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Induction of Resistance in Tea Plants against *Sclerotium rolfsiiby* Application of Bio-control Agents, Plants Extract, Systemic Fungicides and Organic Amendments

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Keywords

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Abstract

Tea is one of the important plantation crops in Nepal. One of the important fungal pathogens *Sclerotium rolfsii* causing seedling blight disease in tea was found to be predominant in the nursery grown plants. Alternations in antigenic patterns following induction of resistance of in susceptible tea plant were detected using immunological assays. These antigenic changes, owing to calixin treatment, that was analyzed using immune-diffusion tests, have some significance in the resistance of tea to *Sclerotium rolfsii*. PTA-ELISA formats and PAb of *Sclerotium rolfsii*, treated and untreated plants exposed to natural inoculum of *S. rolfsii* after 15 and 30 days of soil amendments, detection of *S. rolfsii* in tea root tissues and rhizosphere soil of different treatment with pathogen , bio-control agents and systemic fungicides determined immunologically in both root tissues , soil and antigens prepared from mycelia of *S. rolfsii*, amendment soils tested on nitrocellulose paper PAbs raised against mycelia of S. rolfsii using NBT/BCIP as substrate. Following PTA-ELISA with PAb raised against mycelia of S. rolfsii, it could be inferred that the absorbance (A405) 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 bio-control agents reacted with PAbs of *S. rolfsii*. Trichodermaharzianum and *T. viride* showed the reduction of pathogen population in rhizosphere soil and root. Reaction of various amended soil antigens with of *S. rolfsii*. The amended soil antigens inoculated with *S. rolfsii* showed lesser colour intensity on nitrocellulose membranes than homologous antigen of *S. rolfsii*. In conclusion, by application of bio-control agents, plantextracts, systemic fungicides and organic amendments in tea roots and soil reduce intensity of sclerotial blight disease.

1. Introduction

Tea (*Camellia sinensis*) is the most important hot beverage in the world today. One of the important fungal pathogen isSclerotium rolfsiiwhich causes sclerotial blight in tea. 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. is one of the fungal diseases which appears in the nursery grown tea seedlings. So, it is very important to detect the disease by immunolocal assays. Disease detection by immunological means is gaining ground in case of fungal diseases (Chakraborty and Chakraborty, 2003; Gawandeet 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. 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.Since the application of bio-control agents in rhizosphere soil reduced intensity of sclerotial blight disease, it was decided to investigate this reduction which could also be determined immunological 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.

2. Materials and Methods

Eighteen tea varieties of which 5 from Tocklai (TV-18,TV-22,TV-25,TV-26,TV-30), 6 from UPASI (UP-2,UP-3,UP-8,UP-9,UP-26 and BSS-2) and 7 from Darjeeling (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, Department of Botany,

North Bengal University, were used for experimental purpose.

2.1 Fungal culture

2.1.1 Source of cultures

Virulent culture of Sclerotium rolfsiiSacc (*Corticiumrolfsii Curzi*) was obtained from Immuno-Phytopathology Laboratory, Department of Botany, NorthBengal 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 Fusariumgraminearum (a non pathogen of tea), Trichodermaharzianumand Trichoderma viride (bio-control agents) were also obtained from the laboratory, mentioned above.

2.2.2 Maintenance of stock cultures

The fungus thus obtained was sub-cultured on Potato Dextrose Agar (PDA) slants. After two weeks the culture was stored under three different conditions (5°C, 20°C and at room temperature 25+3°C) apart from weekly transfer for experimental work, the culture of *S. rolfsii* was also examined at regular intervals to test its pathogenicity.

2.3 Inoculum preparation

2.3.1 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 280C for 7 days. The inoculum was mixed with sterile soil at the ratio of 1:8. Fungus soil mixture (100 g) were mixed with the top soil of earthen pots containing tea seedlings and kept for development of disease reaction.

2.3.1 Bio-control agents

Trichodermaspp were 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 tea plants.

