STUDIES ON SCLEROTIAL BLIGHT OF TEA AND ITS MANAGEMENT

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This is to certify that Ms Indramani Bhagat has carried out her research under my supervision. Her thesis entitled "**Studies on Sclerotial Blight of Tea and its Management**" is based on her original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Botany in accordance with the rules and regulations of the University of North Bengal.

(B. N. Chakraborty)

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Contents

Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] is the most important hot beverage in the world today. It occupies a very important position in Indian agriculture being one of the major foreign exchange earners of the country. The world's best quality of tea is produced in Darjeeling. It is also grown in the slopes of Nilgiris and Annamalai hills of Peninsular South India and Bhramhaputra Valley of Assam located 100m. above sea level. Being a perennial, the tea plant possibly interacts with, and samples more environmental problems than do most other plants. A number of fungal pathogens cause diseases of tea which reduces the quality and quantity of tea production. Sclerotial blight caused by *Sclerotium rolfsii* Sacc. (telomorph: *Athelia rolfsii* (Curzi) Tu and Kimbrough = *Corticium rolfsii* Curzi) is one of the fungal diseases which appears in the nursery grown tea seedlings. The fungus is a soil borne rotting pathogen of very aggressive nature and causes considerable damage of young tea seedlings in the nursery which is very common in the plains but rare in the hills.

S. rolfsii affects the lower stems and roots of tea seedlings at or near the soil line. During infection whitish mycelial growth of the fungus can be seen at the junction of the branch with the stem close to the soil level, which is the most favoured point of attack. A dark brown lesion on a tea seedling's stem near the soil line is a very early indicator. With time, the disease progresses and a white mycelial web spreads over the soil and the basal canopy of the plant, followed by the appearance of sclerotia of mustard seed size on the infected areas. In its advanced stage infection becomes prominent in the root system and subsequently the entire shoot withers and falls and finally the plants die. Seedling death usually occurs rapidly. The fungus can remain in the soil as mycelium, living as saprophytes on diverse organic material and grows actively only in moist soil at moderate to high temperature (25-35^{0}C). Light brown to yellow, round sclerotia may remain viable for many years. *S. rolfsii* occurs in diverse soils, has a very wide host range and world wide distribution (Punja, 1985). It infects at least 500 species in about 100 plant families. Diseases caused by *S. rolfsii* are especially rampant where temperatures are sufficiently high to permit the growth and survival of the fungus.

Plants respond to infection by pathogens in a number of ways which are triggered by the initial recognition phenomenon. Successful establishment of the

pathogens in host depends upon some kind of molecular similarity between two partners (De Vay and Adler, 1976). However, only certain key common antigens are important in host parasite compatibility (Chakraborty and Purkayastha, 1983; Chakraborty *et al.*, 1995a; 1997a; 2002d). It has been observed that with increased antigenic disparity, the response of host may prevent further activity of the parasite (Chakraborty, 1988). Disease detection by immunological means is gaining ground in case of fungal diseases (Chakraborty and Chakraborty, 2003; Gawande *et al.*, 2006).Though significant advances have been made in the development of rapid and sensitive assays for fungi in recent years, commercially available techniques are limited to a few pathogens and diseases. Such detection techniques make it possible to detect mircoquantities of the pathogen within a few hours of infection, which is much more advantageous than the conventional techniques involving pathogen inoculation, visible symptoms and microscopy. On the other hand detecting spores of plant pathogens in air samples can be used predictively to forecast the likelihood of important transmission events (Kennedy *et al.*, 1999; 2000). Detection and quantification of pathogen inoculum has considerable applicability in diagnosis and management of existing and emerging plant diseases within agricultural crop production systems (Kennedy and Wakeham, 2006). These have tremendous potential for plant disease control measures since detection of a pathogen at the initial stage of infection can lead to formulation of control measures before much damage has been done.

The art and science of plant disease control has moved in the direction of biological control of plant pathogens, including use of introduced antagonists. It is now widely recognized that biological control of plant pathogen is a distinct possibility for the future and can be successfully exploited in modern agriculture, especially within the framework of integrated disease management systems. Integrated control is a flexible, multi-dimensional approach to disease control utilizing a range of control components such as biological, cultural and chemical strategies needed to hold diseases below damaging economic threshold without damaging the agro-ecosystem.

The interaction between plants and their pathogens is complex and may be very specific to a given combination of the plant and the fungus. The biochemical

mechanisms responsible for containment of fungal pathogens in the resistant interactions are undoubtedly multifold. Many biochemical changes occur in plants after infection, and some of these have been associated with the expression of defenses that are activated after infection (Chakraborty, 2005). Available evidence also indicates that resistance to disease in many cases is the result of activation of more than one biochemical defense mechanisms (Bera and Purkayastha, 1999; Hammerschmidt, 1999; Chakraborty *et al.*, 2004). It has long been recognized that responses are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid (hypersensitive) cell death. The responses include the formation of lignin, the accumulation of cell-wall appositions such as papillae (Prats *et al.*, 2005), and the early accumulation of phenols (Chakraborty *et al.*, 1995b) within host cell walls. Since it is not practicable to consider all the probable factors associated with disease resistance of tea against *S. rolfsii*, a few promising ones have been undertaken in the present investigation.

The basic objectives of the present investigation are (a) screening of tea varieties for resistance to *S. rolfsii;* (b) estimation of host parasite proteins before and after infection; (c) raising of polyclonal antibodies against antigens of *S. rolfsii* and tea roots; (d) detection of serological cross reactivity between *S. rolfsii* and tea varieties using immunoassays and cellular location following immunofluorescence; (e) detection of *S. rolfsii* in artificially inoculated tea roots by ELISA, dot immunobinding assay and indirect immunofluorescence; (f) determination of the level of phenolics in tea roots following infection by *S. rolfsii;* (g) ascertaining the antifungal activity of phenolics associated with differential host response to infection; (h) assay of phenylalanine ammonia lyase, peroxidase and polyphenol oxidase activities in tea roots following inoculation with *S. rolfsii*; (i) *in vitro* studies of *S. rolfsii* with biocontrol agents, plant extracts and systemic fungicides and (j) developing effective integrated management strategies against sclerotial blight disease of tea.

Before going into the details of the present work, a brief review in conformity with this study has been presented in the following pages.

Literature Review

Microorganisms incited plant diseases are numerous, diverse and omnipresent. More than 1,00,000 plant diseases are known to be caused by plant pathogens (fungi, bacteria, viruses, mycoplasmas, viroids etc). They are equally diverse in their ecological niches. Fungi are most damaging and sometimes highly destructive too (Gupta and Mukherjee, 2006). Diseases caused by *Sclerotium rolfsii* are especially rampant in the tropics and subtropics where temperatures are sufficiently high to permit the growth and survival of the fungus. Despite continuous research over the years since the report by Rolfs on *S. rolfsii* on tomato, this pathogen continues to plague growers and cause considerable economic loss. Eruptive germination on soil is greatest at 21-30^{0}C and is less common below 15^{0}C and above 36^{0}C. Although percentage of germination is lower at soil depth below 2.5 cm, than it is at the soil surface and is nil below 8 cm, results from controlled gaseous studies suggest that the inhibition is not due to oxygen depletion or ethylene and carbon dioxide buildup (Punja, 1985). Sclerotia formed in non-sterile soil germinate poorly and less consistently than those produced in laboratory cultures. Mycelium survives better in sandy soils than in fine-texture soils. Sclerotia survive better in soil near the surface rather than buried beneath it. Factors that increase nutrient leakage and the activities of soil microorganisms near sclerotia, or that predispose sclerotia to antagonism, may accelerate the death of sclerotia. The effective plant disease management lies in rapid diagnosis of the disease and detection of its causative agent. The prevalence, the extent of spread and damage is assessed through surveys on the basis of symptoms followed by detection of pathogen in laboratories, which helps to assess, alter and modify the effectiveness of the plant protection measures that are being followed. Apart from the role of detection and diagnosis in plant disease management, detection of pathogen(s) can act as an important tool to localize and prevent the spread of the disease(s). Recognition of the diagnostic potential of such determinants for both experimental and applied investigations in plant pathology has resulted in a bewildering array of techniques being developed referred to as immunoassays. The presence of cross-reactive antigens (CRA) between plant hosts and parasites have been reviewed (De Vay and Adler, 1976; Chakraborty, 1988; Purkayastha, 1994). Immunological techniques used for detection of fungal pathogens in soil, water and plant tissues

have also been reviewed by various workers (Hansen and Wick, 1993; Werres and Steffens, 1994, Chakraborty and Chakraborty, 2003; Gawande *et al.*, 2006). On the other hand detection and quantification of pathogenic inoculum has considerable applicability in diagnosis and management of existing and emerging plant diseases within agricultural crop production systems (Kennedy *et al.*, 1999; 2000). Detecting target particles or spores of plant pathogens in air samples can be used predictively to forecast the likelihood of important disease transmission events. A range of methods have been developed by Kennedy and Wakeham (2006), which can be used to detect inoculum within air samples. In the era of globalization and WTO regime, detection of plant pathogens has an immense role to play because the free movement of materials create chances for introduction of new pathogens across the countries.

A short, selective review is presented on the observation of previous workers in concordance with the present line of investigation on three major aspects (A) Serological relatedness between host and pathogens; (B) Biochemical changes in plants following infection and (C) The effective integrated disease management practices.

Serological relatedness between host and pathogens

Taking advantage of the serological relationship between host and pathogen, the antiserum raised against the pathogen is being used for the detection of the pathogen in the host tissues beginning from the early stages of host pathogen interaction. Commercial diagnostic kits have been offered in recent years for the rapid diagnosis of several fungi in plant tissues, soil and water.

Using the serological techniques of agglutination, gel diffusion and immunofluorescence, Amos and Burrell (1966) identified eight species of the genus *Ceratocystis*. The immunofluorescence technique proved to be the most useful in differentiating among these species. Root antigens from four cotton varieties and isolates of *Fusarium* and *Verticillium* species exhibited common antigen relationship in immunodiffusion tests (Charudattan and De Vay, 1972). Five to eight precipitin bands were observed in the homologous reactions; of these only one or two bands

were present in heterologous reactions. The common antigenic determinant shared by cotton fungal isolates did not appear to be related to the severity of wilt symptoms, but it had affected host-pathogen compatibility during the process of root infection. Using fluorescein isothiocynate (FITC), De Vay *et al.* (1981) demonstrated indirect immunofluorescence in cross-sections of cotton roots. Cross reactive antigenic (CRA) substance was concentrated mainly on xylem elements, the endodermis, epidermal cells and around the cortical tissue. Protoplasts prepared from cross-sections of young cotton roots also contained the CRA concentrated in the region of the plasmalemma. Treatment of conidia and mycelia of *Fusarium oxysporum* with antiserum to cotton and followed by labelling with FITC indicated that CRA was mainly present in hyphal tips and in patch like areas on conidia. Ishizaki *et al.* (1981) investigated the serological cross reactivity of *Sporothrix schenkii* serum with various unrelated fungi by use of immunodiffusion tests. A rabbit anti *S. schenkii* serum was obtained, which related with *Cladosporium werneckii, C. carrionii, C. bantianum, Coccidioides immitis, Phialophora jeanselmi, P. gougerotii, P. dermattidis, Fonsecaea pedrosoi, Asperigillus fumigatus, Histoplasma capsulatum* and *Trichophyton mentagrophytes,* but not with *Saccharomyces cerevisiae* antigens. The serological determinates responsible for the cross-reactions were suggested to be D-galactosyl residue.

Rabbit antisera were raised by Chakraborty and Purkayastha (1983) against the antigens of *Macrophomina phaseolina* (isolate MPI) and roots of soybean cultivars *viz.*, Soymax and UPSM-19, susceptible and resistant to charcoal rot disease respectively. These antisera were used in immunodiffusion and immuno-electrophoretic tests for the presence of common antigens between isolates of *M. phaseolina* and soybean cultivars. Four antigenic substances were found common between the susceptible soybean cultivars and isolates of *M. phaseolina* but no common antigens were detected between resistant cultivars and the fungus. Cross-reactive antigens were also detected between *Phytophthora infestans* and potato cultivars (King Edward and Pentland Dell) using ELISA (Alba and De Vay, 1985). Immunodiffusion, immuno-electrophoretic and cross immuno-electrophoretic analysis of rice antigens using polyclonal antisera raised against *Acrocylindrium oryzae* was done by Purkayastha and Ghoshal (1985). When the antigen preparation

of *A. oryzae* was cross-reacted with its own antiserum or against the antisera of four susceptible rice cultivars, one precipitin band was detected. Precipitin band was detected when antiserum of the resistant cultivar was cross reacted with antigen preparations of three isolates of *A. oryzae*.

Purkayastha and Ghoshal (1987) also compared the antigenic preparations from two isolates of *Macrophomina phaseolina* (causal agent of root rot of groundnut), four non-pathogens of groundnut (*viz., Corticium sasaki, Colletotrichum lindemuthianum, C. corchori* and *Botrytis alii*), and five cultivars of groundnut using immunodiffusion, immunoelectrophoresis and crossed immunoelectrophoresis in order to detect CRA. Common antigens were found among the susceptible cultivars of groundnut and two isolates of *M. phaseolina* but not between non pathogens and groundnut cultivars. No antigenic similarity was found between non pathogens and *M. phaseolina* isolates. Competitive types of two novel enzyme linked immunosorbent assays (ELISA) for *Fusarium* species were developed by Kitagawa *et al.* (1989). Antiserum against a strain (F504) of *F. oxysporum* was elicited in rabbits, and a highly specific, sensitive, and accurate ELISA for the homologous strains was developed by using the antiserum with β-D-galactosidase-labeled anti-rabbit IgG as the secondary antibody and cell fragments of the strain attached to amino-dylark balls as the solid-phase antigen. All other micro-organisms tested, including nine other strains of *Fusarium*, showed little cross-reactivity. When cell fragments of *F. oxysporum* (F504) attached to the balls were used as a solid phase antigen in a heterologous competitive ELISA, the modified system was a general assay for 10 strains of four *Fusarium* species with a high sensitivity. The specificity of the heterologous competitive ELISA was shown to be limited to *Fusarium* species.

Fuhrmann *et al.* (1989) raised antisera from a rabbit immunized with *Penicillium verrucosum* var. *verrucosum*. These antisera were characterized by immunofluorescence and by indirect enzyme-linked immunosorbent assay for their reactivity with 44 strains of moulds. Antigenically, *P. verrucosum* var. *verrucosum* (subgenus *Penicillium*) appeared to be similar to strains belonging to subgenus *Furcatum*, but strongly different from *Penicillium frequentans* (subgenus

Aspergillides) specific absorption of antibodies to antigens confirmed the existence of similar biochemical structures on *Penicillium frequentans, Aspergillus versicolor* and *Aspergillus fumigatus.* Immunological procedures may thus significantly contribute to refine the taxonomic classification of moulds. Purkayastha and Banerjee (1990) studied common antigenic relationships between seven soybean cultivars, their pathogens and non pathogens using immunodiffusion, immunoelectrophoresis and indirect ELISA technique. CRA between susceptible soybean cultivars and the virulent strain of *Colletotrichum dematium* var. *truncate* were detected but no CRA could be detected between avirulent pathogen or non-pathogen and soybean cultivars.

Antibodies to three isolates each of *Armillaria mellea, A. ostoyae, A. tabescens* and *Lentinula edodes* were isolated from eggs of immunized laying hens. The reactivity of each antibody preparation with the isolates was examined using an enzyme-linked immunosorbent assay (ELISA). The cross-reactivity of the antibody preparations to a given *Armillaria* species varied considerably when tested against isolates of other *Armillaria* species. Several antibody preparations were capable of distinguishing isolates of homologous species from isolates of heterologous species. The specificity of the antibodies present in eggs was dependent on time elapses since immunization (Burdsall *et al.,* 1990). Rabbit antisera were raised against one resistant cultivar (UPSM-19), two susceptible cultivars (DS-74-24-2 and PK-327) of soybean and three isolates of *Myrothecium roridum* (M-1, ITCC-1143 and ITCC-1409) for analysis of CRA shared between host and pathogen. Results of immunodiffusion revealed that common antigens were present only between the virulent strain and susceptible host cultivars which was confirmed by presence of one common antigen in immunoelectrophoretic analysis. In case of resistant cultivars (UPSM-19 and DS-73-16) no CRA was detected (Ghose and Purkayastha, 1990).

Sundaram *et al.* (1991) prepared antisera against purified mycelial proteins from *Verticillium dahliae,* the predominant fungus species in the potato early dying complex. The tested antisera against crude mycelial preparations of *Verticillium* spp. using indirect enzyme-linked immunosorbent assay (ELISA) reacted positively with 11 of 12 *V. dahliae* isolates from potato, cotton and soil but negatively with one isolate from tomato. The antisera did not react with mycelial proteins from *Fusarium*

spp. from potato and cotton, with a *Colletotrichum* sp. from potato, or with one isolate of *Rhizoctonia solani* from sugarbeet. Double antibody sandwich (DAS) - ELISA, using polyclonal antisera, detected *V. dahliae* and *V. albo-atrum* in infected roots and stems of potato. Ricker *et al.* (1991) detected water soluble antigens produced by *Botrytis cinerea* in spiked and naturally infected grape juice by using an enzyme immunoassay with an indirect format of antibody horseradish-peroxidase conjugate bound to polyclonal rabbit antibodies directed against *B. cinerea*. Daniel and Nilsson (1991) raised polyclonal antiserum against mycelial extracts from *Phialophora mutabilis a* wood degrading soft rot fungus. In ELISA, the antiserum reacted strongly to moderately with six soft rot *Phialophora* species. With exception of *Cerotocystis albida,* the serum reacted weakly or not at all with 11 other mold, and rot fungi occurring frequently in or on wood. The antiserum was cross-reacted strongly with antigens in extracellular filtrates from *P. mutabilis* cultures that contained about 40mg / ml of protein. Ultrastructural and immunocytochemical studies on wood degraded by *P. mutabilis* showed specific localization of the fungal cell wall and certain intracellular structures. Extracellular labeling within soft rot cavities and sites of erosion decay of wood also were noted. The antiserum was assessed by ELISA for detecting the presence of fungus and soft rot in untreated and preservative treated wood blocks of pine and birch degraded for periods of 1-12 months. *P. mutabilis* was detected in samples from all wood blocks degraded to low or high weight loss. Highest ELISA readings were recorded for wood blocks with highest substrate losses and vice versa.

A polyclonal, enzyme-linked immunosorbent assay of *Phytophthora infestans* has been developed by Beckman *et al.* (1994) for use in the determination of fungal biomass during the early stages of infection of tuber disc of *Solanum tuberosum.* By optimizing the dilution of sample extracts and the dilution of primary anti *P. infestans* antiserum, quantification of the biomass of *P. infestans* in zoospore inoculated tuber disc could be achieved by 8-18h after inoculation. Differences in growth between avirulent and virulent isolates of *P. infestans* on the resistant potato cv. Kennebec were quantified by 32-48h after inoculation. Together with a comparison of growth of the same isolates on the susceptible cv. King Edward, these results comprised on ELISA of the Quadratic Check. On the resistant host, the

growth of the avirulent isolate was essentially arrested by 16h after inoculation, whereas that of the virulent isolate continued throughout the time course. On the susceptible host, however, the avirulent isolate appeared more aggressive than the virulent isolate. These results demonstrated that ELISA, which is often simpler to perform than other procedures for estimating fungal growth, may be used to complement biochemical studies of rapidly induced plant defence response.

Purkayastha and Pradhan (1994) studied serological differences between three strains of *Sclerotium rolfsii* and groundnut cultivars. Among three strains of *S. rolfsii*, 266 was most virulent and exhibited antigenic relationship with susceptible groundnut cultivars (AK-12-24, Gangapuri and J-11). However, resistant cultivars (ICGS-26 and JL-24) showed no antigenic relationship with fungal strains in either immunodiffusion or immunoelectrophoretic test.

Brill *et al.* (1994) produced polyclonal antibodies against immunogen preparations from culture filtrate and mycelial extract of *Phomopsis longicalla* and soybean. The PAbs were purified to the immunoglobulin fraction and tested in indirect ELISA and in DAS-ELISA. The PAbs raised to culture filtrate were more specific but less active in binding to members of the *Diaporthe- Phomopsis* complex than were those raised to the mycelial extract immunogen preparation. DAS-ELISA was more specific and 100-fold more sensitive in detecting members of the complex than was indirect ELISA. Variability in specificity between different PAbs was lower in DAS-ELISA, compared with indirect ELISA. Immunization of one rabbit with culture filtrate over an extended time resulted in maximum anti- *P. longicolla* activity after three immunizations and the activity became constant against most members of the complex at the same time.

CRA were found among the isolates of *Bipolaris carbonum* and susceptible tea varieties (Chakraborty and Saha, 1994a). Such antigens were not detected between isolates of *B. carbonum* and resistant varieties, non pathogens and tea varieties as well as non pathogens and isolates of *B. carbonum*. Indirect staining of antibodies using FITC indicated that in cross sections of tea leaves the CRA were concentrated mainly around epidermal cells. Treatment of mycelia and conidia of

B. carbonum with antisera to tea leaves (TV-18) and indirect staining with FITC indicated the presence of CRA in the young growing hyphal tips of conidia.

Polyclonal antisera were raised against mycelial suspensions of *P. theae* (isolate Pt-2) causal agent of gray blight disease and leaf antigens of Teen Ali 17/1/54 and CP-1 and immunological tests were performed in order to detect CRA shared by the host and parasite (Chakraborty *et al.*,1995a). CRA were found among the susceptible varieties and isolates of *P. theae*. Such antigens were not detected between isolates of *P. theae* and resistant varieties, *B. tetramera* and tea varieties or isolates of *P. theae*. Indirect staining of antibodies using FITC also indicated the presence of CRA in the epidermal cells and mesophyll tissue of tea leaves. CRA was evident in the young hyphal tips of the mycelia and on the setulae and appendages of the conidia of *P. theae*.

Chakraborty *et al.* (1996b) raised polyclonal antisera against leaf antigens to tea varieties (TV-18, Teen Ali 17/1/54 and CP-1) and mycelial antigens of *G. cingulata* (isolate GC-1) separately in white rabbits. CRA were found among the susceptible varieties and *G. cingulata* isolates. Such antigens were not detected between *G. cingulata* and resistant varieties of tea, non pathogen and tea varities as well as *G. cingulata* and non pathogens. In cross sections of tea leaves (TV-18), the CRA was found to be concentrated in epidermal cells, mesophyll tissue and vascular elements. Chakraborty *et al.* (1997b) also determined the presence of CRA between *Fusarium oxysporum* and soybean cultivars. Antigens were prepared from the roots of ten varieties of soybean and mycelium of *F. oxysporum*. Polyclonal antisera were raised against the mycelial suspension of *F. oxysporum* and root antigen of susceptible soybean cultivar (UPSM-19). The immunoglobulin (IgG) fraction of those antisera were purified by ammonium sulfate precipitation & DEAE-Sephadex column chromatography. In enzyme-linked immunosorbent assay, antigens of susceptible cultivars showed higher absorbance values when tested against the purified anti-*F. oxysporum* antiserum. Antiserum produced against UPSM-19 showed cross-reactivity with the antigens of other cultivars. Indirect staining of antibodies using FITC indicated that CRA were concentrated around xylem elements, endodermis and epidermal cells in cross-section of roots (UPSM-19), while in the resistant variety, fluorescence was concentrated mainly around

epidermal cells and distributed in the cortical tissues. CRAs were also present in microconidia, macroconidia and chlamydospores of the fungus. Polyspecific antisera were raised against a plant cell supernatant fraction from homogenized naturally blister infected tea leaf tissue (AV-2) and immunoprecipitated with healthy leaf antigens of AV-2 in order to separate antibodies unique to *E. vexans*. Immunoglobulin (IgG) was purified following ammonium sulphate fractionation and chromatography on DEAE-Sephadex. CRA were detected between the pathogen and the susceptible Darjeeling tea varieties in immunodiffusion tests and enzyme-linked immunosorbent assay (Chakraborty *et al.* 1997a).

Gupta *et al.* (2000) had done immunodetection of teliospores of *Telletia indica* which is a causal agent of Karnal bunt (KB) of wheat, by using fluorescent staining test. Polyclonal antibodies were raised against teliospores and indirect immunofluorescence (IIF) test was developed using anti-teliospores serum and binding was monitored by goat-rabbit antibody conjugated to FITC. The standardization of IIF test was carried out by optimization of dilution of anti-teliospores antibodies, fluorescent probe and exposure time. The teliospores of *T. indica* showed bright green, patchy and ring shaped fluorescence around the teliospore. The spore exhibited uniform distribution in discrete regions of spore probably in spore episporium. Similar fluorescence pattern in the teliospores of KB isolated from infected wheat seeds of cultivars HD 23328, UP 2338, PBW 393, WH 542, as well as RR 21 (susceptible cultivars) respectively, is an indication of the presence of similar antigenic configuration of teliospores. Again, they did not exhibit variation in the expression of teliospore associated molecular pattern during previous and subsequent years of infection. Polyclonal antiserum raised against *T. indica* also reacted strongly in agglutination reaction with intact teliospores of Pantnagar isolate. The wheat grains with different grades of infection could be readily detected by Seed Immunoblot Binding Assay (SIBA). The teliospores of karnal bunt infected wheat seeds when kept for vigour reacting on nitrocellulose paper, formed a coloured imprint after the paper was assayed. The SIBA developed was not only a better indication of teliospores load on seed but also quality of seed in terms of vigour. The developed immunodetection method apparently proved to be useful in routine

monitoring of wheat lots for the presence of karnal bunt pathogen (Kumar *et al.*, 2000)

Viswanathan *et al.* (2000) performed ELISA using PAb raised against *Colletotrichum falcatum* to detect pathogen before the symptom development. When 20 different sugarcane varieties were subjected to ELISA test after pathogen inoculation, it showed a clear variation in disease resistance among them as in field testing. Chakraborty *et al.* (2000) had demonstrated immunological detection of *Sphaerostilbe repens, Trichoderma viride* and *T. harzianum* using DAC-ELISA formats to develop strategies for management of violet root rot of tea. Polyclonal antibody based immunoassay for detecting *Fomes lamaoensis,* causing brown root rot disease of tea has also been developed (Chakraborty *et al.*, 2001c). Eight blood samples were collected and IgG were purified using DEAE cellulose. Immunodiffusion tests were performed in order to check the effectiveness of mycelial antigen preparations of *F. lamaoensis* for raising PAbs. Optimization of PAbs was done using indirect ELISA. Increased activity of PAbs against *F. lamaoensis* could be noticed from second bleedings, which continued up to fourth bleeding. Root antigens prepared from healthy and artificially inoculated (with *F. lamaoensis*) tea plants (Teen Ali 17/1/54, TV-18, TV-22, TV-26, TV-27, TV-28, TV-30, S-449, BSS-2) were analysed following DAC-ELISA format. Such format was also used to detect the pathogen in infested soil. Young mycelia of *F. lamaoensis* gave bright fluorescence in indirect immunofluorescence tests using PAbs and FITC-conjugates of goat specific for rabbit globulin. Such immunological assays developed for detection of *F. lamaoensis* in rhizosphere of tea plantations can enable disease prevention at an early stage.

Immunodiagnostic kits were developed for detection of *Ustulina zonata,* causing charcoal stump rot disease, in the soil and tea root tissues. PAbs were raised separately against mycelial and cell wall antigens prepared form 10-day-old cultures of *U. zonata.* Optimization of PAbs was done using indirect ELISA. Two different ELISA formats such as direct antigen coated (DAC) and double antibody sandwich (DAS) were tested to detect the pathogen in soil and artificially inoculated root tissues. Indirect immunofluorescence using PAbs and FITC conjugates of goat

specific for rabbit globulin were assessed for their potential to detect mycelia and spores in soil (Chakraborty *et al.*, 2002b).

Chakraborty *et al.* (2002d) studied serological cross reactivity between *Glomerella cingulata* and *Camellia sinensis.* PAbs were raised against antigen preparations from mycelia and cell wall of *G. cingulata* (isolate Gc-1), causal agent of brown blight of tea, mycelia of *Fusarium oxysporum* (non pathogen of tea) and leaf antigens of TV-18 and CP-1. CRA were found among the susceptible varieties of tea and isolates of *G. cingulata* (Gc-1, 2 and 3). Such antigens were not detected between resistant varieties of tea and isolates of *G. cingulata* (Gc-1, 2 and 3); non pathogen (*F. oxysporum*) and tea varieties; isolates of *G. cingulata* and *F. oxysporum* and between non host (*Glycine max*, *Cicer arietinum* and *Camellia japonicum*) and *G. cingulata.* Antisera raised against cell wall preparations gave better recognition than that against mycelial preparations as observed in ELISA test with antigens of tea leaves of different ages.

Biochemical changes in plants following infection

Disease develops in individual plants by a series of sequential steps beginning with the arrival of inoculum at the plant surface and ending with the terminal stages of pathogens. There are many defense barriers in plants such as the cuticle, cell wall or constitutive antimicrobial compounds as well as defenses triggered by the invader. The success or failure of infection is determined by dynamic competition and the final outcome is determined by the sum of favourable and unfavourable conditions for both the pathogen and host cells. One of the most important and well documented host responses is the biochemical changes following infection. Phenols in plants which occur constitutively are thought to function as preformed inhibitors associated with non host resistance. Since the phenolic intermediates have a role in the active expression of resistance, an underlying problem in ascertaining that such secondary metabolites are of primary importance has been the localization and timing of the host response (Nicholson and Hammerschmidt, 1992).

In potato tubers chlorogenic acid was reported to accumulate slower following inoculation with *P. infestans* than in non-inoculated controls, regardless of cultivar resistance (Gans, 1978). In contrast, in some susceptible cultivars chlorogenic acid accumulates at an accelerated rate after inoculation (Henderson and Friend, 1979). The differentiation of the responses of plants to pathogens based on host and non host interactions has been argued by Heath (1980).

Chlorogenic acid acts as a reservoir for the caffeoyl moiety that, as an activated phenylpropanoid, could be shunted to the synthesis of other phenolics possibly involved in containment of the pathogen (Friend, 1981). The accumulation of chlorogenic acid may represent a general rise in phenolic biosynthesis which can ultimately result in the accumulation of compounds with sufficient toxicity to be involved in resistance. When carrot root slice is infected with *Botrytis cinerea*, the infection leads to the production of inhibitors such as 6-methoxymellein, p-hydroxybenzoic acid and falcarinol (Harding and Heale, 1981). Oat produces nitrogen containing phenolic phytoalexins, the avenalumins, and these compounds accumulate only in incompatible host pathogen interactions (Mayama *et al.*, 1981).

Mayama and Tani (1982) took advantage of the UV-absorbance and auto fluorescence spectra of the avenalumins and used microspectrophotometry to reveal the presence of intense fluorescence only in cells immediately associated with the infection site. Rapid accumulation of phenols may result in the effective isolation of the pathogen (or non pathogen) at the original site of ingress (Legrand, 1983; Ride, 1983). In potato, phenols accumulate as an initial response to infection (Hammerschmidt, 1984; Hachler and Hohl, 1984). The accumulation of polymerized phenols also occurs as a rapid response to infection. Hydroxycinnamic acids and their derivatives are thought to contribute to the discoloration and autofluorescence of host tissues at the site of infection (Farmer, 1985; Bolwell *et al.*, 1985).

Werder and Kern (1985) demonstrated resistance of maize to *Helminthosporium carbonum* and subsequent changes in host phenolics and their antifungal activity. Maize inbreds Prl (resistance) and Pr (susceptible) to *B. zeicola* race 1 were inoculated and phenolic material was extracted from maize leaf tissue. The components were then analyzed and resistance was studied with respect to

phenol metabolism and accumulation of fungitoxic compounds. Host responses could be differentiated by changes in content of phenolic compounds. The pattern of changes of total phenolic content (hydrolyzed and unhydrolyzed ethylacetate soluble phenols) of resistant and susceptible inbreeds did not differ much between 0 h and 96 h. after inoculation. However, phenolics content in the resistant inbreed increased between 96 and 120 h after inoculation to a level two to three times higher than that of susceptible and non-infected control in breeds. They isolated four antifungal compounds, A, B, C and D from hydrolyzed maize leaf extracts. All four compounds were fungitoxic to *B. zeicola* in spore germination and chromatographic bioassays. Compounds A and B were inhibitory to *B. zeicola* only in high concentrations. The investigators suggested a role of the phenol metabolism in the resistance of maize to *B. zeicola* based on different content of total phenolics in resistance and susceptible inbreeds. The compounds C and D were supposed to play a role in the resistance mechanism as fungitoxic components.

Saxena *et al.* (1986) evaluated the changes in phenolics of two each of resistant and susceptible varieties of wheat leaves in response to *Puccinia recondita* causing brown rust. They found that resistant varieties exhibited higher concentration of phenolics than the susceptible ones. Esterification of phenols to cell-wall materials has been considered as a primary theme in the expression of resistance (Fry, 1986; 1987). Biochemical analysis of pea varieties resistant and susceptible to *Erysiphe polygoni* causing powdery mildew disease revealed that the quantity of total phenol and ortho-dihydroxyphenol was higher in stem and leaves of resistant varieties as compared to susceptible ones which decreased as the age of plant increased in all the varieties (Parashar and Sindhan, 1987).

The temporal and spatial differences in the accumulation of phenylalanine ammonia lyase (PAL) mRNA occurred as a response on incompatible race of fungus, whereas a significantly different profile of mRNA accumulation occurred in interactions involving a compatible race (Cuypers *et al.*, 1988). The kinds of phenolic compounds that accumulate prior to the active defence response as well as their origin has been addressed by Matern and Kneusel (1988) using parsley leaves with *P. megasperma* f. sp. *glycinea* (Pmg) or treatment of parsley cell suspensions with a Pmg elicitor results in the accumulation of substantial concentrations of

coumarin phytoalexins as well as esterification of phenylpropanoids, in particular ferulic acid, to cell walls. Treatment of parsley cells with the Pmg elicitor causes the synthesis of the coumarin phytoalexins isopimpinellin, psoralen, bergapten, xanthotoxin and graveolone. The healthy leaves of *Morinda tomentosa* contained the two methoxyflavonols 4'–OMe Kaempferol and 3', 4' – dia OMe quercetin, and the four phenolic acids – vanillic, syringic, gentisic and ferulic. The *Colletotrichum gloeosporoides* infected leaves contained the hydroxyflavonols kaempferol and quercetin along with four phenolic acids found in healthy leaves. The diffusates of both the pathogen and non-pathogen (*F. solani*) treated leaves contained quercetin and kaempferol (Abraham and Daniel, 1988).

Matern and Kneusel (1988) have proposed that the defensive strategy of plants exists in two stages. The first is assumed to involve the rapid accumulation of phenols at the infection site, which function to slow (or even has) the growth of the pathogen and to allow for the activation of "secondary" strategies that would more thoroughly restrict the pathogen. Secondary responses would involve the activation of specific defenses as the *de novo* synthesis of phytoalexins or other stress-related substances. They argue that the initial defense response must occur so rapidly that it is unlikely to involve *de novo* transcription and translation of genes, which would be characteristic of the second level of defence. The sequence of events in a defence response can be thought to include – host cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic substituents or physical barriers such as appositions or papillae, and finally, synthesis of specific antibiotics such as phytoalexins.

Prasada *et al.* (1988) also reported that after infection total phenol increased in green and ripe tomato fruits in case of rotting due to *Sclerotium rolfsii*. There is often a greater increase in phenolic biosynthesis in resistant host species than in susceptible hosts and it is sometimes postulated that the increase in phenolic compounds is part of the resistance mechanism. Some of these compounds are toxic to pathogenic and non-pathogenic fungi and have been considered to play an important role in disease resistance (Vidyasekharan, 1988). Changes in phenol contents were determined by Oke (1988) in healthy and *Colletotrichum nicotianae* infected leaves of tobacco. After infection the quantity of total phenols and ortho

dihydroxy phenol increased in both stem and leaves of susceptible and resistant varieties.

The changes in phenolic and nitrogen metabolism were investigated by Tore and Tossi (1989) in healthy and infected (with *Thielaviopsis basicola*) tobacco roots and leaves. The cholorgenic acid content increased in infected root and leaves compared with the control beginning on the 8^{th} day after inoculation. Polyphenol content in sweet cherry bark was drastically changed after infection by *Cercospora personii* (Bayer, 1989). Infected tissue and closely neighboring areas were characterized by the appearance of phenolic aglycones which inhibited growth of both the pathogen. Mechanically wounded bark tissues showed different phenolic patterns than infected ones.

Etenbarian (1989) detected quantitative changes in phenolic compounds at different time intervals on barley varieties inoculated with *Puccinia hordei*. Luthra (1989) detemined the levels of total phenol in sorghum leaves, resistant and susceptible to *Ramulispora sorghicola* at 15-day-intervals after 25 day of sowing. Resistant varieties exhibited high phenol content in comparison to susceptible ones at all stages of growth.

