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Immunodetection of root pathogen and bioinoculants following induction of immunity in *Camellia sinensis*

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Screening for resistance of ten tea varieties (TV-25, TV-26, TV-9, TV-20, TV-18, T-17, T-78, AV-2, UP-3 and UP-26) against *Sclerotium rolfsii* was carried out in sick plot developed specifically for this pathogen. Among the tested tea varieties, TV-9, TV-20, TV-25, and TV-26, were found to be moderately susceptible in comparison with other tea varieties.Morphological, cultural characteristics and molecular analysis of the pathogen (*S. rolfsii*) were done. The BLAST query of the 18S r DNA sequence of the *S. rolfsii* against GeneBank database confirmed the identity as *Athelia rolfsii* (anamorph *Sclerotium rolfsii*). The sequences have been deposited in NCBI, Gen Bank database under the accession no. JQ429785. A multiple sequence alignment of ITS gene sequences of *S. rolfsii* and phylogeny was conducted. Scanning electron microscopic observation of bioinoculants (*Trichoderma harziaum*, *Rhizophagus fasciculatum* and *Gigaspora gigantea*) were also done. Using serological techniques such as immunodiffusion test, dot immunobinding assay, western blot analysis and indirect immunofluorescence, immunodetection of *S. rolfsii* have been successfully demonstrated. The young and old sclerotia along with the mycelia treated with PAb of pathogen and labeled with FITC showed apple green fluorescence which was more intense on young sclerotia. By raising PAbs against bioinoculants, BCA (*T. harzianum*) and AMF(*R. fasciculatum* , *G. gigantea*), we have demonstrated first time immunodetection of bioinoculants using their respective PAb and FITC labeling. Bright apple green fluorescence were evident in hyphae and phialides of *T. harzianum* , spores of *R. fasciculatum* with hyphal attachments, successful tea root colonization with *R. fasciculatum,* spores of *G. gigantea* with hyphal attachments following indirect immunofluorescence. Induced resistance in tea plants against *S. rolfsii* following root colonization with AMF and application of BCA have been demonstrated. Accummulation of defense enzymes (PAL, peroxidase, glucanase and chitinase) were maximum in *T. harzianum* treated plants than *R. fasciculatum* and *G. gigantea* which were further confirmed in tea leaf tissues following indirect immunofluorescence.

Keywords : *Sclerotium rolfsii*, tea, immunoassays, induced resistance, *Rhizophagus fasciculatum, Trichoderma harzianum, Gigaspora gigantea*

INTRODUCTION

Traditional detection of phytopathogens and disease diagnosis methods using conventional methods are often slow and inefficient. In many cases—especially with root diseases—visible symptoms above ground appear only after the infection has progressed too far for effective intervention. Since many pathogenic fungi grow slowly, early confirmation of their presence is difficult. Another major issue is that simply detecting a pathogen does not prove it is responsible for the disease.

Plants can host multiple microorganisms, including fungi that are not actively causing harm. For instance, culturing a plant sample may yield several pathogens, but not all of them are contributing to the observed symptoms. This makes diagnosis complex, as the diagnostician must determine which organism is actually causing the disease and how significant its role is.

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As an alternative to conventional methods, serological diagnostic methods for early detection of phytopathogens have been introduced. Following the development of monoclonal antibodies, standardization of many serological assays have revolutionized detection of phytopathogens (Chakraborty and Chakraborty, 2002 ; Lopez *et al,* 2003; Gawande *et al.* 2006; Chakraborty and Chakraborty, 2012; Chakraborty, 2018). Subsequent to this, attempts have been made for constant advancement of our knowledge in the field of molecular biology, sequencing, nanotechnology and computer sciences for specific pathogen detection (Chakraborty and Chakraborty, 2021).

Soil and rhizopshere microbiome such as *Trichoderma* sp., AM fungi and plant growth promoting rhizobacteria (PGPR) are frequently associated with many nutrient and biological processes at root level and these are being used as bioinoculants (Chakraborty and Chakraborty, 2019). The sustainability of the bioinoculants applied in the soil has to be ascertained prior to recommending their application. Association and diversity of AM fungi with plantation crops have been documented (Chakraborty and De, 2013). Mycorrhizal association and root colonization of *Citrus reticulata*, *Citrus medica* and *Citrus limonia* grown in Darjeeling hills have also been extensively studied (Allay *et.al.* 2021). Immunodetection of *Trichoderma harzianum* and dominant AM Fungi and using their specific antisera can be used to ascertain their presence in soil and tea root tissues after varying periods of time.

