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Serological detection of *Sclerotium rolfsii* in Tea root tissue

INDRAMANI BHAGAT[1] AND BISHWANATH CHAKRABORTY[2*]
[1]Post graduate campus Biratnagar, Department of Botany, Tribhuvan University, Nepal
[2] Department of Biological Sciences, Aliah University, New Town, Kolkata 700 016

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Among 18 varieties of tea tested against *Sclerotium rolfsii*, UP-8, Teen Ali-17/1154 and B-157 were found most susceptible while K1/1 and HV-39 were resistant. Polyclonal antibody was raised against mycelial antigen of *S.rolfsii.* The immunoglobulin fraction of the antibody was purified by ammonium sulfate precipitation and Sephadex column chromatography. Effectiveness of raising antibody against the pathogen was confirmed by agar-gel double diffusion test and optimization of antigen and antibody concentration was done using PTA-ELISA format. The pathogen could be detected in root tissues after inoculation with *S. rolfsii* using PTA-ELISA. Cellular localization of the pathogen was evident as bright fluorescence mainly in the epidermis, cortical and endodermal layers reacting with PAb of the *S.rolfsii*and labeled with FITC.

Key words: *Sclerotium rolfsii*, *Camellia sinensis*, PTA-ELISA, Dot immunbinding assay, indirect immunofluorescence

INTRODUCTION

Tea is an important plantation crop grown in tropical agroclimatic regions. India is the major producers, consumers and exporters of tea. The tea bush, like any other living plant, during the various stages of growth and development is susceptible to attack by diseases, more so as it has been subjected to various cultural treatments which are widely at variance with the natural conditions of growth (Chakraborty and Chakraborty,2018). Sclerotial blight caused by *Sclerotium rolfsii* is one of the important fungal diseases which appear in the nursery grown tea seedlings in Terai region of North Bengal. The pathogen produces initially a dark brown lesion on a tea seedling's stem near the soil line. Later the pathogen produces a white mycelial web over the soil and basal canopy of the plant,followed by the appearance of dark brown mustard seed size sclerotia on the infected areas. In its advanced stage, the infected tea seedlings ultimately topped down and finally the plants died. In pure culture, initially white mycelial growth of *S.roflsii*s visible which gradually creamy white in appearance became. The aerial hyphae appear as dense tufts dispersed all over the culture medium.Often the hyphae aggregate into strands of rhizomorph like structure. The hyphae are hyaline thin walled and septate. Later on scletotia are formed from hyphal strands. Mature sclerotia are dark brown in colour (Fig. 1a). Plant disease detection by immunological means is gaining ground in case of fungal diseases Acharya *et al.,* 2015). Both disease detection and diagnosis of plant disease is rapidly increases by using immunological assay formats (Chakraborty and Chakraborty, 2012). In the present investigation attempts have been made to develop polyclonal antibody based immunotechniques for detection of pathogen (*S.rolfsii*) in soil and tea root tissue and to determine their cellular localization following indirect immunofluorescence.

*Corresponding author : bncnbu@gmail.com

MATERIALS AND METHODS

Plant material

Eighteen tea varieties of which 5 from Tocklai Experimental Station (TV-18, TV-22, TV-25, TV-26, TV-30),6from UPASI (UP-2, UP-3,UP-8,UP-