Phenolic compounds inhibitory to the germination of spores of *Colletotrichum graminicola* were shown to leach from necrotic lesions on corn leaves caused by the fungus. Primary components of the phenolic mixture were identified as esters and glycosides of p-coumaric and ferulic acids as well as the free compound themselves. Spores of *C. graminicola* produced in acervuli of infected leaves were shown to be surrounded by a mucilaginous matrix as in the case when the fungus is cultured *in vitro*. It is suggested that the mucilage protects spores from the inhibitory effects of the phenols by the presence of proline rich proteins that have been shown to have a high binding affinity for a variety of phenols (Nicholson *et al.*, 1989). The relatively non-specific disruptive effects on cells that result from wounding lead almost immediately to a variety of physiological changes, including oxidation of secondary metabolites. The accumulation of these esters preceded the onset of visible necrosis of infection sites, the concentration of the compound fell substantially after the onset of necrosis both of which strengthen the argument for

their involvement in the browning response (Bostock and Stermer, 1989). Toxic phenylpropanoids, such as ferulic acid, can form rapidly without the involvement of the traditionally accepted route of phenyl-propanoid synthesis and conversion to CoA esters (Hahlbrock and Scheel, 1989). It has long been recognized that responses are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid (hypersensitive) cell death (Fernandez and Heath, 1989).

Baker *et al.* (1989) examined specific race interaction with clones of resistant and susceptible genotypes and they found greater accumulation of phenolic compounds in resistant reaction than in susceptible reaction. They suggested that accumulation of phenolics may play a role in natural and induced interaction involving *Colletotrichum trifolii* and *Medicago sativa.*

Kumar *et al.* (1990) analysed certain biochemical changes in the pearl millet shoots infected with downy mildew pathogen (*Sclerospora graminicola*). The estimation revealed that the total phenol and free amino acids content were found to be low both in diseased shoot and roots of pearl millet (*Pennisetum glaucum*). A marked accumulation of two caffeic acid esters after inoculation of maize with *Glomerella graminicola* or *Cochliobolus heterostrophus* was reported in both compatible and incompatible combinations (Lyons *et al.*, 1990). One compound was identified as caffeoyl glucose, whereas the other was a caffeoyl ester of an unknown organic acid moiety. Although neither compound was fungitoxic, a pattern of rapid accumulation followed by a sharp decrease in the amount of both compounds in the tissue suggested that they may serve as a pool of phenols required for diversion to other products. Seasonal changes in the phenolic constituents of jack pine seedlings (*Pinus banksiana*) in relation to the purpling phenomenon were studied by Nozzolillo *et al.* (1990).

Mansfield (1990) has proposed that cell death results from irreversible membrane damage that may occur in response to pathogen recognition or as a result of activated host response. Niemann *et al.*, (1991) demonstrated that low molecular weight phenols, such as benzoic acids and the phenylpropanoids, are formed in the initial response to infection. Early after infection, low molecular weight phenols

accumulate in both incompatible (resistant) and compatible (susceptible) interactions. Whether these compounds, are significant in the ultimate host response presents a perplexing problem. Bruzzese and Hasan (1991) demonstrated that accumulation of phenols at the infection site occurred as early as 3h after inoculation, indicating an association of phenols with the initial stages of the response. The contents of phenols, o-dihydroxy phenols and peroxidase activity in healthy and *Curvularia andrepogonis* infected leaves of *Java citronella* (*Cymbopogan winterianus*) were determined by Alam *et al.* (1991). As a result of infection the content of phenols and peroxidase increased two and four fold respectively, in necrotic lesions compared to healthy leaves. It has been suggested by Permulla and Heath (1991) that the accumulation of phenolics as an initial response to infection may reflect a general increase in host metabolism as well as an accumulation of relatively non-toxic secondary metabolites, which could ultimately serve as precursors for compounds essential for expression of resistance. In the interaction of potato tubers with *Verticillium dahliae* hypersensitive browning and suberization are characteristic of the initial events in resistance rather than production and accumulation of phytoalexins (Vaughn and Lulai, 1991).

The *Fusarium* sp. infected leaves of *Trianthema portulacastrum* contained 6, 7, dimethoxy-3, 5, 4' – trihydroxy flavone, vanillic acid, p-hydroxybenzoic acid, quercetin and ferulic acid. By using drop diffusate technique it was found that the pathogen induces the formation of quercetin and ferulic acid (Darshika and Daniel, 1992). Changes in carbohydrates, amino acid and phenolic contents in jute plant on inoculation with *Macrophomina phaseolina, Colletotrichum corchori* and *Lasiodiplodia theobromae* were studied by Sahabuddin and Anwar (1992). Total sugars, non-reducing sugars, starch and total free amino acids were found to decrease on inoculation with all the three test pathoges of jute, while reducing sugars, total phenols and orthodihydric phenols increased.

Fifteen isolates of *Phytophthora parasitica,* nine from tobacco (causing black shank disease) and six from other host plants were compared by root inoculation with regard to their pathogenicity to young tobacco plants. A progressive invasion of the aerial parts over 1 week was observed only with the black shank isolates, while the non-tobacco isolates induced leaf necrosis within 2 days. Similar necrosis

occurred when the roots of tobacco plants were dipped in diluted culture filtrates from non-tobacco isolates, but not in those from tobacco isolates. The necrosis inducing filtrates contain 10 kDa protein band which was not present in the other filtrates. This protein (named parasiticein) was purified by ion exchange chromatography to homogenicity in SDS-PAGE and reverse phase HPLC. Parasiticein was serologically related to cryptogein, a member of the elicitin family of proteinaceous elicitors. Like the other elicitins, parasiticein induced necrosis in tobacco plants and protected them against black shanks. It most closely resembled little leaf necrosis. Ricci *et al.* (1992) suggested that the absence of parasiticein production by the black shank isolates might be a factor involved in their specific pathogenicity to tobacco.

A glycoprotein elicitor of phytoalexin accumulation in leaves of *Phaseolus vulgaris* produced well before lysis in the medium of cultures of *Colletotrichum lindemuthianum* was purified to homogeneity by Coleman *et al.* (1992). The glycoprotein was a monomer of M.W. 28 kDa. The glycosyl side chains which accounted for 43% of the weight of the holoprotein, were composed principally of galactose, mannose and rhamnose exhibited a minimum degree of polymerization of eight and were apparently O-linked to abundant serine and / or threonine residues of the peptide backbone. In a *P. vulgaris* leaf infection bioassay the purified glycoprotein had activity easily detectable at nanomolar concentrations and induced browning of the treated tissue and also the accumulation of both phenylalanine ammonia-lyase and the isoflavanoid phytoalexins phaseollin isoflavone. For these three linked defence responses, sub optimal concentrations of the glycoprotein induced respectively 4.2, 7.6 and 9.7 fold more activity in the cultivar resistant to race delta (cv. Kievit) than in a cultivar susceptible to that race (cv. Pinto). Protein integrity was not required for elicitor activity and glycosyl side-chains isolated from the protein were shown to be active elicitors. The effects of an elicitor from *Colletotrichum graminicola* was studied by Ransom *et al.* (1992). Roots of sorghum (*Sorghum biocolor*) accumulated 3-deoxyanthocyanidin phytoalexins in response to CG elicitor. Elicitation of the phytoalexins prior to treatment with the elicitor did not prevent infection and development of disease symptoms in susceptible seedlings inoculated with conidia of *Periconia circinata*. However, treatment of roots with the

CG elicitor enhanced the synthesis of 16 kDa proteins in both resistant and susceptible genotypes without expression of disease symptoms.

Effect of the elicitor and the suppressor from a pea pathogen, *Mycosphaerella pinodes*, on polyphosphoinositide metabolism in pea plasma membrane were examined *in vitro* by Toyoda *et al.* (1992). Lipid phosphorylation in the isolated pea plasma membrane was drastically stimulated by the elicitor, but markedly inhibited by the suppressor. A similar inhibitory effect was observed by the treatment with orthovande or K-252a that blocked pisatin production induced by the elicitor. Neomycin, an aminoglycoside antibiotic that interacts with the polyphosphoinositide metabolism, also affected the lipid phosphorylation *in vitro* and blocked the elicitor induced accumulation of pisatin *in vivo*. Rapid changes of polyphosphoinositide metabolism in pea plasma membranes, in one of the indispensable processes during the elicitation of defence responses. Cell walls of germ tubes from wheat stem rust (*Puccinia graminis* f. sp. *tritici*) contain a glycoprotein with a residues in the carbohydrate moiety because periodate, but not trypsin or pronase destroyed activity. These results suggest that the Pgt elicitor is released from hyphal cell walls into the wheat protoplast during stem rust infection.

The elicitor induced incorporation of phenylpropanoid derivatives into the cell wall and the secretion of soluble coumarin derivatives (phytoalexins) by parsley (*Petroselinum crispum*) suspension cultures can be potentiated by pretreatment of the cultures with 2, 6-dichloroisonicotinic acid or derivatives of salicylic acid. The cell walls and an extra cellular soluble polymer were isolated by Kauss *et al.* (1993) from control cells or cells treated with an elicitor from *Phytophthora megasperma* f. sp. *glycinea*. After alkaline hydrolysis, both fractions from elicited cells showed a greatly increased content of 4-coumaric, ferulic, and 4-hydoxybenzoic acid, as well as 4-hydroxybenzaldehyde and vanillin. Two minor peaks were identified as tyrosol and methoxy tyrosol. The pretreatment effect is most pronounced at a low elicitor concentration. Its specificity was elaborate for coumarin secretion. When the parsley suspension cultures were pre-incubated for 1 day, with 2, 6-dichloroisonicotinic, 4- or 5-chlorosalicylic, or 3, 5- dichlorosalicylic acid, the cells exhibited greatly increased elicitor response. Pretreatment with isonicotinic, salicylic, acetylsalicylic, or 2, 6-dihydroxybenzoic acid was less efficient in enhancing the response, and some

other isomers were inactive. This increase in elicitor response was also observed for the above mentioned monomeric phenolics, which were liberated from cell walls upon alkaline hydrolysis and for "lignin like" cell walls polymers determined by the thioglycolic acid method. It was shown for 5-chlorosalicylic acid that conditioning most likely improves the signal transduction leading to the activation of genes encoding phenylalanine ammonia lyase and 4-coumarate: coenzyme A ligase. The conditioning thus sensitizes the parsley suspension cells to respond lower elicitor concentration. If a similar mechanism were to apply to whole plants treated with 2, 6-dichloroisonicotic acid, a known inducer of systemic acquired resistance, one can hypothesize that fungal pathogens might be recognized more readily and effectively.

The elicitor molecules that function *in vivo* for phytoalexin elicitation in soybean (*Glycine max*) infected with *Phytophthora megasperma* f. sp. *glycinea* have been identified as β-1, 6-and β-1, 3-linked glucans that are released from fungal cell walls by β-1,3-endoglucanase contained in host tissue. Yoshikawa and Sugimoto (1993) identified the putative receptor like target sites for glucanase-released elicitor in soybean membranes. The binding was dependent on the pH of the incubation chamber, as well as on the duration and temperature of the incubation. The binding of the glucanase released elicitor to membranes was abolished by both heat and proteolytic enzymes. Therefore, the binding site was probably composed of proteinaceous molecules.

Resistance or virulence are modelled by multiple biochemical components of two living organisms. *Costus speciosus* a major sapogenin bearing medicinal plant was severely affected by *Drechslera rostrata* causing leaf blight disease. An interesting interaction phenomenon was noticed by Kumar *et al.* (1995). The HPLC analysis indicated the accumulation of glyceollin II and III as potent phytoalexins by *C. speciosus* in response of non pathogenic *D. longirostrata.* Further the presence of a polysaccharide elicitor or mycelial wall component seems to be detrimental cause of phytoalexin accumulation. The same elicitor was also present in mycelial wall of pathogenic *D. rostrata* but in much lower concentration. Additionally it was associated with another polysaccharide component with different identity. The bioassay method of elicitor preparation was expressed in terms of antimicrobial activity mediated through glyceollins. It was determined to be 88.6% in incompatible

which was considerably low (13.7%) in pathogenic reaction. During the pathogenesis of *D. rostrata* the susceptibility was not only exercised with low concentration of elicitor but also being mediated with the association of additional carbohydrate component of mycelial wall hence expressing the involvement of multiple biochemical components to regular susceptibility. The non specific elicitors (which include proteins, glycoproteins, various types of oligosaccharides and unsaturated fatty acids) are more difficult to assign a role in the induction of phytoalexin production by pathogens (Hahn, 1996). A race specific elicitor has been isolated from *Uromyces vigna*. This elicitor can induce phytoalexin production in cowpea resistant to this race of the pathogen based on hypersensitive response (HR) – like symptoms induced by treatment of resistant cowpea leaves with the elicitor (D'Silva and Heath, 1997). The presence of phenolic acids in cell wall - esterified p-coumaric acid and ferulic acids bound to cell wall polysaccharides are widespread in Gramineae. Cell wall bound phenolics in resistance to rice blast disease was demonstrated by Kumar *et al.* (1997). The relative roles of glyceollin, lignin and the hypersensitive response (HR) in pathogen containment and restriction were investigated in soybean cultivars that were inoculated with *Phytophthora sojae*. Incompatible interactions in leaves and hypocotyls were characterized by HR, phenolic and lignin deposition and glyceollin accumulation. The uncoupling of glyceollin synthesis is a major factor in restriction of the pathogen during these interactions (Mohr and Cahill, 2001).

The response of bavistin on disease incidence, phenolic compounds and their oxidative enzymes, non-structural carbohydrates, different forms of nitrogen and mineral content in cowpea roots susceptible to *Rhizoctonia solani* and *R. bataticola* was reported by Kalim *et al.* (2000). Bavistin (0.2%) as seed treatment significantly reduced the incidence of root rot of cowpea to the extent of 57.5 and 58.9 percent in case of *Rhizoctonia solani* and *R. bataticola,* respectively. Reduction in disease incidence has been attributed to the increased activities of polyphenol oxidase (PPO) and peroxidase (PO) along with higher amounts of total phenols. PO activity was several times more as compared to PPO specific activity. Contrary to PPO and PO the specific activity of catalase declined sharply. Bavistin seed treatment also caused

an increase in reducing sugars, Cu, Zn and Mn but a decrease in o-dihydric phenols, flavanols, total soluble sugars, non-reducing sugar and Fe contents.

The effect of phenolics and related compounds on pectinolytic enzymes of *Sclerotinia sclerotiorum,* a phytopathogenic fungus causing white rot in pea (*Pisum sativum*) had been studied by Sharma *et al.* (2001). Activities of both pectinases (polygalacturonase and pectin methyl esterase) from *S. sclerotiorum* increased with the growth period of fungus upto 7 days of growth and declined as the growth period was further progressed. Polygalacturonase (PG) and pectin methyl esterase (PME) had pH optima of 5.2 and 5.0 and maximum activity at 35^0C and 45^0C temperatures respectively. Activities of these enzymes were in general inhibited by divalent metal ions. However, Mg^{++} stimulated activities of both the enzymes. Both PG and PME were inhibited by phenolic compounds viz. m-coumaric, homo-vanillic and protocatechuic acid. The activities of these enzymes also decreased when phenolic extracts of resistance variety of pea seeds and neem leaves were incorporated in the culture medium. These results suggest the role of phenolics in disease resistance.

Biochemical study on peroxidase (PO) and polyphenol oxidase (PPO) activity; reducing, non reducing and total sugar; total phenol and potash content before and after powdery mildew infection in seven mungbean genotype was carried out by Gawande *et al.* (2002) to know their role in host parasite interaction. Resistant genotype had higher activities of PO and PPO, total phenol and potash content before and after infection and lower level of sugars than observed in susceptible genotype. Activity of enzymes total phenols and potash content were positively associated with resistance, whereas sugars had negative association with disease resistance.

Ten cultivars of soybean were tested for their disease reactions against *Fusarium oxysporum* Schlecht the causal agent of root rot disease. The different cultivars exhibited varying degrees of susceptibility with Soymax being the most and JS-2 and UPSM-19 being the least resistant. Seed bacterization with *Bradyrhizobium japonicum* reduced root rot intensity significantly. Application of *Trichoderma harzianum* to soil also reduced root rot intensity. Combined application of *B. japonicum* and *T. harzianum* gave the most significant disease reduction.

B. japonicum did not exhibit any antagonistic reaction against *F. oxysporum in vitro*, whereas *T. harzianum* inhibited growth of *F. oxysporum*. Phenylalanine ammonia layse and peroxidase activities were assayed in both resistant and susceptible cultivars following the different treatments. Activities were significantly higher in the infected roots in comparison to healthy ones. PAL activity was higher in the resistant cultivar but bacterization with *B. japonicum* prior to inoculation with *F. oxysporum* enhanced PAL activity in both the cultivars. Persoxidase activity did not show any increase following pre-inoculation with *B. japonicum*. Glyceollin accumulation which was significantly higher in the resistant cultivar also registered a marked increase due to pre-inoculation with *B. japonicum*. *T. harzianum* did not affect enzyme activities or glyceollin accumulation (Chakraborty *et al.*, 2003).

Accumulation of total and o-dihydroxy phenols in three maize varieties (Malan, Ganga-5 and VL-42) infected with *Helminthosporium maydis* and *H. turcicum* was recorded as compared to their healthy counterparts. Reaction of these varieties to both the pathogens varied significantly in terms of accumulation of phenolics. Ganga-5 showed three-fold increase in phenolic contents due to infection by *H. maydis* while double amount of total phenols was recorded in VL-42. *H. turcicum* induced maximum accumulation of phenolics in variety VL-42 followed by Ganga-5 and Malan. An increase in the activity of peroxidase, polyphenol oxidase and IAA-oxidase was noticed in all the three varieties of maize under infection of *H. maydis* and *H. turcicum*. The results have suggested that the accumulation of phenolics was higher in resistant varieties like 'Ganga-5' and "VL-42' as compared to susceptible Malan. Corresponding increase in the activities of oxidative enzymes suggested active metabolic reaction of the host to the pathogenesis and their possible role in an increased level of phenolics (Sukhwal *et al.*, 2003).

Six apple rootstocks, namely M7, M9, M25, MM103, MM104 and MM115 showed different reactions to *Pythium ultimum* causing collar rot of apple. The maximum amount of total and ortho-dihydroxy (OD) phenols and high activity of phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) and polyphenol oxidase (PPO) were detected in highly resistant healthy (uninoculated) apple rootstock (MM115) and minimum in highly susceptible ones (MM103 and MM104).

The peroxidase activity was, however, maximum in M9 (susceptible) and minimum in M25 (resistant). On infection, the levels of total phenols and activities of their synthesizing (PAL and TAL) and oxidizing enzymes (PPO and PO) increased rapidly in resistant root stocks (MM115 and M25) at the initial stages of pathogenesis and subsequently declined rapidly. The activity of these enzymes also continued to increase gradually with pathogenesis (up to 20^{th} or 25^{th} day of inoculation) in highly susceptible root stocks (MM103 and MM104). In resistant root stocks, the level of phenols and activities of enzymes (except peroxidase) remained higher during pathogenesis in comparison to that of susceptible ones (Sharma, 2003).

During cavity spot disease of carrot (*Daucus carota*), the surface of the root is penetrated by the fungus *Pythium violae* causing surface lesions and cell breakdown. Commercial varieties range from the very susceptible Bertan, to the less susceptible Bolero with Narbonne intermediate while the gene bank cultivar Purple Turkey was much less susceptible. Examination of the colonization process *in vitro* by scanning electron microscopy of Narbonne showed that fungal proliferation occurred in the first 2 days of colonization but this species had disappeared from lesions by day 7. No lesions were evident on Purple Turkey although the fungus had penetrated the root which itself was composed of small regularly arranged cells. Examination of the activity of defence related enzymes during *in vitro* colonization showed that phenylalanine ammonia lyase and chitinase activities remained low throughout the first 7 days of infection of commercial cultivars, Bolero and Bertan. Peroxidase and β-glucosidase activity in Bolero increased briefly on day 3 but otherwise were uniformly low. Enzyme activities were generally higher in Purple Turkey. The small cell size within the root and higher constitutive levels of the enzymes may constitute the basis for resistance in Purple Turkey. Potentially this cultivar may provide a source of germplasm for improving the resistance of commercial carrots to cavity spots (Cooper *et al.*, 2004).

Alternaria blight disease of cluster bean is caused by *Alternaria cucumerina* var. *cyamopsides*. The disease appears year after year in mild to severe form to cause yield losses, as the pathogen is seed borne in nature. An investigation was attempted to quantify biochemical changes in cluster bean using highly susceptible (IC 116835)

and moderately resistant (IC 116903) genotypes. The catalase activity (65 DAS) decreased with the increase in disease intensity in both genotypes. Activity of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) as well as quantity of phenols and lignin increased with the increase in disease intensity, indicating thereby that these enzymes play important roles in the defense mechanism against *Alternaria* blight in clusterbean (Joshi *et al.*, 2004).

A study was carried out by Sharma and Chakraborty (2004) on the association of defense enzymes with resistance in tea plants triggered by *Exobasidium vexans*. Results revealed significant changes in the level of enzymes mainly β-1, 3 glucanase and chitinase exhibiting antimicrobial activity. A wide variety in the activities of the enzymes involved in phenol metabolism including phenylalanine ammonia lyase, peroxidase and polyphenol oxidase were seen in compatible and incompatible interactions. Multicomponent coordinated responses of tea plants under biotic stress with special reference to *E. vexans* causing blister blight disease of tea have also been demonstrated by Chakraborty *et al.* (2004). The possibility of inducing resistance in susceptible tea varieties was achieved following foliar spray with salicylic acid and results established its potential in immunizing tea plants which was confirmed by immunoassays and immunolocalization of chitinase in tea leaf tissues by employing polyclonal antibodies raised against chitinase and labeled with FITC after induction of resistance. The accumulation of defense enzymes in tea plants in response to salicylic acid treatment suggests its role in the cellular protection mechanism which was also confirmed (Chakraborty *et al.*, 2005b).

The effective integrated disease management practices

As the management of some diseases is not possible through only one approach, efforts are being made to reduce environmental effects and rationalize the use of pesticides and manage diseases more effectively. This led to the emergence of the new discipline called Integrated Disease Management (IDM), which is an ecologically based, environmentally conscious method that combines or integrates

biological and non-biological control techniques to suppress weeds, insects and diseases (Anahosur, 2001). The five components of an IDM program are prevention, monitoring, correct disease and pest diagnosis, development and use of acceptable thresholds, and optimum selection of management tools. For sustainable crop production the components involved should be eco-friendly, so that beneficial organisms would be safe and IDM practices would go a long way helping stabilized crop production. Integrated disease management is the selection and hormonal combination of appropriate techniques to suppress the disease to a tolerable level (Gupta and Mukherjee, 2006).

Integration of *Trichoderma harzianum*, *Rhizobium* and carbendazim remarkably reduced the root rot of groundnut, caused by *Sclerotium rolfsii*. The antagonistic population increased with increasing time, and maximum population was recorded 75 days after sowing. At this time, while the native soil recorded only $2.8x10^3$ cfu/ g of *Rhizobium*, the seed treatments with *T. harzianum* and *Rhizobium* plus soil inoculation with *T. harzianum* recorded $44x10^3$ cfu/ g after 6 days, indicating a 16 fold increase. Maximum number of plants survived when the antagonist was applied as seed treatment or applied to soil at sowing. The nodule number and maximum population of $37x10^3$ cfu/g were recorded when *T. harzianum* inoculum was added to soil at sowing. Addition of *T. harzianum* inoculum at 2 and 5% were at par with 88 and 92% surviving plants, respectively. Addition of inoculum at 5% recorded a slight increase in nodule number. When the dose of inoculum was increased from 2 to 10%, there was no corresponding increase in population of antagonist in all intervals tested. In all experiments, plants died within 45 days after sowing in pathogen alone inoculated soils (Muthamilan et *al.*, 1996).

Crop losses caused by *Sclerotinia sclerotiorum*, *S. minor* and *S. rolfsii* were evaluated in 41 commercial peanut (*Arachis hypogaea*) fields located in the southern region. The incidence of either disease was generally higher in fields in which the sequencing of crops was the same during the last 15 years. Furthermore, the incidence of 'blight' and 'wilting' was often higher in peanut crops where the preceding crop had been peanut, soybean (*Glycine max*) or sunflower (*Helianthus annus*) than in peanut crops preceded by sorghum (*Sorghum bicolor*), maize (*Zea mays*), alfalfa (*Medicago sativa*), lovegrass (*Eragrostis curvula*) or grassland

(Marinelli *et al.*,1998). The soil borne diseases of crops incited by species of *Sclerotium, Rhizoctonia, Fusarium* and *Pythium* are difficult to be managed through one method of approach *viz.*, cultural practices or fungitoxicants or host plant resistance or bio-agents. Among them the disease caused by *Sclerotium rolfsii* are predominant under rainfed and assured moisture conditions and cause considerable loss to field crops, vegetables, fruit crops and plantation crops.

Fourteen isolates of *Trichoderma* and *Gliocladium* species were tested *in vitro* against *Sclerotium rolfsii*, the causal organism of root / collar rot of sunflower. Two isolates of *T. viride* four isolates of *T. harzianum*, one each of *T. hamatum, T. koningii, T. polysporum, G. virens, G. deliquescens* and *G. roseum* inhibited mycelial growth of the pathogen significantly. Among *Trichoderma* species, *T. harzianum* isolates PDBCTH-2 gave 61.4% inhibition of mycelial growth followed by PDBCTH-8 (55.2%) and PDBCTH-7 (54.9%). Among *Gliocladium* isolates, *G. virens* gave maximum (39.9%) inhibition of mycelial growth. Suppression of sclerotial production by the antagonists ranged from 31.8 to 97.8%. Complete inhibition of sclerotial germination was obtained with the culture filtrates of *T. harzianum* (PDBCTH-2, 7 and 8), *T. pseudokoningii* and *G. deliquescens*. The three *T. harzianum* isolates and the *T. viride* isolate (PDBCTV4) were superior under greenhouse conditions with PDBCTH 8 showing maximum (66.8%) disease control followed by PDBCTH 7 (66.0%), PDBCTH 4 (65.4%), PDBCTH 2 (61.6%) and were even superior to the fungicide - captan. *G. deliquescens* gave maximum (55.7%) disease control among *Gliocladium* spp. (Prasad *et al.*, 1999).

The antagonistic microbes *viz. Trichoderma harzianum, T. viride, Gliocladium virens, Penicillium* spp. *Bacillus subtilis, Pseudomonas fluorescens*, mycorrhizae and few others have been extensively evaluated as seed dress, soil application or plant spray or spot application against soil borne diseases *viz.*, root rots, foot rots, wilt; damping off of seedlings caused by *Sclerotium rolfsii, Pythium debarianum, Macrophomina phaseolina, Rhizoctonia solani and Fusarium* spp. As bio-control agents alone may not completely and effectively manage disease, it can be used as one of the components in the integrated management of diseases. (Anahosur,1999).

Broad spectrum biological control of diseases caused by soil borne plant pathogens such as *Pythium, Phytophthora,* and *Rhizoctonia solani* requires the introduction into or presence of edaphic sources of organic nutrients in soil for sustenance of biological agents. The decomposition level of organic matter critically affects the composition of bacterial taxa as well as the populations and activities of biocontrol agents. Composition, antibiosis, parasitism, and systemic induced resistance are all affected. Highly stabilized sources of *Sphagnum* peat consistently fail to support sustained biological control, even when inoculated with biocontrol agents. Composts, on the other hand, can serve as an ideal food base for biocontrol agents and offer an opportunity to introduce and establish specific biocontrol agents into soils, which in turn leads to sustained biological control based on the activities of microbial communities (Hoitink *et al.,* 1999). Species of *Trichoderma, Gliocladium* and *Pseudomonas* have been employed and some success has been achieved in selected crops against a few pathogens in restricted soil temperature and pH (Sen, 2000).

Three biocontrol agents (BCAs) were evaluated individually and in combinations, and in integration with bavistin seed treatment in pathogen infested soil in pots, for suppression of dry root rot pathogen *Rhizoctonia solani* in bell Pepper (*Capsicum frutescens* cv. California Wonder). Seed treatment with the bio-control agents was as effective as bavistin seed treatment. Integration of seed and soil application of individual BCA resulted in higher germination and reduced mortality due to disease. Combination of two biocontrol agents, particularly of *Trichoderma harzianum* and *T. aureoviride* was better than the individual ones. Population of BCAs in chilli rhizosphere and soil was directly related to suppression of *R. solani.* Application of mixture of *T. harzianum* and *T. aureoviride* as seed and soil treatment was the most promising in increasing the germination and suppression of chilli root rot pathogen and the disease (Bunker and Mathur, 2001).

Six varieties of ginger along with five fungicidal treatments were examined to find out the best ginger variety giving highest yield and it has been observed that 'Moran' variety yielded 114.08 q ha^{-1} which was followed by 'Rio-de-genario' (106,47 q ha^{-1}). Per cent disease incidence recorded was less in these two varieties. Effect of different fungicides on disease incidence showed that Ridomil MZ-72 @

0.2% effectively reduced the disease with only 18.63 per cent disease incidence. Ridomil MZ-72 treated rhizome also showed maximum germination as well as the highest yield. It was followed by copper oxychloride @ 0.3%, Bordeaux mixture @ 1%, mancozeb (indofil M-45) @ 0.25% and captan @ 0.25% (Das *et al.*, 2001).

Agricultural soils suppressive to soil borne plant pathogens occur world wide and for several of these soils the biological basis of suppressiveness has been described. Two classical types of suppressiveness are known. General suppression owes its activity to the total microbial biomass in soil and is not transferable between soils. Specific suppression owes its activity to the effects of individual or select groups of microorganisms and is transferable. The microbial basis of specific suppression to four diseases, *Fusarium* wilts, potato scab, apple diseases, and take-all, is discussed. One of the best described examples occurs in take-all decline soils. In Washington State, take-all decline results from the build up of fluorescence *Pseudomonas* spp. that produce the antifungal metabolite 2, 4-diacetyl phloro-glucinol. Producers of this metabolite may have a broader role in disease suppressive soils worldwide. By coupling molecular technologies with traditional approaches used in plant pathology and microbiology, it is possible to dissect the microbial composition and complex interactions in suppressive soils (Weller *et al.*, 2002).

Upmanyu *et al.* (2002) studied root rot and web blight (*Rhizoctonia solani*). Soil amendment with cotton, mustard and neem cakes was found effective in reducing the root rot (pre- and post-emergence) incidence both under glass house and field conditions and increased the yield. Foliar sprays of carbendazim (0.1%) and tebuconazole (0.05%) were most effective in reducing web blight severity while seed treatment with carbendazim (0.2%) in combination with foliar sprays were found effective the root rot incidence and web blight severity as well as increased the yields. *Trichoderma harzianum* and *T. virens* as seed treatment and foliar sprays were found effective in reducing the disease and resulted in increased pod yield. Among the bioagents, *T. viride* showed the maximum tolerance to carboxin, tebuconazole and carbendazim followed by *T. virens*, *T. harzianum* and *A. niger* and were used in integrated disease management along with fungicides and oil cakes both under glass house and field conditions. Soil amendment (cotton cake) + *T. virens* and carboxin (ST), mustard cake + *T. virens* and carbendazim (ST), seed

treatment with combination of *T. viride* and carboxin, *T. harzianum* + tebuconazole and soil amendment (mustard cake) + carbendazim (ST) were found effective in containing the root rot under glasshouse conditions while soil amendment (mustard cake) + carbendazim (ST) + carbendazim (FS) were found highly effective in reducing pre-and post – emergence root rot while web blight severity was best contained by soil amendment (mustard cake) + carbendazim (ST+FS) followed by tebuconazole + *T. virens* (ST) + carbendazim (FS). Sprays of carbendazim (0.1%) on mulch and foliage were found highly effective but pine needle mulch impregnated with floor application of carbendazim was also quite effective in avoiding the contamination with fungicide residues.

Biologically and chemically treated chickpea seeds observed after one month showed that vitavax (carboxin @ 1g kg^{-1} seed) did not significantly affect the spore viability of *Gliocladium virens* (Gv). Application of carboxymethyl cellulase (CMC) with *G. virens* powder (10^9 spores per g) in combination with vitavax provided maximum protection (81.9%) to the crop against chickpea root rot and collar rot pathogens in glasshouse. Chickpea seeds treated with Gv powder + CMC + vitavax significantly increased seedling emergence (47.9%); final plant stand (85.8%) and grain yield (79.7%) which was statistically at par with the treatment Gv powder + vitavax and Gv suspension + vitavax in a sick plot (Tiwari and Mukhopadhyay, 2003).

Ashwagandha (*Withania somnifera*) is an important medicinal plant and a major source of alkaloid and steroidal lactones (withanolide), which are regularly used in pharmaceutical industries. Plant growth retardation and gall formation in the root system indicated the presence of root – knot nematode, which was confirmed as *Meloidogyne incognita* race-2. Green house trials were conducted to determine the influence of different inoculum levels of *M. incognita* Chitwood on growth and yield of *W. somnifera*. Various organic materials *viz.* neem compound *Mentha* distillate, *Murraya koengii* distillate, *Artemisia annua* and vermicompost, bio-agents *viz.*, *Glomus aggregatum* and *Trichoderma harzianum* were tested individually as well as in different combinations for the management of root-knot nematode, *M. incognita* on *W. somnifera*. The experimental results indicated that most of the bio-agents and organic materials alone as well as in combination were root-knot nematode

suppressive and enhanced the growth and yield of *W. somnifera*. Maximum root-knot suppression was noticed in vermicompost and *T. harzianum* combination followed by *Mentha* distillate and *G. aggregatum*. Maximum increase in plant yield was noticed when the soil was amended with *Mentha* and *Murraya koengii* distilled *waste* along with bio-agents (Pandey *et al.*, 2003).

Sharma and Gupta (2003) reported soil solarization of infested soil with single and double polyethylene mulch alone and combination with soil amendment (mustard cake) increased soil temperature as compared to unmulched plots. This rise in temperature in mulching for 30 and 50 days eliminated the pathogen from 5 and 10 cm in soil depth, respectively. They also found antagonistic activity of *Bacillus subtilis, Trichoderma longibrachiatum* and *T. harzianum* against *Rhizoctonia solani*. Soil application of *B. subtilis* reduced pre and post-emergence root rot. Integrated management practices was done by using a combination of soil solarization + soil amendment (mustard cake) + combination of *A. sativum* and *B. subtilis* was found effective in reducing the incidence of root rot while web blight severity was best contained by combination of soil solarizatioin + soil amendment (mustard cake) + *A. sativum* (ST + FS) followed by soil solarization (SS) + soil amendment (mustard cake) + combination of *A. sativum* and *B. subtilis* + bavistin (0.1%) foliar sprays and increased green pod yields.

Rhizoctonia solani [*Thanatephorus cucumeris*] causes sheath blight, one of the most widely distributed and destructive diseases of rice. Three fungal bioagents *Trichoderma viride*, *T. harzianum* and *T. virens* and nine antagonistic rhizospheric bacteria were evaluated *in vitro* by dual culture method for their antagonistic activities against *Rhizoctonia solani*. A maximum inhibition in growth of *Rhizoctonia solani* was obtained by WRPf (62.96%) followed by WRb 8 (56.67%) and *T. virens*. Out of 4 plant extracts, extracts of *Allium sativum* inhibited maximum (100%) mycelial growth and sclerotial production at 10% concentration *in vitro* condition. Increase in epicotyl and hypocotyl length associated with plant growth promotion was observed in seed treatment with $FeCl_3$ (1mM) and IAA (0.001%) as compared to water treated control. Among abiotic elicitors, maximum reduction in sheath blight incidence was observed by seed treatment and seedling dipping with K_2HPO_4 (20mM) followed by $FeCl_3$ (10mM). On the other hand, among bioagents

maximum reduction in sheath blight incidence was observed in soil treated with WRPf alone or in combination with other bioagents and soil amendments. But, maximum reduction in sheath blight incidence as well as severity was observed in soil treated with *T. virens* + WRPf + *Gliricidia* compost (93% less disease) which was closely followed by combined application of soil amendments with *T. virens* and neem cake with seed and root dipping with K_2HPO_4 (20mM) in pot experiment under glass house condition (Chowdhury *et al.*, 2003).

Aqueous extracts of mustard cakes (5%), neem cake (1%), pine needles (5%), deodar needles (3%) and neem oil (3%) respectively, led to reduced *in vitro* germination of sclerotia of the pathogen *Sclerotium rolfsii* causing seedling blight disease in apple nurseries, as compared to control. Combinations of mustard cake (5%) with neem oil (3%), neem cake (1%) with deodar needles (3%) and neem oil (3%); and mustard cake (5%) with neem cake (1%), pine needles (5%) and neem oil (3%) resulted in total inhibition of sclerotial germination (Sonali and Gupta, 2004).