In the present investigation attempts have been made to use reliable serological techniques to detect soil borne plant pathogen, *Sclerotium rolfsii* causing root disease of tea seedlings as well as to develop serological format for detection of dominant AM Fungi and *Trichoderma harzianum* (bioinoculants) in soil and root tissues and utilization of bioinoculants for induction of resistance in nursery grown tea plants and confirmation using serological format of successful root colonization with AMFungi and also cellular localization of defense enzymes in tea leaf tissues.

MATERIALS AND METHODS

Preparation of inoculum and inoculation technique

Culture of *Sclerotium rolfsii* was grown in sterilized autoclavable plastic bags containing sand- maize meal medium (maize meal: sand: water- 1:9:1.5 w:w:v, sterilized at 20 lbs. pressure for 20 min) for a period of three weeks at 28°C until the mycelia completely covered the substrate. Selected varieties of tea plants were then inoculated by adding 100g of prepared inoculum of *S. rolfsii* to the rhizosphere of each plant.

Disease assessment in tea plants

To determine the disease assessment sick plot was prepared. For this, roots of infected plants were chopped and mixed with the soil, along with inocula of *S.rolfsii.* Separate plots were earmarked for control set. The percentage of disease incidence was calculated by dividing the number of diseased plants by total number of plants and then multiplying by hundred while disease intensity was calculated by using 0 - 6 scale as adopted by Bhagat and Chakraborty (2020) after 15, 30 and 45 days of inoculation. The disease intensity was recorded following 0-6 scale, where 0 = no symptoms; 1= small roots turn brownish and start rotting; 2 = leaves start withering and 20 - 30 % of root turns brown; 3 = leaves withered and 50 % of leaves affected; 4 = shoot tips also start withering and 60-70 % root affected; 5 = shoot withered with defoliation of lower withered leaves and 80 % roots affected; 6 = whole plant die with upper withered leaves still remaining attached and roots fully rotted.

Isolation of AM fungi and mass multiplication

AM fungal spores were isolated from rhizosphere soil of tea plant by wet sieving and decanting method as described by Gerdemann and Nicolson (1963). Purification of AM fungal spores was done following sucrose gradient centrifugation method of Daniels and Skipper (1982). Single spore culture of AMF spores isolated from tea rhizosphere were maintained in laboratory conditions and then finally applied to the sterile soil of maize for mass multiplication in pots. Root colonization percentage and spore mass produced per gm soil per pot were recorded. Microscopic observations of the root inoculated with AMF showed profuse colonization especially in the feeder roots.

T. harzinaum formulation

Wheat bran based formulation of T.harzianum was prepared as described by Chakraborty *et al.* (2025).

Scanning Electron Microscopy of AMF . T. harzinaum

Selected AMF spores were sonicated under 35 MHz followed by washing five times in sterile distilled water, surface disinfected with 4% (wt/vol) chloramine-T and 300 ppm of streptomycin for 1 h, and then rinsed a further five times in sterile distilled water and were stored in eppendorf's tube in room temperature. AMF spores and hyphae with spores of *T. harzianum* were placed within separate aluminium "disc cup" (20 mm dia x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold coated samples were examined with a Philips 505 scanning electron microscope operating at 9.5-r5 Kev.

SDS-PAGE Analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of soluble proteins of the pathogen (*S. rolfsii*) was carried out on 10% gel. Protein extract along with standard molecular weight markers were loaded on the gel and separated at 18 mA for 3 h. 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 blue in 45ml 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.

Immunological studies

Fungal antigen

Mycelial proteins from *Sclerotium rolfsii* and *Trichoderma harzianum* were prepared following the method as outlined by (Chakarborty *et al.* 1995). Mycelial mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite and 0.5 mM MgCl2 in ice bath. The homogenate mixture was kept for 2h or overnight at 4 ºC and then centrifuged at 10.000rpm for 30 min at 4 ºC to eliminate cell debris. The supernatant was collected and stored in -20 ºC and used as antigen for the preparation of antiserum.

Antigen of AMF spores

Dominnat spores of *Rhizophagus fasciculatum* and *Gigaspora gigantea* from tea rhizosphere were isolated by wet sieving and decanting method as described before. With the help of a dissecting microscope parasitized spores, plant debris etc were separated and clean spores of *R. fasciculatum* and *G. gigantea* were isolated. Spores were sonicated with 0.1% normal saline under the frequency range of 70-75 mhz as impulse. The supernatant was used as antigen source.

