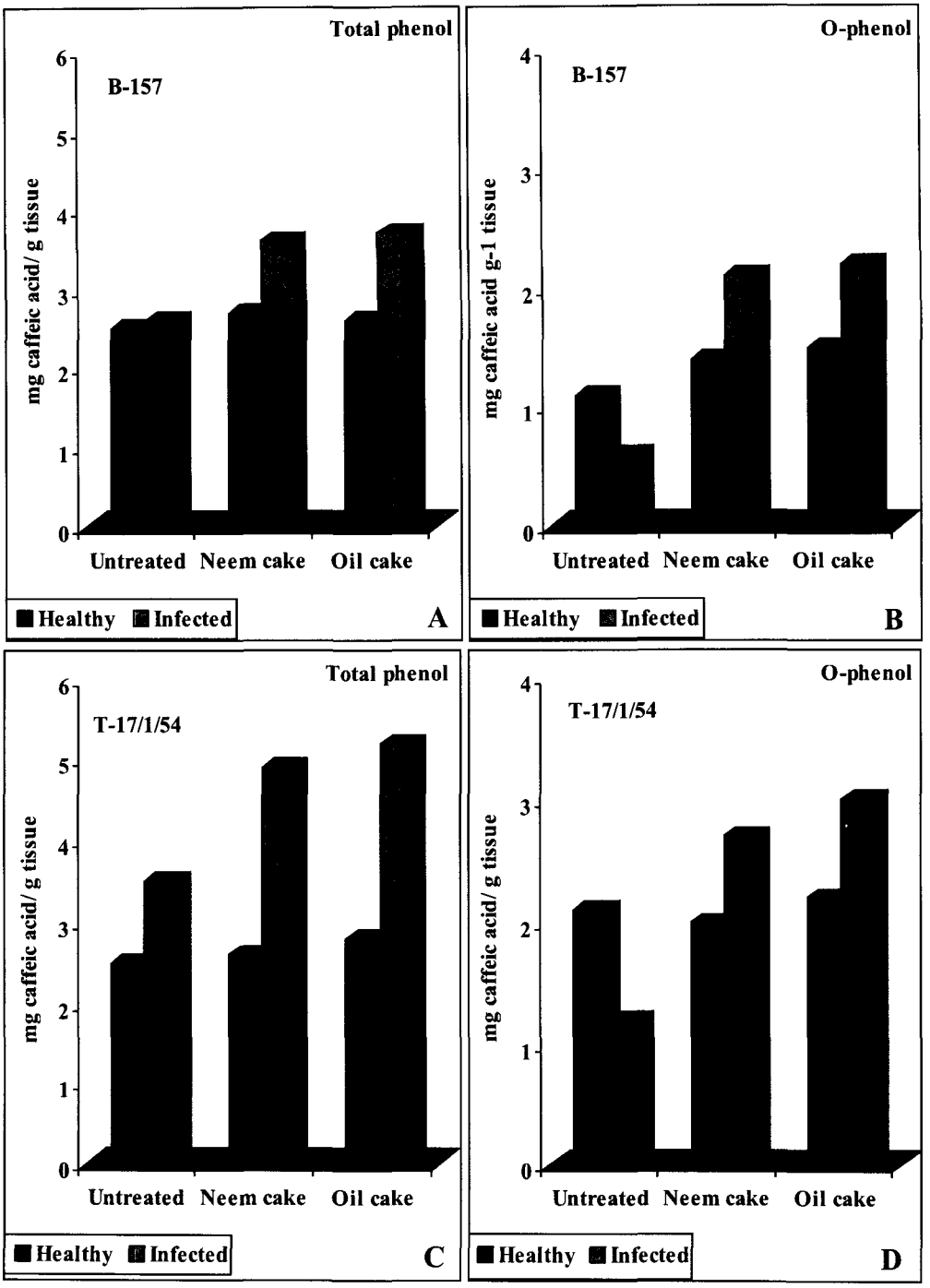

Fig.17 (A-D).Total phenol and ortho dihydroxyphenol content in treated tea varieties following inoculation with *S. rolfsii.*

Changes in the level of phenolics were also determined in two varieties of tea plants (UP-3, B-157 and K 1/1) grown separately in soil amended with cowdung, rabbit manure and chicken manure following inoculation with *S. rolfsii*. Results revealed that total phenol content decreased in untreated plants of two susceptible varieties (UP-3 and B-157) 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 (Tables 35 and 36; Figs. 18 A-F).