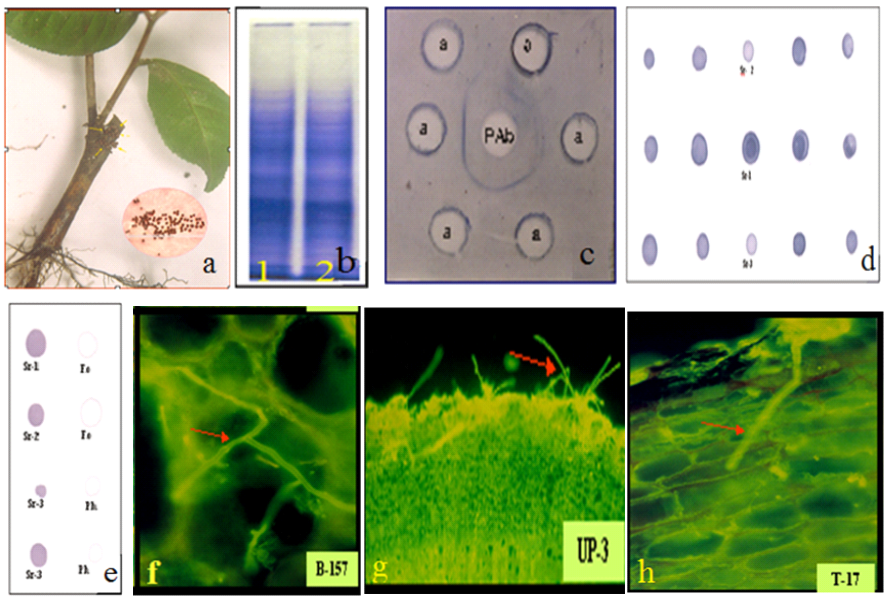

Fig.1. (a)Naturally infected tea seedling showing sclerotia of *Sclerotium rolfsii*on collar region; (b)SDS-PAGE analysis of mycelial antigens of *S.rolfsii* (lanes1 and2); (c) Agar gel double diffusion with mycelial antigen(peripheral wells) andPAb of *S.rolsii* (central well);(d) Dot immunobinding assay with antigens of*S.rolfsii* isolates (Sr-1,Sr-2 and Sr-3); (e) Dot blot reaction with homologous (*S. rolfsii* isolates, Sr-1,Sr-2 and Sr-3) and heterologous (*Fusarium oxysporum*and*Poriahypobrumea*) antigens reacted with PAb of*S.rolfsii*; (f-h) Indirect immunofluorescence ofT.S. of infected root tissues of tea varieties (B-157,UP-3andT-17)treated with homologous PAb and labeled with FITC-conjugateshowing hyphal penetration of*S.rolfsii*

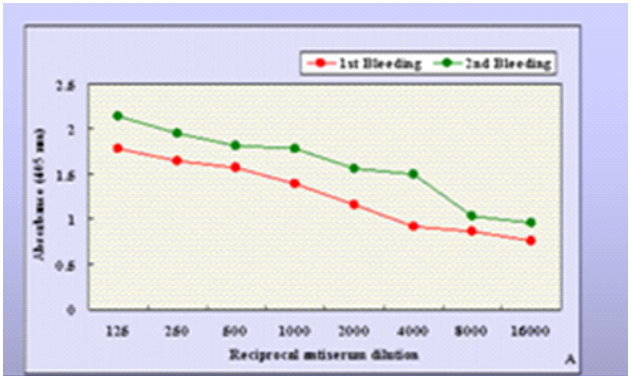

Fig 2. Optimization of PAb raised against mycelia of *S. rolfsii*with homologous antigen

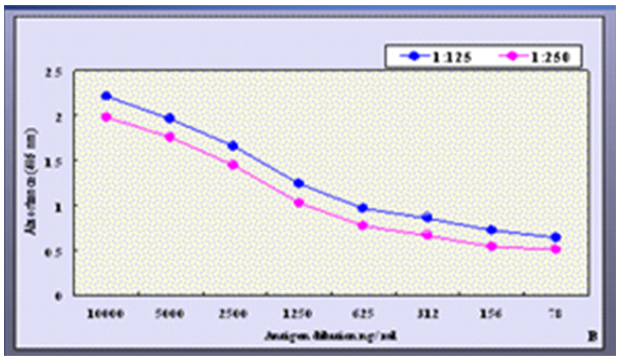

Fig 3. Optimization of antigen concentration of *S. rolfsii* with homologous PAbs using PTA-ELISA format.