Production of polyclonal antibody

Polyclonal antibodies were raised separately against fungal antigen (*S. rolfsii*), bioinoculants (*Trichoderma harzianum, Rhizophagus fasciculatum* and *Gigaspora gigantea*) in New Zealand white male rabbit accordingly to method as described by Chakraborty *et.al*. (1995). Prior to immunization, normal serum was collected from each rabbit by marginal ear vein puncture. Antisera were stored at -20^{0}C until required. Immunoglobulin G (IgG) of all four samples were purified by ammonium sulphate precipitation and ion-exchange chromatography using diethylainoethyl (DEAE) cellulose column and concentration of each IgG preparation was calculated.

Agar gel double diffusion test

Immunodiffusion test was carried out using antigen and antiserum as described by Chakraborty and Das Biswas (2008).

Dot blot analysis

Dot immunobinding assay was performed on nitrocellulose membrane filters using Bio-dot apparatus (Bio-Rad). PAb raised against *S. rolfsii* was reacted against its homologous antigen as oulined by Chakraborty *et.al.* (2016a). Colour intensity of dots were noted.

Western blot analysis

Immunoblotting was also determined using Western Blot technique as described by Wakeham and White (1996). Following the SDS-PAGE, the gel was transferred in prechilled transfer (Towbin) buffer for 1h. The nitrocellulose membrane (BIO-RAD, 0.45µm) and the filter paper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves and soaked in Towbin buffer for 15min. The transfer process was done in Trans-Blot SD Semi-Dry Transfer cell (BIO-RAD) through BIO-RAD power pack. After the run the membrane was removed and dried on a clean piece of 3mm filter paper for 1h and processed for immunological probing.

Fluorescence antibody staining and microscopy

Fluorescence antibody staining and microscopy were done following the method of (Chakraborty and Saha, 1994). Fungal mycelia were grown in liquid Richards's medium as described earlier. After five days of inoculation young mycelia were taken out from flask and taken in Eppendorf tube and washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1 h at room temperature. The mycelia was washed thrice with PBS- Tween (pH 7.2) as mentioned above and treated with Goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45 min at room temperature. After incubation mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were then ready to observe. Indirect fluorescence staining of AMF spores, arbuscules under tea roots were also done using FITC and RITC labeled goat antirabbit IgG. PAbs raised against sonicated spores of *Rhizophagus fasciculatum* and *Gigaspora gigantea* and goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC) were used for indirect immunofluorescence study to detect the AMF spores as well as in rhizosphere root samples and to determine the cellular location of major cross reactive antigens (CRA) shared by AMF in healthy tea root tissues. Besides root colonization and cellular location of AMF was also observed in tea root tissue.

Assay of defense enzymes

Estimation of b-1, 3-glucanase activity (E.C.3.2.3.39) was done by following the laminarin dinitro salicylate method described by Pan *et al.* (1991). The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissue. Chitinase activity (E.C.3.2.1.14) was measured according to the method described by Boller and Mauch (1988). The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released min^{-}1 g^{-1} fresh wt. tissue. Phenylalanineammonialyase(E.C.4.3.1.5) and Peroxidase(E.C.1.11.1.7) were extracted and estimated following the protocol described by Chakraborty *et al.*(1993).

RESULTS

Incidence of sclerotial blight disease of tea

Sclerotial blight caused by Sclerotium rolfsii Sacc. [telomorph: *Athelia rolfsii* (Curzi), Tu and Kimbrough = *Corticium rolfsii* (Curzi)], is a soil borne plant pathogen causing diseases on a wide range of agricultural and horticultural crops due to its prolific growth rate and the production of oxalic acid and cell wall degrading enzymes. It is among one of the most aggressive root pathogens which causes considerable damage to young tea saplings in the nursery. The first visible symptom of sclerotial blight disease is observed as yellowing and wilting of lower leaves. The fungal mycelia first appears at the base near the soil line. The pathogen then grows upwards covering the stem with a cottony white mass of mycelia. Later on, water soaked and grey lesions appear on the tea seedlings that turn brown, resulting in the death of the whole plant. A large number of small light brown, mustard like sclerotia develops in the collar zone. After the pathogen establishes itself, its subsequent advancements in production of mycelia and sclerotia is quite rapid. The infected tea seedlings ultimately topple down and die.

Screening for resistance of tea varieties against *S. rolfsii* was carried out in sick plot developed specifically for this pathogen (Fig.1). Varietal resistance test of tea against *S. rolfsii* was carried out in ten tea cultivars including five Toklai varieties (TV-25, TV-26, TV-9, TV-20, TV-18), two Teen Ali varieties (T-17 and T-78), two UPASI varieties (UP-3 and UP-26) and one Assam variety (AV-2). Three year old plant roots were inoculated with *S. rolfsii* and disease assessment was done on the basis of visual observation of symptoms and disease index (0-6 scales) was calculated after 15, 30 and 45 days following inoculation as well as on the basis of histopathological studies of the infected root (Fig. 2).