A survey of 277 farmers in three major potato growing areas in Kenya was conducted by Nyankanga *et al.* (2004) with the aim of assessing farmers' current perception and knowledge of late blight and practices for its management and identifying points of potential intervention in the development of integrated disease management (IDM) programmes. The problem of late blight was one of several constraints that growers faced such as lack of quality seed, markets, storage and prevalence of bacterial wilt. Most farmers (5.1%) regarded late blight as a serious biotic constraint upon production. Many farmers (79%) were able to recognize foliar symptoms of late blight but there was an evident lack of knowledge of tuber and stem infection, causes of leaf, stem and tuber infection, different inoculum sources, and accurate diagnosis of the disease. Most (81%) farmers associated the disease with cold weather. Farmers overwhelmingly (98%) relied on application of fungicides, mostly mancozeb (Dithane M45) and metalaxyl (Ridomil) as the main control methods, with most farmers knowing of no other method. High cost of fungicide, poor application techniques, and preference of susceptible cultivars were among the reasons contributing to inadequate control of late blight. Very few farmers showed the elements of IDM strategies, probably due to their limited knowledge of the biology of late blight. These results suggest that improvement of

late blight control could be achieved by enhancing farmers knowledge and developing and deploying IDM practices involving a multidisciplinary approach, which encompasses addressing other production constraints.

Crown rot (*Sclerotium rolfsii*) of French bean was found to be more severe in Solan district than Kullu of Himachal Pradesh. The disease incidence ranged from 11 to 56 per cent. Characteristic symptoms appeared as dark brown, water soaked lesions on the stem and collar region just below the soil surface followed by yellowing of leaves and production of a white mouldy growth interspersed with sclerotia at the base of the stem, under the surface of leaves and pods. *In vivo* evaluation of fungicides against the pathogen showed that penconazole, hexaconazole, propineb and mancozeb inhibited mycelial growth. Propineb was found to be the most effective in reducing disease incidence on crown and pods. Among the biocontrol agents *Gliocladium virens* and *Trichoderma viride* were found to be the most effective against the pathogen (Gupta and Sharma, 2004).

According to Bhatnagar *et al.*, (2004) cumin wilt, a serious disease induced by *F. oxysporum* f. sp. *cumini* causes heavy losses to the crop. A few compounds of plant origin have been provided to be possible alternatives to pesticides use. Out of 17 species tested plant extract from Datura (1.3 cm) and Isabgol (1.5 cm) were effective in reducing the radial growth of *F. oxysporum* f. sp. *cumini*. In a similar study, Ghasolia and Jain (2004) evaluated four commonly used fungicides, two bio-agents, two phyto-extracts and two physical seed treated agents, in both *in vitro* and *in vivo* conditions for fungitoxicity against *F. oxysporum* f. sp. *cumini*. Carbendazim (0.2%), thiram (0.25%), captan (0.25%), tebuconazole (0.2%), *Trichoderma viride*, *Euphorbia antiquorum* and hot water gave higher seed germination and vigour index and minimum pre – and post – emergence seedling mortality over check. Before maturity, all treatments showed reduced number of seedlings showing wilt symptoms in the field.

A number of plant species (*Azadirachta indica, Lantana camera, Dryopteris filix-mas*, *Eichhornia* sp.) have been reported to possess some natural substances in their leaves which were toxic to foliar fungal pathogens (*Pestalotiopsis theae, Glomerella cingulata*) of tea causing brown blight and grey blight disease

respectively. Attempts have also been made to use aqueous extracts of selected plants (*A. indica* and *Catharanthus roseus*) on tea plants for induction of resistance against *Alternaria alternata*, a newly recorded foliar fungal pathogen, causing leaf blight disease of tea as well as *E. vexans* causing blister blight of tea with special reference to the involvement of defense enzymes such as β-1,3- glucanase, chitinase and phenylalanine ammonia lyase and antifungal phenols. These extracts enhanced the level of defense enzymes, developed acquired resistance in tea plants and reduced blister blight disease incidence (Chakraborty *et al.*, 2004). Tea varieties treated with aqueous leaf extracts of *A. indica* exhibited high level of all three defense enzymes along with rapid and distinct accumulation of antifungal phenolics in comparison with *C. roseus*. Reduction in disease incidence by application of these extracts was also evident. Plant extracts from *A. indica* seem to act at various points in the defense activating networks and mimic all or part of the biological activities of resistance. The results support the hypothesis that neem extract may act indirectly by inducing plant defense reactions and it may be useful in integrated management of foliar disease of tea (Chakraborty *et al.*, 2005d).

Volatile and non-volatile metabolites of *Trichoderma* spp. significantly reduced the mycelial growth and germination of *Sclerotinia sclerotiorum*. Among the delivery systems evaluated by Kapil and Kapoor (2005) for controlling the white rot, sodium alginate pellet formulation (800 No./m^2) followed by soil application of wheat bran based *Trichoderma viride* formulation @ 11.2g /m^2 were found to be most effective delivery systems. Six neem based biopesticides were evaluated at two concentrations (0.5 and 0.1%) against *S. sclerotiorum*. Results revealed, Wanis as the most effective at both the concentrations followed by Neemgold. Out of eight organic substrates evaluated for mass multiplication of bioagents, maximum multiplication of bioagents was found in FYM followed by in *Lantana camara* and wheat bran. Population dynamics studies of *T. viride* revealed significant increase in population even after 60 days of application of bioagent.

Stalk rot of cauliflower caused by *S. sclerotiorum* could be reduced to 9.7% with 1.5 g/l bavistin 50% followed by 15.3% with 5.25g/l sailaxyl-MZ 72%, 16.7% with 8.34 g/l mancozeb 75%, 20.8% with 1.08 g/l topsin-M 70% and 34.7% with 6

ml/l neem extract 25%. Bavistin 50% also gave highest seed yield. Among bioagents, culture filtrate of *Trichoderma harzianum* was most effective in reducing disease incidence to 23.6% as compared to *Aspergillus niger*, kalisena or booty. The seed production was increased to 200.8 kg/ha after treatment with *T. harzianum*. The application of *A. niger* (194.1, 314.8 kg/ha), kalisena (192.2, 308.5 kg/ha), *T. harzianum* + *A. niger* (190.8, 302 kg/ha) booty + kalisena (186.3, 297 kg/ha) and booty (165.4, 258.5 kg/ha) also increased seed production. Intercropping with one and two rows of garlic reduced disease index significantly over control in first cropping season. However, intercropping with one and two rows of onion reduced disease index significantly in second season (Zewain *et al.*, 2005).

A severe crown and root rot of Chinese gooseberry (*Actimidia deliciosa*) caused by *Sclerotium rolfsii* was observed in the nursery at Kullu, Himachal Pradesh. The incidence was more pronounced in the areas previously occupied by strawberry or apple nursery. The infected plants showed water soaked areas at the base of the stem at soil level and drooping of the lower most leaf of the plant showed drooping as initial symptoms. White fan shaped mycelium developed which later disappeared and mustard-shaped sclerotia were formed. The fungus was isolated, and its pathogenecity was proved. In two years field tests *Trichoderma viride* (talc formulation 1.0%) proved very effective in providing 74 per cent disease control. Treatment with thiram (0.4%) and *Trichoderma viride* (0.5%) in combination was the best in controlling the disease (Khosla *et al.*, 2005).

Induction of chitinase in suspension-cultured tea cells following inoculation with *E. vexans* or treatment with hexaconazole, calixin and aqueous leaf extracts of *Catharanthus roseus* were characterized biochemically and immunologically in order to understand the mechanism of plant-pathogen recognition and the complex signaling networks mediating the activation of defense responses. The knowledge gained by such studies provides a base for the development of novel agrochemicals for disease control and also for the development of disease resistant crops by regulating the system in plants by integrated management that leads to development of systemic acquired resistance (Sharma and Chakraborty, 2005).

Materials and Methods

3.1. Plant material

Eighteen tea varieties which includes five Tocklai varieties (TV-18, TV-22, TV-25, TV-26 and TV-30), six UPASI varieties (UP-2, UP-3, UP-8, UP-9, UP-26 and BSS-2) and seven Darjeeling varieties (Teen Ali -17/1/54, AV-2, HV-39, T-78, T-135, K-1/1 and B-157) being maintained in Tea Germplasm Bank at the Department of Botany, North Bengal University were used for experimental purposes. These were originally collected from Tocklai Experimental Station, Jorhat, Assam, UPASI Tea Research Station, Valparai, Tamilnadu and Darjeeling Tea Research Centre, Kurseong, Darjeeling, respectively.

3.1.2. Growth and maintenance

Seedlings of tea varieties were propagated by cutting in the nursery under a green agro house. Soil mixture was prepared using sand (75%) and soil (25%) and pH was adjusted ranging between 4.5- 4.9. Polythene sleeves (9"x 6") were filled up with the prepared soil and stacked in rows in beds and watered thoroughly. Beds were arranged in two rows, fifteen in each row. One hundred and fifty cuttings of each of the 18 varieties were allowed for rooting in individual sleeves subsequent to dipping them in rooting hormone. Sleeves of each bed were covered with polythene cloches and maintained by pouring water frequently at an interval of two days. Sleeves were further treated with nutrient mixture as recommended by Ranganathan and Natesan (1987). The composition of this mixture is as follows:

Ammonium phosphate sulphate (16:20)	-	35 parts by weight
Ammonium sulphate	-	8 parts by weight
Magnesium sulphate	-	3 parts by weight
Potassium sulphate	-	15 parts by weight
Zinc sulphate	-	3 parts by weight .

Thirty grams of the above mixture was dissolved in 1 litre of water and 50 ml was given in each sleeve once in 15 days up to 12 months. Tea plants were then transferred from sleeves to earthen pots and maintained [Plates 1 and 2] in glass

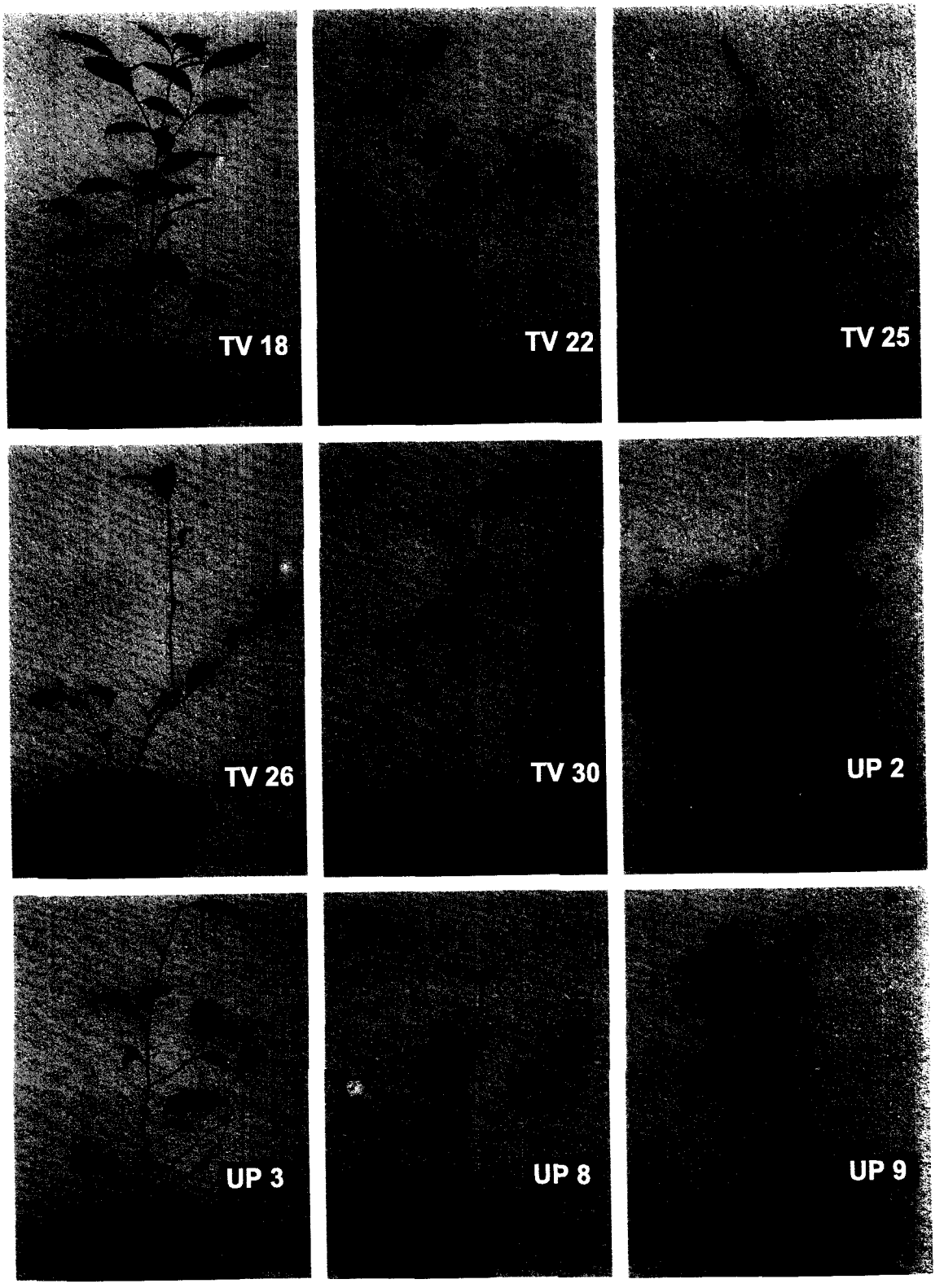

Plate 1. Tocklai and UPASI varieties of tea *(Camellia sinensis)* being maintained in Tea Germplasm Bank.

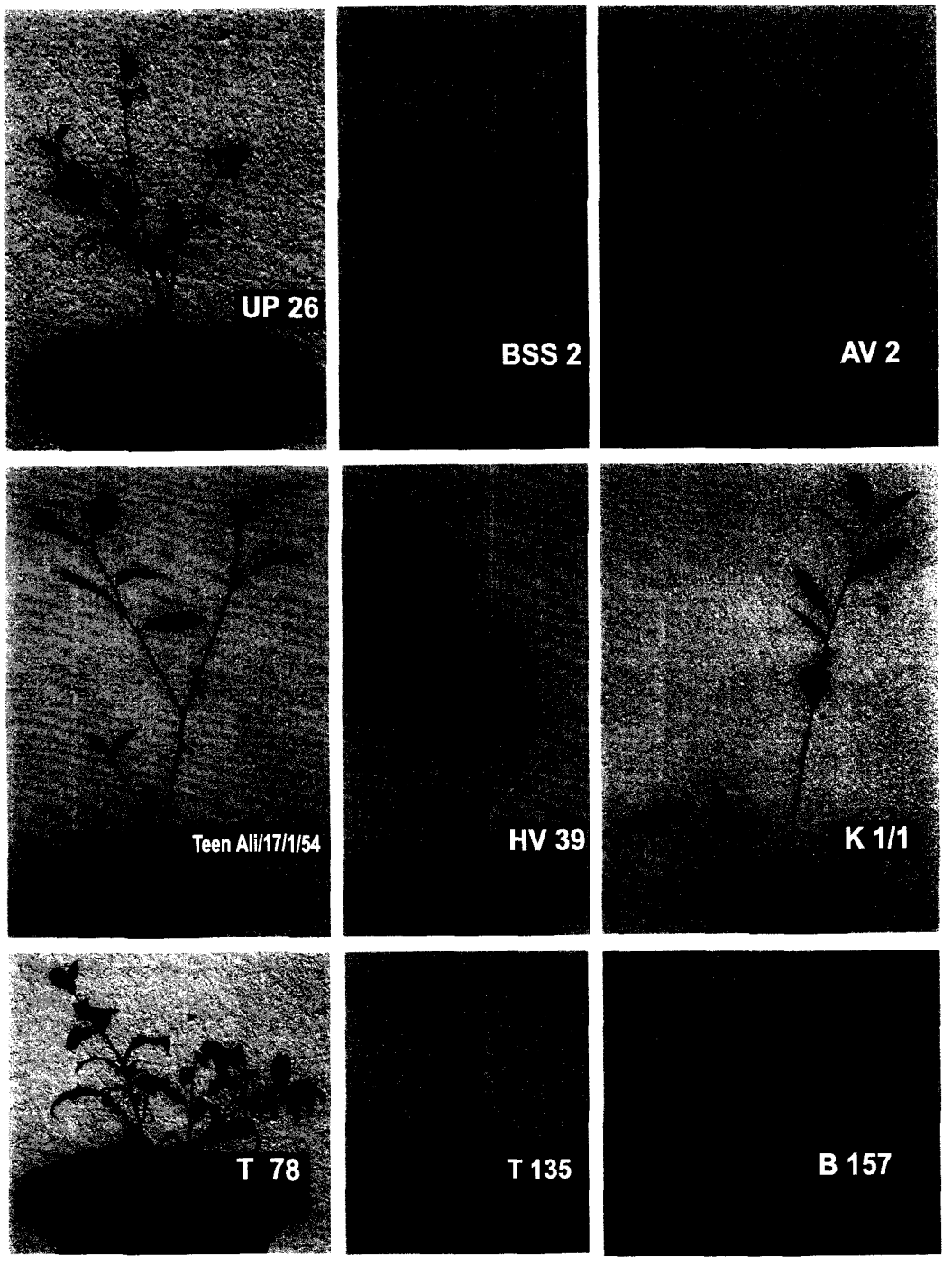

Plate 2. UPASI and Darjeeling varieties of tea (*Camellia sinensis*) being maintained in Tea Germplasm Bank.

house under natural condition of day light and temperature and watered on alternate days with ordinary tap water by sprinklers.

3.2. Fungal culture

3.2.1. Source of cultures

Virulent culture of *Sclerotium rolfsii* Sacc (*Corticium rolfsii* Curzi) was obtained from Immuno-Phytopathology Laboratory, Department of Botany, North Bengal University. This was originally isolated from Teen Ali-17/1/54 and after completion of Koch's postulate, the organism was identified by the Global Plant Clinic, Diagnostic and Advisory Service, CABI Bioscience UK and designated as Sr-1. Besides, two more isolates (Sr-2 and Sr-3) of *S.rolfsii* which were used in this investigation were isolated from infected tea roots of TV-25 and UP-8 respectively. Cultures of *Fusarium graminearum* (a non pathogen of tea), *Trichoderma harzianum* and *Trichoderma viride* (biocontrol agents) were also obtained from the laboratory, mentioned above.

3.2.2. Completion of Koch's postulates

Tea seedlings (1-year-old) were inoculated with 5 ml suspensions of the pathogen (*S. rolfsii*) prepared in sterilized distilled water from 15-day-old cultures grown on PDA containing one sclerotium per ml as described by Sonali and Gupta (2004). Infected roots were collected, washed, cut into small pieces, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water and transferred to PDA slants. After 7 days, the isolated organism was examined, compared with the original stock culture of *S. rolfsii* and its identity was confirmed.

3.2.3. Maintenance of stock cultures

The fungus thus obtained was sub-cultured on PDA slants. After two weeks the culture was stored under three different conditions (5^0C, 20^0C and at room temperature $25^{\pm}$ 3^0C). Apart from weekly transfer for experimental work, the culture of *S. rolfsii* was also examined at regular intervals to test its pathogenicity.

3.2.4. Assessment of mycelial growth

The mycelial growth on both solid and liquid media for various experimental purposes were as follows -

3.2.4.1. Solid media

To assess the growth of *S. rolfsii* in solid media, the fungus was first grown in petridishes, each containing 20 ml of PDA and incubated for 7 days at room temperature. Agar blocks (6 mm dia) containing the mycelia were cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20ml of sterilized solid media. Growth of *S. rolfsii* was studied in six different solid media *i.e.*, potato dextrose agar (PDA), potato sucrose agar (PSA), carrot juice agar (CJA), Richard's agar (RA), Czapek-Dox agar (CDA) and yeast extract agar (YDA) as described by Dhingra and Sinclair (1985).

Potato dextrose agar		**Potato sucrose agar**	
Peeled potatoes	40 g	Peeled potatoes	40 g
Dextrose	2 g	Sucrose	2 g
Agar	2 g	Agar	2 g
Distilled water	100 ml	Distilled water	100 ml

Richard's agar		**Carrot juice agar**	
KNO_3	1.00g	Carrot	2g
KH_2PO_4	0.50g	Agar	2g
$MgSO_4.7H_2O$	0.25g	Distilled water	100ml
Sucrose	3.00g	**Czapek Dox agar**	
KCl	0.05g	$NaNO_3$	0.20g
Agar	2.00g	K_2HPO_4	0.10g
Distilled water	100ml	KCl	0.05g
Yeast dextrose agar		$FeSO_4.7H_2O$	0.05g
Yeast extract	0.75g	Sucrose	3.00g
Dextrose	2.00g	Agar	2.00g
Agar	1.50g	Distilled water	100ml
Distilled water	100ml		

All the petriplates were then incubated at 28^0C and colony diameter of the fungi were studied at 2 –day-intervals for 8 days.

3.2.4.2. Liquid media

To assess mycelial growth of *S. rolfsii* in liquid culture, the fungus was first grown in a petridish, containing 20 ml of PDA medium and incubated for 8 days at 28 ± 1^0C. From the advancing zone of the mycelial mat, agar blocks (6 mm dia) containing the mycelia, were cut with a sterilized cork borer and transferred to Erlenmeyer flasks (250 ml) each containing 50 ml of sterilized medium for the desired period at 28 ± 1^0C. Finally the mycelia were strained through muslin, collected in aluminium foil cups of known weight, dried at 60^0C for 96 h, cooled in a desiccator and weighed.

3.3. Inoculation technique

3.3.1. Inoculum preparation

3.3.1.1. Fungal pathogen:

According to Chowdhury and Sinha (1995), sand maize meal medium was prepared in the ratio of 3:1 (sand : maize). In the prepared sand maize meal medium fungal pathogen (*S. rolfsii*) was inoculated and incubated at 28^0C for 7 days. The inoculum was mixed with sterile soil at the ratio of 1:8. Fungus soil mixture (100 gm) were mixed with the top soil of earthen pots containing tea seedlings and kept for development of disease reaction.

3.3.1.2. Biocontrol agents

Trichoderma species prepared in several media *viz.*, wheat bran media (wheat-bran : sand 1:1, and 25 ml of water for 150 g of inoculum in each polythene packet); Saw dust media (saw dust and water), tea waste media (tea waste and water). Media were autoclaved and inoculated as above.

3.3.2. Inoculation of healthy tea seedlings in pot

One year tea seedlings were planted in earthen pots containing 1 kg soil and allowed to be established. Regular watering was done for two weeks and then 100 g

of pathogen inoculum was added carefully in the rhizosphere of each plant. Disease assessment was done after 2-week- intervals and up to 45 days of inoculation.

3.4. Disease assessment

The external symptoms were assessed thrice (15, 30 and 45 days) after inoculation with *S. rolfsii*. Disease intensity was assessed on the basis of above ground and underground symptoms together on a scale of 0 - 6; 0-no disease; 1 - small roots turn rotten; 2 - 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%; 6 - whole plants die.

3.5. Soluble protein

3.5.1. Extraction

For extraction of soluble proteins from tea roots, the method of Alba and Devay (1985) was followed with modification. Roots were collected from the experimental garden and washed thoroughly. Root tissue (1 gm) was homogenized with 0.05 (M) sodium phosphate buffer pH 7.2 containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2 mM soluble polyvinyl pyrrolidine (PVP) and 2 mM polymethyl sulphonyl fluoride (PMSF) in a mortar with a pestle using sea sand and insoluble PVP at 4^0C. Homogenates were centrifuged at 12,000 *g* for 15 minutes at 4^0C. The supernatant was collected and after recording its volume, was used immediately for estimation and analysis or stored at - 20^0C for further use.

3.5.2. Estimation

The protein estimation was done following by the method of Lowry *et al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent (0.5 ml of 1% $CuSO_4$, 0.5 ml of 2% Sodium-potassium-tartarate and 50 ml of 2% Na_2CO_3 in 0.1 N NaOH) was added, mixed thoroughly and incubated for 15 minutes at room temperature. Then to it 0.5 ml of Folin-Ciocalteau's reagent (diluted 1:1 with distilled water) was added and again incubated for 20 min after mixing well for colour development.

Then optical density was measured at 690 nm in a colorimeter. Using bovine serum albumin as standard the protein concentrations were computed.

3.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total protein

This technique is used for analyzing protein mixtures quantitatively. It is useful for monitoring protein purification and also used to determine the molecular mass of proteins. Denaturation of the proteins by SDS makes all the proteins negatively charged. Electrophoresis in this leads the movement of proteins according to their molecular weight.

3.6.1. Preparation of stock solutions

Stock solutions: For the preparation of gel the following stock solutions were prepared as described by Sambrook *et al.* (1989)

A. Acrylamide and N-N^{1}-methylenebisacrylamide

Acrylamide	-	29 gm
N, N^{1} – methylenebisacrylamide	-	1 gm
Distilled water	-	100 ml

A stock solution containing 29% acrylamide and 1% bisacrylamide was prepared in warm water. The solution was filtered in dark, kept in a brown bottle and stored at 4^{0}C. It was used within one month.

B. Sodium dodecyl sulphate (SDS)

A 10% stock solution of SDS was prepared in distilled water and stored at room temperature.

C. Tris buffer

(a) Lower gel buffer (1.5 M Tris)

1.5 M Tris buffer was prepared for resolving gel (pH adjusted to 8.8 with concentrated HCl and stored at 4^{0}C) until use.

(b) Upper gel buffer (1M Tris)

1.0 M Tris buffer was prepared for stacking and loading buffer (pH adjusted to 6.8 with concentrated HCl).

D. Ammonium per sulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris-glycine electrophoresis buffer (reservoir buffer)

25 mM Tris base, 250 mM glycine (pH 8.3) and 0.1% SDS, 18.8 g glycine, 10 ml of 10% SDS in 1 L of distilled water.

F. SDS loading buffer

Tris buffer containing 50 mM Tris HCl (pH 6.8) 10 mM β-mercaptoethanol, 2% SDS, 10mg bromophenol blueb and 1 ml glycerol in 6.8 ml of distilled water was prepased.

3.6.2. Slab gel preparation

Glass plates of the same size (8 cm x 5 cm) were degreased with alcohol, wiped with blotting paper and dried. Then 1.5 mm thick spacers were placed between the glass plates at the two edges and the two sides of the glass plates were sealed with gel sealing grease uniformly and were kept in a gel casting unit. Resolving gel solutions (10% and 12%) were prepared mixing the stock solutions in the following order :

Preparation of 10% resolving gel

Solution	**Amount**
Distilled water	2.85 ml
30% Acrylamide mixture	2.55 ml
1.5 M Tris (pH 8.8)	1.90 ml
10% SDS	0.075 ml
10% APS	0.075 ml
TEMED	0.003 ml

Preparation of 12% resolving gel

Solutions	Amount
Double distilled water	2.45 ml
30% Acrylamide mixture	3.0 ml
1.5 M Tris (pH 8.8)	1.9 ml
10% SDS	0.07 ml
10% APS	0.07 ml
TEMED	0.003 ml

The mixture was poured into the set-up leaving sufficient space for the well-comb. After pouring the resolving gel solution it was immediately over layered with water and kept for polymerization for 45 min. After polymerization of the resolving gel the water overlayer was decanted off and a seven welled 1.5 mm thick comb was inserted.

Preparation of 5% stacking gel

Solutions	Amount
Double distilled water	2.10 ml
30% Acrylamide	0.50 ml
1 M Tris (pH 6.8)	0.38 ml
10% SDS	0.03 ml
10% APS	0.03 ml
TEMED	0.003 ml

The stacking gel solution was poured carefully up to a height of 1 cm over the resolving gel and finally overlayered with water. The gel was then kept for 30 min for polymerization.

3.6.3. Sample preparation

34 µl sample was prepared by mixing the sample protein with 1xSDS gel loading buffer (16µl) in a cyclomixer. Samples were floated in a boiling water bath for 3 min which denatured the sample.

3.6.4. Electrophoresis

Electrophoresis was performed at 18 mA current for a period of 2 h until the dye front reached the bottom of the gel.

3.6.5. Fixing and Staining

After electrophoresis, the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R250) in 45 ml methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

The gel was removed from the fixer and was stained by staining solution for overnight and finally soaked in destaining solution containing methanol, water and acetic acid (4.5:4.5:1).

3.7. Extraction and estimation of phenolics

3.7.1. Extraction

Phenols were extracted and assayed as described by Mahadevan and Sridhar (1982), with slight modification. 1 gm of root tissue was cut into small pieces and immediately immersed in boiling absolute alcohol which was then boiled on a boiling water bath for 5-10 minutes, using 4 ml of alcohol / 1gm of tissue. After cooling, the tissues were crushed with mortar and pestle using 80% alcohol and were filtered. Residues were re-extracted as before for 3 minutes and filtered. Both the filtrates were mixed and final volume was made up with 80% alcohol. Extracts were stored at 4^0C in separate vials, covered with brown paper. The whole extraction was done in dark to prevent light induced degradation of phenol.

3.7.2. Estimation

3.7.2.1. Total phenol

Total phenol was estimated by Folin–Ciocalteau's method as described by Mahadevan and Ulaganathan (1991). To 1 ml of phenolic extract, 1 ml of Folin - Ciocalteau's reagent and 2 ml 20% Na_2CO_3 was added, shaken properly and heated on a boiling water bath for 1 min and the volume was raised to 25 ml with double distilled water. Absorbance was measured in a Systronics photoelectric colorimeter Model-101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.7.2.2. Ortho-dihydroxy phenol

Ortho-dihydroxy phenol was estimated as described by Mahadevan and Ulaganathan (1991). In 1 ml of each phenolic extract, 2 ml of 0.5 (N) HCl, 1 ml Arnow's reagent ($NaNO_3$ – 10 g; Na_2MoO_4 – 10 g; distilled water 100 ml) and 2 ml of 1(N) NaOH was added. These were then diluted with distilled water up to 25 ml. The tubes were shaken well and absorbance was recorded by Systronics photoelectric colorimeter Model-101 at 515 nm. Quantity of ortho-dihydroxy phenol was estimated using caffeic acid as standard.

3.8. Extraction of antifungal phenolics

Antifungal phenolics from root samples were extracted following the method as described by Daayf *et al.*, (1995) with modification for the determination of free and glycosidically linked phenolics. Root samples (10 g) were mixed with 80% methanol at 10 ml / g tissue and homogenized by blending for about 1 min. Samples were extracted for 48 h on a rotary shaker in a conical flask at 40 r.p.m covered with aluminum foil for protection from light. Methanolic extracts were then collected by filtration on a Whatman No.1 filter disc and concentrated by evaporation to a final volume of 20 ml (aqueous fraction). Concentrates were first partitioned against equal volume of anhydrous diethyl ether three times which was treated as Fraction I. The aqueous fraction was partitioned secondly with equal volume of ethyl acetate three times and the ethyl acetate fraction was considered as Fraction II. Acid hydrolysis of

the remaining aqueous fraction was done with 4(N) HCl to yield phenolic aglycones as suggested by Daayf *et al.* (1997). Aglycones were recovered by partitioning hydrolysates against an equal volume of ethyl acetate (three times), which was treated as Fraction III. All the fractions were evaporated to dryness and finally dissolved in 3 ml of the respective solvents.

3.8.1. Chromatographic analysis

Ethyl acetate fractions of both healthy and infected tea roots were analysed by thin layer chromatography (TLC) on silica gel G. The development of the chromatograms was carried out at room temperature using a solvent system (chloroform: methanol:; 9:1 v/v) as suggested by Chakraborty and Saha (1994a; 1995). Following evaporation of the solvent, thin layer plates were observed under UV light and sprayed separately with Folin-Ciocalteau's phenol reagent (Harborne, 1973). Colour reactions and Rf values were noted.

3.8.2.Bioassay of antifungal phenols

3.8.2.1. Radial growth

Radial growth inhibition assay was performed as described by Van Etten (1982). Ethyl acetate fractions of healthy and infected extracts (0.2 ml) were initially taken separately in sterile Petri plates and allowed to evaporate. In control sets, only ethyl acetate (0.2 ml) was taken and allowed to evaporate. Subsequently 10 ml sterilized PDA was poured in each Petri plate, thoroughly mixed and allowed to solidify. Agar blocks (3 mm dia) were cut with a sterilized cork borer from the advancing zone of a 4-day-old culture of *S. rolfsii* grown in PDA and was placed in the center of each Petri plate. Radial growth of *S. rolfsii* was recorded after 3 days of incubation at $28\pm2^{0}C$

3.8.2.2. Sclerotial germination

Sample solution was placed on a clean grease free slide, and it was dried. Mature sclerotia of *S. rolfsii* were placed on the test solution. Slides were kept on bent rods in moist Petri plates (100% humidity) and incubated for 24 h . In control

sets sclerotia were placed on sterile distilled water. Slides were observed under the microscope and percentage of germination were determined. Another set up was made for determination of sclerotial germination. In Petri plates black paper was kept and sterilized. These papers were soaked either with sample solution or sterile distilled water and in each Petri plate 10-20 mature sclerotia were placed on the top of the soaked black papers and incubated for 48 h. Germination percentage was computed and photographs were taken.

3.8.3. UV- spectrophotometric analysis

For spectral analysis of antifungal phenols extracted from healthy and *S. rolfsii* inoculated roots, initially ethyl-acetate fractions were plotted on TLC plates and developed in chloroform-methanol (9:1 v/v) solvent. Silica gel from corresponding antifungal zones as detected in chromatogram inhibition assay as well as sclerotial germination assay were scrapped off and eluted separately in spec methanol. These were re-spotted on TLC plates and developed in the same solvent, were diluted with spec. methanol and taken for UV-spectrophotometric analysis at a range of 200-400 nm.

3.8.4. HPLC analysis

Analysis of antifungal phenolics extracted from healthy and *S. rolfsii* inoculated tea plant roots were carried out on C 18 hypersil column using methanol as mobile phase in isocratic system. The elution was complete after a total of 15 min. Flow rate was fixed at 1 µl/min, sensitivity 0.5 aufs, injection volume 20 µl and monitored at 220 nm (Shimadzu, Japan).

3.9. Extraction of enzymes

Three enzymes – phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) involved in phenol metabolism were extracted from healthy and *S. rolfsii* infected tea plants to determine their activities.

3.9.1. Phenylalanine ammonia lyase (EC 4.3.1.5)

For the extraction of phenylalanine ammonia lyase the method of Bhattacharya and Ward (1987) was followed with modifications. Tea root tissue (1 *g*) was crushed in a mortar with pestle in 5 ml of 0.1 M sodium borate buffer (pH 8.8) containing 2 mM β-mercaptoethanol in ice. The crushed material was centrifuged at 12,000 *g* for 20 min at 4^0C. The supernatant was collected, volume recorded and then immediately used for assay.

3.9.2. Peroxidase (EC 1.11.1.7)

To extract peroxidase the method of Chakraborty *et al.* (1993) was followed with modification. Tea root tissue (1 g) was crushed with 0.1 M sodium borate buffer (pH 8.8) containing 2 mM β-mercaptoethanol in mortar with pestle on ice. The crushed material was centrifuged at 12,000 *g* for 20 min at 4^0C. The supernatant was collected and its final volume was measured and used immediately for assay.

3.9.3. Polyphenol oxidase (EC 1.10.3.2)

For the extraction of polyphenol oxidase the method of Mahadevan and Sridhar (1982) was followed with modification. Root tissue (1 g) was cut into pieces. The pieces were then crushed with mortar and pestle in ice cold condition with 5 ml of 0.2 M sodium phosphate buffer (pH 6.6). The slurry was immediately centrifuged at 12,000 *g* for 20 min at 4^0C. The supernatant was decanted and after recording its volume was used immediately for assay.

3.10. Assay of enzyme activities

Enzyme activities were assayed following specific procedure in each case.

3.10.1. Phenylalamine ammonia lyase

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture (total volume 3 ml) contained 0.3 ml 300 μM sodium borate (pH 8.8), 0.3 ml 30 μM L-phenylalanine and 0.5 ml of enzyme extract and 1.9 ml of double distilled water

Blank was prepared in same way but with water instead of enzyme extract. Then the tubes were incubated at 37^0C for 1h in water bath. After 1 h absorbance was noted at 295 nm in UV-VIS-spectrophotometer (SICO, Digispec – 200 GL) against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid / min.

3.10.2. Assay of peroxidase

For the assay of PO activity, 100 µl of freshly prepared crude enzyme was added to the reaction mixture containing 1 ml of 0.2 (M) sodium phosphate buffer pH 5.4, 100 µl of 4 mM H_2O_2, 100 µl of orthodianisidine (5mg /ml methanol) and 1.7 ml of double distilled water. PO activity was assayed spectrophotometrically at 495 nm by monitoring the oxidatioin of o-dianisidine in the presence of H_2O_2. Specific activity was expressed as the increase in absorbance at 495 nm / g tissue / min

3.10.3. Polyphenol oxidase

For the determination of PPO activity, 1.9 ml of 0.1 (M) sodium phosphate buffer pH 6.5, 0.1 ml enzyme extract and 0.1 ml of 0.025 (M) catechol solution (0.014 g in 5 ml sodium phosphate buffer pH 6.5). The reaction mixture was incubated at room temperature in the dark for the prevention of photo-oxidation of the enzyme. Initial absorbance was noted at 495 nm at 0 min. Further reading was taken at 1 min intervals. PPO activity was expressed as the increase in absorbance at 495nm / g tissue / min, when the substrate catechol was oxidized due to the enzyme activity from 1 g of tissue.