Table 35: Changes in the level of total phenol content in tea roots grown in soil amended with organic additives following inoculation with *Sclerotium rolfsii*

Tea variety	Phenol content (mg /g)[a]	
	Healthy	Infected
UP – 3 Untreated	3.0	2.1
Treated		
Cowdung	3.1	3.2
Rabbit manure	3.5	5.6
Chicken manure	3.3	3.5
B – 157 Untreated	3.5	2.6
Treated		
Cowdung	2.6	3.9
Rabbit manure	4.1	4.7
Chicken manure	4.6	4.9
K-1/1 Untreated	2.8	6.7
Treated		
Cowdung	5.6	8.4
Rabbit manure	4.4	8.5
Chicken manure	4.1	4.9

[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 in case of UP-3 and B-157, whereas level of ortho-dihydroxyphenol increased in plants (B-157 and K 1/1) grown in soil amended with rabbit manure following inoculation with the pathogen

Table 36: Changes in the level of ortho-dihydroxy phenol content in tea roots grown in soil amended with organic additives following inoculation with *Sclerotium rolfsii*

Tea variety	Ortho-dihydroxy content (mg /g)[a]	
	Healthy	**Infected**
UP – 3 Untreated	2.1	1.5
Treated Cow dung	2.4	2.6
Rabbit manure	2.3	2.5
Chicken manure	2.2	2.4
B – 157 Untreated	1.1	0.6
Treated Cow dung	1.8	1.9
Rabbit manure	1.9	2.1
Chicken manure	1.7	1.8
K-1/1 Untreated	0.9	2.3
Treated Cow dung	1.7	2.4
Rabbit manure	3.0	4.5
Chicken manure	1.7	2.6

[a] Average of 3 replicates.