Results presented in Table-1 shows that among the tested tea varieties, TV-25, TV-26, TV-9 and TV-20 were found to be less susceptible in comparison with other tea varieties. Defoliation of leaves following infection with *S. rolfsii* was evident in TV-18, T-17, AV-2,T-78,UP-3,UP-26 and TV-25 after 30 days of inoculation in relation to healthy control plant. Disease symptoms occurred in those varieties within 7days after transplantation following death of the plants, were selected as highly susceptible varieties, whereas the varieties showing resistant reactions were also categorized.

Morphological and cultural characteristics of Sclerotium rolfsii

Sclerotium rolfsii was grown in different media *i.e.* Potato dextrose agar (PDA), Potato sucrose agar (PSA), Richard's Agar (RA), Carrot juice agar (CJA) Czapek-Doxagar(CDA), and Yeast extract- dextrose agar (YDA). Results revealed that the maximum growth was recorded in PDA. Colonies of *S. rolfsii* are readily distinguished by rapidly growing silky-white hyphae that tend to aggregate into rhizomorphic cord like structure. In culture, the whole area of a Petri plate is rapidly covered with mycelium, including aerial hyphae which may cover the lid of the plate. Both in culture and in plant tissue, a fan-shaped mycelia may be observed growing out ward and branching acutely. At least two types of hyphae are produced. Sclerotia (0.5-2.0 mm diameter) begin to develop after 6-7 days of mycelial growth. Initially a felt like white appearance then sclerotia quickly changes to a dark brown coloration (Fig. 3). Irrespective of the media used, maximum growth was observed at the temperature range lying between 25 ºC - 28ºC. *S. rolfsii* was grown in PDA medium for a period of 30 days, mycelial growth was recorded after 5,10,15, 20, 25, 30, 35 and 40 days of growth and incubated at 25± 1ºC. Maximum growth was recorded after 25 days of incubation after which it declined (Table 2).

18S rDNA sequence and BLAST analysis of S.rolfsii

Genomic DNA of *S. rolfsii* isolate - RHS/T-381 was isolated and purified. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free was around 1.80 Kb. ITS-PCR was performed with the help of ITS specific universal primer pair where a uniform product of 250 bp was obtained. The amplicons were sequenced and was further analyzed. The BLAST query of the 18S r DNA sequence of the isolate RHS/T-381 against GeneBank database confirmed the identity of the isolate as *Athelia rolfsii* (anamorph *Sclerotium rolfsii*). The sequences have been deposited in NCBI, Gen Bank database under the accession no. JQ429785 **(**Fig. 4).

Multiple sequence alignment and phylogeny

A multiple sequence alignment of ITS gene sequences of *S. rolfsii* was conducted. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Fig. 5). Phylogenitic analysis of *S. rolfsii* was carried out with the Ex-type strain sequences obtained from NCBI Genbank Database which showed maximum homology with the isolate TG1 (Table 3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 0.85088554 with 176 positions in final data set have been shown (Fig. 6). The evolutionary distances were computed

Fig 1: Different stages of disease development in tea varieties showing disease incidence after inoculation with *S. rolfsii* in sick plots

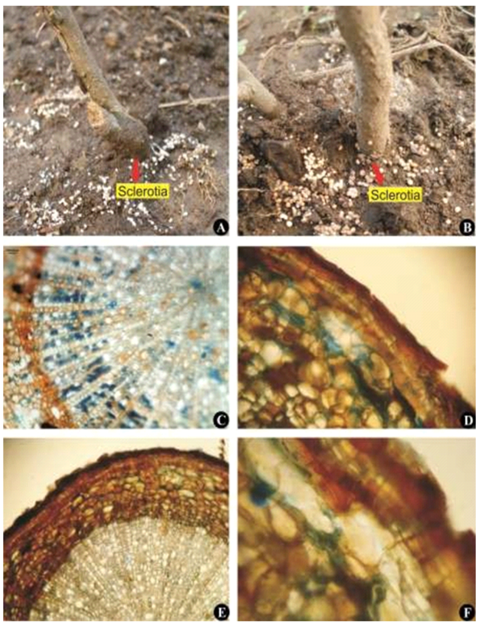

Fig 2: (A&B) Tea root rhizosphere showing development of sclerotia around the severely infected plant root and collar regions. (C-F) T.S. of tea root showing affected zones and parenchyma tissue blocked by the hyphae of *S. rolfsii* and progression towards the vascular regions