3.11. Preparation of antigens

3.11.1. Root antigen

Root antigens were extracted from healthy and *S. rolfsii* infected tea roots following the method of Chakraborty and Saha (1994a). Healthy and infected plants were uprooted after 2-week-intervals, washed with cold water and kept at 15^0C for 1 h. Finally, roots (20 g fresh wt.) were crushed with sea sand in mortar and pestle in

cold (4^0C) and kept at -15^0C for 1 h and homogenized with 20 ml of 0.05 M sodium phosphate buffer\ (pH 7.2) supplemented with 10 mM sodium metabisulphite and 0.5 mM magnesium chloride. Homogenate was strained through cheese cloth and then centrifuged (12,000 *g*) at 4^0C for 1 h. A known quantity of ammonium sulphate was added to it for 100% precipitation (Green and Hughes, 1995), and was kept at 4^0C. Precipitate was dissolved in the same extractive buffer (pH 7.4) and dialysed against 0.05 M phosphate buffer for 24 h at 4^0C. During this period 10 changes were given. The dialysate (*i.e.* soluble protein) was used for antisera production and for gel electrophoretic studies.

3.11.2. Mycelial antigen

Mycelial antigen was prepared following the method of Chakraborty *et al.* (1995b). Initially fungal mycelia (6 mm disc) were transferred to 250 ml Erlenmeyer flasks each containing 50 ml of sterilized liquid Richards medium and incubated for 10 days at 28 ± 1^0C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (30 g fresh wt.) were homogenized with 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 10 mM sodium metabisulphite and 0.5 mM $MgCl_2$ and 0.85% NaCl with mortar and pestle and sea sand. Cell homogenates were kept overnight at 4^0C and then centrifuged (15000 *g*) for 30 min at 4^0C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4^0C. After this period the mixture was centrifuged (15000 *g*) for 30 min at 4^0C, the precipitate was dissolved in 10 ml of 0.05 M sodium phosphate buffer (pH 7.2). The preparation was dialysed for 72 h through cellulose tubing (Sigma) against 1 L of 0.005 M sodium phosphate buffer (pH 7.2) with 10 changes. Then the dialysed material was stored at -20^0C and used as antigen for the preparation of antiserum.

3.11.3. Soil antigen

1 gm of soil was crushed in 2 ml of 0.05 M sodium phosphate buffer (pH 7.2) in mortar with pestle and kept overnight at 4^0C. Next day it was centrifuged at 12,000 *g* for 10 min. Supernatant was used as antigen for experimental purpose.

3.12. Serology

3.12.1. Maintenance of rabbit

New Zealand white male rabbits (one and half kg wt.) were used for immunological works. The rabbits were observed at least for one week before starting the immunization schedule. They were regularly fed with green grass, soaked gram seeds and green vegetables in the morning and evening. Cages were cleaned everyday in the morning for better hygenic conditions. After each bleeding they were given saline water for 3 consecutive days.

3.12.2. Immunization

Before immunization normal serum was collected from each rabbit. Polyclonal antibodies were raised against mycelial antigen of *S. rolfsii* and tea root antigen. The rabbit was injected subcutaneously once a week at 7-day-intervals with 1 ml antigen mixed with 1 ml of Freund's complete adjuvant (Difco, USA) for first two injections and the next emulsified with incomplete adjuvant for 5 weeks.

3.12.3. Bleeding

Bleeding of the rabbits was performed by marginal ear vein puncture, 3 days after the first six injections and then after every fourth injection.The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol, the ear vein was irritated by the application of xylene and an incision was made with the help of a sharp blade and 5 to 10 ml of the blood samples from each rabbit were collected in sterile graduated glass tubes. After collection, all the precautionary measures were taken to stop the flow of the blood from the puncture. The blood samples were kept as such for 1 h at 37^0C for clotting then the clot was loosened with a sterile needle. Finally, the serum was clarified by centrifugation (2000 *g* for 10 min at room temperature) and distributed in 1ml vials and stored at – 20^0C for further use.

3.12.4. Purification of IgG

3.12.4.1. Precipitation

IgG was purified as described by Clausen (1988). Crude antiserum (2 ml) was first diluted with two volumes of distilled water and an equal volume of 4 M ammonium sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16 h at 22^0C. The precipitate thus formed was collected by centrifugation at 10,000 *g* at 22^0C for 1 h. Then the precipitate was dissolved in 2 ml of 0.02 M sodium phosphate buffer (pH 8.0).

3.12.4.2. Column preparation

Eight grams of DEAE cellulose (Sigma Co., USA) was suspended in distilled water and kept for overnight. The water was poured off and DEAE cellulose was suspended in 0.05 M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30 cm high) and allowed to settle for 2 h. After the column material had settled, 25 ml of 0.02 M sodium phosphate buffer (pH 8.0) washing was given to the column material.

3.12.4.3. Fraction collection

At the top of the column, 2 ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and with a continuous change in molarity from 0.02 M to 0.3 M. The initial elution buffer (1) was 0.02 M sodium phosphate buffer (pH 8.0) where as final elution buffer (2) was 0.3 M sodium phosphate buffer (pH 8.0). Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV Spectrophotometer (SICO, Digispec – 200GL).

3.13. Immunodiffusion

3.13.1. Preparation of agarose gel plates

Glass plates (6 cm x 6 cm) were degreased in 90% (v/v) ethanol, ethanol: diethylether (1:1 v/v) and then dried in a hot air oven. After drying, plates were

sterilized. Agarose gel was prepared in Tris barbiturate buffer (pH 8.6) at 90^0C using 0.9% agarose (Sigma, USA), placed on a water bath and stirred till the agarose solution became clear. Then 0.1% (w/v) sodium azide was added in it. After pouring 10 ml of molten agarose on each plate, it was kept for solidification. After that, 3-7 wells were cut with a sterilized cork borer at a distance of 6 mm dia from the central well.

3.13.2. Diffusion

Agar gel double diffusion tests were performed following the method of Ouchterlony, 1967. The antigens and undiluted antisera (50 µl per well) were pipetted directly into pre-determined wells and allowed to diffuse in a moist chamber for 48-72 h at 25^0C.

3.13.3. Washing, staining and drying of slides

After immunodiffusion, the plates were washed with aquous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 72 h with six hourly changes to remove unreacted antigens and antiserum widely dispersed in the agarose. After that the plates were stained with Coomassie blue (Sigma R250) for 10 min at room temperature. After staining, these were destained with methanol:water:acetic acid (45:45:10) until the background become clear. Lastly, the slides were washed with distilled water and dried in a hot air oven for 3 h at 50^0C.

3.14. Enzyme linked immunosorbent assay (ELISA)

Following buffers were prepared for indirect ELISA

(A) Antigen coating buffer (0.05 M Carbonate-bicarbonate buffer, pH 9.6)

Stock

a.	Sodium carbonate	5.29 g
	Distilled water	1000 ml
b.	Sodium bicarbonate	4.2 g
	Distilled water	1000 ml.

160 ml of stock solution "A" was mixed with 360 ml of stock solution "B" pH was adjusted to 9.6.

(B) 0.15M Phosphate buffered saline pH 7.2 (PBS)

Stock

a.	Sodium dihydrogen phosphate	23.40 g
	Distilled water	1000 ml
b.	Disodium hydrogen phosphate	21.29 g
	Distilled water	1000 ml

With 280 ml of stock solution 'A', 720 ml of stock solution 'B' was mixed and the pH of the mixed solution was adjusted to 7.2. Then 0.8% NaCl and 0.2% KCl was added to the solution.

(C) 0.15 M Phosphate buffered saline – Tween pH 7.2 (PBS-T)

To 0.15 M phosphate buffered saline, 0.05% Tween 20 was added and the pH was adjusted to 7.2.

(D) Blocking reagent (0.05 M Tris buffer saline, pH 8.0)

Tris	0.657g
NaCl	0.81g
KCl	0.023g

Distilled water was added to make up the volume up to 100 ml. The pH was adjusted to 8.0 and 0.05% Tween 20 and 1% bovine serum (BSA) was added.

(E) Antisera dilution buffer

In 0.15 M PBS -Tween, pH 7.2, 0.2% BSA, 0.02% polyvinyl polypyrrolidone (PVPP-10), and 0.03% Sodium azide (NaN_3) was added.

(F) Substrate solution

This solution was prepared by addition of the substrate, p-nitrophenyl phosphate (10 mg / ml), to alkaline phosphatase buffer (0.05 m Tris – HCl buffer, pH 9.8) containing 1% diethanolamine.

(G) Stop solution

A 3 M NaOH solution was used to stop the reaction.

3.14.1. Plate trapped antigen - ELISA

PTA- ELISA was performed following the method as described by Chakraborty *et al.* (1996a). Plant and fungal antigens were diluted with coating buffer and the diluted antigens were loaded (200 µl/well) in a Nunc 96 well ELISA plate. After loading, the plate was incubated 25^{0}C for 4 h. The plate was then washed three times under running tap water and once with PBS-Tween and each time the plate was shaken dry. Subsequently, 200 µl of blocking agent was added to each well for blocking the unbound sites and the plate was incubated at 25^{0}C for 1 h. After incubation, the plate was washed as mentioned earlier. Purified antiserum (IgG) was diluted in antisera dilution buffer and loaded (200 µl/well) to each well and incubated at 4^{0}C overnight. After further washings 200 µl of anti rabbit IgG goat antiserum labeled with alkaline phosphatase was added and incubated at 37^{0}C for 2 h. Plate was washed, dried and loaded with 200 µl of p-nitrophenyl phosphate substrate in each well and incubated in the dark at room temperature for 30-45 min. Colour development was stopped by adding 50 µl / well of 3 N NaOH and absorbance was determined in an ELISA reader at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.15. Immunoblotting

3.15.1. Dot-immunobinding assay

Dot-immunobinding assay was performed following the method suggested by Hammond and Jordan (1990) for detection of pathogen. Following buffers were used for dot-blot.

(i) **Coating buffer** - 0.05 M Carbonate – bicarbonate, pH 9.6.

(ii) **Washing buffer** - 10 mM Tris buffer saline, pH 7.4 (TBS) with 0.9% NaCl and 0.5% Tween – 20

(iii) **Blocking solution** -10% (w/v) skimmed milk powder (casein hydrolysate, SRL) in TBST (0.05 M Tris – HCl, pH 10.3, 0.5 M NaCl, 0.5% (v/v) Tween-20. Nitrocellulose membrane (NCM) (7 cm x 10 cm), pore size 0.45 µm,

Millipore) was first cut carefully into the required size and placed inside the template; 2 µl of coating buffer was loaded in each well of the template over the NCM and kept for 30 min to dry.

Following this 2µl of test samples (antigens) were loaded into the template wells over the NCM and kept for 1 h at room temperature. The template was removed and blocking was done with 1% non fat dry milk (Casein) prepared in TBS for 30-60 min on a shaker. Polyclonal antibody (IgG 1:500) was added, directly in the blocking solution and further incubated at 4^0C for overnight. The membrane was then washed gently with washing buffer for 3 min (three times) in TBS (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase-conjugated goat antirabbit IgG (Sigma) diluted at 1:10,000 in alkaline phosphatase buffer, for 2 h at 37^0C. The membrane was washed as before. Substrate BCIP-NBT tablet (Sigma) dissolved in 10 ml double distilled water, was added and colour development noted. Colour development was stopped by washing the NCM with distilled water and colour development was categorized on the intensity of the dots. Finally floating the NCM in deionized water stopped the reaction.

3.15.2. Western blotting

Immunoblotting was also determined using Western Blot technique as described by Wakeham and White (1996). The following buffers were used for western blotting.

[A] All the solutions for SDS-PAGE were prepared as mentioned earlier under 3.6

[B] Transfer buffer (Towbin buffer) - 25 mM Tris, 192 mM glycine in 20% reagent grade Methanol, pH 8.3.

[C] 0.15 M phosphate buffer saline, pH 7.2 (PBS) was made as mentioned previously.

[D] Blocking solution 5% Casein hydrolysate in PBS, containing 0.02% Sodium Azide and 0.02% Tween –20

[E] Washing buffer (50 mM Tris HCl, 150 mM NaCl pH-7.5)

[F] Alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$ pH-9.5)

[G] Enzyme Alkaline phosphatase tagged with antirabbit goat IgG alkaline phosphatase buffer enzyme diluted (1:1000) in alkaline phosphatase buffer

[H] Substrate – one tablet of BCIP-NBT (Sigma) in 10 ml double distilled water

[I] Stop solution – EDTA sodium salt (0.0372 g in 200 µl distilled water) added in 50 ml of PBS.

3.15.2.1. SDS-PAGE of protein

Protein extraction, estimation and SDS-PAGE was carried out as mentioned earlier.

3.15.2.2. Blot transfer process

Following SDS-PAGE, the gel was transferred in prechilled (Towbin buffer) for 1 h. The nitro-cellulose membrane (Bio-Rad, 0.45 µm) and the filter paper (Bio--Rad, 2 mm thickness) were cut to gel size, wearing gloves, and soaked in Towbin buffer for 15 min. The transfer process was done in Trans-Blot SD Semi-Dry. Transfer cell (Bio-Rad) through a power pack (Bio-Rad). The presoaked filter paper was placed on the platinum anode of the unit. A pipette glass (or glass rod) was rolled over the surface of the filter paper to exclude all air bubbles. The pre-wetted membrane was placed on top of the filter paper. Then the equilibrated gel was carefully placed on the membrane and air bubbles were rolled out. Finally another pre-soaked filter paper was placed on the top of the gel and air bubbles were removed. The cathode was carefully placed on the sandwich and pressed to engage the latches with the guide posts without disturbing the filter paper stack. The blot unit was run for 45 min at a constant volt (15 V). After the run the membrane was removed and dried on a clean piece of 3 mm filter paper for 1 h and proceeded for immunological probing.

3.15.2.3. Immunoprobing

Following drying, blocking was done by 5% non fat dried milk in a heat sealable plastic bag and incubated for 90 min with gentle shaking on a platform

shaker at room temperature. Subsequently the membrane was incubated with purified IgG (diluted in PBS). The bag was sealed leaving space for few air bubbles and incubated at 4^0C overnight. All the processes were done with gentle shaking following incubation the membrane was washed thrice in 250 ml PBS. Final washing was done in 200 ml TBS to remove azide and phosphate from the membrane before the enzyme coupled reactions. The enzyme, alkaline phosphatase tagged with antirabbit goat IgG (Sigma) diluted (1:10,000) in alkaline phosphatase buffer, was added and incubated for 1 h at room temperature. After the enzyme reaction, the membrane was washed four times in PBS. Then 10 ml the substrate was added and the reaction was monitored carefully. When bands were observed up to the desired intensity the membrane was transferred to a tray containing 50 ml of the stop solution.

3.16. Florescence antibody staining and microscopy (Immunofluorescence)

Indirect immunofluorescence staining of cross-section of tea roots, fungal sclerotia and mycelia were done using FITC labeled goat antirabbit IgG following the method of Chakraborty *et al.*(1995b).

3.16.1. Mycelia

Fungal mycelia were grown in Richards agar media. After 4 days of inoculation young mycelia were taken out from the flask and kept in grooved slides. After washing with PBS (pH 7.2), mycelia were treated with normal sera or antisera diluted with PBS (1:125) and incubated at 27^0C for 30 min. Then mycelia were washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with FITC (Sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 30 min at 27^0C. After incubation, mycelia were washed thrice in PBS (pH 7.2) and mounted in 10% glycerol. A cover glass was placed on mycelia and sealed. These slides were observed and photographed under both phase contrast and UV fluorescence condition for comparison of treatment using Leica Leitz Biomed microscope with fluorescence optics equipped with Ultra Violet (UV) I3 filter block.

3.16.2. Cross-section of tea roots

Initially, cross sections of healthy and infected tea roots were cut and immersed in phosphate buffer saline (PBS) pH 7.2. These sections were treated with normal serum or antiserum diluted in PBS (1:125) and incubated for 1 h at room temperature. After incubation, for 1 h at room temperature and transferred to 40 µl of diluted (1:40) anti-rabbit goat IgG conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min in the dark. After that sections were washed thrice with PBS-Tween and mounted on a grease free slide with 10% glycerol. Fluorescence of the root sections was observed under UV light using Leica Leitz Biomed microscope equipped with I3 filter block ideal for FITC fluorescence. Tissue sections were photographed under both phase contrast and UV-fluorescent conditions for comparison of treatment.

3.17. Inducing agents and their application

3.17.1 *In vitro* test

(a) Biocontrol agents: Antagonistic properties of *T. harzianum* and *T. viride* against *S. rolfsii* were studied through dual plate method. Mycelial blocks (4 mm dia) cut from the margin of 3-day-old cultures of both the test pathogen (*S. rolfsii*) and biocontrol agents (*T. harzianum* and *T. viride*) were placed opposite to each other on PDA in petri plates (10 cm dia). The distance between inoculum blocks was 7 cm. Inoculated petri plates were incubated at 28^0C. To examine the inhibitory effect of culture filtrates of *T. harzianum* and *T. viride* against *S. rolfsii*, initially these were grown in potato dextrose broth (PDB) at 28^0C with vigorous shaking on a platform shaker at 175 r.p.m. for 7 days. Mycelia were harvested and culture filtrates were centrifuged at 12,000 *g* for 20 min and the supernatants were filter sterilized separately by passing through milipore filter. Then 45 ml of PDB media and 5 ml of culture filtrate were taken in 250 ml conical flask, while in control set 45 ml PDB media and 5 ml of sterile distilled water was taken. Each flask was then inoculated with 4 mm dia agar block of *S. rolfsii* and incubated at 28^0C for 10 days. Finally mycelial dry weights were taken.

(b) Plant extracts: For *in vitro* evaluation of plant extracts of *Azadirachta indica* and *Catharanthus roseus*, these were separately mixed with 15 ml of molten PDA (v/v 5:15) and poured into sterilized glass Petri plates. After solidification, all the plates were inoculated individually with a 4 mm diameter culture disc taken from the advancing region of *S. rolfsii* grown on PDA . PDA plates without plant extracts but inoculated with *S. rolfsii* served as control. The plates were then incubated at 28 ± 2^0C. The colony diameter of the fungus was measured after six days of incubation and compared with the colony growth of the fungus in control.

(c) Fungicides: The fungitoxicity of the fungicides were tested using slide germination and poisoned food techniques. For *in vitro* fungicidal evaluation, five systemic fungicides *viz.*, thiodan, calixin, captan, carbendazim (Bavistin) and Indofil M-45 were screened at 4 different concentrations (0.1%, 0.05%, 0.025% and 0.0125%) and inhibitory effect on the mycelial growth was assessed using poisoned food technique. Three replicates of each fungicide concentration were prepared by pouring 15 ml PDA in each petri plate. After solidification of media, mycelial blocks (4 mm dia) from actively growing culture of pathogen (*S. rolfsii*) was inoculated at the center. Control plates without any fungicides were also inoculated for comparison. The inoculated plates were incubated 28^0C till the fungus covered the PDA in control plates. The growth of the fungus was recorded.

(d) Organic additives: To evaluate the survival of *S. rolfsii* in organic amendments the sand-maize meal media was prepared with 2% saw dust. The different organic amendments (cow dung, chicken manure, rabbit manure, mustard oil cake and neem cake) were powdered in a grinder separately. The sand maize meal media and amended substance were mixed in 1:3 ratio and water added as required and sterilized. The mixture was plated in 9 cm dia Petri dishes and inoculated with 4 mm mycelial discs of *S. rolfsii* and incubated at 28^0C. The growth of the fungus was recorded and compared.

3.17.2. *In vivo* test

Mature leaves (500 g) each of *Azadirachta indica* and *Catharanthus roseus* were harvested, washed thoroughly with running tap water, rinsed with distilled

water, air dried and macerated separately homogenized in a electric blender. The leaf extract was filtered through double – layered muslin cloth and centrifuged at 10,000g for 30 minutes. The supernatant was collected and filtered through Whatman No.1 filter paper. Each filtrate was further filter sterilized and preserved as stock (100%) solution aseptically in bottles at 5^0C for further use. Leaf extracts were diluted (1:10) with distilled water, drops of Tween-80 was mixed and spayed on tea plants with the help of sprayer. The control plants were sprayed with distilled water mixed with Tween 80. Spray was done four times at 7-day intervals. Both treated and untreated plants were inoculated with *S. rolfsii* and disease assessment was made.

Mustard oil cakes and neem cakes were allowed to decompose separately for a week in a clay pot covered with polythene. After decomposition, 100 ml of decomposed oil cake solution was added in each tea seedlings pots. The pots were then inoculated with *S. rolfsii*. Untreated control was kept for comparison. Growth behaviour also observed up to two months. Organic additives (cow dung, rabbit manure and chicken manure), 100 gm of each were taken separately and mixed in 1 kg of soil. These soil mixtures were separately kept in each pot. Tea seedlings were planted in each pot containing different organic components. After one week, 100 gm of pathogen (*S. rolfsii*) inoculum was added in the rhizosphere of each tea seedling.

Mass cultures of *T. harzianum and T. viride* were prepared on carrier medium comprising of wheat bran and sawdust (WBSD) in 3:1 ratio. Five hundred grams of the contents of carrier medium moistened with 20 percent (w/w) distilled water was filled in each bag. These polythene bags were sterilized at 15 lb pressure for 1 h for 2 consecutive days. Each polythene bag was then inoculated with 4-6 days old bits (0.3 cm) of pure culture either of *T. harzianum* and *T. viride* and incubated at 28 ± 1^0C. During incubation, these bags were gently hand shaken to promote uniform sporulation over the carrier medium and to avoid clusters. Addition of biocontrol agents in soil was done 10 days prior to inoculation with *S. rolfsii*.

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

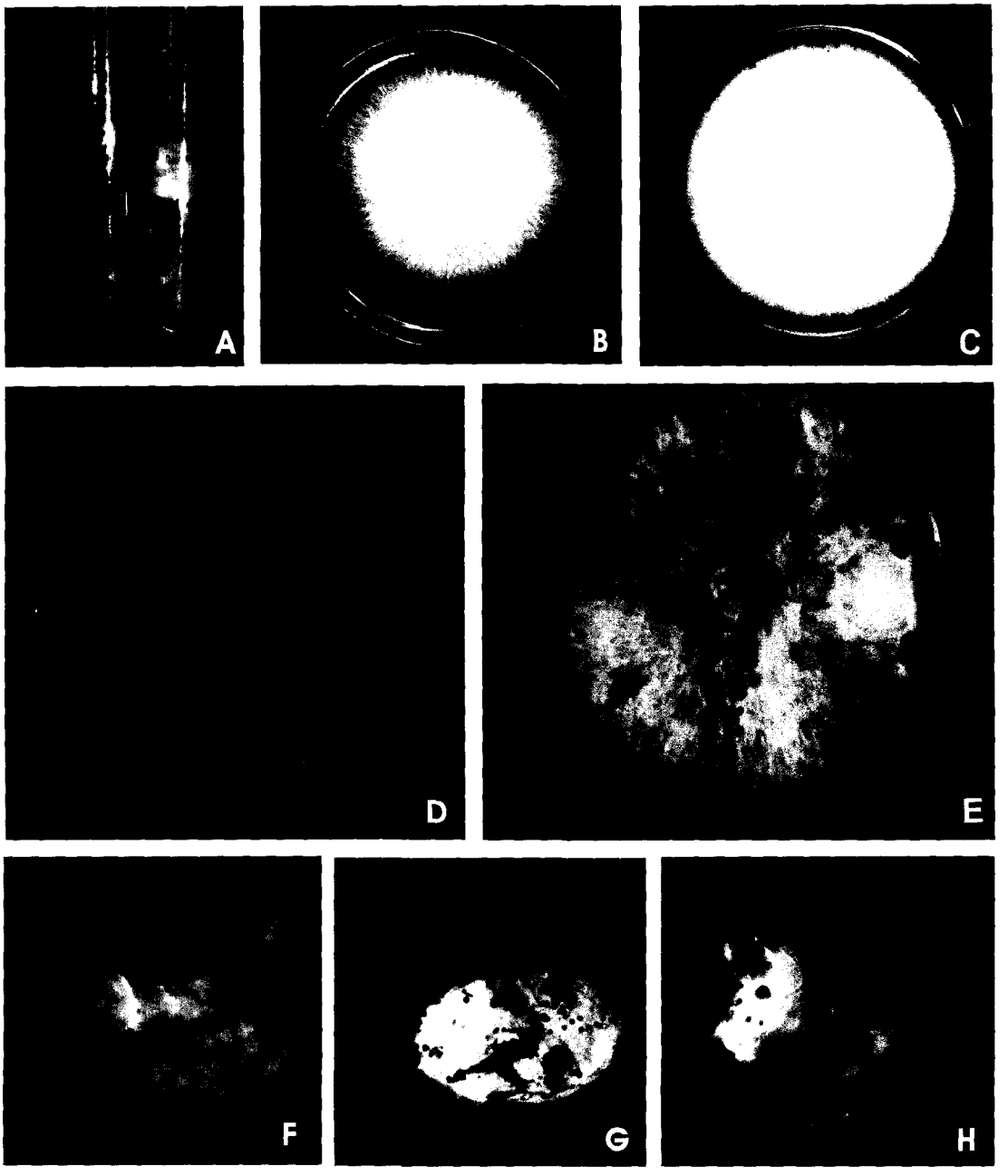

Plate 4 (figs. A-H). Growth of *S. rolfsii* isolates on potato dextrose agar (A-C,E) and potato dextrose broth (D, F-H). Isolate Sr-1(A-F), isolate Sr-2 (G) and isolate Sr-3 (H).

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).

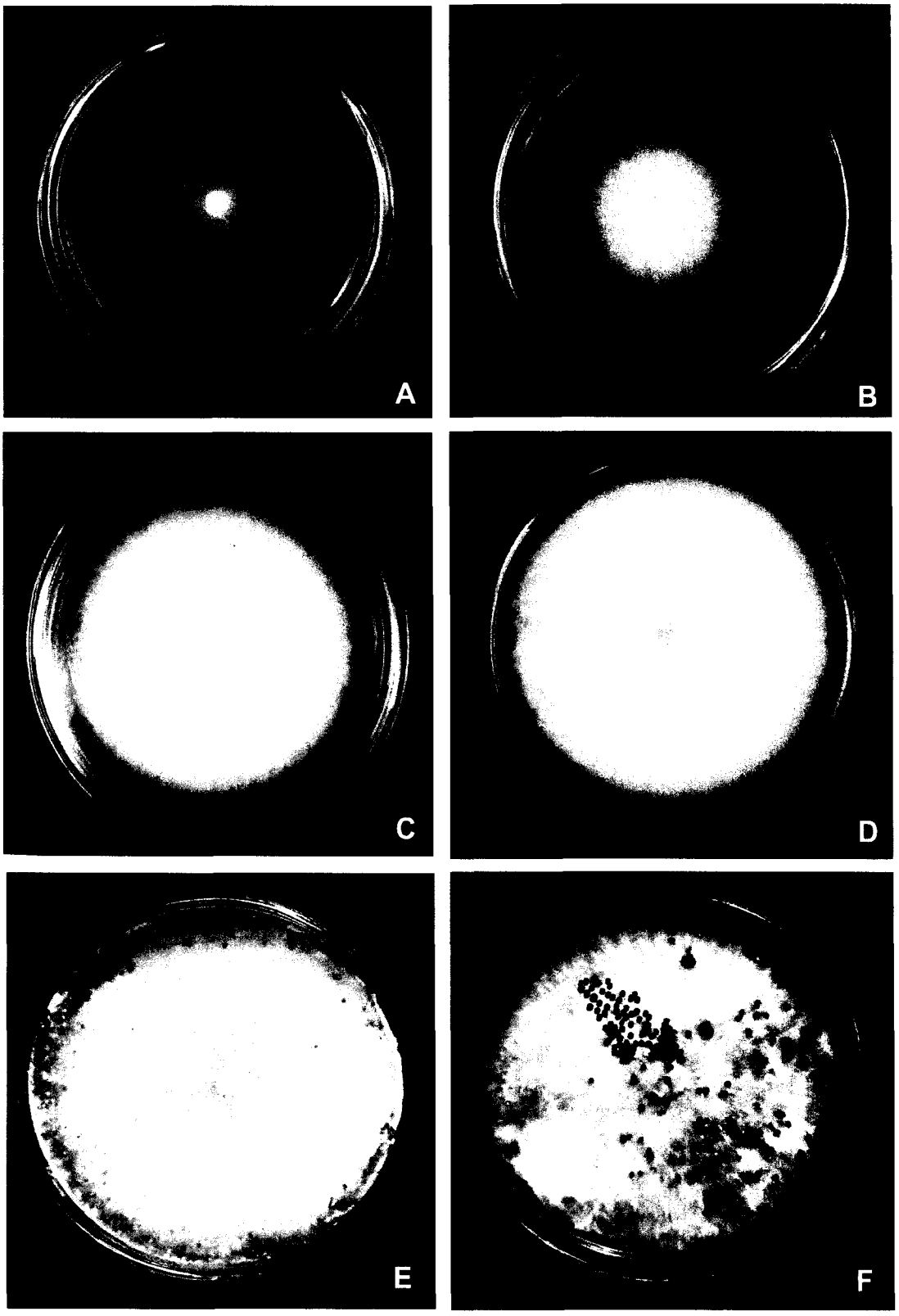

Plate 5 (figs. A-F). Mycelial growth of *S. rolfsii* on different media. (A) Richard's Agar, (B) Carrot juice Agar (C) Potato Sucrose Agar (D) Yeast Extract Dextrose Agar (E&F) Potato Dextrose Agar.

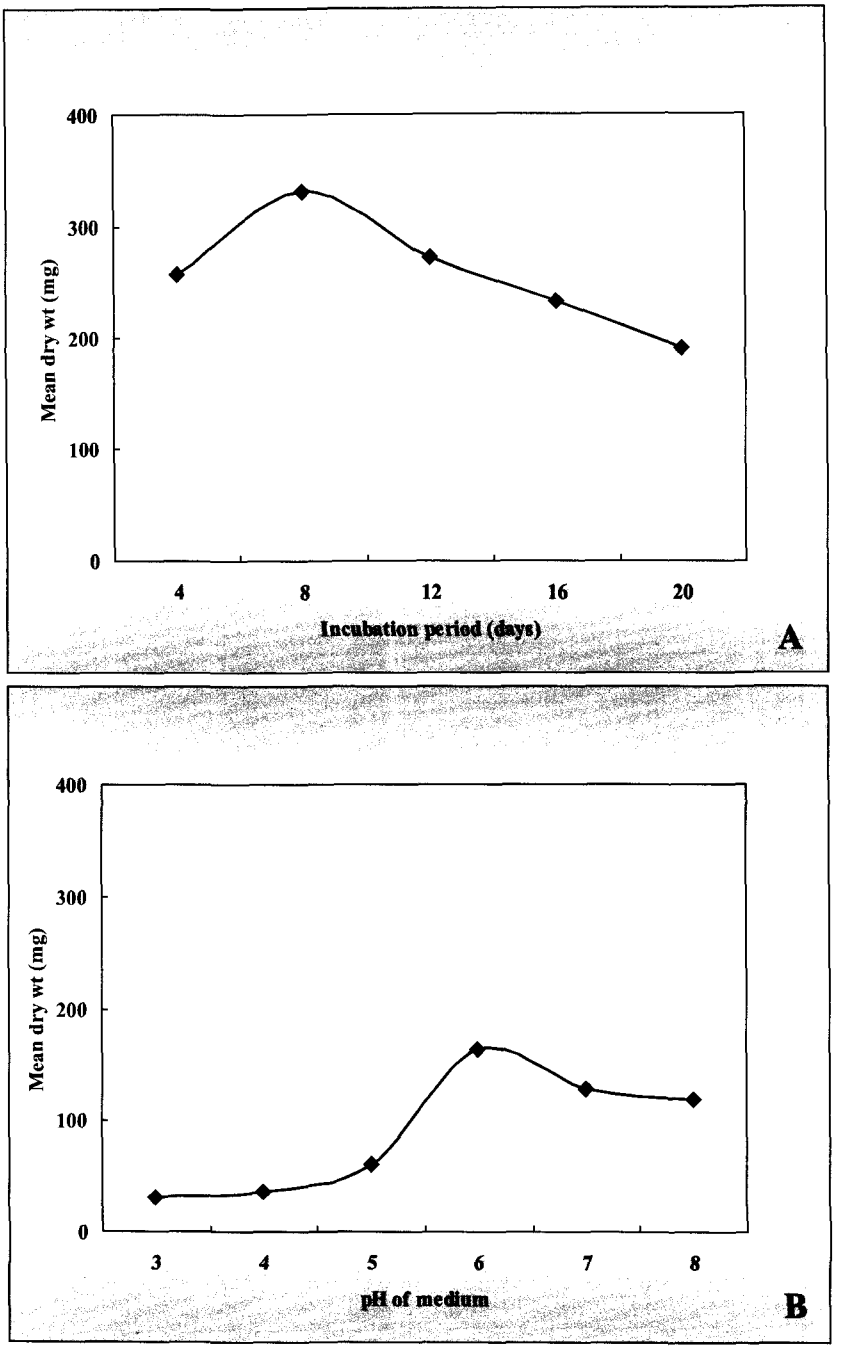

Fig. 1(A&B). Effect of incubation period (A) and pH (B) on mycelial growth of *S. rolfsii.*

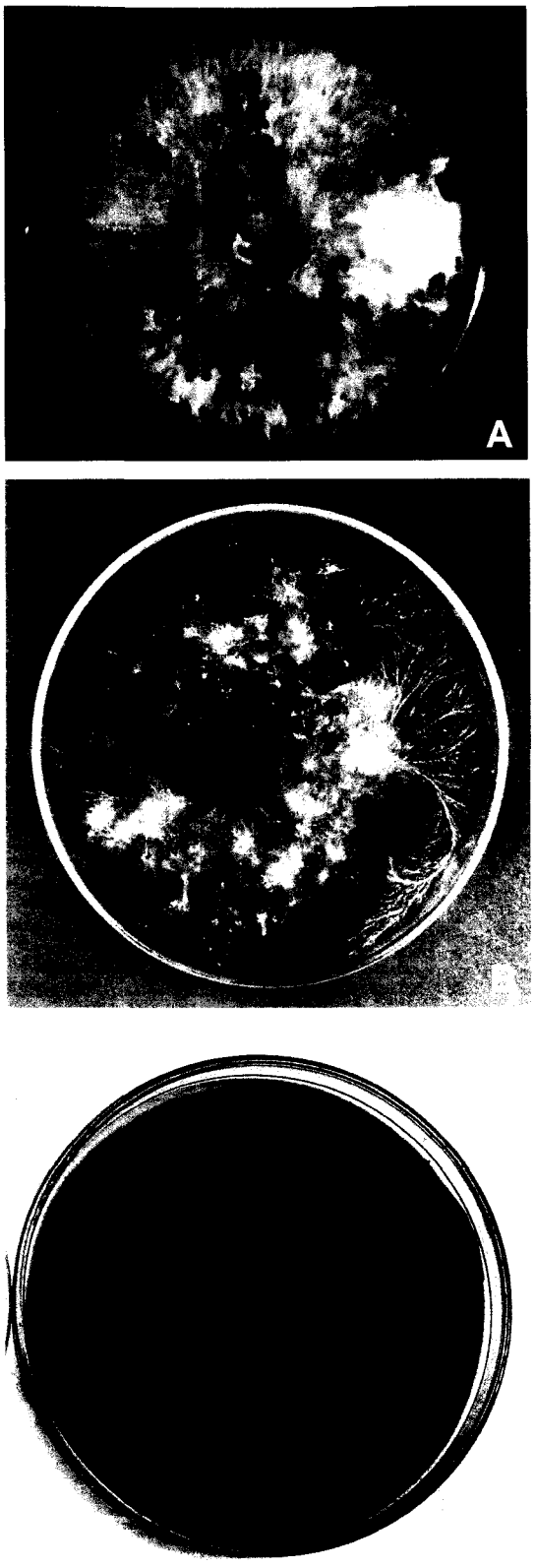

Plate 6 (figs. A-C). Germinated sclerotia of *S. rolfsii*

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).