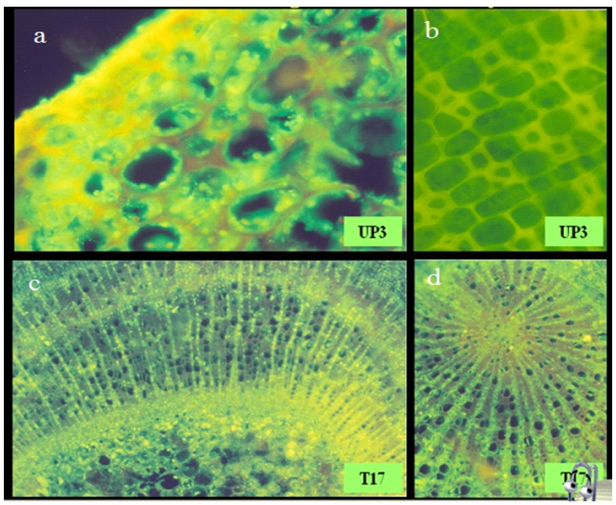

Fig 4. Cross section of tea root tissues (UP-3 and T-17) treated with PAb of *S.rolfsii* and labeled with FITC conjugates

9,UP-26 and BSS-2) and 7 from Darjeeling Tea Research Centre (Teen Ali-17/1/54, AV-2, HV-39, T-78, T-135, K-1/1 and B-157) were used for experimental purpose.

Fungal culture

A virulent culture of*S.rolfsii* isolated from naturally infected tea plant (Teen Ali-17/1/54) was identified from the Diagnostic and Advisory Service, CABI Bioscience UK. The culture was maintained on Potato Dextrose Agar (PDA) slants.

Inoculation technique and disease assessment

The mass culture of fungal pathogen (*S.rolfsii*) was prepared in sterilized sand maize meal medium containing maize, in the ratio of 3:1(sand: maize). The two week old cultures were planted in earthen pots containing 1 kg soil and allowed to be established.Then 100 g of pathogen inoculums was added carefully in rhizosphere of each plant. Regular watering was done to ensure the successfully establishment of the pathogen.

Disease assessment

Diseases assessment was done on on the basis of above ground and underground symptoms of wilting that developed on the artificially inoculated tea roots 15, 30 and 45 days following inoculation with *S.rolfsii*.Disease index was calculated on the basis of above ground and underground symptoms together on a scale of 0 – 6, where 0 = no symptoms; 1 = small roots turn brownish and start rotting; 2 = leaves start withering and 10-20% of the roots turn brown; 3 = leaves withered and 50% of leaves affected; 4 = extensive rotting at the collar region of root 60-70% of the roots and leaves withered 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 dead. Percentage of disease incidence was calculated by dividing the number of diseased plants by total number of plants and then multiplying by hundred.

Preparation of antigens

Antigens were prepared from healthy and *S. rolfsii* inoculated tea roots as well as from mycelia of *S.rolfsii* following the method of Chakraborty and Saha (1994). These were stored at -20^{o}C and used as root and mycelial antigens.

Extraction and estimation of protein

Roots were collected from the experimental garden and washed thoroughly. For extraction of soluble proteins from tea roots, root tissue (1g) 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^{0}C. Homogenates were centrifuged at 12,000/*g* for 15 minutes at 4^{0}C. The supernatant was collected and after recording its volume, was used immediately for estimation and analysis or stored at - 20^{0}C for further use. The protein estimation was done using bovine serum albumin (BSA) as standard.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis of fungal antigen was carried out on 10% gel. Protein extract (50 g/ml) along with standard molecular weight markers were loaded on the gel and separated at 18 mA for 3h. The gel was fixed overnight in glacial acetic acid:methanol:water (1:2:7),stained in coomassie blue R_{250} (0.25 g of coomassie brilliant blue in 45 ml of methanol)and destained in methanol: water: acetic acid(4.5:4.5:1)at 40^{0}C with constant shaking till the background was clear.

Polyclonal antibody(PAb) preparation

Polyclonal antibodies were raised against 8 d-old fungal antigen (*S.rolsfii*.) in New Zealand white male rabbit according to the method of Chakraborty and Saha (1994). Prior to immunization, normal serum was collected from the rabbit by marginal ear vein puncture. Antisera were stored at-20^{o}C until required. Immunoglobulin G(IgG) was purified by ammonium sulphateprecipitation and ion-exchange chromatography using diethylaminoethyl (DEAE) cellulose column and concentration of IgG was calculated.