2.3.2 Inoculation of healthy tea seedlings in pot

One year tea seedlings were planted in earthen pots containing 1 kg soil and let the seedlingsgrew well in pots. Regular watering was done for two weeks and then 100g of pathogen inoculums was added carefully in the rhizosphere of each plant.

2.3.3 Preparation of antigens

Antigens were prepared from healthy and S. rolfsii inoculated tea roots as well as from mycelia of the pathogen (Chakraborty and Saha 1994). Root antigen is also prepared. They were stored at -20°C and used as root and mycelial antigens.

2.4 Production and purification of Polyclonal antibody

Polyclonal antibodies were raised against 8 days old fungal antigen (*S. rolfsii*) in New Zealand white male rabbit according to the method of Chakraborty and Purkayastha (1983). Normal Sera were collected from the rabbit by ears vein puncture before immunization. Antisera were stored at –20°C until required following the protocol of Clausen (1988, immunoglobulin G (IgG) was purified by DEAE-Sephadex column Chromatography. Concentration of IgG was calculated according to the method of Jayaraman (1996). 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 sterile surgical 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°C for clotting then the clot was loosened with a sterile needle. Finally, the serum was centrifuged (2000g for 10 min at room temperature) and distributed in 1ml vials and stored at – 200C for further use.

2.5 Immunodiffusion

Agar gel double diffusion tests were performed using Polyclonal antibody (PAb) arose against S. rolfsii following the method of Ouchterlony, 1967. After immunodiffusion, the slides were washed and stained with coomassive blue (R-250, Sigma) followed by destaining.

2.6 ELISA

PTA – trapped antigen- enzyme linked immunosorbent assay was performed following the method as described by Chakraborty et al. (1996). Plant and fungal antigens were diluted with coating buffer. Purified antiserum (IgG) was diluted to 1:125 in antisera dilution buffer with PBS–Tween containing 0.5 % BSA Goat anti rabbit immunoglobulin (IgG) whole molecule conjugated with alkaline phosphates and p- nitrophenyl phosphate substrate, as enzyme – substrate, were used or ELISA tests. Absorbance- values were measured at 405 nm multiskan EX (Thermo Electron) ELISA reader. Absorbance values in wells not coated with antigens were coated with considered as blanks.

2.7 Inducing agents and their application

2.7.1 In vivo test

Mature leaves (500 g) each of Azadirachtaindica and Catharanthusroseus 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°C 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 behaviorwas 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 g of pathogen (*S. rolfsii*) inoculums 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) at 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°C. During incubation, these bags were gently hand shaken to promote uniform sporulation over the carrier medium and to avoid clusters. Addition of bio control agents in soil was done 10 days prior to inoculation with S. rolfsii.

3. Results and Discussion

In the present investigation, using PTA-ELISA formats and PAb of Sclerotiumrolfsii, treated and untreated plants exposed to natural inoculum of *S. rolfsii* after 15 and 30 days of soil amendments, detection of *S. rolfsii* in tea root tissues and rhizosphere soil of different treatment with pathogen , bio-control agents and systemic fungicides determined immunologically in both root tissues, soil and antigens prepared from mycelia of *S. rolfsii*, amendment soils tested on nitrocellulose paper PAbs raised against mycelia of *S. rolfsii* using NBT/BCIP as substrate.

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 1 and 2.

Table1. PTA-ELISA reaction of PAbS. rolfsii with root antigens of tea varieties following treatment with bio-control 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 2.PTA- ELISA reaction of PAbs of pathogen and bio-control 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 (A405) 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 3). This result has definitely opened new horizons for testing various other eco-friendly plant extracts for the management of the disease.

3.1 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 bio control agents. Results presented in Table 4 revealed that PAb of S. rolfsii reacted very strongly with the antigens from soil infested with S. rolfsii 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. In cases where soil was treated with other organic amendments (cow dung and chicken manure) less positive reactions were evident.