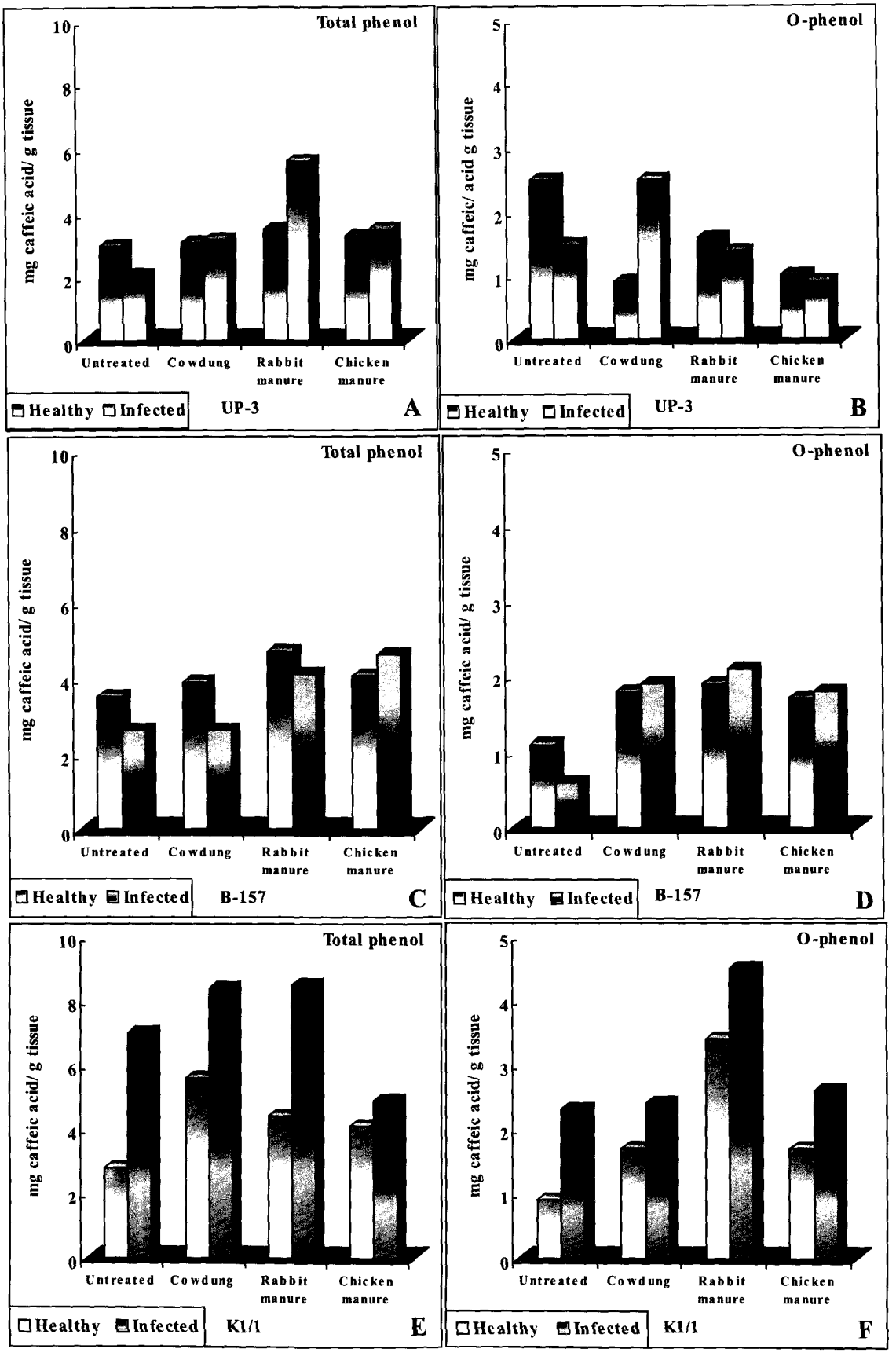

Fig.18 (A-F). Total phenol and orthodihydroxy phenol content in treated tea varieties following inoculation with *S. rolfsii.*

4.10.2. Serological changes

4.10.2.1. Immunodiffusion test

In the present investigation an attempt was made to induce disease resistance in tea plants (TeenAli-17/1/54) applying fungicides. Among the tested fungicides carboxin and thiodan were found to be highly effective in reducing disease intensity. Alteration in antigenic patterns after induction of resistance by carboxin was also worked out since both are believed to be associated with developing immunity in plants. Polyclonal antibodies raised against the pathogen (*S. rolfsii*) were used for such immunological studies. Agar gel double diffusion tests were performed using root antigens prepared from untreated and carboxin treated tea roots of TeenAli-17/1/54. Results (Table-37) revealed that strong precipitin reactions occurred when PAb of *S. rolfsii* was reacted against its own antigens as well as root antigens of an untreated susceptible tea root variety (T-17/1/54). However, cross reaction between PAb of the pathogen and antigens of treated roots failed to developed even weak precipitin bands, which indicates antigenic disparity between pathogen and treated plant roots.

Table 37. Immunodiffusion tests tea root tissues (TeenAli-17/1/54) before and after treatment with carboxin using PAb of *Sclerotium rolfsii*

Antigens	PAb		
	Untreated root	**Treated root***	***S. rolfsii***
Tea variety T-17/1/54 Untreated	+	+	+
Treated*	±	+	-
Pathogen *S. rolfsii*	±	-	+

* Plants treated with carboxin (0.1%)

Common precipitin band : (+) present (±) weak band (–) band absent.

4.10.2.2. PTA-ELISA

Since the application of biocontrol agents in rhizosphere soil reduced intensity of sclerotial blight disease, it was decided to investigate this reduction which could also be determined immunologically in both root tissues and soil. For this purpose PTA-ELISA was carried out. ELISA reactions were performed with root antigens from different treatment as well as soil antigen. PTA-ELISA format was developed using polyclonal antibody (PAb) raised against *S. rolfsii* in order to screen the infection. Healthy, untreated inoculated, treated tea root antigens were prepared on PTA-ELISA format. Root antigens were prepared from uprooted plants (1year old) of different treatments after 30 days of pathogen inoculation. These antigens were reacted in PTA-ELISA using PAb of *S. rolfsii*. Results showed that ELISA values of roots treated with *T. harzianum* and *T. viride* were significantly lesser than *S. rolfsii* alone. The results and means of three experimental sets are shown in Tables 38 and 39.

Table 38. PTA-ELISA reaction of PAb *S. rolfsii* with root antigens of tea varieties following treatment with biocontrol agents

Antigen source	Absorbance at 405 nm	
	TV-18	AV-2
Control plant	0.583±0.01	0.561±0.01
Treatments		
S. rolfsii + *T. harzianum*	0.102±0.01	0.180±0.02
S. rolfsii + *T. viride*	0.292±0.01	0.285±0.01
S. rolfsii	0.919±0.09	0.834±0.06
T. harzianum	0.582±0.09	0.586±0.01
T. viride	0.594±0.02	0.583±0.01

Antigen concentration 100 µg/ml

IgG source –PAb of *S. rolfsii*

30 days after pathogen inoculation

± Standard error.