Immunodiffusion

Agar gel double diffusion tests were carried out using antigen and antiserumas described by Chakraborty and Das Biswas (2008).

PTA-ELISA

Plate trapped antigen coated enzyme linked immunosorbent assay (PTA-ELISA) was performed as described by Chakraborty *et al* (1996). Antigens from host root and fungal pathogen were diluted with coating buffer and IgGs were diluted to 1:125 with PBS-Tween containing 0.5 % BSA. Goat anti-rabbit IgG (Whole molecule), alkaline phosphate (Sigma) conjugate and 4-nitrophenyle phosphate (pNPP), as enzyme-substrate, were used for ELISA tests. Absorbance values were measured at 405 nm in an ELISA reader (Multiskan EX, Labsystems). Absorbance values in wells not coated with antigens were considered as blanks.

Dotimmunobinding assay

Antigens prepared from healthy and *S.rolfsii*inoculated roots were loaded on nitrocellulose membrane filters using Bio-Dot apparatus (Bio-Rad). Dot immunobinding assay was performed using PAb of *S.rolfsii* as outlined by Chakraborty *et al.* (2016)Colar intensity of each dot was noted.

Immunofluorescence

PAb of *S.rolfsii* and goat antisera specific to rabbit globulins conjugated with fluorescent isothiocyanate (FITC) were used for indirect immunofluorescence study to detect the cellular location of pathogen in healthy as well as *S.folfsii* inoculated tea roots following the method of Acharya *et al*. (2015) Cross section of healthy and artificially inoculated with *S.rolfsii* root samples were cut and immediately immersed in PBST containing 0.01 % PVP-10 and 1% BSA, washed in PBS (pH 7.2), treated with PAb of *S.rolfsii* (1:10) and incubated for 2h at 25°C in grooved incubated for 30 min at 25°C, washed and observed using a Biomed microscope (Leitz) equipped with an 13 filter block ideal for FITCfluorescence under UV light in the dark. Photographs were taken by Leica WildMPS 48 camera on Kodak 800 ASA film.

RESULTS

Among 18 tea varieties screened, resistance to *S.rolfii* UP-8 and Teen Ali-17 were highly susceptible whereas K1/1 and HV-39 werefound to be the most resistant (Table 1).

Table1: Varietal resistance of tea towards *Sclerotium rolfsii*

Tea Varieties	Days	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 20 inoculated plants
[b]Days after inoculation
±Standard error

Table 2 : PTA-ELISA response of healthy inoculated tea root antigensusing PAb of *S. rolfsii*

Antigens	Absorbance values at 405 nm[a]	
(40 µg/ml)	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

Protein of *S. rolfsii*was characterized. SDS-PAGE analysis of the proteins revealed 24 protein bands ranging in molecular weights ca from 6.5 to 205 kDa (Fig.1b, lanes 1 and 2).Effectiveness of antigen in raising polyclonal antibody against mycelia of *S.rolfsii* when testedagainst the1st and 2nd bleed by immunodiffusion agar plate showed

strongprecipitation band.It was observed in agar gel double diffusion tests with antigens and antibody of *S.rolfsii* (Fig.1c)). Optimization of ELISA was done initially prior to ELISA tests by considering two variables, dilution of antigen extract and of antiserum to a maximum sensitivity.PAb at a dilution of 1:125, antigen conc. of 40 µg/ml and IgG alkaline phosphate at 1:10,000 gave optimum results. Absorbance values decreased with increasing dilution (Fig. 2). Homologous antigens at conc. as low as 78 ng/ml could be detected in indirect ELISA in both antiserum dilution (Fig.3).

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*and 15 days after inoculation antigens were prepared from healthy as well as infected tea roots of five varieties (AV-2, Teen Ali-17, UPASI-8 and UPASI-9 and TV-30) and tested by using PTA-ELISA formats. Results revealed that higher absorbance values were recorded in all the tested inoculated susceptible varieties (after 15 days of inoculation) in comparison to healthy root antigens (Table 2).