Table 1 : Disease development in different tea varieties observed during varietal resistance test against *Sclerotium rolfsii* in sick plot

Tea varieties	Disease index^{ab}		
	15days	30days	n45days
TV-25	2.64±0.01	3.02±0.03	3.6±0.04
TV-26	0.53±0.02	2.08±0.05	3.48±0.06
TV-9	1.64±0.02	3.11±0.06	3.48±0.05
TV-20	2.52±0.03	3.02±0.03	4.06±0.08
TV-18	1.67±0.03	3.53±0.09	4.06±0.08
T-17	1.65±0.02	3.46±0.04	4.09±0.08
AV-2	0.22±0.01	4.63±0.09	5.71±0.06
T-78	2.52±0.03	4.91±0.02	5.41±0.01
UP-3	0.66±0.01	3.65±0.04	4.08±0.06
UP-26	3.9±0.08	4.94±0.04	5.43±0.01

[a]Results are in average of 10 inoculated plants of each variety.
[b]Days after inoculation ± Standard error

Key to disease index : Disease intensity was assessed as rot index on a scale of 0-6, depending on both underground and above ground symptoms as follows: 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 3 : Growth of *Sclerotium rolfsii* on PDA medium. (A&B) 7and10days old (C)10 days old culture grown in conical flask (D) Enlarged view of C showing sclerotia

Table 2: Effects of incubation period on mycelia growth of *Sclerotiumrolfsii*

Incubation period (Day)	Mean mycelia dry weight (mg)[a]
5	07.60±0.42
10	19.5± 0.80
15	37.3± 0.25
20	69.6± 0.40
25	77.10±0.50
30	74.8± 0.51
35	66.2±0.40
40	67.2±0.37

[a]Average of three replicates;
±standard error, Temp.25±1°C,pH-5

using the Maximum Composite Likelihood method (Tamura *et. al,* 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd +3rd +Noncoding. Phylogenetic analyses were conducted in MEGA-4 (Tamura *et. al.* 2007)

Serological detection of Sclerotium rolfsii

Using serological techniques such as immunodiffusion test, dot immunobinding assay (DIBA), western blot analysis and indirect immunofluorescence, immunodetection of *S. rolfsii* have been successfully demonstrated. The effectiveness of the purified antigen of *S.rolfsii* in raising PAbs was checked by homologous cross reaction following agar gel double diffusion tests. The precipitin reaction done with PAb raised against mycelial protein yielded sharp band which was stained blue (Fig. 7A). Dot immunobinding assay was performed using total soluble proteins of *S. rolfsii* used as homologous antigen source. Antigens were carefully spotted on nitrocellulose paper and probed with PAb of *S. rolfsii*. Results presented in Fig. 7 B, shows clear and intense colour reactions.

SDS-PAGE analysis of soluble proteins were done (Fig 7C) and following Western blot analyses using polyclonal antibody of *S. rolfsii* revealed that the PAb collected after successful immunization of the rabbits for consecutive six weeks could show different levels of homologous reactions with the antigen of *S. rolfsii.* A sharp and intense band at 35 Kda was obtained on the nitrocellulose membrane after enzymatic reaction with NBT-BCIP (Fig.7D). Efficacy of polyclonal antibodies raised against the mycelial proteins used as antigen source was further tested with the help of indirect immuno fluorescence of young and mature sclerotia of *S. rolfsii.* The young and old sclerotia along with the mycelia treated with PAbs and labeled with FITC showed apple green fluorescence where fluorescence was more intense on young sclerotia (Fig. 8.). Neither mycelia nor sclerotia showed any type of auto-fluorescence nor they showed any fluorescence when treated with normal serum followed by FITC.

Tea Root colonization of Arbuscular Mycorrhizal Fungi

AMF associations in tea plants from three geographically different tea gardens *viz,* Happy Valley, Darjeeling Hills (27°32 003 N 88°162 003 E, 2,100 meters above sea level), Hasqua Tea Garden, Siliguri (26°36’58"N 88°18’45"E, 126 meters above sea level) and Karala Valley Tea Garden, Jalpaiguri (26°31’3"N 88°42’13"E, 75 meters above sea level) were studied. Eleven different species of *Glomus*, six different species of *Acaulospora*, three different species of *Gigaspora* and four species of *Scutellospora* were found to be dominant in all the soil samples collected from different tea gardens. In addition few species of *Sclerocystis* were also documented. Microscopical views of the spores are presented in Fig 9.