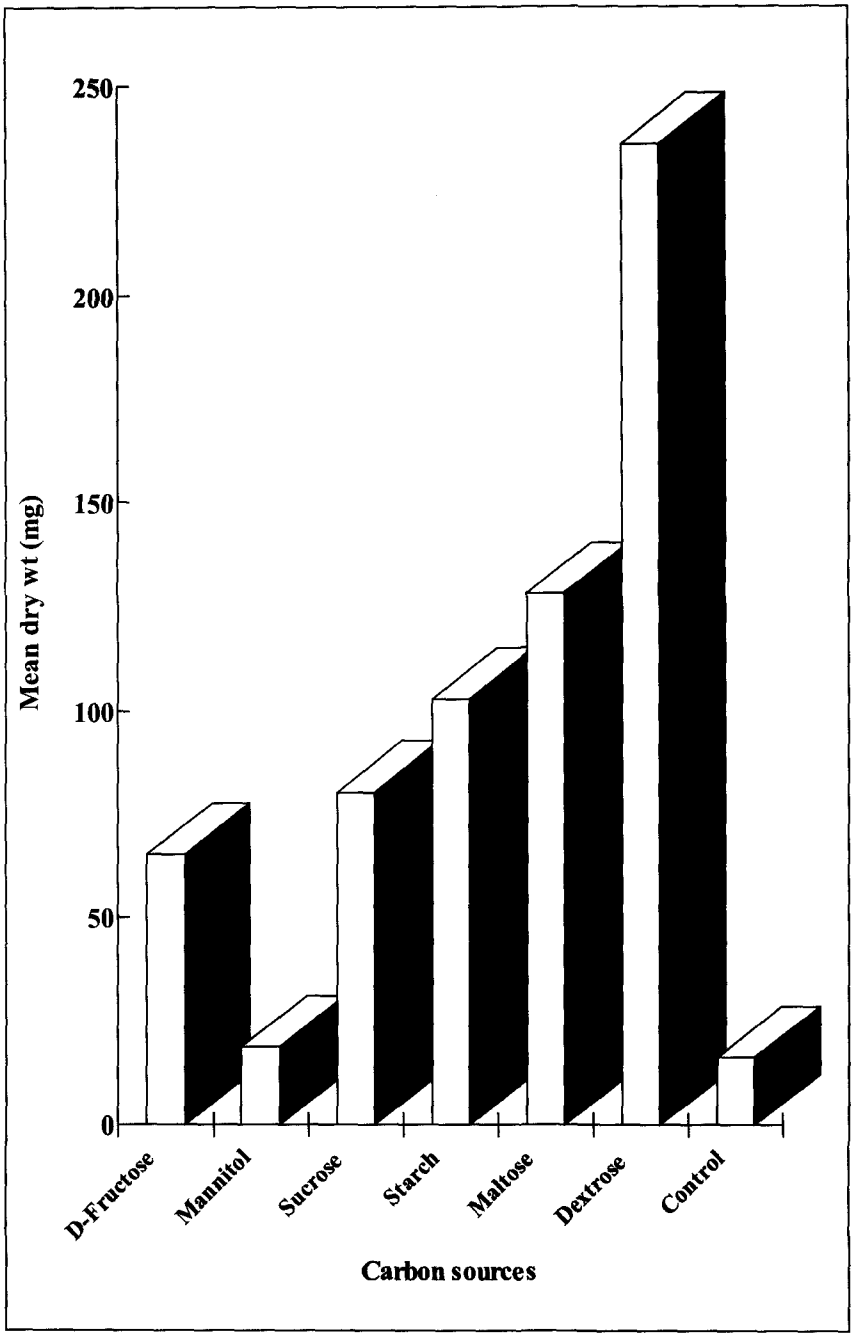

Fig. 2. Effect of different carbon sources on mycelial growth of *S. rolfsii*

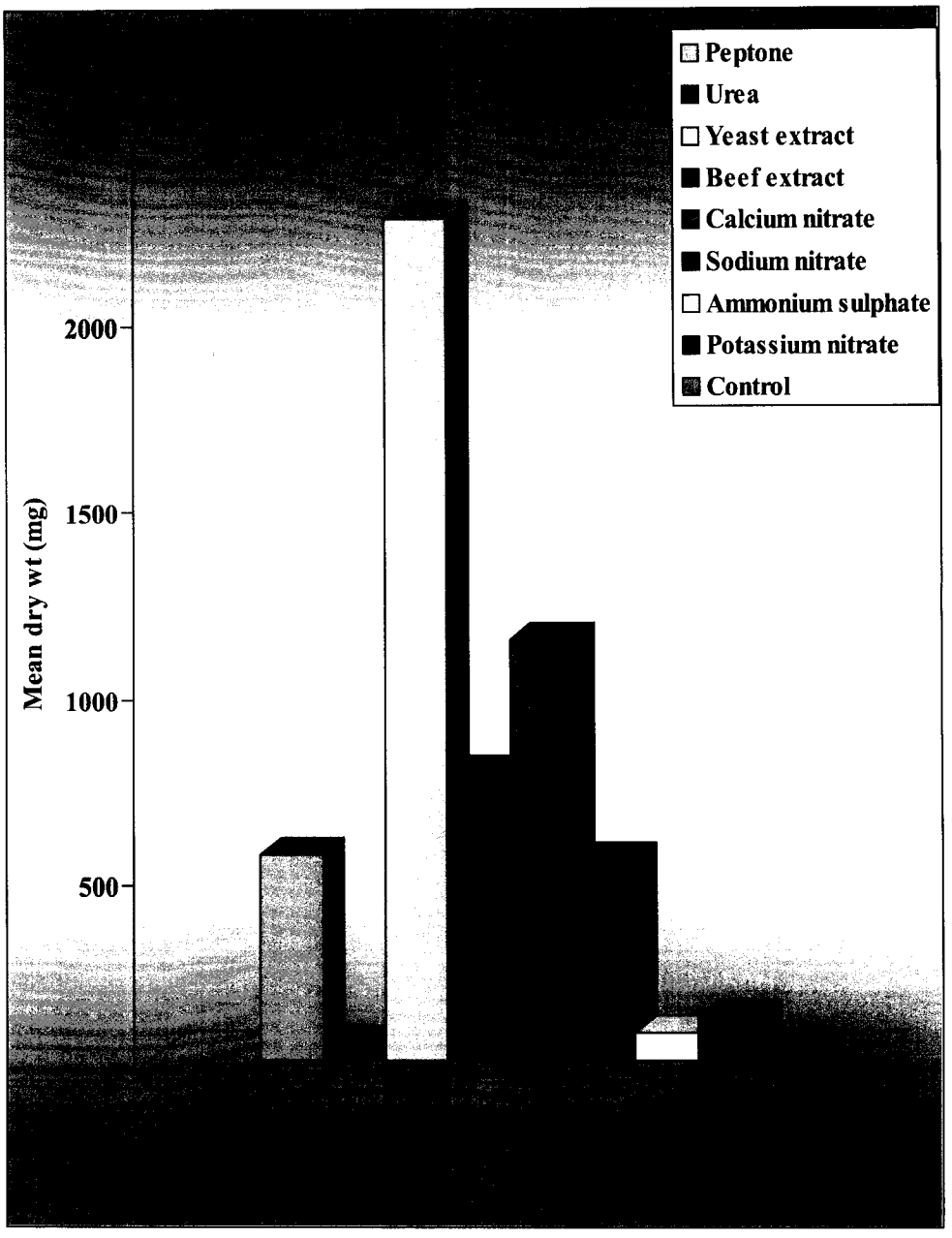

Fig. 3. Effect of different organic and inorganic nitrogen sources on mycelial growth of *S. rolfsii.*

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).

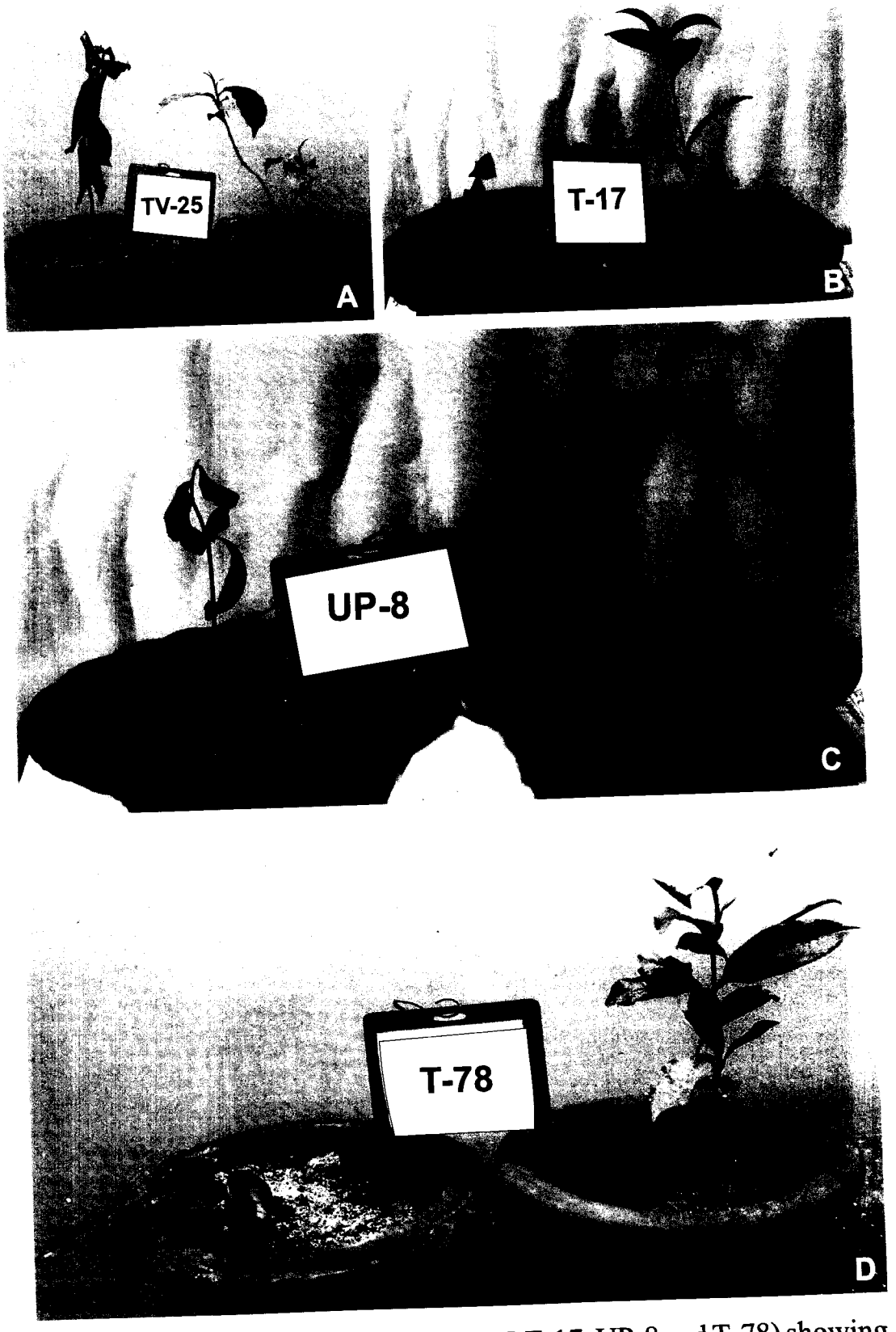

Plate 7 (figs. A-D). Tea varieties (TV-25,T-17, UP-8 and T-78) showing symptoms following inoculation with *S. rolfsii.*

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.

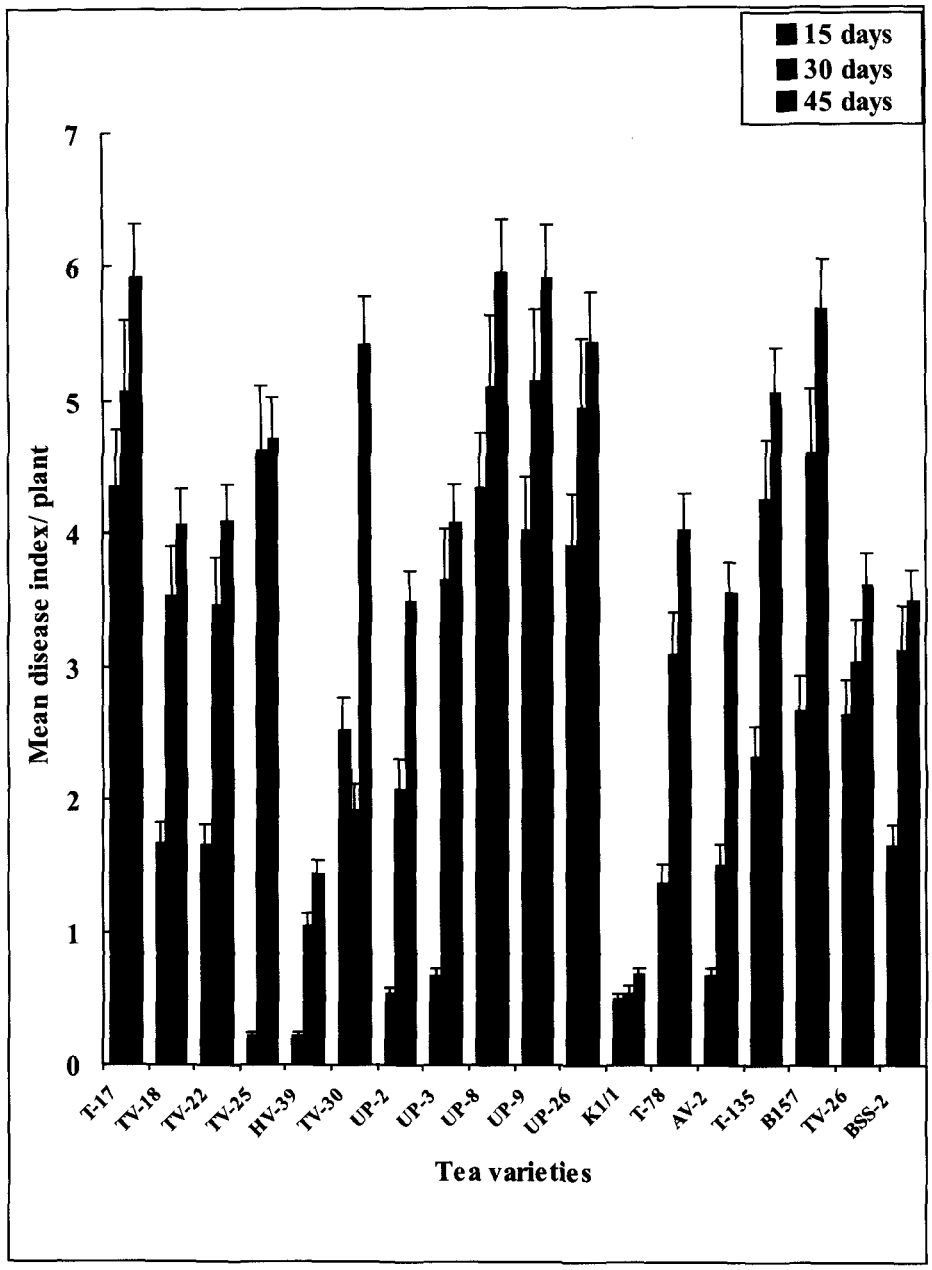

Fig. 4. Screening of tea varieties for resistance against *S. rolfsii*.

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.

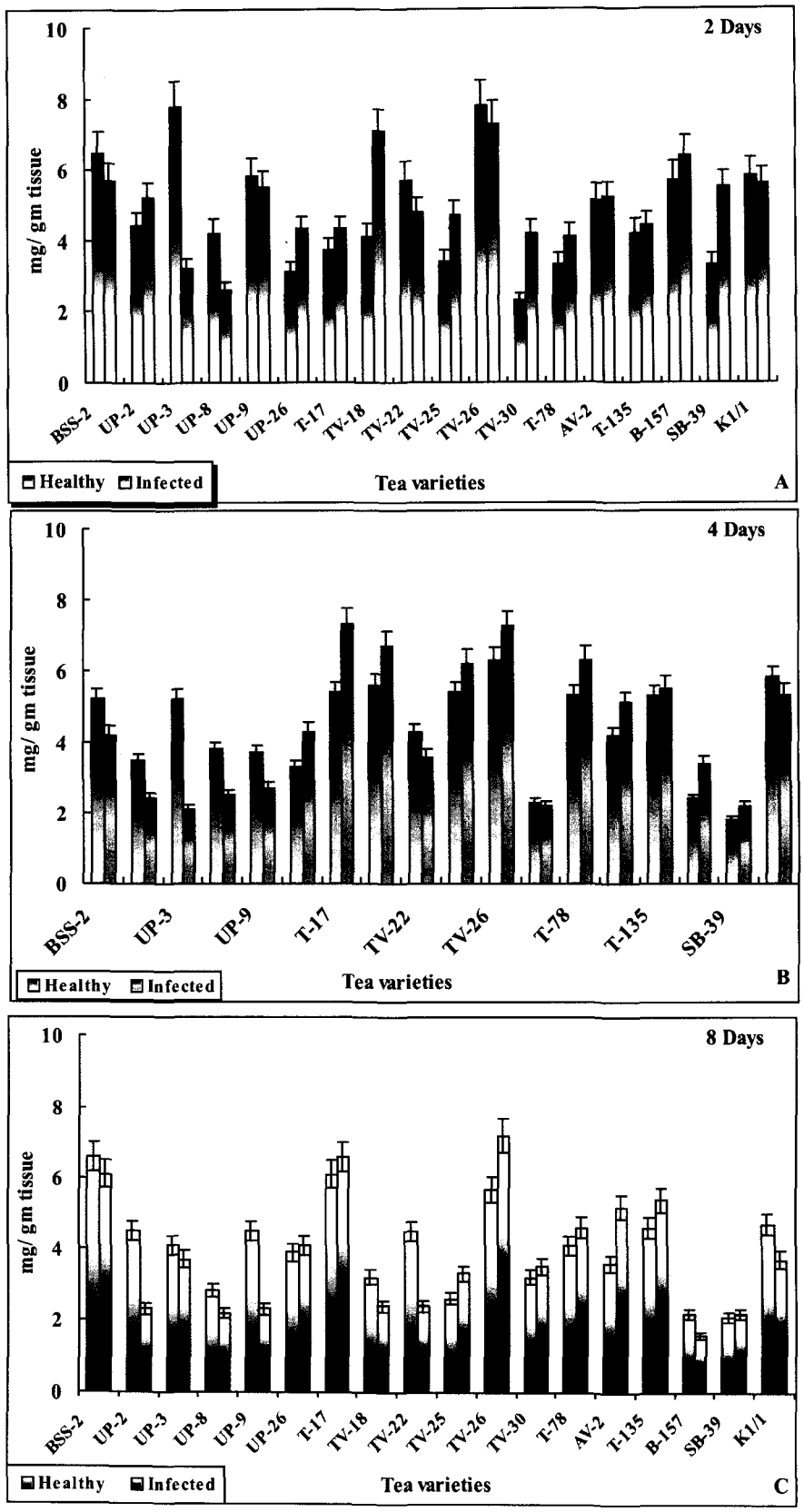

Fig. 5 (A-C). Protein content in healthy and *S. rolfsii* inoculated root of tea varieties.

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.

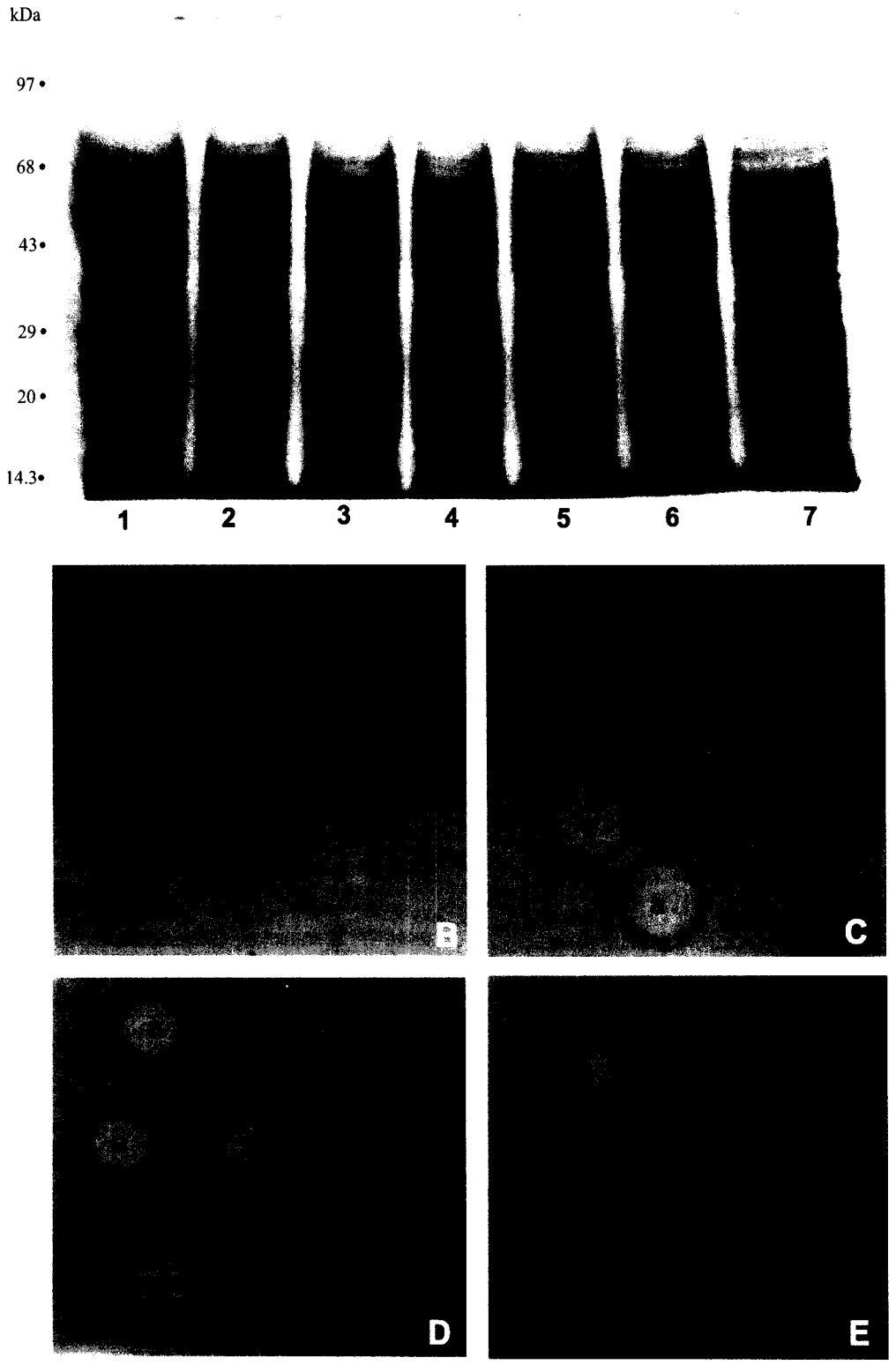

Plate 8 (figs. A-E). Mycelial antigens of *Sclerotium rolfsii.* (A) SDS-PAGE analysis (B-E) Agar gel double diffusion test with PAb of *S. rolfsii* (central wells) and homologous antigen (peripheral wells).

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

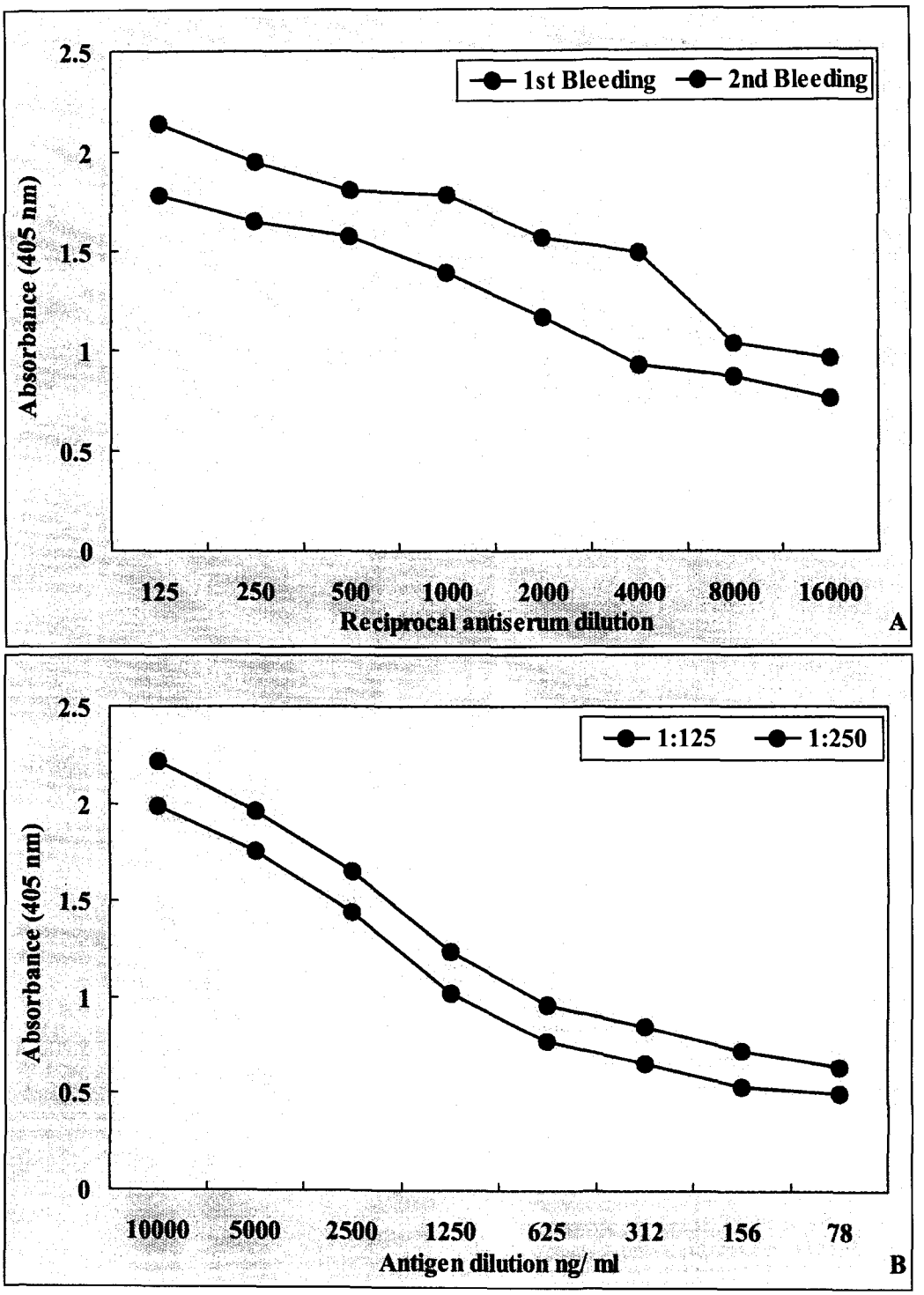

Fig. 6 (A&B). Effect of dilution of antiserum (A) and antigen (B) of *S. rolfsii* on homologous reaction using PTA-ELISA format.

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.

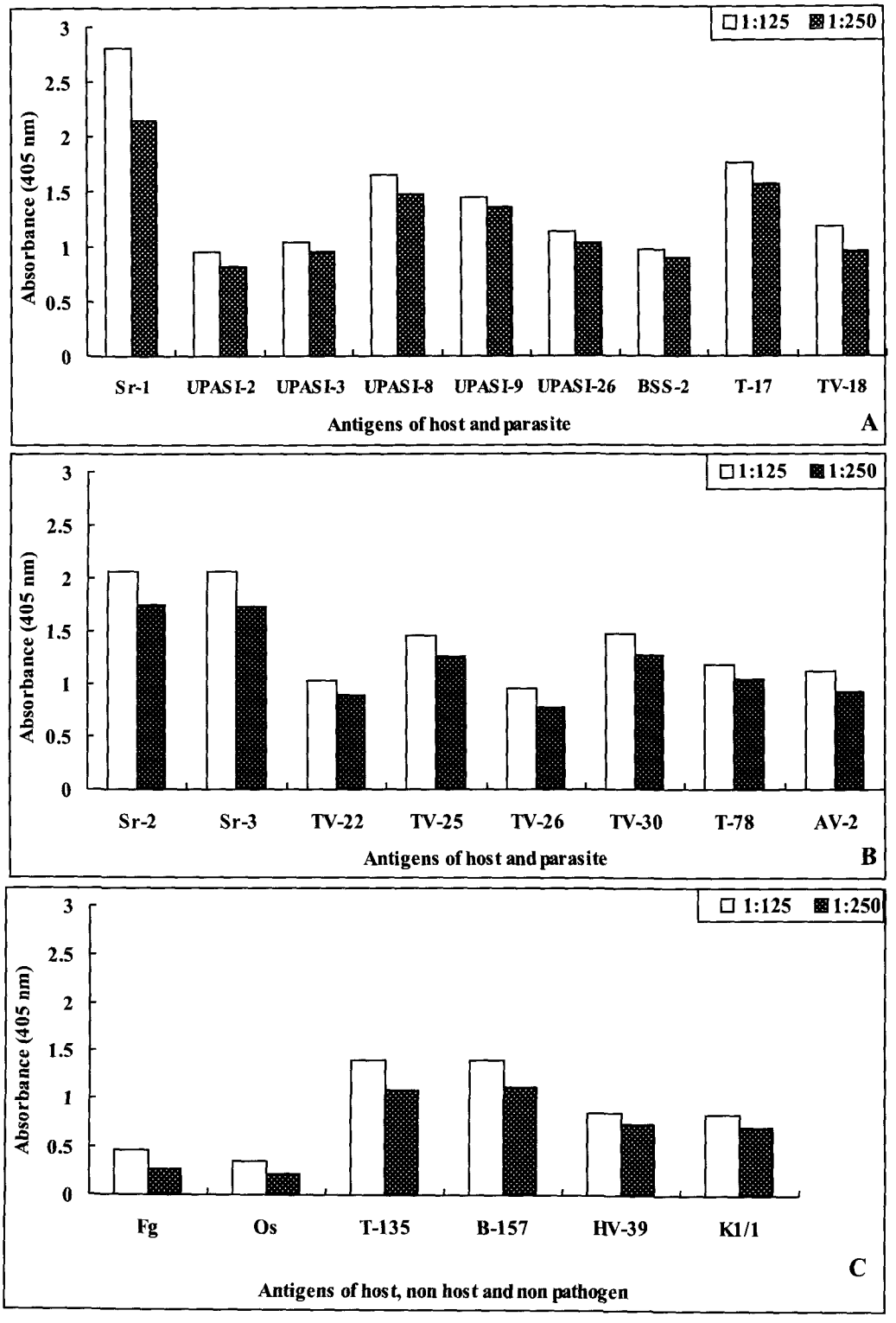

Fig. 7. PTA-ELISA responses of healthy root antigens of different tea varieties against PAb of *S. rolfsii.*

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.

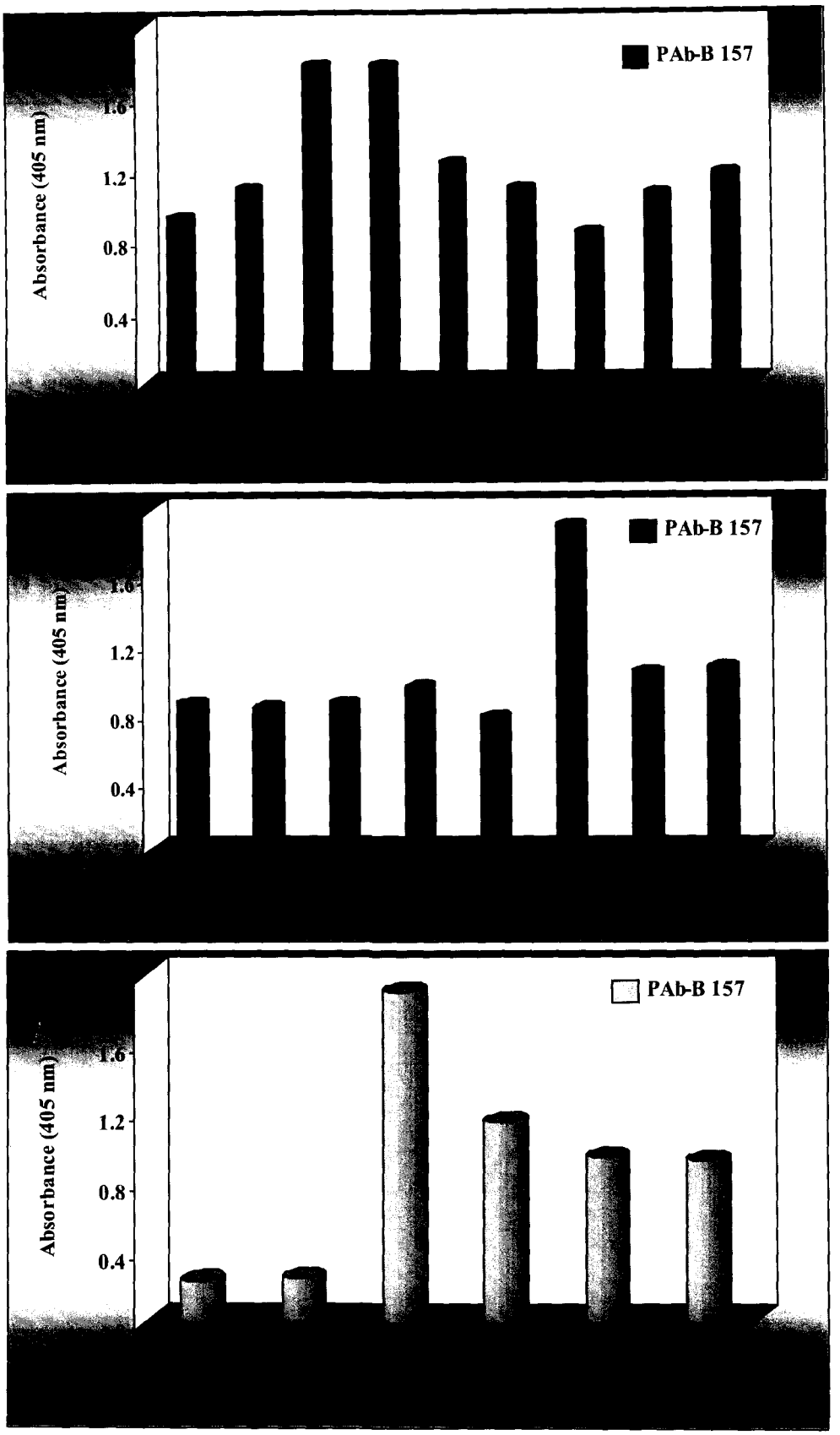

Fig. 8. Reciprocal cross reaction of root antigens of tea varieties (host), non host, mycelial antigen of pathogen and non pathogen with PAb of tea variety (B-157) using PTA-ELISA format.

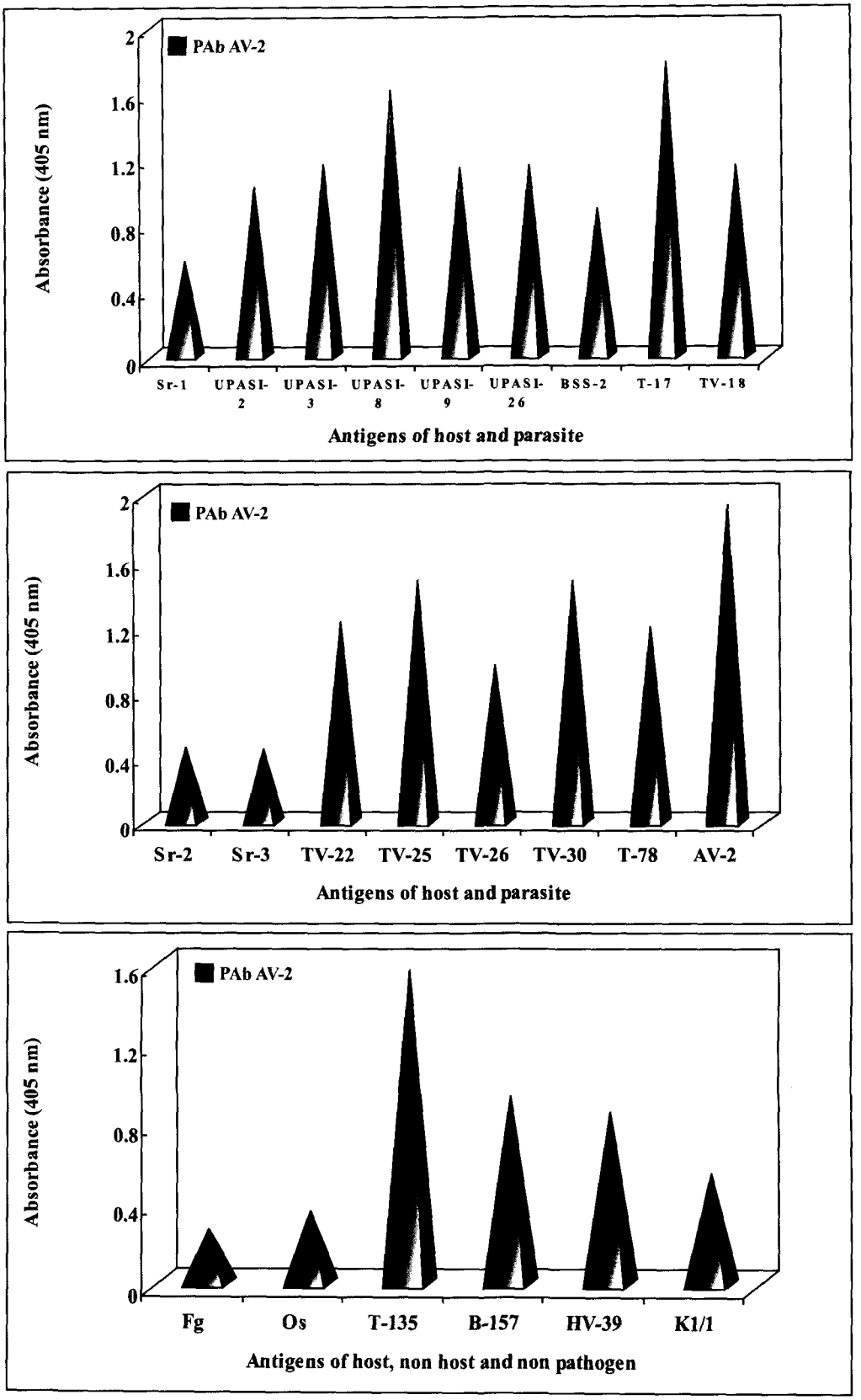

Fig. 9. Reciprocal cross reaction of root antigens of tea varieties (host), non host, mycelial antigen of pathogen and non pathogen with PAb of tea (AV-2) using PTA-ELISA format.