Table 3 : Dot immunobinding assay of different fungal antigens reacted with PAb of *S.rolfsii*

Antigen source	Colour intensity [a]
Fusarium oxysporum	+
S. rolfsii(Sr-1)	++++
Poriahypobrumea	+
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

Histopathological observations of cross sections of sclerotial blight affected tea roots (Fig1-f,g,h) revealed a bright apple green fluorescence of hyphal penetration in the root tissue on the epidermis, cortical tissue and endodermal layers (Fig. 4).Similarly when mycelia and sclerotia of *S.rolfsii* were treated with its PAb, bright apple green fluorescence was observed that was intense in young hyphal tips and sclerotia of *S.rolfsii* . Cross section of three susceptible varieties of tea (UPASI-3, Teen Ali-17 and B-157)20 days following inoculation with *S.rolfsii.,* treated with PAb raised against the pathogen and labeled with FITC conjugates also showed a bright apple green fluorescence on the epidermis, cortical tissue and endodermal layers. Fungal hyphal penetration within the tissue elements was visible in all three varieties tested (Fig.1, f,g,h).

DISCUSSION

If the microorganism is successful, disease is endresult; but more often than not, the host emerges the winner as the invader is successfully warded off.Visible outcome of a compatible host pathogen interaction may be obtained in many cases only after a few days of infection, by which time the pathogen would be well established in the hosttissues.Plants have well developed defense mechanisms which enable them to defend themselves against penetration, intracellular growth and development of parasites in their tissues. Visible outcome of a compatible host pathogen interaction may be obtained in many case only after a few days of infection, by which time the pathogen would be well established in the host tissues.

Enzyme linked immunosorbent assay (ELISA) is probably one of the most sensitive serological techniques for the detection of pathogen in host tissues. Various formats of ELISA using polyclonal antiserum have found widespread applications in plant pathology and are routinely used for detection and identification purposes. It is also reported that antiserum raised against *Pestalotiopisis thea* could detect homologus antigen at 25 ng/ml. Antiserum dilution 1:16,000 was effective for the detections of pathogen. It has also been determined that the tissue and cellular location of major cross reactive antigens (CRA) shared by tea leaf tissue and *Exobasidium vexans.* Cellular location of CRA in tea leaf tissues shared by four foliar fungal pathogens such as *Bipolaris carbonum* ; *Pestalotiopsis thea*, *Exobasidium vexans* and *Corticiumin visum* (Chakraborty and Das Biswas, 2008) have been demonstrated.

In the present findings, efficient immunodiagnostic kits based on either ELISA or dot immunobinding assay have been developed for screening the blight infection. Dot immunobinding assay showing a visual difference between healthy and *S.rolfsii*

inoculated tea roots can be used instead of ELISA with relative accuracy and ease for field application. A microtitreimmunospore trapping device, which uses a suction system to trap air particulates directly by impaction into microtitre wells, has been used successfully for the rapid immune detection and quantification of ascospores of *Mycosphaerella brassicicola* and conidia of *Botrytis cinerea*. Similarly, indirect ELISA has also proved to be valuable in screening commercially cultivated varieties of tea (Chakraborty and Sharma, 2007).

Detection of pathogen in host tissues using antibody based immunofluorescent technique has been reported. On the basis of immunofluorescence studies, chlamydospores, basidiospores and mycelia of *Phaseolus schweinitzii* contained molecules antigenically related to species specific antigens secreted by mycelia grown in liquid culture. The presence of mycelium and chlamydospores in naturally and artificially infested soil samples have been demonstrated using this technique. Different test formats including indirect ELISA, western blotting, dot blot and indirect immunofluorescence were assessed for their potential to detect *Fomes lamaoensis*in soil. Serological detection of *Pestalotiopsis disseminata*ca using grey blight disease of som plant (Acharya *et al*. 2015) as well as *Bipolaris sorokiniana* causing spot blotch disease of wheat have also been demonstrated (Chakraborty *et al.* 2016). These techniques can be used for early detection of fungal pathogens of different crops for developing disease management strategies.

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