Among all the genera, the genus *Glomus* was predominant followed by *Acaulospora*, *Gigaspora*, *Scutellospora*, *Sclerocystis* and *Entrophospora*. *Glomus aggregatum*, *G. constrictum*, *G. mosseae, G. intraradices, G. fasciculatum* are most abundant species of the genus *Glomus* in tea rhizosphere collected from all the sites. The most common species of *Acaulospora* were found to be *A. bireticulata, A. capsicula* and *A. spinosa*. Large dark red *Scutellospora* spores, mainly *S. calospora*, *S. rubra* and *S. pellucida* are present throughout the year. Sporocarps of

Table 3 : Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *S. rolfsii* that showed homology with the isolate RHS/T-381

AccessionNo.	StrainNo	rDNASequence	Country	Organisms
KC293992	BeanScRs1	663bp	Italy	*Athelia rolfsii*
JF966208	KACC:45155	685bp	Korea	*Athelia rolfsii*
JN241565	1112	646bp	USA	*Athelia rolfsii*
JN241564	176	646bp	USA	*Athelia rolfsii*
JN241563	AS-1	648bp	USA	*Athelia rolfsii*
JN241562	3083	649bp	USA	*Athelia rolfsii*
JN241561	138	649bp	USA	*Athelia rolfsii*
JN241560	1125	649bp	USA	*Athelia rolfsii*
JN241559	1810	647bp	USA	*Athelia rolfsii*
JN241557	WM913	649bp	USA	*Athelia rolfsii*
JN241556	3095	649bp	USA	*Athelia rolfsii*
JN241555	SR2	649bp	USA	*Athelia rolfsii*
JN241558	185	649bp	USA	*Athelia rolfsii*
JN241554	SR1	649bp	USA	*Athelia rolfsii*
JN241553	3082	649bp	USA	*Athelia rolfsii*
JN241552	3087	613b	USA	*Athelia rolfsii*
HQ420816	SR001	684bp	Korea	*Athelia rolfsii*
HM355751	KACC42087	684bp	Korea	*Athelia rolfsii*
FJ968783	S-07	683bp	India	*Athelia rolfsii*
GQ148561	S-08	641bp	India	*Athelia rolfsii*
JQ429785	RHS/T-381	235bp	India	*Athelia rolfsii*

Partial sequence of 28S ribosomal RNA gene

TTATAAATTTTTTTAAATTATAGCCTTTAGAGGAAATACACATTTTCCCTTTTAAGGTTTCAGTCAAGTA
CGAAATAATATAAAAACAAAGGGGGGTAAAAAGTAAAATCCCCATCCGGAAGGGGGATTCTAGCTTGTA
TGTACTACTTATAATATCATGCGCATATATTAGCCTATAAGTGCATATATGGCCATTGACTCAAATCAGT
TGTACCGTTCACTATGGTTCCCTCC

Sequence deposited: NCBI Title: Athelia rolfsii strain RHS/T-381 28S ribosomal RNA gene, partial sequence