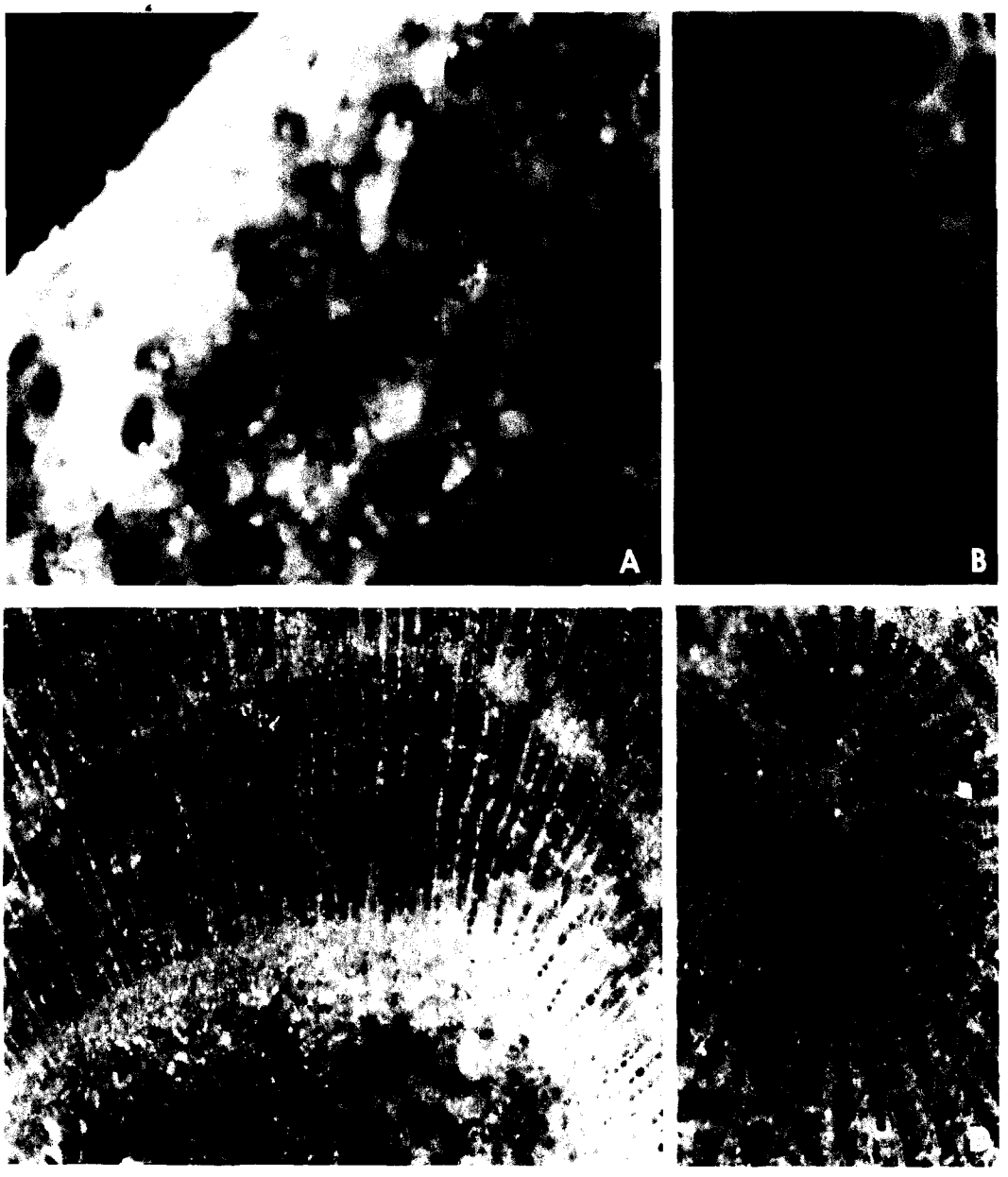

Plate 9 (figs. A-D). Fluorescent antibody staining of tea root tissues for cross reactive antigens shared with *S. rolfsii*. Healthy root tissues treated with PAb of *S. rolfsii* and labeled with FITC antibodies of goat specific for rabbit globulin (A&B) UP-3; (C&D) T-17.

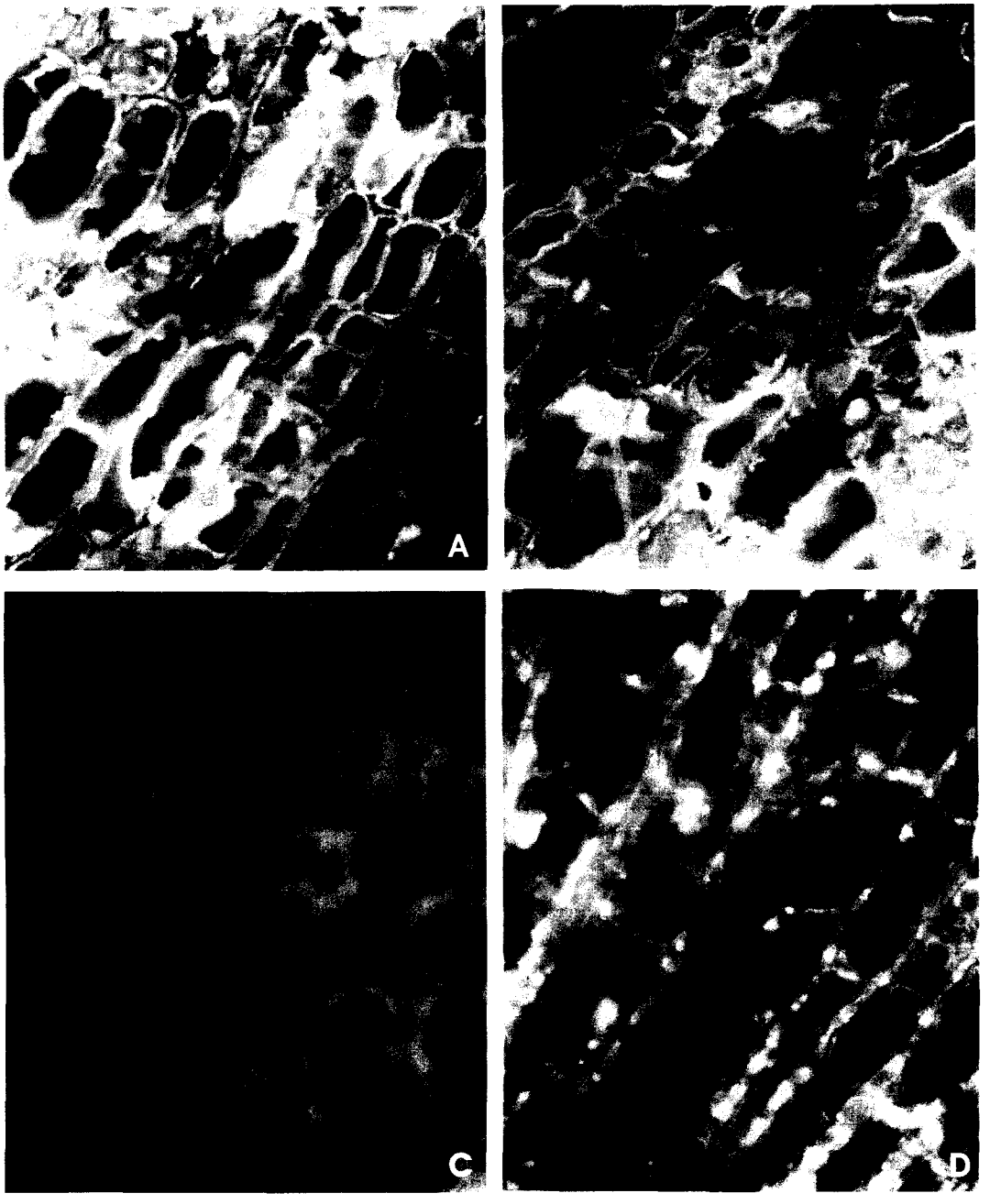

Plate 10 (figs. A-D). Fluorescent antibody staining of tea root tissues (B-157) for cross reactive antigens shared with *S. rolfsii.* Healthy root tissue treated with PAb of *S. rolfsii* and labeled with FITC antibodies of goat specific for rabbit globulin

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).

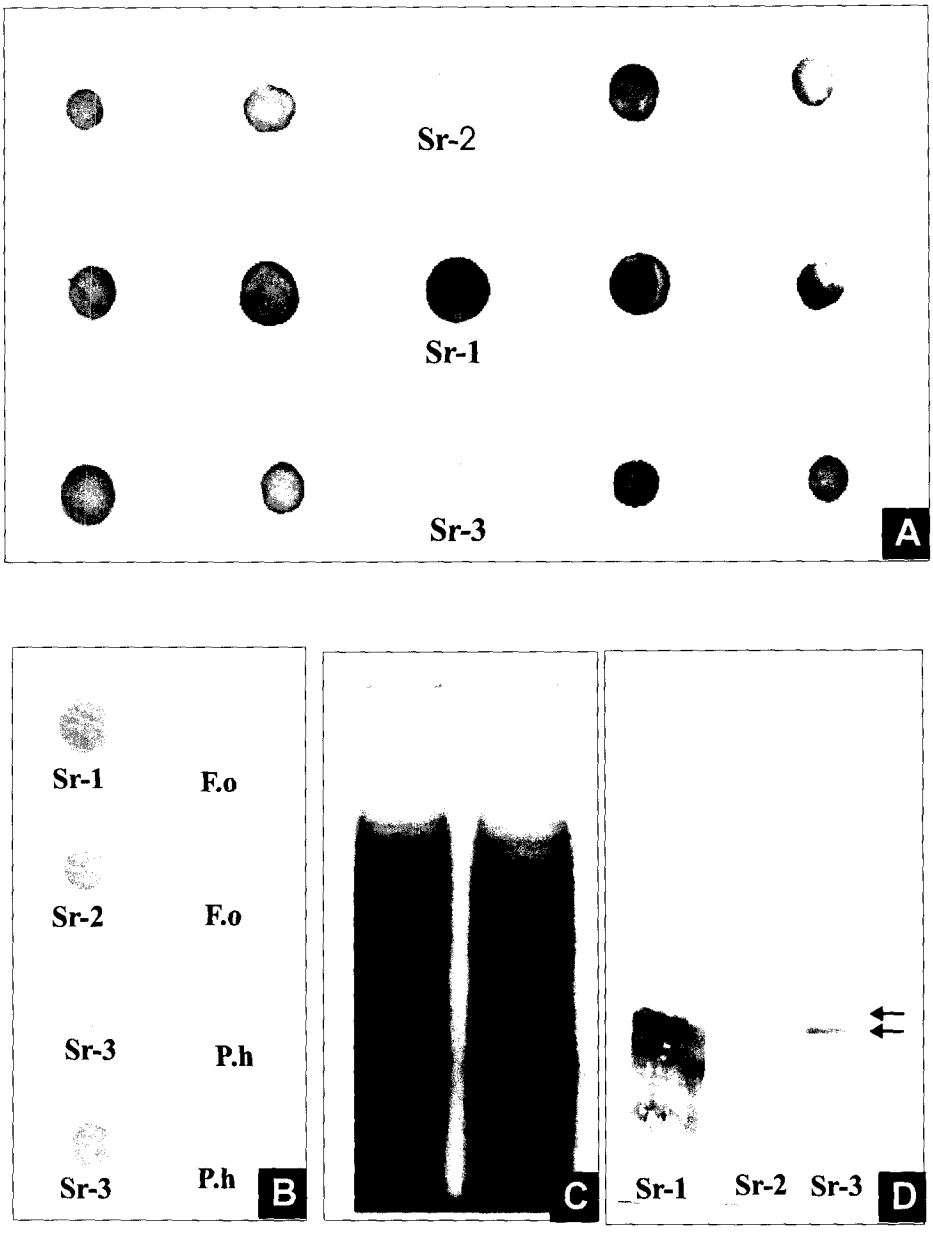

Plate 11 (figs A-D): Dot immunobinding assay and Western blot analysis of mycelial antigens with PAb of *S. rolfsii*. (A&B) Antigens of *S.rolfsii* (isolates Sr-1, Sr-2 & Sr-3), *F.oxysporum* f.sp. *lycopersici, Poria hypobrumea* reacted with PAb of *S. rolfsii*. (C) SDS-PAGE analysis of mycelial antigens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

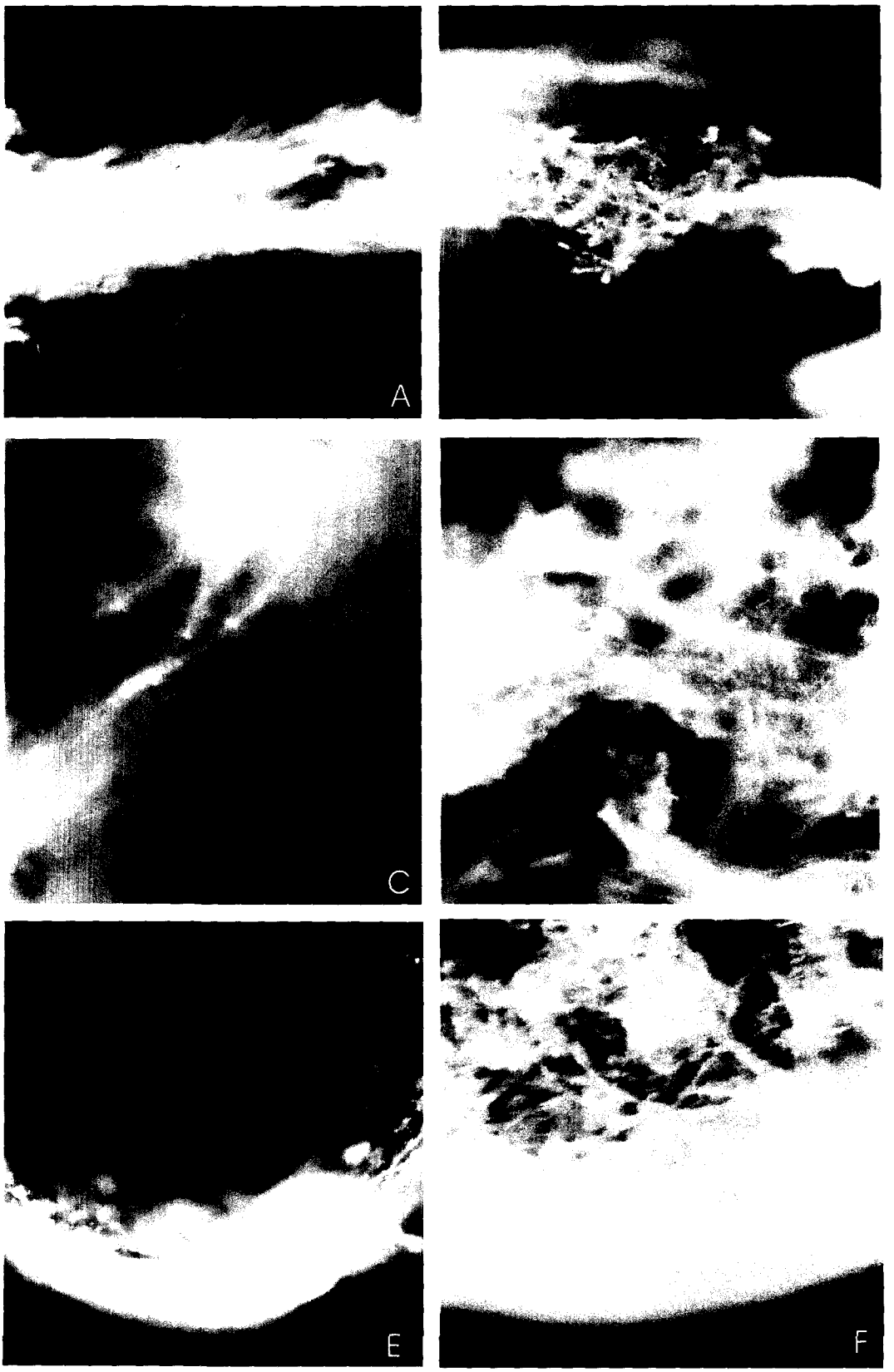

Plate 12 (figs. A-F). Indirect immunofluorescence of hyphae (A-D) and Sclerotia (E-F) of *S. rolfsii* treated with PAb of *S. rolfsii* and FITC labeled antibodies of goat specific for rabbit globulin.

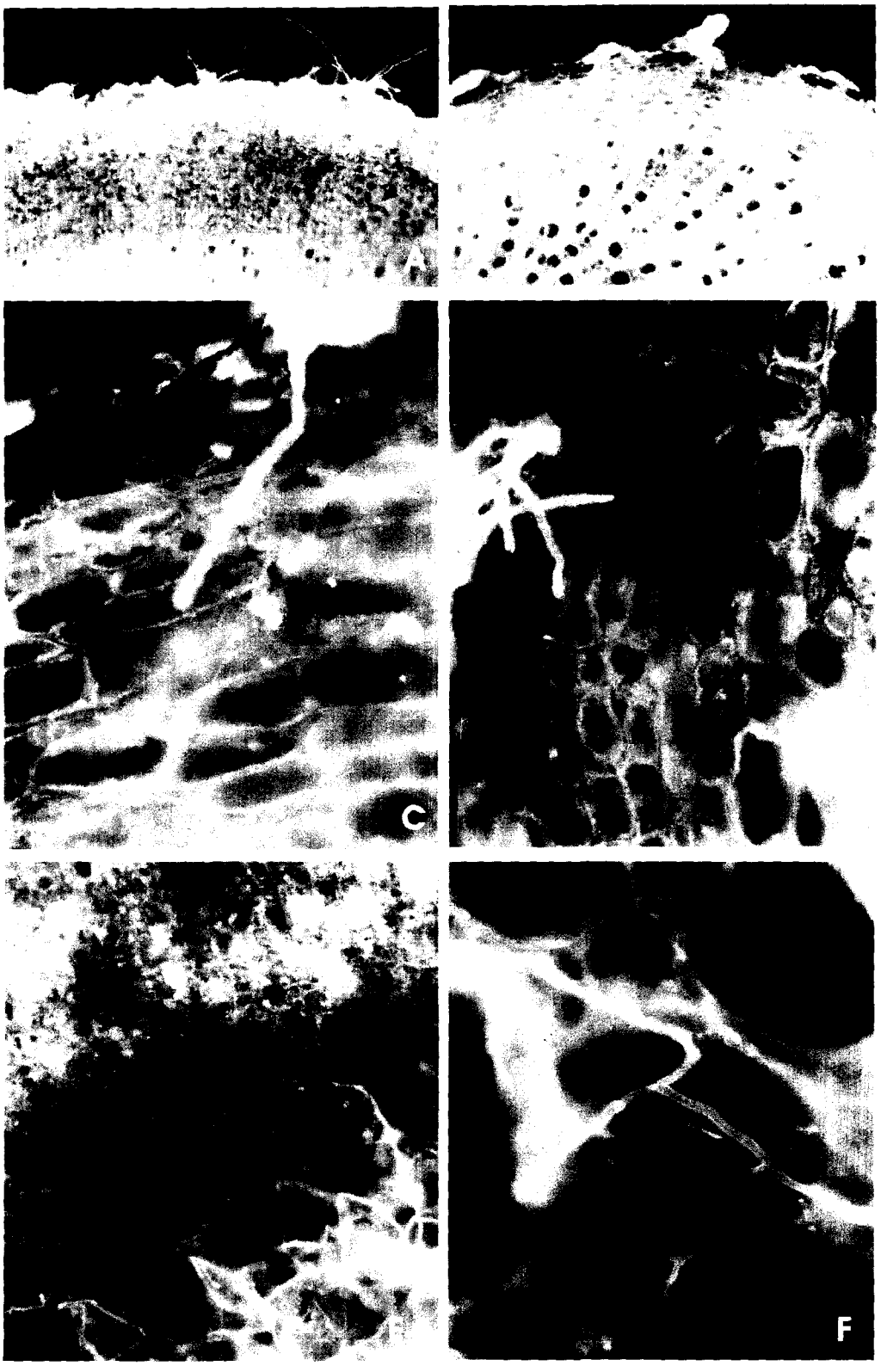

Plate 13 (figs. A-F). Fluorescent antibody staining of infected tea root tissues (A&B: UP-3; C&D: T-17 and E&F: B-157) treated with PAb of *S. rolfsii* and labeled with FITC antibodies of goat specific for rabbit globulin.

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.*

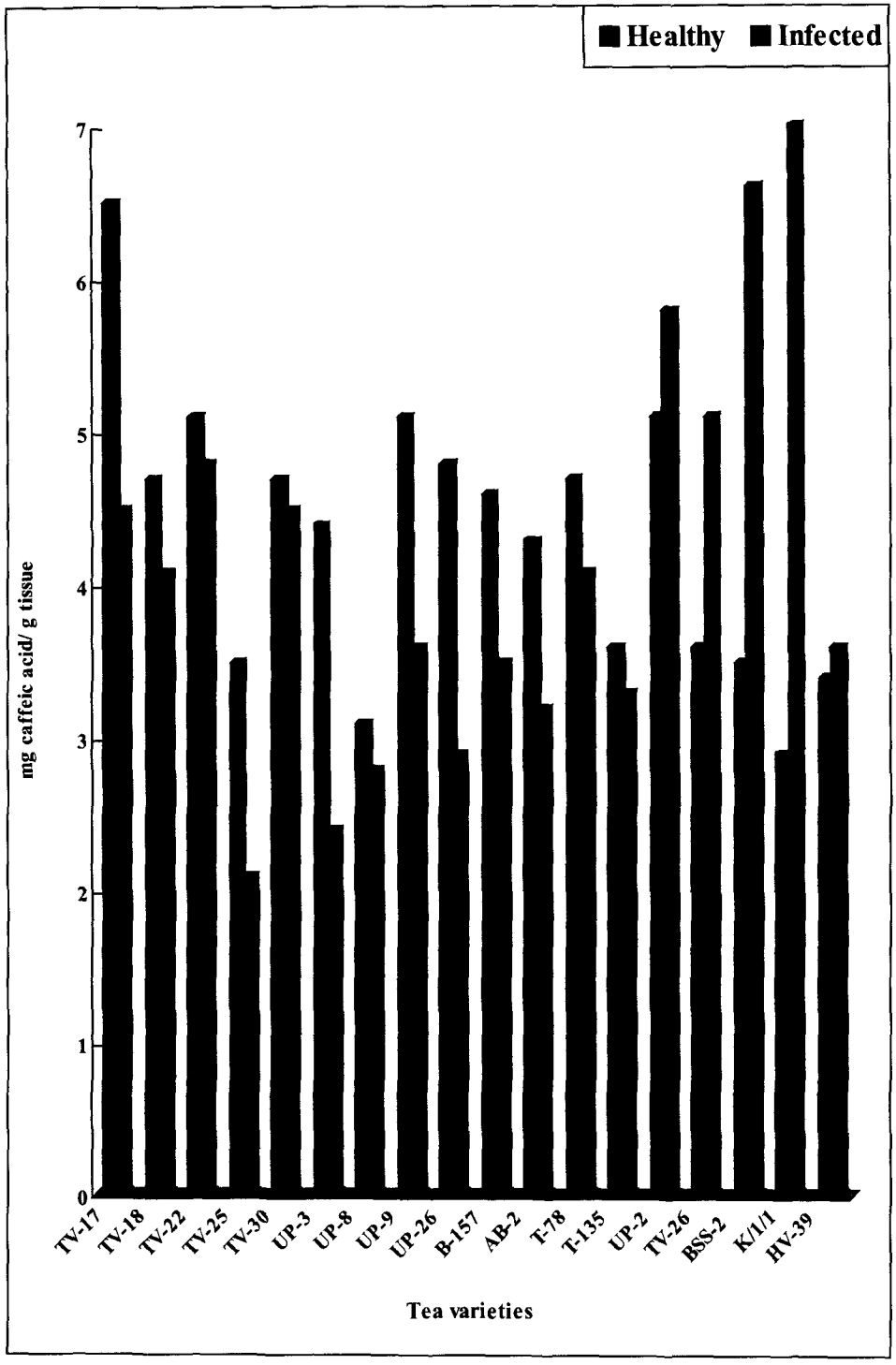

Fig. 10. Total phenol content in tea varieties following inoculation with *S. rolfsii*

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*

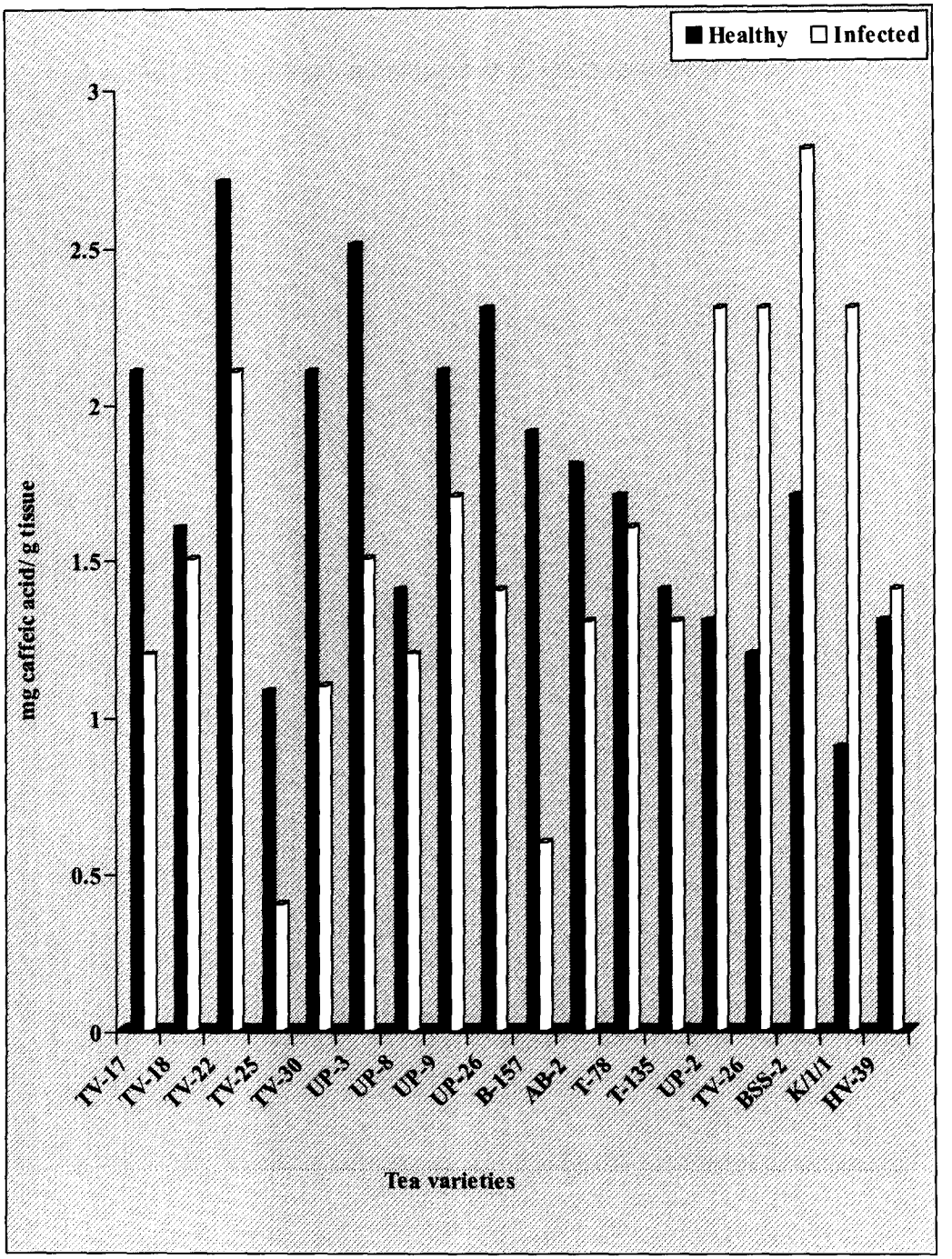

Fig. 11. Orthodihydroxy phenol content in healthy and *S. rolfsii* inoculated roots of tea varieties.

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

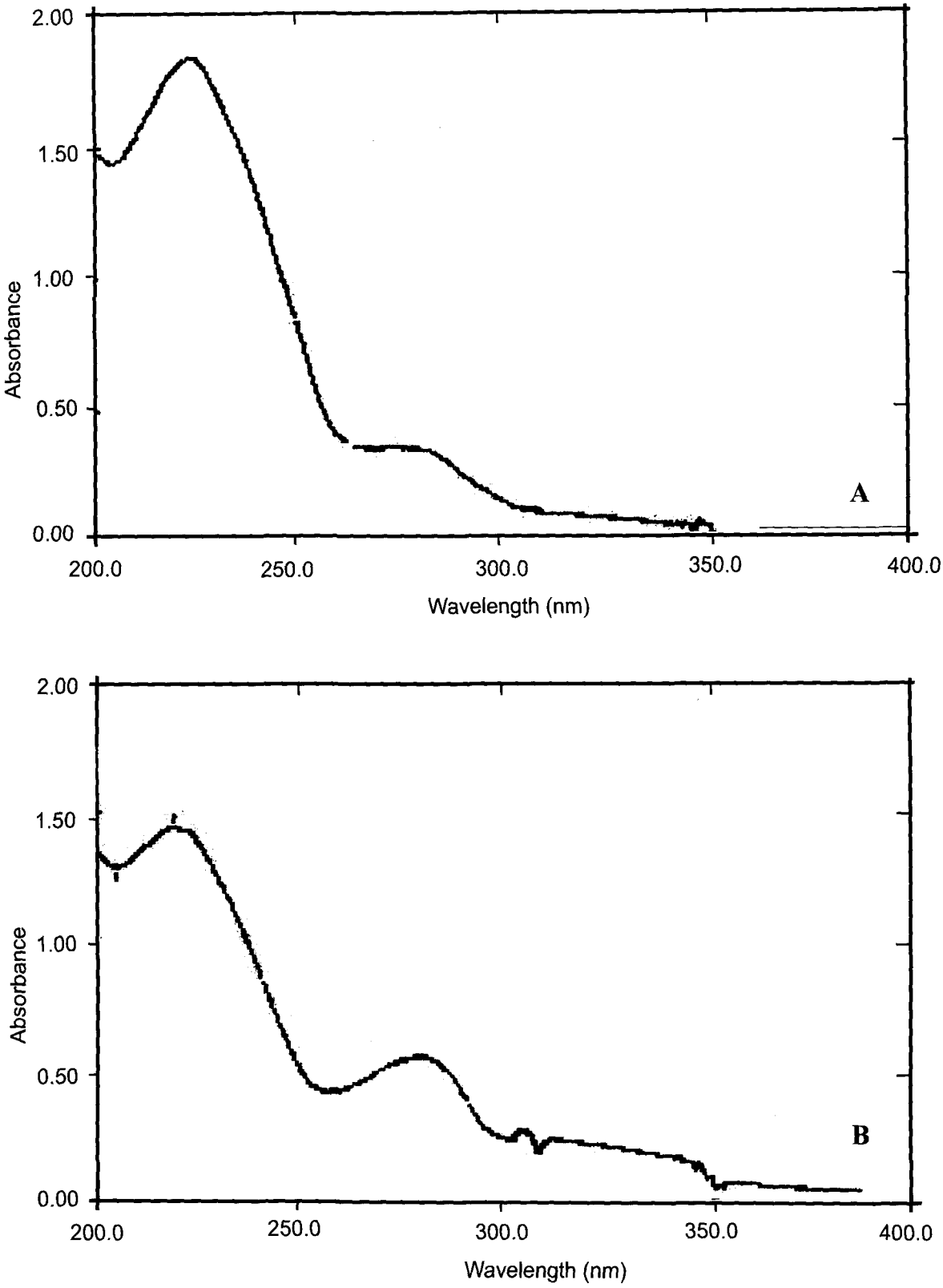

Fig. 12. (A&B): UV-spectrophotometric analysis of antifungal phenolics of tea plants(K1/1) [A] Healthy root, [B] *S. rolfsii* inoculated root.