Table 39. PTA- ELISA reaction of PAbs of pathogen and biocontrol agents with root antigens of tea varieties following treatment and after inoculation with pathogen

Antigen source	*S. rolfsii*		*T. harzianum*		*T. viride*	
	T-78	**UP-26**	**T-78**	**UP-26**	**T-78**	**UP-26**
Control Plant	0.572±0.04	0.593±0.05	0.363±0.03	0.353±0.04	0.322±0.03	0.383±0.06
Treatment *S. rolfsii* + *T. harzianum*	0.581±0.01	0.602±0.01	0.390±0.09	0.363±0.02	0.385±0.04	0.374±0.02
S. rolfsii + *T. viride*	0.559±0.03	0.578±0.02	0.330±0.02	0.350±0.03	0.387±0.03	0.382±0.01
S. rolfsii	1.345±0.05	1.396±0.04	0.400±0.01	0.423±0.02	0.524±0.07	0.542±0.04
T.harzianum	0.571±0.02	0.580±0.02	0.384±0.01	0.380±0.01	0.360±0.03	0.367±0.02
T. viride	0.575±0.03	0.592±0.01	0.334±0.08	0.325±0.01	0.380±0.01	0.384±0.01

Antigen concentration 100 µg/ml

IgG source –PAb of *S. rolfsii*

30 days after pathogen inoculation

Age of plants five years

± standard error.

It is very interesting to note that untreated and sclerotial blight infected roots show very high absorbance (A_{405}) values when compared to the treated root antigens. Treatment with systemic fungicides gave the lowest O.D. value followed by *A. indica* and *C. roseus* (Table 40). This result has definitely opened new horizons for testing various other eco-friendly plant extracts for the management of the disease.

Table 40: Indirect ELISA reaction of treated (systemic fungicide and plant extract) and untreated tea roots before and after inoculation with *Sclerotium rolfsii* against PAb of *Sclerotium rolfsii*

Antigen Concentration (40 µg/ml)	**PAb of *Sclerotium rolfsii* (1:250 dilution)**					
Treatment [a]	**1st harvest**			**2nd harvest**		
	Exp.1 [b]	**Exp.2**	**Exp.3**	**Exp.1**	**Exp.2**	**Exp.3**
Untreated Healthy	0.639±0.01	0.684±0.01	0.674±0.01	0.605±0.06	0.670±0.01	0.678±0.01
Untreated Infected	0.849±0.01	0.854±0.03	0.795±0.08	0.754±0.03	0.744±0.02	0.826±0.01
Treated Carboxin H [c]	0.475±0.01	0.459±0.03	0.467±0.01	0.454±0.03	0.454±0.02	0.452±0.03
Carboxin I [d]	0.487±0.02	0.485±0.01	0.485±0.04	0.498±0.905	0.493±0.01	0.485±0.03
Carbendazim H	0.605±0.01	0.0609±0.01	0.625±0.02	0.628±0.01	0.610±0.01	0.608±0.01
Carbendazim I	0.609±0.01	0.622±0.02	0.628±0.02	0.643±0.03	0.625±0.01	0.621±0.02
Indofil H	0.593±0.05	0.590±0.03	0.601±0.01	0.607±0.01	0.599±0.01	0.595±0.01
Indofil I	0.595±0.01	0.592±0.01	0.606±0.01	0.609±0.01	0.606±0.01	0.602±0.01
Captan H	0.588±0.01	0.581±0.01	0.589±0.01	0.602±0.01	0.581±0.02	0.580±0.02
Captan I	0.604±0.01	0.602±0.01	0.607±0.01	0.606±0.01	0.583±0.02	0.601±0.01
Thiodan H	0.492±0.01	0.458±0.03	0.467±0.01	0.460±0.02	0.461±0.03	0.452±0.03
Thiodan I	0.492±0.01	0.485±0.02	0.499±0.03	0.480±0.02	0.487±0.01	0.484±0.01
C. roseus H	0.655±0.06	0.617±0.001	0.670±0.05	0.701±0.03	0.618±0.01	0.681±0.01
C. roseus I	0.657±0.02	0.731±0.08	0.668±0.09	0.762±0.09	0.657±0.01	0.708±0.01
Neem H	0.629±0.04	0.550±0.03	0.627±0.05	0.654±0.02	0.647±0.06	0.653±0.04
Neem I	0.802±0.03	0.708±0.04	0.638±0.05	0.738±0.03	0.762±0.04	0.662±0.05

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

[b] Each experiment is mean of 3 replicates and 3 experiments were performed for each treatment per harvest

[c] H – healthy

[d] I – infected.