ACCESSION:**JQ429785**
VERSION:JQ429785.1
GI:384872367

```
ORIGIN
        1 ttataaattt ttttaaatta tagcctttag aggaaataca cattttccct tttaaggttt
       61 cagtcaagta cgaaataata taaaaacaaa ggggggtaa aaagtaaaat ccccatccgg
      121 aaggggatt ctagcttgta tgtactactt ataatatcat gcgcatatat tagcctataa
      181 gtgcatatat ggccattgac tcaaatcagt tgtaccgttc actatggttc cctcc
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Fig 4: Sequence deposition of 18 S rDNA of *Athelia rolfsii* (anamorph *Sclerotium rolfsii*) strain RHS/T-381

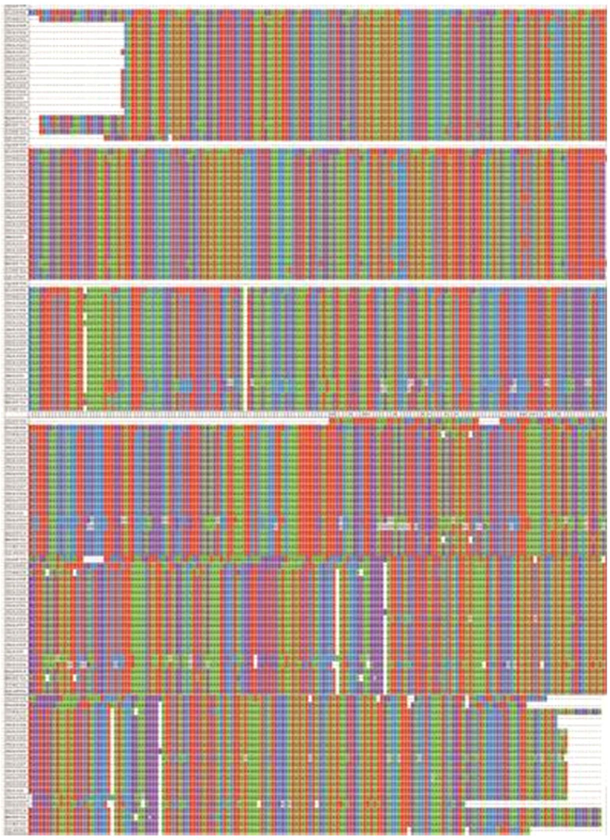

Fig 5: 18S r DNA sequence alignments of *Athelia rolfsii* (anamorph *Sclerotium rolfsii*) (JQ429785) with other ex types isolates. The conserved regions of the gene are demonstrated in different colour.

Sclerocystis with its conspicuous beaded amorphous structures are also present.

Scanning Electron Microscopy of dominant AMF spores and Trichoderma harzianum

Scanning electron microscopic observation was made of three most abundant AMF genera *viz Glomus, Gigaspora* and *Acaulospora*. Different species of *Glomus*, *Glomus mosseae*, *Glomus constrictum* and *Glomus intraradisces* obtained from tea rhizosphere showed difference in their wall characters and ornamentations. There basal attachments of all the different species were distinct which varied in their shape and size from one another . Similarly *Glomus mosseae*, *Gigaspora gigantea*, *Rhizophagus fasciculatum* and *Acaulospora* sp. isolated from tea root rhizosphere showed adhered hyphae with its sloughed and eroded outer hyaline layer covering the whole surface area (Fig. 10). *G. constrictum* shows attached hyphae and few pores in the spore surface where MHB (Mycorrhiza Helper Bacteria) might persist. Image of *Gigaspora gigantea* reveals the outer hyaline layer and the conspicuous curved hyphal attachment. *Acaulospora bireticulata* with ornamentation consists of hyaline to round-tipped polygonal structures and the attached sporiferous sacule. One of the potential strain of *Trichoderma harzianum* originally isolated from tea rhizosphere was also selected as bioinoculant for its evaluation for biocontrol of sclerotial blight disease of tea. Mycelial growth with sporulation and scanning electron microscopic view of spores of *T. harzianum* have been presented in Fig 11.

Indirect immunofluorescence of tea root tissue colonized with AMFungi and AMF spores using PAb of R. fasciculatum