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*

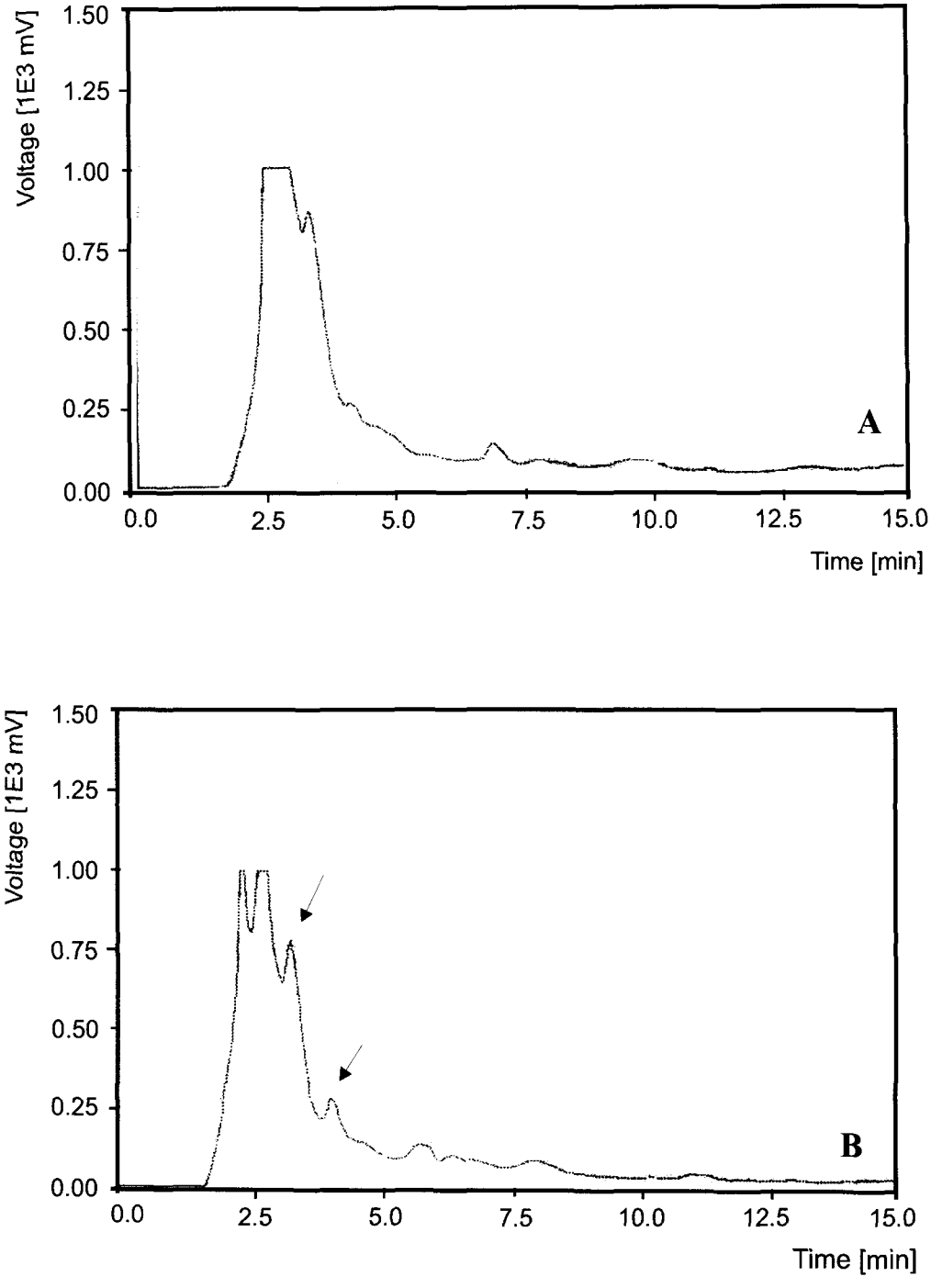

Fig. 13 (A & B). HPLC elution profiles of antifungal phenolics of tea plants (K1/1) (A) Healthy root (B) *S. rolfsii* inoculated root

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

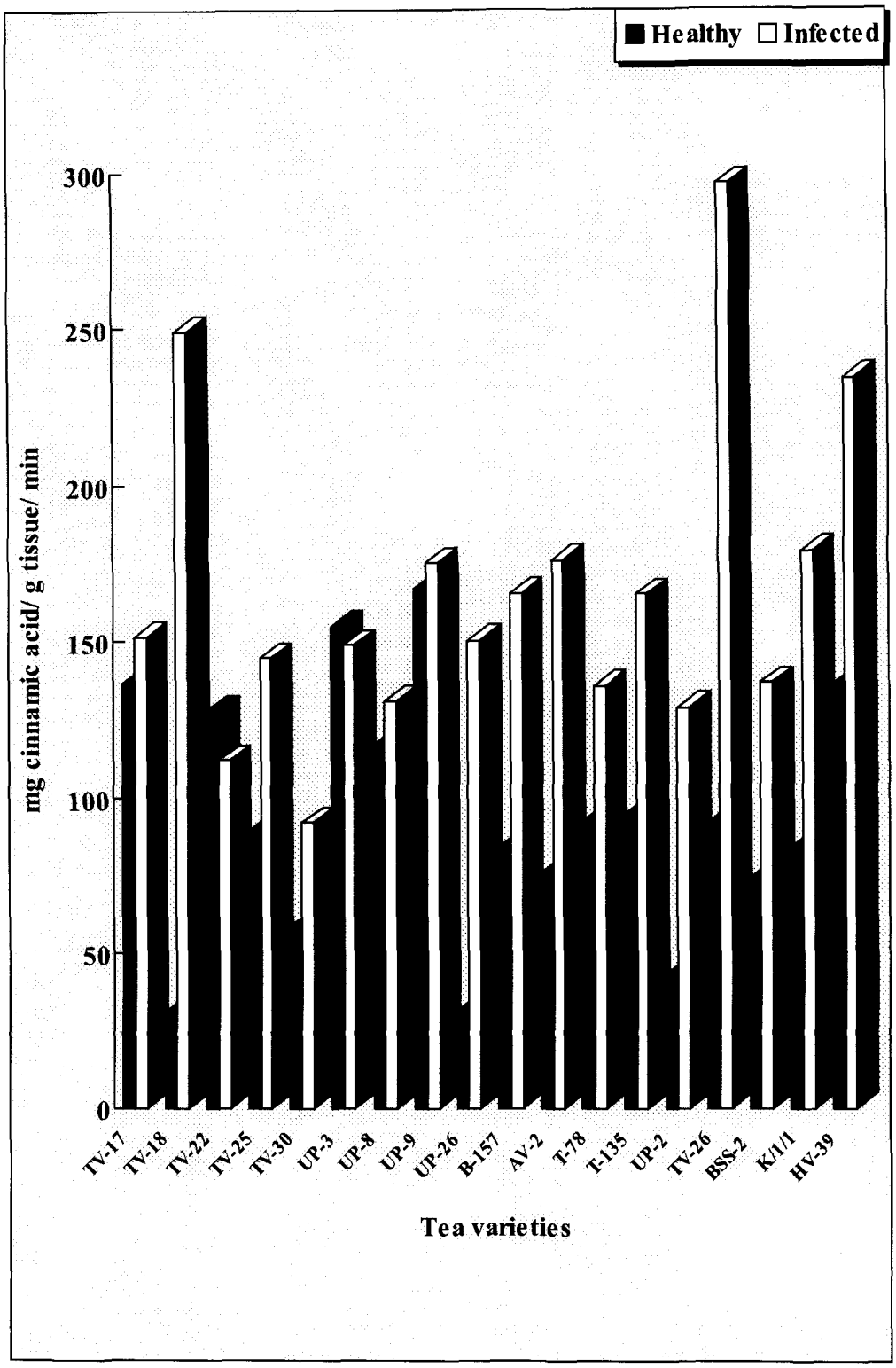

Fig. 14. Phenylalanine ammonia lyase (PAL) activity in tea varieties following inoculation with *S. rolfsii.*

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

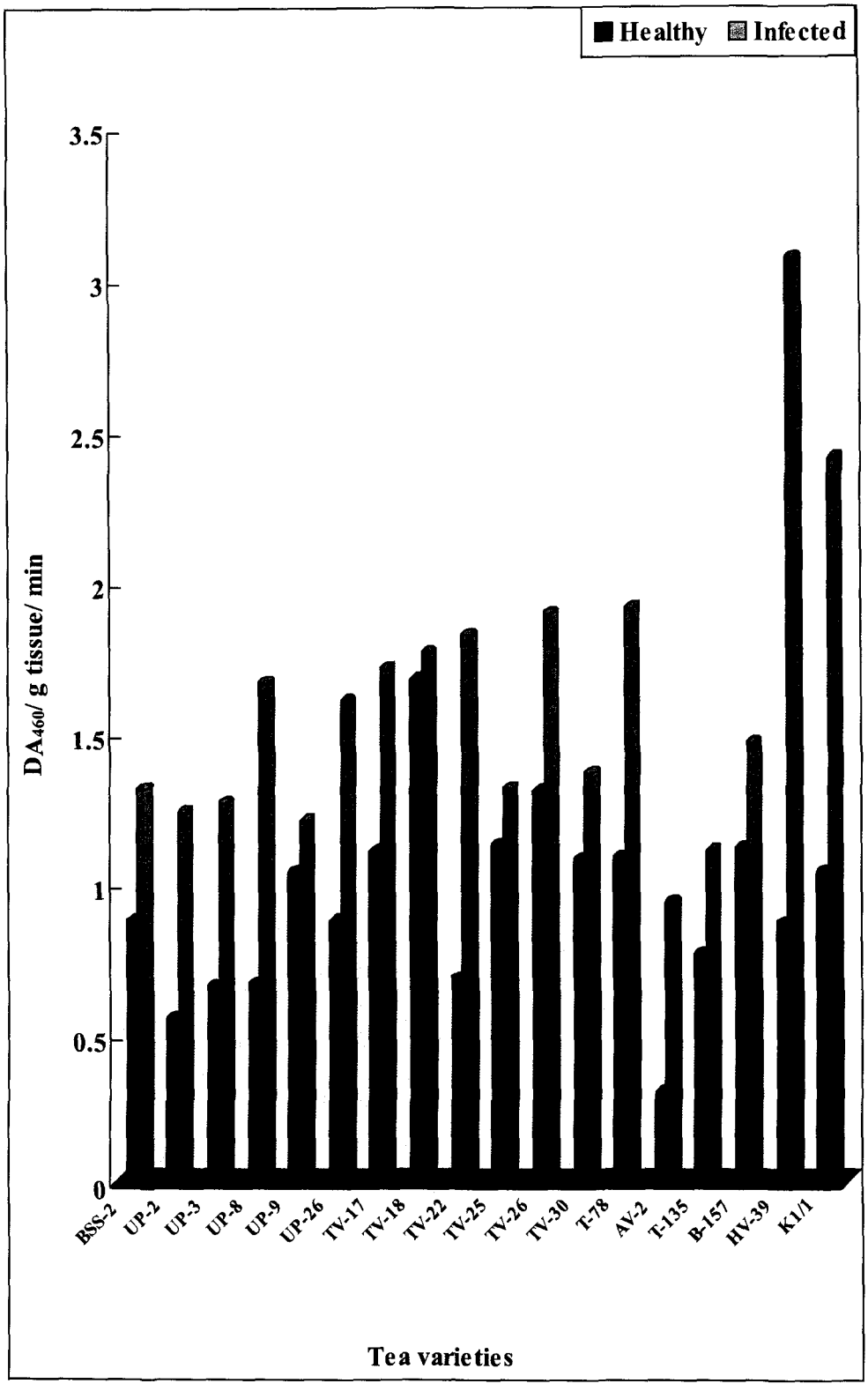

Fig. 15. Peroxidase (PO) activity in tea varieties following inoculation with *S. rolfsii.*

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

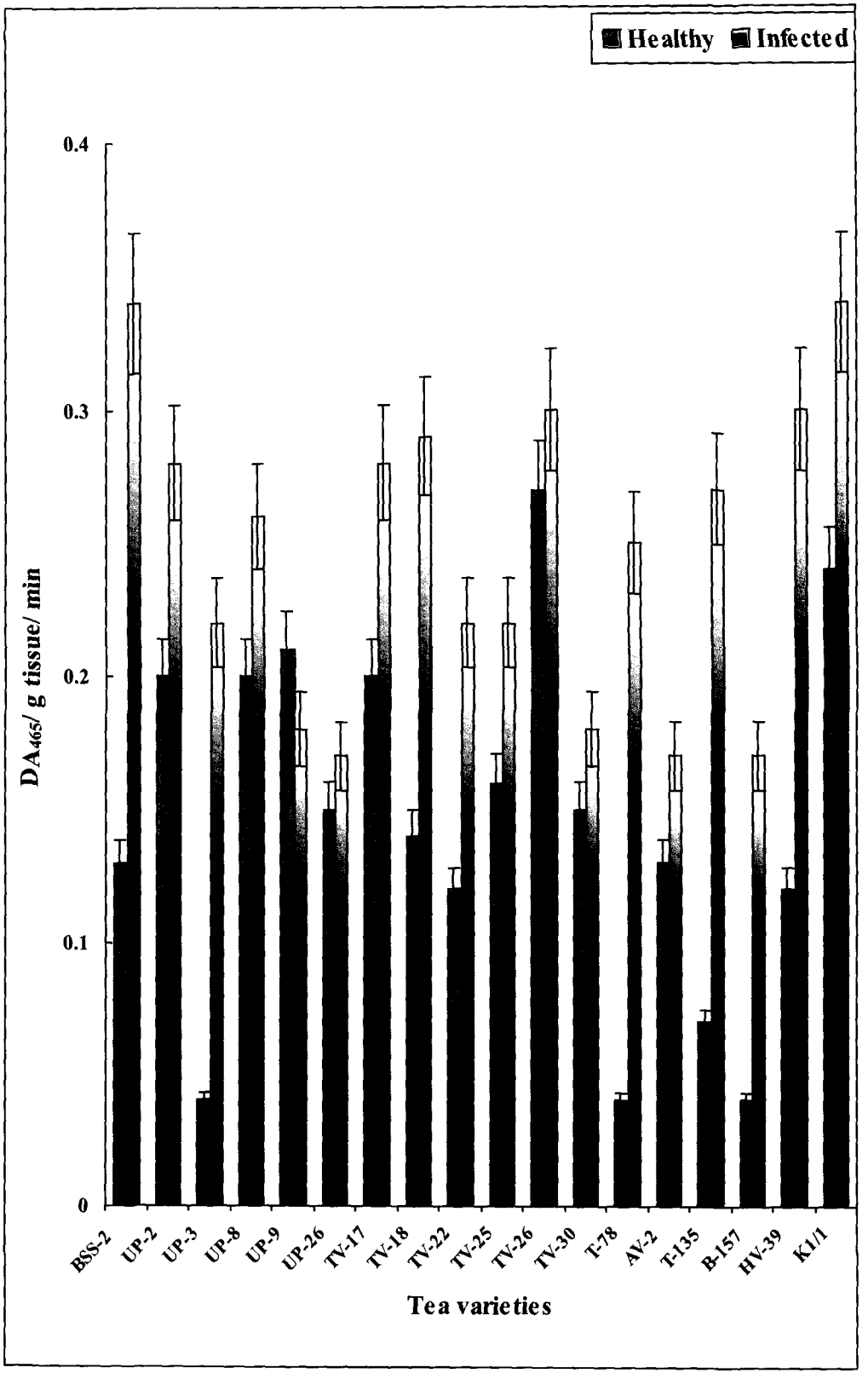

Fig. 16. Polyphenol oxidase (PPO) activity in tea varieties following inoculation with *S. rolfsii*

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).

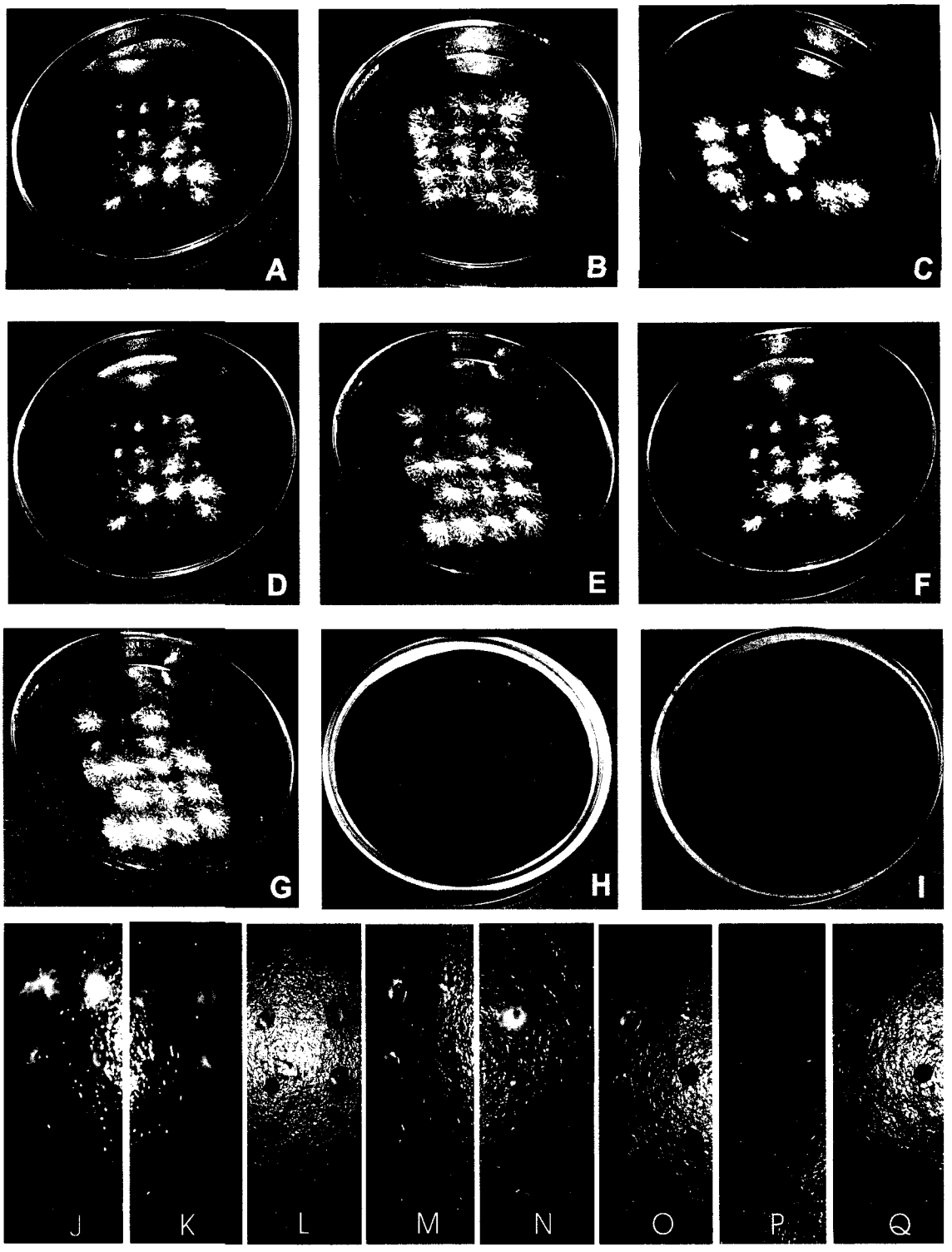

Plate 14 (figs. A-Q) Sclerotial germination bioassay of *S. rolfsii* against plant extracts and fungicides. (A-D) *Catharanthus roseus,* (E-G) *Azadirachta indica,* (H&I) Distilled water control, (J&K) Captan, (L&M) Carbendazim, (N&O) Thiodan, (P&Q) Calixin

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.

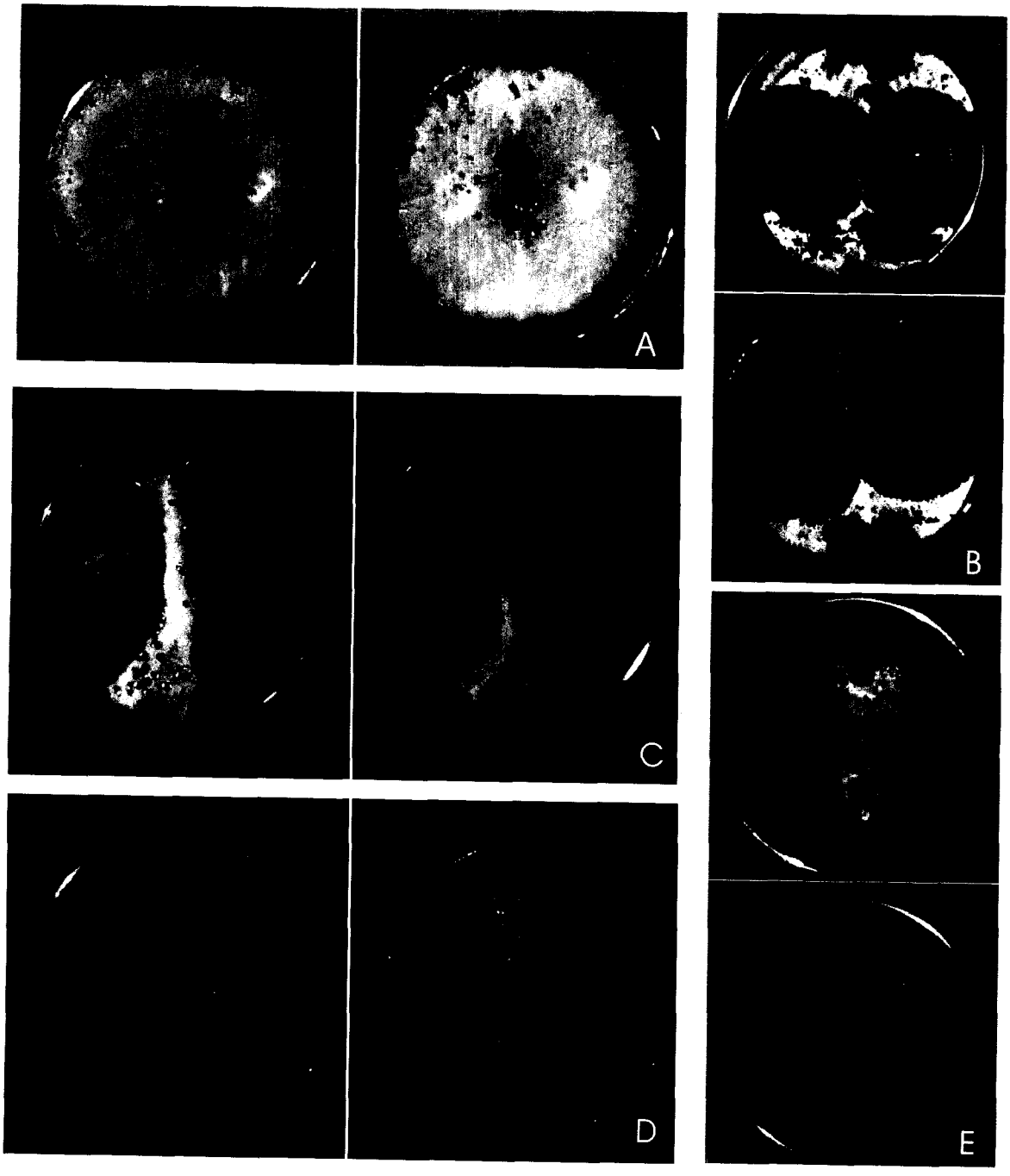

Plate 15 (figs. A-E): Pairing of *S. rolfsii* with *Trichoderma harzianum* and *Trichoderma viride.* Homologous pairing of *S. rolfsii* (A), *T. harzianum* (B) and *T. viride* (E) respectively. Pairing of *S. rolfsii* with *T. harzianum* (C) and with *T. viride* (D).

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

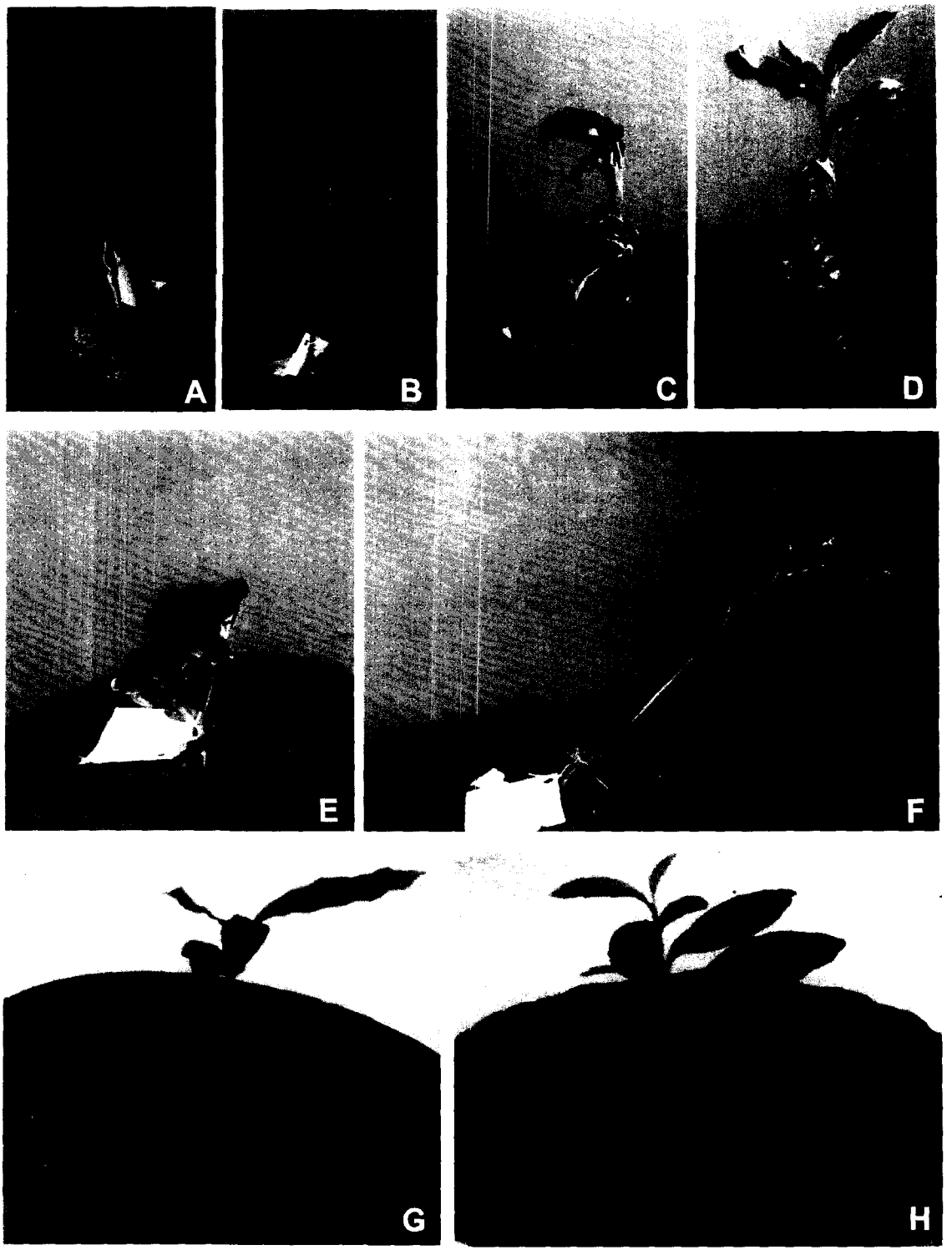

Plate 16 (figs. A-H). Tea plants following treatment with biocontrol agents and organic amendments. **(A)** Untreated inoculated with *S. rolfsii* **(B)** Untreated healthy **(C-E)** Ammended with chicken manure **(F)** Ammended with rabbit manure **(G)** *T. harzianum* inoculated **(H)** *T. viride*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

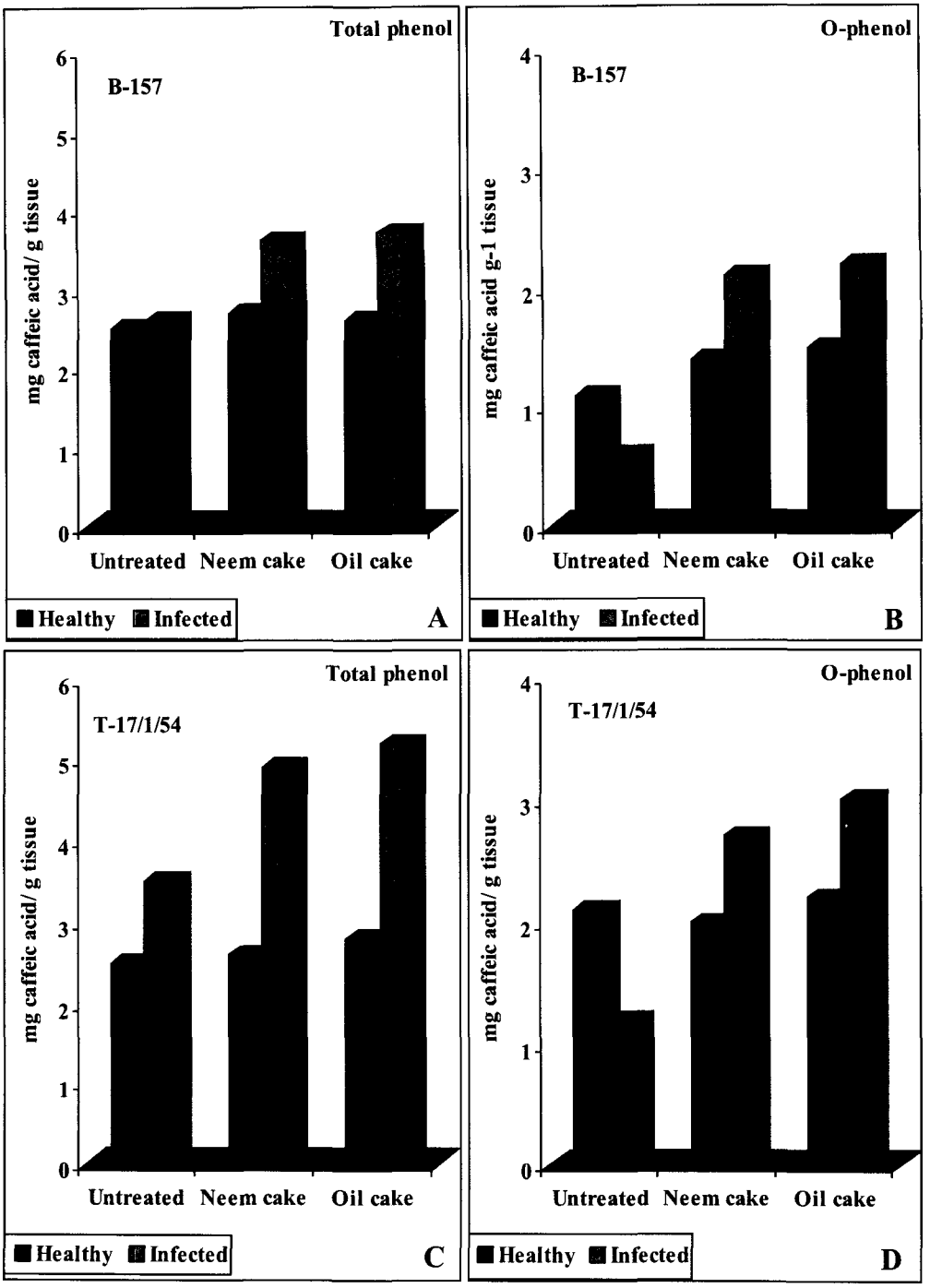

Fig.17 (A-D).Total phenol and ortho dihydroxyphenol content in treated tea varieties following inoculation with *S. rolfsii.*

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.

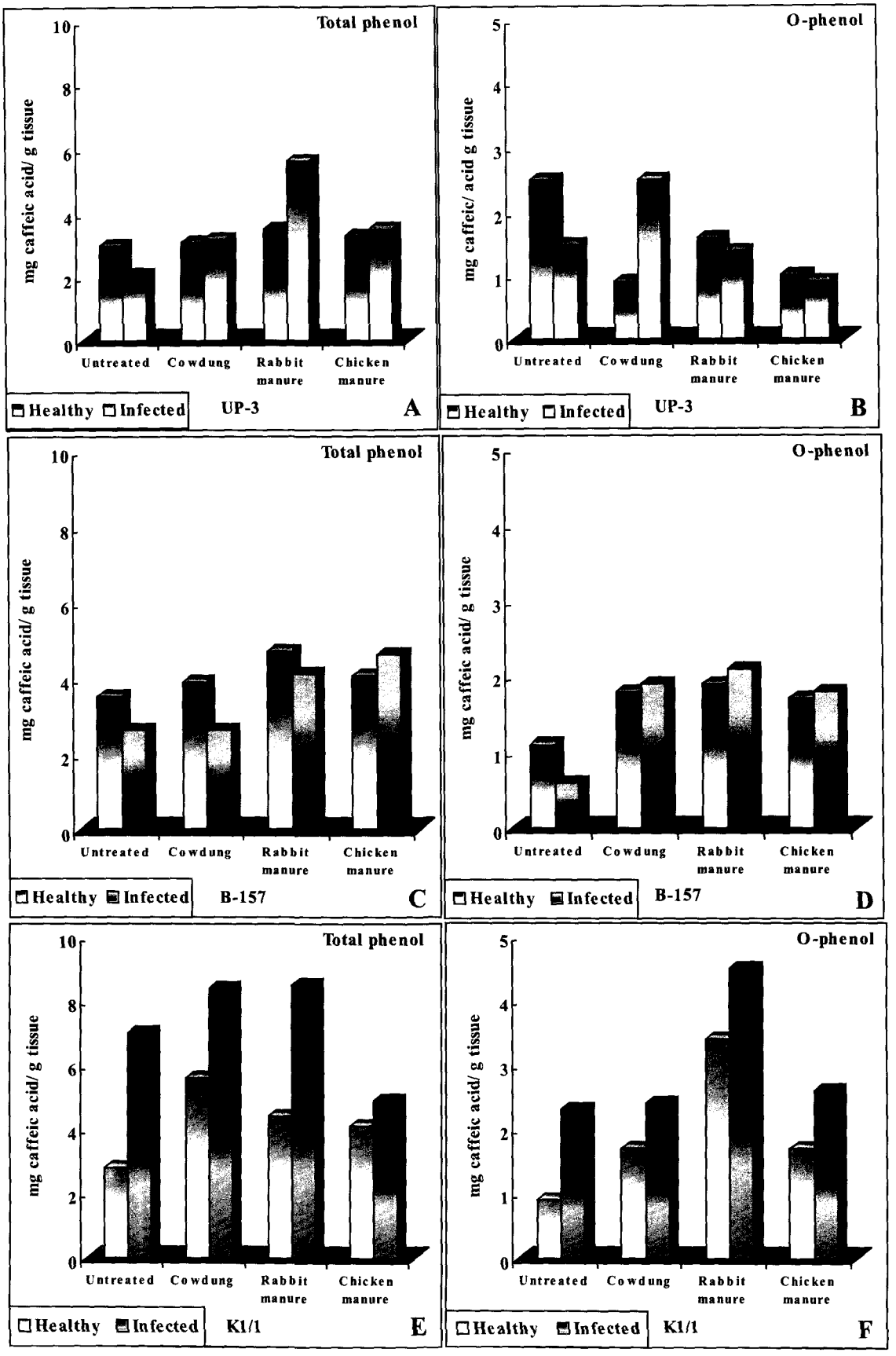

Fig.18 (A-F). Total phenol and orthodihydroxy phenol content in treated tea varieties following inoculation with *S. rolfsii.*

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*	+

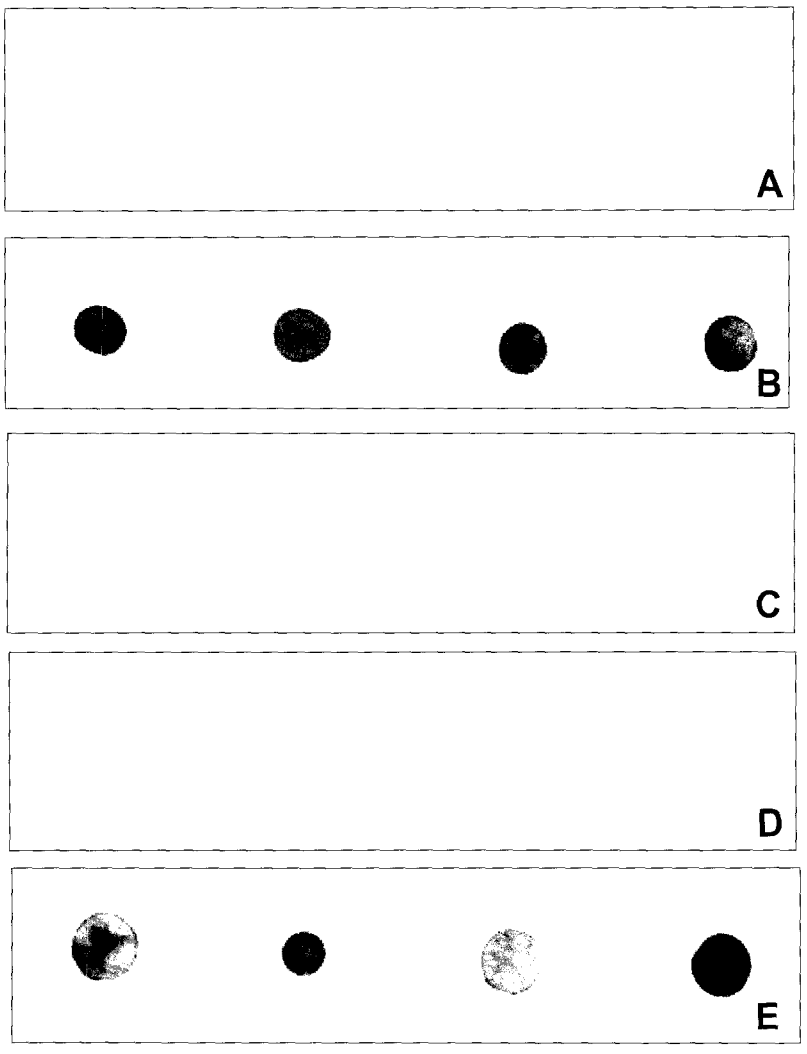

Plate 17 (figs. A-E). Dot blot analysis of tea rhizosphere soil of different treatments probed with PAb of *S. rolfsii* (A) soil treated with *T.harzianum* (B&E) Soil inoculated with *S. rolfsii* (C) soil amended with rabbit manure and inoculated with *S. rolfsii* (D) soil amended with *T. harzianum*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

Discussion

Tea is subjected to varying environmental conditions throughout its life, as well as to numerous attacks by pests and fungal pathogens, which in turn are influenced by various environmental conditions. If the microorganism is successful, disease is the end result; but more often than not, the host emerges the winner as the invader is successfully warded off. Plants have to make necessary metabolic and structural adjustments to cope with stress conditions (Ho and Sachs, 2000). The aerial surfaces of tea plants are usually inhabited by a variety of microorganisms, many of which are capable of influencing the growth of foliar pathogens (Chakraborty and Chakraborty, 1997). The interactions between these microorganisms might result in the suppression of pathogen activity. Besides, it is likely that the tea plant, in the course of its adjustment to varying environments, has evolved a very effective defense mechanism, which successfully wards off most of the fungal pathogens. The common plant pathogens induce some type of resistance in plants to subsequent challenges, both to the original and as well as to other biotic agents. In general, the defenses of higher plants against any form of stress, whether biotic or abiotic, fall under two categories: preformed and induced.

In the present investigation, 18 tea varieties were screened against *S. rolfsii*. Among all the tested varieties, UP-8 and T-17/1/54 were found to be most susceptible while K1/1 and HV-39 were found to be most resistant. Although less in known with certainty about the specific recognition events that predict incompatible host-pathogen interaction, considerable genetic and biochemical evidence indicates that constitutive specificity imparting molecules must exist in the incompatible pathogen and the resistant host plants that dictate the ultimate accumulation of antifungal compounds at the infection site. Cell recognition has been defined as the initial event of cell-cell communication which elicits morphological, physiological and biochemical response. Surface molecules of eukaryote cells have been involved in cell-cell recognition and/or adhesion and as receptors for various effects. Many of these specificity imparting molecules are glycoproteins, and fungi are known to possess them on their cell-walls and plasma membranes (Keen and Legrand, 1980; Ransom *et al.*, 1992).