Table 3: Indirect ELISA reaction of treated (systemic fungicide and plant extract) and untreated tea roots before and after inoculation with Sclerotiumrolfsii against PAb of Sclerotiumrolfsii

Antigen Concentration (40 µg/ml)	PAb ofSclerotiumrolfsii (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						
CarboxinH 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
CarboxinI 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 har vest
[c]H – healthy
[d]I – infected.

3.2 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 bio control agents. Results presented in Table 4 revealed that PAb of *S. rolfsii* reacted very strongly with the antigens from soil infested with S. rolfsii 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. In cases where soil was treated with other organic amendments (cow dung and chicken manure) less positive reactions were evident.

Table 4:Dot-blot of soil antigen of different treatment with combination

Antigen source	Colourintensity[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	+

[a]Colour intensity of dots : - no colour; + very light violet ; ++++ deep violet.
PAb concentration: 40 µg / ml; NBT/BCIP used as substrate and soil reduce intensity of sclerotial blight disease.

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 Plasmodiophorabrasicain 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 make it possible to detect micro-quantities 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.

References

Borthakur, B. K. and Dutta, P. K. 1992. Proceeding of the 31stTocklai Tea Conference Jan 20-21, Tocklai, pp. 163-168.

Chakraborty, B. N. and Chakraborty, U.2003.Immunodetection of plant pathogenic fungi.In: Frontiers of Fungal Diversity in India (Eds. G.P.Rao, C. Manoharachari, D. J. Bhat, R. C. Rajak and T. N. Lakhanpal) International Book Distributing Co. Lucknow p. 23-42.

Chakraborty, B. N. and Saha, A. 1994. Detection and cellular location of cross-reactive antigens shared by Camellia sinensisand Bipolariscarbonum. Physiol. Mol. Plant. Pathol.,44: 403-416.

Chakraborty U., Basu, P., Das, R., Saha, A. and Chakraborty, B. N. 1996. Evaluation of antiserum raised against Pestalotiopsistheae for detection of grey blight disease of tea by ELISA. Folia Microbiol., 41: 413-418.

Chowdhury, A. K. andSinha, A. K. 1995.Trop. Agric. 68: 109-116.

Clausen, J. 1988. Laboratory techniques in Biochemistry and Molecular Biology. Vol. 1 part III. (Eds. By.R. H. Burdon and P. H. Van.Knippenberg). pp. 64-65.

Gawande, S. J., Chimote, V. P., Shukla, A. and Gadewar, A. V. 2006. Serological and molecular diagnosis of plant pathogens In:Plant Protection in New Millennium (Eds: A.V. Gadewar and B.P. Singh) 1: 563-588.

Hazarika, D. K., Phookan, A. K., Saikia, G. K., Borthakur, B. K. and Sarma, D. 2000. Management of charocoal stump rot of tea with biocontrol agents. J. Plantn. Crops.28: 149-153.

Jayaraman J. 1996.Laboratory Manual in Biochemistry, New Age Publishers, 79p.megasperma f. sp. glycinea. Physiol. Plant Pathol.17 : 175-192

Kennedy, R., Wakeham, A. J. and Cullington, J. E. 1999. Production and immunodetection of ascospores of Mycosphaerellabrassicicola: ringspot of vegetable crucifers. Plant Pathol.48: 297-307.

Ouchterlony, O. 1967. In: Handbook Experimental Immunology (ed. D.M. Wier) Blackwell Scientific Publications, Oxford and Edinburgh. Londeon.p. 655.

Wakeham, A. J. and White, J. G. 1996. Serological detection in soil of Plasmodiophorabrassicaeresting spores. Physiol. Mol. Plant Pathol.,48: 289-303.

Walsh, J. A., Merz, U. and Harrison, J. G. 1996. Serological detection of spore balls of Spongosporasubterranea and quantifion in soil. Plant Pathol.45: 884-895.

Watabe, M. 1990. Immunofluorescent antibody technique for detecting Phytophthorain soil.Phytopathol. Soc. Japan, 56: 269-272.