4.10.2.3. Dot immunobinding assay

Soil samples of the rhizosphere of different treatment *viz.*, soil amended with cow dung, rabbit manure, chicken manure, biocontrol agents (*T. harzianum* and *T. viride*) were collected separately at a depth of 6-9 inches from soil surface. *S. rolfsii* was evaluated through dot immunobinding assay by reacting the antigens from collected soils after 30 days of pathogen inoculation on nitrocellulose paper with the PAbs of *S. rolfsii*. Control set was prepared from uninfested sterile soil. In PTA-ELISA results from soil treated with *S. rolfsii* and *T. harzianum* or *S. rolfsii* and *T. viride* reacted with PAb of *S. rolfsii* showed significantly lower absorbance values than that of soil antigen treated with *S. rolfsii* alone. This indicated that population of *S. rolfsii* soil had been reduced by the biocontrol agents. Results presented in Table 41 and Plate 17 figs A-E revealed that PAb of *S. rolfsii* reacted very strongly with the antigens from soil infested with *S. rolfsii* (Plate 17, figs. B&E), however reactions were very poor on the nitrocellulose paper when reacted with soil antigens amended either with rabbit manure or *T. harzianum* and inoculated with the pathogen (Plate17, figs. A, C & D). In cases where soil was treated with other organic amendments (cowdung and chicken manure) less positive reactions were evident.

Table 41: Dot-blot of soil antigen of different treatment with combination of *Sclerotium rolfsii*

Antigen source	Colour intensity[a]
	PAbs raised against *S. rolfsii*
Sterile soil	-
Soil inoculated with *S. rolfsii*	+++++
Treated Cow dung + *S. rolfsii*	+
Rabbit manure + *S. rolfsii*	+
Chicken manure + *S. rolfsii*	+
T. harzianum + *S. rolfsii*	+
T. viride + *S. rolfsii*	+

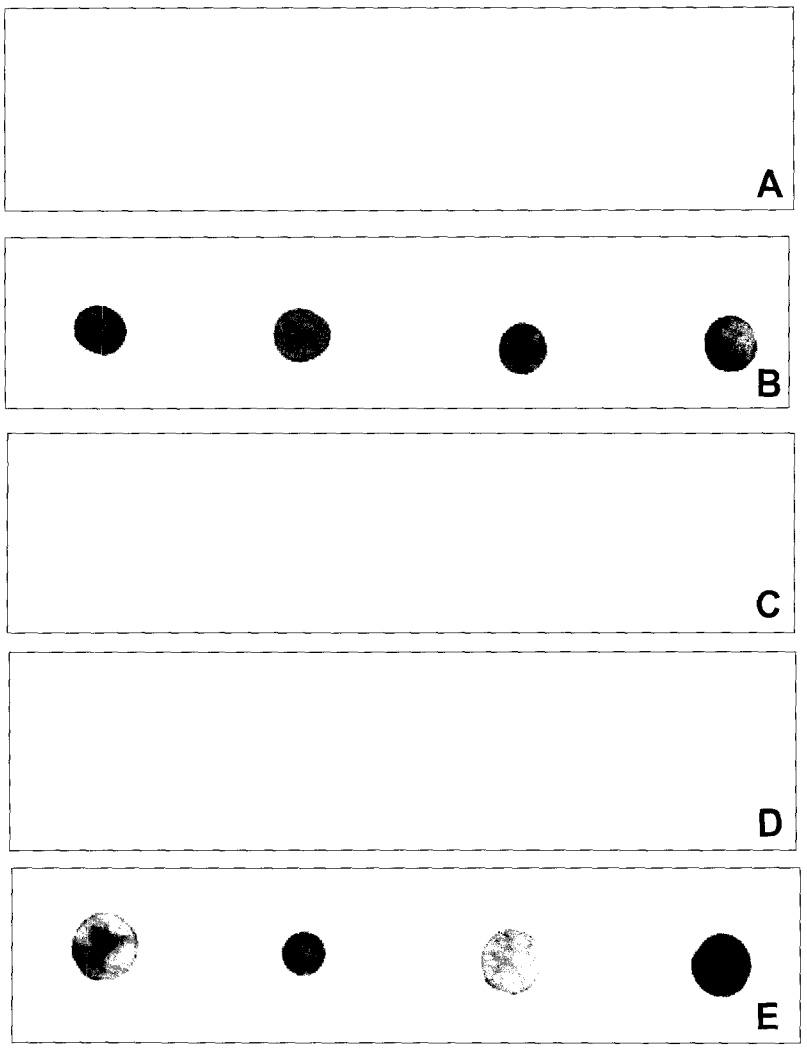

Plate 17 (figs. A-E). Dot blot analysis of tea rhizosphere soil of different treatments probed with PAb of *S. rolfsii* (A) soil treated with *T.harzianum* (B&E) Soil inoculated with *S. rolfsii* (C) soil amended with rabbit manure and inoculated with *S. rolfsii* (D) soil amended with *T. harzianum* and inoculated with *S. rolfsii.*