In the initial stage of infection at the cellular level the exchange of molecular signals between host and parasite is considered to be one of the mechanisms resulting in the specificity of such interactions. The genetic information contained in nucleic acid is expressed in the cell via protein synthesis. Several proteins function as enzymes in the metabolic pathways, which synthesize or breakdown cellular components. When plants containing various kinds of proteins are infected by pathogens, the proteins in the penetrated plant cells are changed chemically and physically. Thus qualitative and quantitative changes in proteins are related to both plant and pathogen. A protein competition model was proposed by Jones and Hartley (1999) for predicting total phenolic allocations and concentration in leaves of terrestrial higher plants. They suggested that protein and phenol synthesis compete for the common limiting resource-phenylalanine and hence protein and phenolic allocations are inversely correlated.

In the present investigation changes in the protein content was noted in the *S. rolfsii* inoculated leaves of susceptible varieties in relation to their healthy control. Increased protein level was also detected after infection of susceptible bean leaves by *Uromyces phaseoli*. The greater accumulation of protein in susceptible host after inoculation may also be attributed to the total proteins of both host and parasite. However, it is difficult to separate the relative contribution of host and parasite to the total protein content. It is evident from the above statement that some changes occur in proteins of infected plants. However, these changes are not always significant. Sometimes protein content of the host remains more or less similar even after inoculation but isozyme pattern may change (Sako and Stahman, 1972).

Advances made in the formulation of concepts and techniques of modern, quantitative cell biology in recent years have paved the way for a basic understanding of the physiology and biochemistry of plant host pathogen interactions. The success or failure of infection is determined by the dynamic competition and the final out come is determined by the sum of favourable and unfavourable conditions for both the pathogen and host cells. At the same time the potential host may be able to detect or recognize a fungal pathogen and use the initial act of recognition to trigger a range of induced resistance (Purkayastha, 1994). The initial cellular recognition is followed by communication between its components.

This exchange of information is generally mediated by soluble antigens located on or near the cell surface (Chakraborty, 1988). The significance of antigenic relationship between plant hosts and pathogenic organisms with regard to disease susceptibility has been recognized by many investigators. Whenever an intimate and continuing association of cells of host and pathogen occur it has been observed that partners of this association have unique serological resemblance to one another involving one or more antigenic determinants (Chakraborty *et al.*, 2002d).

Enzyme linked immunosorbent assay (ELISA) is probably one of the most sensitive serological techniques for the detection of pathogen in host tissues (Chakraborty and Chakraborty, 2003). In the present study polyclonal antibody was raised against mycelia of *S. rolfsii*. The antisera obtained were purified to minimize non specific binding. At the beginning, the sensitivity of the assay was optimized. Root antigens of 18 tea varieties, one non host (*O. sativa*) and one non pathogen of tea (*F. graminearum*) were cross reacted separately with PAb of *S. rolfsii*. Presence of cross reactive antigens (CRA) between *S. rolfsii* and tea varieties (T-17/1/54, TV-30, UP-3, UP-8, UP-9, UP-26, T-135 and B-157) was evident in immunodiffusion test. However, weak precipitation reaction was observed with antigens of tea varieties (TV-18, TV-22, TV-25, TV-26, UP-2, T-78, AV-2 and B-157). No common antigenic substance was found between *S. rolfsii* and other varieties. The presence of CRA and their involvement in various host parasite combinations have been observed. These are cotton and *Fusarium oxysporum* f. sp. *vasinfectum* (Charudattan and De Vay, 1970); cotton and *Verticillium alboatrum* (Charudattan and De Vay, 1972); sweet potato and *Ceratocystis fimbriatae* (De Vay *et al.*, 1972); potato and *Phytophthora infestans* (Palmerly and Callow, 1978, Alba and De Vay, 1985); coffee and *Hemilea vastatrix* (Alba *et al.*, 1983); soybean and *Macrophomina phaseolina* (Chakraborty and Purkaystha, 1983); soybean and *Colletotrichum dematum* var. *truncata* (Purkaystha and Banerjee, 1986), jute and *Colletotrichum corchori* (Bhattacharya and Purkaystha, 1985); soybean and *Myrothecium roridun* (Ghose and Purkayastha, 1990); groundnut and *S. rolfsii* (Purkayastha and Pradhan, 1994); tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994a); tea and *Pestalotiopsis theae* (Chakraborty *et al.*, 1995a); tea and *Glomerella cingulata* (Chakraborty *et al.*, 2002d), soybean and *Fusarium oxysporum*

(Chakraborty *et al.*, 1997b). In the present study PTA-ELISA readily detected CRA between tea root antigens and *S. rolfsii* at a concentration of 1:250 antiserum dilution. Alba and De Vay (1985) also detected CRA in crude preparations and in purified preparations from mycelia of *Phytophthora infestans* (races 4 and 1.2.3.4.7) using antisera of two potato cultivars (King Edward and Petland Dell) at concentrations lower than 50 µg/ml protein in indirect ELISA. Among the 18 tea varieties tested with PAb of *S. rolfsii*, very high absorbance values were obtained in case of UP-8, UP-9, T-17/1/54, TV-30, T-135, B-157 and UP-2, BSS-2, TV-26, HV-39 and K1/1 showed very low absorbance values.

Visible outcome of a compatible host pathogen interaction may be obtained in many cases only after few days of infection, by which time the pathogen would be well established in the host tissues. Recent trends have developed highly specific techniques for the detection of pathogen at a very early stage. Various formats of ELISA using polyclonal antiserum have found widespread application in plant pathology and are routinely used for detection and identification purposes (Lyons and White, 1992; Hansen and Wick, 1993, Chakraborty *et al.*, 1995a; 1996b; Kennedy *et al.*, 1999; 2000; Chakraborty *et al.*, 2002d).

It is also important in the studies on host parasite relationship to determine the cellular location of the pathogen. For this purpose in this study, indirect immunofluorescence tests were conducted with cross sections of healthy and artificially inoculated (with *S. rolfsii)* tea roots, and mycelia. Transverse sections from infected roots were made and PAb raised against mycelial antigens of *S. rolfsii* were used for probing the fungal hyphae which penetrate the root tissues. Bright fluorescence was observed in the cross sections of tea roots. De Vay *et al.* (1981) determined the tissue and cellular location of major cross reactive antigens (CRA) shared by cotton *and F. oxysporum* f. sp. *vasinfectum*. Cellular location of CRA in tea leaf tissues shared by three foliar fungal pathogens such as *Bipolaris carbonum* (Chakraborty and Saha, 1994a); *Pestalotiopsis theae* (Chakraborty *et al.* 1995a) and *Exobasidium vexans* (Sharma and Chakraborty, 2004) have been demonstrated. Besides detection of pathogen in host tissues using antibody based immunofluorescent technique has been reported by several previous authors

(Warnock, 1973; Reddy and Ananthanarayan, 1984). On the basis of immunofluorescence studies, Dewey *et al.* (1984) suggested that chlamydospores, basidiospores and mycelia of *Phaseolus schweinitzii* contained molecules antigenically related to species specific antigens secreted by mycelia grown in liquid culture. They also demonstrated the presence of mycelium and chalmydospores in naturally and artificially infested soil samples, using this technique. Different test formats including indirect ELISA, western blotting, dot blot and indirect immunofluorescence was assessed for their potential to detect resting spores of *Plasmodiophora brassica* (Wakeham and White 1996) as well as *Fomes lamaoensis* (Chakraborty *et al.*, 2002a) in soil.

The dot immunobinding technique has also been found to be a rapid and sensitive method for detection of fungal pathogens. In the present study, antigens were prepared from sclerotial blight infected tea plants. Healthy and artificially inoculated (with *S. rolfsii*) tea roots were tested on nitrocellulose paper. Infected and artificially inoculated root antigens gave intense dots when compared to the healthy control confirming the presence of fungal pathogens. Blake *et al.* (1984) has described a method using the alkaline phosphates substrate 5-bromo-4-chloroindolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) to detect the precipitated indoxyl group. When the substrate 5-bromo 4-chloroindolyl phosphate is used, the phosphate is cleaved by the enzyme and the indolyl group precipitates. The hydroxyl group of the indigo then tautomerizes forming a ketone, and under alkaline conditions dimerization occurs, forming a dehydroindigo. In the process of dimerizing, it releases hydrogen ions and reduces the nitroblue tetrazolium which precipitates, forming an intense blue deposition of diformazan. So, early detection of disease is an important requisite for development of management strategies. A microtitre immunospore trapping device, which uses a suction system to trap air particulates directly by impaction into microtitre wells, has been used successfully for the rapid immunodetection and quantification of ascospores of *Mycosphaerella brassicicola* and conidia of *Botrytis cinerea* (Kennedy *et al.*, 2000, Kennedy and Wakeham, 2006).

Plants have well developed defense mechanisms which enable them to defend themselves against parasites in their tissues. The biochemical basis for this

resistance against microbial attack consists of both preformed and post-infectional ones. Preformed defenses are often regarded as general or unspecific as compared to inducible defense systems which are highly specific. Though the versatile multicomponent defense is adequate to provide them protection against most of their potential pathogens, only a few of them can overcome this defense and cause disease. Varieties within the host species are resistant when they possess one or more resistant gene(s) and susceptible when they lack any such gene. To account for the observed specificity and degree of variability of host parasite system, the fungal receptor must have high information content which involves recognition between the host and pathogen both at the cellular and subcellular level. A cell reacts in a special way as a consequence of an association with another cell because it acquires information, which is conveyed through chemical or physical signals in the process or recognition. The spatial and temporal deployment of plant defense responses involves the complex interplay of signal events, often resulting in superimposition of signaling processes. In spite of lacking immune responses like animals, plants have nevertheless evolved immune mechanisms of various types by which they can account for the advance of foreign organisms. The result is that disease tends to be specific, a given pathogen usually infecting a distinct range of host plants.

Environmental effects in phenolics are all the more long-lasting, as they have to cope of such conditions year after year. In a similar study on tea with the fungus *Glomerella cingulata* which causes brown blight of tea, it was reported that high humidity and rainfall were the most important factors predisposing the plants to infection (Chakraborty *et al.*, 2002e). Phenols are also known to play definite roles in a plant defense. Considering this in the present study phenol contents of the healthy and artificially inoculated (with *S. rolfsii*) plants were determined. It has been reported previously that quinones in plant tissues react with proteins to form melanin and other tannins leading to the discoloration of damaged tea leaves (Sudhakran *et al.*, 2000). Many studies have demonstrated the importance of phenolic compounds in plant defense. In general, plant phenolics have a diverse range of biological activity, depending on their structure, degree of polymerization, stereoisomeric differences etc. interaction between phenolic compounds and environmental conditions determines their action. Polyphenols have a distinctive

ability to engage in molecular recognition, or formation of intermolecular complexes with each other and with other molecules (Haslam, 1999). In the present study, total phenol content decreased with pathogen infection. The decrease was most significant in UPASI varieties. However, in these varieties, PAL activity increased significantly. In the present study, the level of antifungal phenolics (pyrocatechol) in healthy and *S. rolfsii* infected tea varieties were estimated. In resistant varieties accumulation of pyrocatechol increased sharply following inoculation with *S. rolfsii* in relation to healthy plants. Accumulation of pyrocatechol in susceptible varieties were not greater than the resistant one. Increased level of pyrocatechol may be associated with the host response to resistant reaction. One of the reasons for the observed tolerance of certain varieties to fungal attack could be their ability to maintain higher levels of phenolics in the face of attack.

Accumulation of defense enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (PO), in tea varieties following inoculation with *S. rolfsii* were determined. PPO usually accumulated upon wounding in plants. PPO transcript levels systemically increased in tomato when mature leaflets were injured (Thipyapong and Steffens, 1997). Increased activity of PPO and PO was demonstrated in the cucumber leaf in the vicinity of the lesions caused by some foliar pathogens or by phosphate application (Avdiushko *et al.*, 1993). Moreover PPO could be induced by jasmonic acid (Constabel and Ryan, 1998). Among all the stress related enzymes, the role of peroxidase has been most thoroughly worked out. PO is a metallo-enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrates including phenols, aromatic amines, amino acids and inorganic compounds (Balasimaha, 1982). These are ubiquitous to plants and are characterized by a large number of isozymes. Various naturally occurring and synthetic substances, growth regulator and environmental factors markedly influence the activity of these peroxidases. Akhtar and Garraway (1990) observed increased PO activity in susceptible cultivars compared with the resistant one when both were treated with sodium bisulphate prior to inoculation with *Botrytis maydis*. On the other hand there are also reports of increased PO activity due to induction of resistance (Chen *et al.*, 2000; Chakraborty *et al.*, 2005d). Curtis *et al.* (1997) also reported the induction of PO activity by pathogens and methyl

jasmonate. The existence of multiple molecular forms of peroxidase in tea has also been reported (Sharma and Chakraborty, 2004).

Previous reports indicate that oxidative enzymes such as PPO and PO as well as those involved in phenolic biosynthesis such as PAL are involved in defense reactions in plants (Chen *et al.*, 2000). Considering the importance of phenol metabolism in tea plants, those three enzymes were selected for studies. Results showed that the constitutive enzyme activities under no stress conditions of the different clones varied. Matsumato *et al.*, (1994) reported that Japanese green tea cultivars belonging to the variety 'sinensis' could be divided into three groups on the basis of their PAL cDNA cloning. Assam hybrids could not be placed into any specific groups because complex patterns were produced. They confirmed the existence of many kinds of PAL genes, expressing of which varied depending on the varieties. An elevation in the level of activity of PAL has been frequently demonstrated to be one of the earliest responses of plants to biotic (Chakraborty *et al.*, 1993; Shiraishi *et al.*, 1995) or to other environmental stresses (Eckey-Kaltenbach *et al.*, 1997). It was reported by Orczyk *et al.*, (1996) that in sorghum, naturally occurring high levels of PAL activity induced by light should be differentiated from the activity induced as a response of attempted fungal infection. Bhattacharya and Ward (1987) reported that PAL activity in soybean was enhanced in the resistance response of soybean hypocotyls to *Phytophthora megasperma* f.sp. *glycinea*. Considering that PAL is a key enzyme in the biosynthesis, not only of phytoalexins, but also of phenolic compounds in general, and melanins, all of which have been associated with resistance responses in various host plants, it may be suggested that activity of PAL could be useful indicators of the activation of defense related enzymes.

Polyphenolics are major constituents of tea leaves and it is expected that they would be affected by the different abiotic and biotic stresses (Chakraborty *et al.*, 2005a; 2005e). In case of temperature stress it was observed that there was a correlation between the inherent phenol contents in the tea variety and its increase following exposure to elevated temperatures. In general, in those varieties with high inherent phenol content, accumulation of phenols kept increasing till 50^0C. A wide variation in the phenol contents in the different tea varieties was also evident

(Chakraborty *et al.*, 2001a). The observed trend could be explained by the fact that phenols are considered to be involved in plant's defense to various stresses. When subjected to temperature stress, varieties with low inherent phenol content increased its accumulation while those that already had a higher content did not have to increase synthesis (Chakraborty *et al.*, 2005e). In case of tea, polyphenols are also known to vary seasonally (Zakoskiva *et al.*, 1991). Thus phenol biosynthesis seems to be well regulated to help the tea plant to overcome various stresses. Similarly, in case of drought too, phenol content increased initially up to 8 days of stress after which there was a decline (Chakraborty *et al.*, 2001b).

Alteration of phenol metabolism following fungal infection has been observed in many diseases and phenolics have been implicated in the defense reaction in several instances (Mahadevan, 1991). There is often a greater increase in phenolic biosynthesis in resistant host species than in susceptible hosts and it is sometimes postulated that the increase in phenolic compounds is part of the resistance mechanism. Some of these compounds are toxic to pathogenic and non-pathogenic fungi and have been considered to play an important role in disease resistance. The involvement of phenol in the defence strategies of tea plants against foliar fungal pathogens e.g *Bipolaris carbonum*, *Pestalotiopsis theae*, *Glomerella cingulata* has been described by Chakraborty *et al.*, (1995b) and Chakraborty (1996). Biochemical responses to tea plants exposed to biotic stress due to blister blight infection caused by *Exobasidium vexans* in the levels of phenols and enzyme activities were studied (Chakraborty *et al.*, 2002c; Sharma and Chakraborty, 2004).

In the present study, the levels of phenolics in leaves of resistant and susceptible tea varieties were estimated following inoculation with *S. rolfsii*. Host responses could be differentiated by changes in content of phenolic compounds. In both the cases total phenol and orthodihydroxy phenol content increased in resistant varieties but decreased in susceptible varieties in comparison to their healthy controls. Hammerschmidt and Nicholson (1977) demostrated a clear difference between resistant and susceptible interaction of maize to *Colletotrichum graminicola* based on accumulation of phenols. Sridhar and Ou (1974) reported diffences in total phenolics accumulation in the interaction of *Pyricularia oryzae* with rice. However, no differences were found in the phenolic content in the interaction *of*

Helminthosporium maydis race T (Macri *et al.*, 1974). On the other hand, a resistant cotton cultivar contained fairly high amount of total as well as orthodihydroxy phenol than susceptible cultivar. In the present study, greater accumulation of orthodihydroxy phenol in resistant interaction of *S. rolfsii* and tea varieties indicated that this might play a role in disease resistance mechanism. Orthodihydroxy phenols play a major role in disease resistance and disease development. They are easily oxidized to highly reactive quinones which are effective inhibitors of sulphydryl enzymes, thereby preventing the metabolic activities of host and parasite cells (Kalaichelvan and Mahadevan, 1988). There are ample evidences that an increased production of phenolic compounds is involved in phytoalexin accumulation (Mansfield 2000). The UV spectra from both the healthy and *S. rolfsii* inoculated tea roots were analysed at 290 nm. A sharp peak at retention time 2.6 was present in both the compounds but in the healthy extracts the peak height was much smaller than the inoculated one. Other small humps and shoulders were also evident in both the cases.

It is known that catechin is oxidatively cleaved to some simpler phenols and phenolic acids like catechol, phloroglucinol and protocatechuic acid. Sambandam *et al.*, (1982) isolated an enzyme (catechin 2-3 dioxygensis) from *Chaetomium cupreum* which cleaved catechin into simpler phenols. It is not unreasonable to speculate that the antifungal compound cleaved to some simpler phenols in the present study. In the susceptible variety, the breakdown of catechin was almost complete while traces were evident in the resistant variety even after 48 h of inoculation. Accumulation of pyrocatechol in resistant varieties increased after 48 h of inoculation with *S. rolfsii*. Increased level of pyrocatechol may be associated with the differential host responses to disease production. Nagahulla *et al.* (1996) reported the production of antifungal compounds in tea leaves following infection with blister pathogen (*Exobasidium vexans*). HPLC analysis of the catechins from healthy and blister infected tea leaves showed marked differences and some quantitative changes (Chakraborty *et al.*, 2002c; 2004).

Research in biological control has progressed from the initial discovery and evaluation phase to the development of practical application techniques. With the emergence of numerous strains or isolates of bacterial and fungal agents, researchers

have investigated methods that will lead to the practical implementation of biocontrol agents. With increased understanding into the mechanisms by which these agents control plant diseases, selection and screening criteria as well as new technology in fermentation and formulation can be evaluated. Several fungi have exhibited biological control activity against plant pathogenic fungi (*Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Alternaria brassicae*) of rapeseed and canola. These include *Trichoderma* spp., *Gliocladium virens*, *Myrothecium verrucaria*, *Talaromyces flavus*. Strategies used for control of these diseases included reducing initial inoculum, reducing secondary inoculum and spread and controlling infection in the rhizosphere and phyllosphere (Boyetchko, 1999). One of the most successful media for sporulation and growth of *T. harzianum* was shown to be wheat bran which acted as both a food base and carrier. Conidia viability and shelf life were significantly improved. By adding peat to the wheat bran at a 1:1 (v/v) ratio, the pH of the medium was more effectively controlled and was found to be a better carrier than wheat bran alone (Sivan *et al.*, 1984). Several *Trichoderma* spp. have been used to reduce the number of sclerotia in soil. *T. viride* and *T. harzianum* parasitized sclerotia and mycelium of *S. sclerotiorum* by penetrating into host mycelia and prodcing lytic enzymes such as β-1,3-glucanases (Jones *et al.*, 1974).

In the present investigation, experiments were conducted using *T. harzianum* and *T. viride* as a biocontrol agents *in vitro*. These were also evaluated against *S. rolfsii* in laboratory by dual culture filtrate test. Consequent to the study, experiments were conducted *in vivo* for the management of the disease. Both antagonists over grew the pathogen and restricted the growth of *S. rolfsii in vitro* but *T. harzianum* was the most effective. Similar observations were reported on wilt of potato caused by *S. rolfsii* (Rao *et al.*, 2004). Studies were made by them to understand the antagonistic activity of microorganisms on mycelial growth, sclerotial production, and inhibition zone against *S. rolfsii*. This result led to their application in integrated disease management practices.

There are several reports on the ability of *T. harzianum* and *T. viride* to inhibit the growth of pathogen in *in vitro* condition. Patel and Anahosur (2001) tested antagonistic potential of *T. harzianum* against four soil borne pathogens

isolated from chickpea plants viz. *Fusarium oxysporum, F. solani, Macrophomina phaseolina* and *S. rolfsii in vitro*. In dual culture, the mycoparasite over grew the pathogen and inhibited their growth by producing antibiosis through the production of some antifungal substances. Sharma and Sharma (2001) reported that among the antagonists tested by them *T. harzianum* and *T. viride* were found most effective in inhibiting mycelial growth of *Dematophora netrix* in dual culture. Prasad *et al.*, (1999) obtained three *T. harzianum* isolates that were highly effective in controlling root / collar rot of sunflower caused by *S. rolfsii* under green house conditions.

In the present investigation, the efficacy of systemic fungicides and plant extracts were also investigated *in vitro*. A selective one of each was tested *in vivo* for the management of sclerotial blight diseases. Results of *in vitro* tests revealed that the systemic fungicides (thiodan, calixin, captan, carbendazim and idofil M-45) were significantly superior over control in checking the mycelial growth of *S. rolfsii*. However, thiodan and calixin completely arrested the growth of pathogen at concentration as low as 0.0125% in *vitro*. Venkata Ram (1974) also reported calixin, a systemic fungicide against blister blight (*Exobasidium vexans*) on tea plants. There are several reports on the efficacy of fungicides on pathogen to inhibit the growth of pathogen *in vitro* condition. The efficacy of different fungicides was studied by Tiwari and Sing (2004) against *Rhizoctonia solani, S. rolfsii,* seed mycoflora and their non-target effect on *T. harzianum* and *Rhizobium leguminosarum*. It was found by them that fungicides *viz.* carboxin, epoxiconasole, hexaconazole, propicanazole and triadimetox which were found highly effective against *R. solani* and *S. rolfsii* can be formulated as seed dresser either with thiram or mancozeb to control both collar rot or rot root as well as seed mycoflora effectively. They also advised to integrate *T. harzianum* and *R. leguminosarum* with these fungicides for seed and seedling protection. Similarly *in vitro* evaluation of fungicides against *S. rolfsii* was studied by Gupta and Sharma (2004). It was observed by them that penconazole, hexaconazole, propineb and mancozeb inhibited mycelial growth of *S. rolfsii*. Propineb was found to be the most effective in reducing disease incidence on crown and pods. Among the biocontrol agents, *G. virens* and *T. viride* were also found to be the most effective against the pathogen. Evaluation of fungicides and plant extracts against *Fusarium solani* leaf blight in *Terminalia catappa* was reported by Mamatha

and Rai (2004). Leaf extracts of *Lantana camara* followed by *Azadirachta indica* and *Acalypha indica* were found to be equally effective in inhibiting the growth of *F. solani in vitro*.

In this investigation, the efficacy of antifungal effect of *Azadirachta indica* and *Catharanthus roseus* were tested *in vitro*. Results revealed that both plant extracts were inhibitory to the mycelial growth of *S. rolfsii* however extract of *A. indica* showed superior upon *C. roseus* extract *in vitro*. It was also observed that as the concentration of extracts increased in the medium the effectiveness of extracts also increased and maximum growth inhibition was recorded at 100% concentration. Similar observation was observed by Sharma and Bohra (2003) in the investigation carried in laboratory and in the field to study the effect of extracts of three medicinal plants species for their antifungal activity against cumin wilt pathogen. Evaluation of plant extracts against *Rhizoctonia solani* incitant of black scurf disease in potato was investigated by Shinde and Patel (2004). It was obtained that garlic extract at 10% concentration showed a complete inhibition of growth (100%). Investigation on the effects of aqueous leaf extracts on neem in inducing resistance against the leaf stripe pathogen by barley, *Drechslera graminis* (Paul and Sharma, 2002) had also been reported.

In another set of experiments different organic amendments were treated in different tea seedlings to observe growth promotion and percentage increase in shoot length in healthy and treated tea seedling varieties. Results revealed that the growth promotion and percentage increase in shoot length in tea seedlings treated with neem cake and oil cake were more in seedlings inoculated with *S. rolfsii* (after treatment) in comparison to the treated uninoculated tea seedlings. Total phenol and orthodihydroxy phenol contents were also increased in treated inoculated tea varieties with neem cake and oil cake. This is due to the decomposition of organic matter that helps in alteration of the physical, chemical and biological conditions of the soil and the altered conditions probably reduce the inoculum potential of soil-borne pathogens (Singh, 1983). In addition, the practice also improves soil structure, which promotes root growth of the host. Various antibiotics and phenols are released during decomposition, which induces resistance in the root system and increases over all growth of the plant. In case of organic amendments, *i.e.*, cowdung, rabbit

manure and chicken manure, the growth promotion as well as percentage increase in shoot length in different tea seedlings varieties were higher in uninoculated tea seedlings than the treated inoculated ones. But total phenol and orthodihydroxy phenol contents were higher in treated inoculated tea seedling varieties as in oil cake and neem cake treated tea seedling. This observation may correspond to the fact that microorganisms being present in soil enhance the decomposition processes releasing phenols which increases total and orthodihydroxy phenol contents in treated inoculated tea seedling varieties with cowdung, rabbit manure and chicken manure.

Further in this investigation, effective integrated management practices against *S. rolfsii* were tested *in vivo*. In this experiment, under pot culture conditions *T. harzianum* alone and in combination with neem cake, oil cake, aquous extract of *A. indica* and calixin (0.1%) provided a total control of seedling blight in all the three modes of application *viz*, simultaneous, repeated and post infection. Similar results were obtained by Sonali and Gupta (2004) when *T. viride* alone and in combination with neem oil, neem cake and deodar needles used in radial growth of *S. rolfsii* resulted in a total control of the disease. But repeated application of neem cake, oil cake with various combinations of cowdung, rabbit manure and chicken manure were found to be less significant. Finally it was observed that *T. harzianum* and in combination with neem cake, oil cake, neem extract and calixin (0.1%) were found most effective in reducing disease incidence on tea seedling plants *in vivo*.

There are several reports on the management of disease by Integrated Disease Management (IDM). Management of chickpea root rot and collar rot against *S. rolfsii* by integration of biological and chemical seed treatment was reported by Tiwari and Mukhopadhyay (2003). They observed that application of carboxymethyl cellulose (CMC) with *G. virens* powder (10^9 spores per g) in combination with vitavax provided maximum protection (81.9%) to the crop against chickpea root rot and collar rot pathogens in glasshouse. Chickpea seeds treated with GV powder + CMC + vitavax significantly increased seedling emergence (47.9%); final plant stand (85.8%) and grain yield (79.7%) which was statistically at par with the treatment GV powder + vitavax and GV suspension + vitavax in a sick plot. Upamanyu *et al.*, (2002) reported the management of root rot and web blight caused by *Rhizoctonia solani*. They obtained that *T. viride* showed the maximum tolerance to carboxin,

tebuconazole and carbendazim followed by *T. virens, T. harzianum* and *A. niger* when used in integrated disease management along with fungicides and oil cakes both under glass house and field conditions. Soil amendment (cotton cake) + *T. virens* and carboxin (ST), mustard cake + *T. virens* + tebuconazole and soil amendement (mustard cake) + carbendazim (ST) were found effective in containing the root rot under glass house conditions while soil amendment (mustard cake) + carbendazim (ST) + carbendazim (FS) were found highly effective in reducing pre- and post- emergence root rot and web blight. Severity was best contained by soil amendement (mustard cake) + carbendazim (ST+FS) followed by tebuconazole + *T. virens* (ST) + carbendazim (FS).

In the present investigation, using PTA-ELISA formats and PAb of *S. rolfsii*, treated and untreated plants exposed to natural inoculum after 15 and 30 days of soil ammendments were compared. The absorbance (A_{405}) values were always reduced in treated root tissues than untreated ones. It indicates clearly that in the treated root tissues the establishment of the pathogen (*S. rolfsii*) was not successful due to the application of bioresources. Detection of *S. rolfsii* in tea root tissues and rhizosphere soil of different treatment with pathogen and biocontrol agents was also determined immunologically in both root tissues and soil. For this purpose, PTA-ELISA format was carried out. Results showed that ELISA values of root tissues treated with *T. harzianum* and *T. viride* were significantly lesser than with *S. rolfsii* alone. The same trend of results was obtained in infested rhizosphere soil through PTA-ELISA analysis. This result is in conformity with that of Hazarika *et al.* (2000) who reported that planting of tea seedlings after dipping roots in spore suspension of *T. harzianum* reduced 56.6% mortality of plant due to *U. zonata* infection. However they observed that the reduction of mortality of plant increased to 62.2% when *T. harzianum* were applied soil to soil drench. Significant control of charcoal stump rot of tea with antagonistic microflora obtained previously by Borthakur and Dutta (1992). The role of *T. harzianum* and *T. viride* as biocontrol crop is well established. In the present study, antigens prepared from mycelia of *S. rolfsii*, amendment soils and 4 different soil fungi were prepared and tested on nitrocellulose paper PAbs raised against mycelia of *S. rolfsii* using NBT/BCIP as substrate. Antigens of homologous source showed deep coloured dot when compared with soil antigens prepared from treated

organic amendments. Other tea root pathogens responded slightly reactivity with *S. rolfsii* walsh *et al.* (1996) also performed western blotting using the raw serum of *Spongospora subterranean* spore balls. Watabe (1990) demonstrated the presence of mycelium and chlamydospores in naturally and artificially infested soil samples, using this technique. Different test formats including indirect ELISA, western blotting, dot blot and indirect immunofluorescence was assayed by Wakeham and White (1996) for their potential to direct resting spores of *Plasmodiophora brasica* in soil. In conclusion, it can be stated that sclerotial blight can cause severe damage to tea plants, particularly to those growing sandy soil. Such detection techniques makes it possible to detect microquantities of the pathogen within root tissue and rhizosphere soil before much damage cause by the pathogen. Therefore, an accurate, rapid and cost-effective diagnosis is the cornerstone of efficient field disease management. Rapid detection of the pathogen is important to take preventive steps.

A possible long-term benefit of increased implementation of microbial control would be reduced input into agriculture, particularly if seasonal colonization and introduction-establishment come into widespread use. Initially, inputs due to implementation of microbial control are more likely to increase than decrease. There is potential for yield increase in the near future. Microbial control is simply one of the best potential alternatives for disease control that could be made available in a relatively short time period. A successful disease control program depends on a crop production system which is closely aligned with the goals of disease management. Integrated Disease Management (IDM) as applied to disease means using all the tactics available to the grower (cultural, biological, host plant resistance and chemical) that provides acceptable yield and quality at the least cost and is compatible with tenets of environmental stewardship.

Summary

A review of literature has been presented in combination with serological techniques for the detection of cross reactivity between host and parasite, effective integrated disease management practices and biochemical changes following induced resistance in plants.

The materials used in this investigation and the experimental procedures followed have been discussed in detail. Factors influencing mycelial growth of *S. rolfsii* were studied with special reference to their growth in different media, variable pH and variable sources of carbon as well as organic and inorganic nitrogen sources. Maximum growth of pathogen occurred after 8 days of inoculation at pH 6. Dextrose was the most effective carbon source and of the nitrogen sources, yeast extract (organic source) was found most optimum for growth of *S. rolfsii*. Organic nitrogen sources were found to be better than inorganic nitrogen sources.

Resistance of eighteen tea varieties (TV-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 screened against *S. rolfsii*. Among these B-157, UP-8 and TeenAli-17/1/54 were found to be highly susceptible, whereas K1/1 and HV-39 were found to be resistant.

Protein content of healthy and artificially inoculated tea root tissues from 18 different tea varieties as well as mycelia of *S. rolfsii* was estimated. Mycelial protein of *S. rolfsii* exhibited 24 bands with molecular weights ranging from 205 to 6.5 kDa in SDS-PAGE analysis.

Polyclonal antibodies (PAbs) were raised against antigen preparations from mycelia of *S. rolfsii* and tea roots. Serological cross reactivity among tea varieties and *S. rolfsii* isolates (Sr-1, Sr-2 and Sr-3) were determined following immunodiffusion test, enzyme linked immunosorbent assay, dot immunobinding assay and indirect immunofluorescence. Optimum conditions for PTA-ELISA reaction with PAb of *S. rolfsii* were determined. The antiserum dilution 1:125 and enzyme (alkaline phosphatase) dilution 1:10,000 proved to be most optimum. PTA-ELISA detected antigen up to a concentration of 10 μg/ml in homologous reaction. Major cross reactive antigens (CRA) shared between isolates of *S. rolfsii* and tea

varieties were determined following PTA-ELISA using PAb of *S. rolfsii*. Cellular location of major CRA was determined following indirect immunofluorescence test. Detection of pathogen (*S. rolfsii*) in artificially inoculated tea roots (AV-2 and B-157) using PTA-ELISA formats and immunofluorescence tests were developed. The reaction of mycelial antigens prepared from various fungi has also been determined on nitrocellulose papers following dot immunobinding assay using PAb of *S. rolfsii*. Specificity of PAb of *S. rolfsii* against all three isolates of *S. rolfsii* was also determined through western blot analysis.

Biochemical changes following inoculation with *S. rolfsii* were investigated. Both total and ortho-dihydroxy phenol contents increased following inoculation with *S. rolfsii* in resistant varieties while there was a decrease in susceptible varieties. Among 5 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* Antifungal compounds were extracted separately from healthy and *S. rolfsii* inoculated tea roots of resistant tea variety that inhibited mycelial growth of *S. rolfsii* in solid media. UV-analysis and HPLC profile clearly showed the presence of antifungal compounds in infected tea roots. It is interesting to note that extracts from *S. rolfsii* inoculated root tissue gave a peak at 274 nm. Maximum absorption peak measured at 274nm was identical to an authentic sample of pyrocatechol. In resistant varieties, higher accumulation (525-678 µg/g fresh wt) of pyrocatechol was detected, than in the susceptible varieties (212-290 µg/g fresh wt) following 96h of inoculation with *S. rolfsii*. Concentration of this compound in healthy root tissues were very low (60-93 µg/g fresh wt). Phenylalanine ammonia lyase (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. Peroxidase (PO) and polyphenol oxidase (PPO) activities in tea roots increased markedly after 4 days of inoculation with *S. rolfsii* in all the varieties tested.

In vitro interaction of *S. rolfsii* with *Trichoderma harzianum* and *T. viride* was studied. Both bioagents inhibited the growth of *S. rolfsii*. The efficacy of fungicides and plant extracts were also tested against *S. rolfsii in vitro*. Thiodan and calixin completely arrested the growth of the pathogen at a concentration as low as

0.0125%. Increase in growth of the plant was evident when grown in soil amended with organic additives. Phenol contents were found to be high in treated plants in comparison to untreated healthy control plants. Effective integrated management practices were adopted using plant extract, biocontrol agents, organic additives along with selected fungicides for control of seedling blight disease . *In vivo* trials demonstrated that *Trichoderma harzianum* alone as well as in combination with neem cake, oil cake, aqueous extract of *Azadirachta indica* and calixin (0.1%) provided a total control of sclerotial blight disease. Changes in the level of phenolics were also determined in tea plants (UP-3, B-157 and K 1/1) grown separately in soil ammeded with cow dung, rabbit manure and chicken manure following inoculation with *S. rolfsii*. Phenolics 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 orthodihydroxy phenol content increased in comparison with untreated healthy control

Alterations in antigenic patterns following induction of resistance in susceptible tea plants were detected using immunological assays. These antigenic changes, owing to calixin treatment, that was analysed using immunodiffusion tests, have some significance in the resistance of tea to *S. rolfsii*. Following PTA-ELISA with PAb raised against mycelia of *S. rolfsii*, it could be inferred that the absorbance (A_{405}) values were always lesser in treated root tissues in comparison to healthy untreated ones. PTA-ELISA of tea root tissues and rhizosphere soil of different treatment with pathogen and biocontrol agents, reacted with PAbs of *S. rolfsii*, *T. harzianum* and *T. viride* showed the reduction of pathogen population in rhizosphere soil and root. Reaction of various amended soil antigens with of *S. rolfsii* was also detected through dot-blot using PAb of *S. rolfsii*,. The amended soil antigens inoculated with *S. rolfsii* showed lesser colour intensity on nitrocellulose membranes than homologous antigen of *S. rolfsii*.

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