Mass multiplication of *R. fasciculatum* was done in Maize plants (Fig12A) . Successful maize root colonization with *R. fasciculatum* was confirmed (Fig 12C) and microscopic observation was made (Fig 12 E). Harvested spores of AMF were further inoculated in tea roots for their colonization which were also confirmed using PAb of *R. fasciculatum* and FITC conjugates following

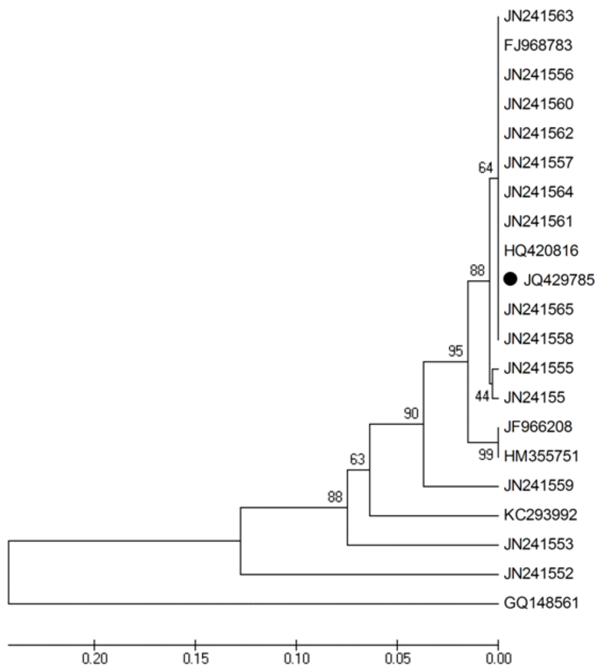

Fig 6 : Phylogenetic placement of *Athelia rolfsii* (anamorph *Sclerotium rolfsii*) JQ429785 with other ex-type strains obtained from NCBI GenBank database by MEGA4 software

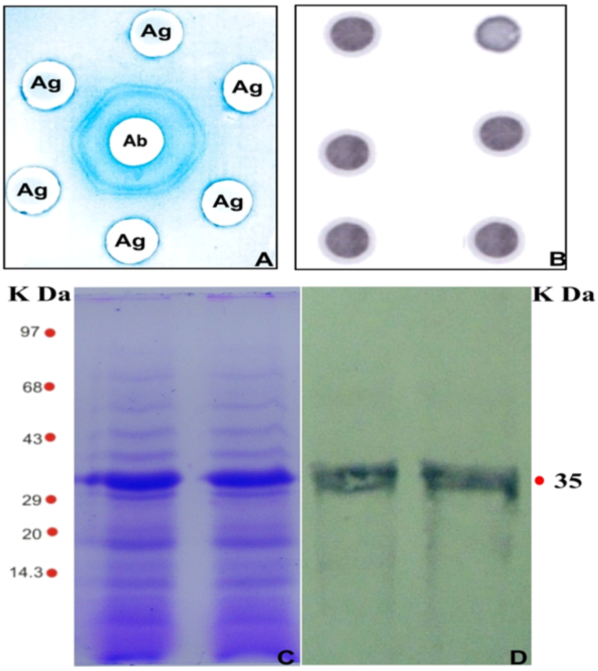

Fig 7: Serological assays of *S.rolfsii*; (A) Immunodiffusion, (B) Dot-immunobinding assay using PAb of *S. rolfsii*; (C) SDS-PAGE and (D)Western blot analysis of *S.rolfsii* using PAb of the pathogen.

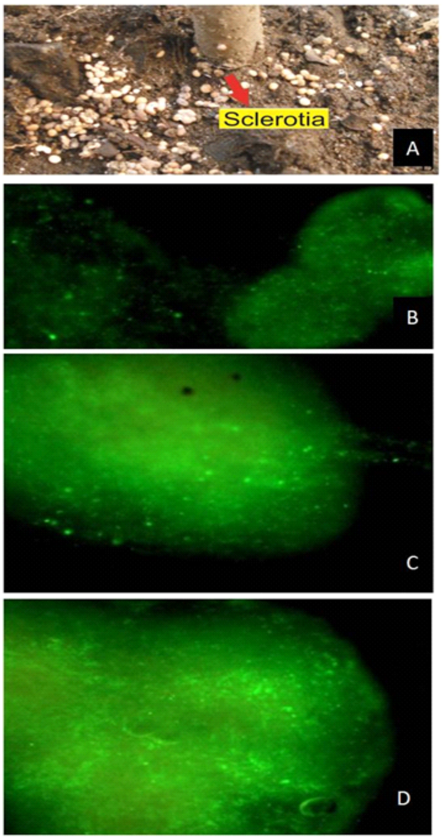

Fig 8 : Indirect immunofluorescence of sclerotia (A) of *S. rolfsii*; young (B) and mature (C&D) sclerotia treated with PAbs of *S. rolfsii* and labelled with FITC showing bright apple green fluorescence.

Fig 9: Compound microscopic observations of Arbuscular Mycorrhizal Fungal spores obtained from different tea gardens. (A) General view of tea garden canopy located at Hasqua TE; (B)Young spore of *Glomus mosseae,* (C) Mature spore of *Glomus mosseae,* (D) *G. intraradisces,* (E) *G. fasciculatum,* (F) *G. constrictum,* (G) *G. ambiosporum* (H) *Glomus* sp. (I) *Glomus badium* (J) *Glomus* sp. (K) *Glomus* sp. (L) *Glomus* sporocarp, (M) *Glomus* sp. (N) *Acaulospora* sp. (O) *Acaulospora bireticulata*, (P) *Acaulospora* sp. (Q) *Acaulospora* sp. (R) *Gigaspora* sp. (S) *Scutellospora* sp.

indirect immunoflurescence. Health root tissue showed major cross reactive antigen showing its compatibility and recognition with PAb of AMF (Fig 12 B). Successful colonization of tea root tissue with AMF as bright apple green fluorescence were evident (Fig 12 D,F& G-J). Bright field compound microscopic views (13A-C) of AMF spores (*Glomus mosseae, Glomus intraradisces* and folded and partially broken spores of *Glomus* sp. collected from tea rhizosphere were also probed with PAb of *R. fasciculatum* and labelled with FITC conjugates. Apple green fluorescence were evident only on the outer walls indicating cross reactive antigen shared between PAb of *R. fasciculatum* and cell surface antigen of AMF spores (Fig 13 D-F).

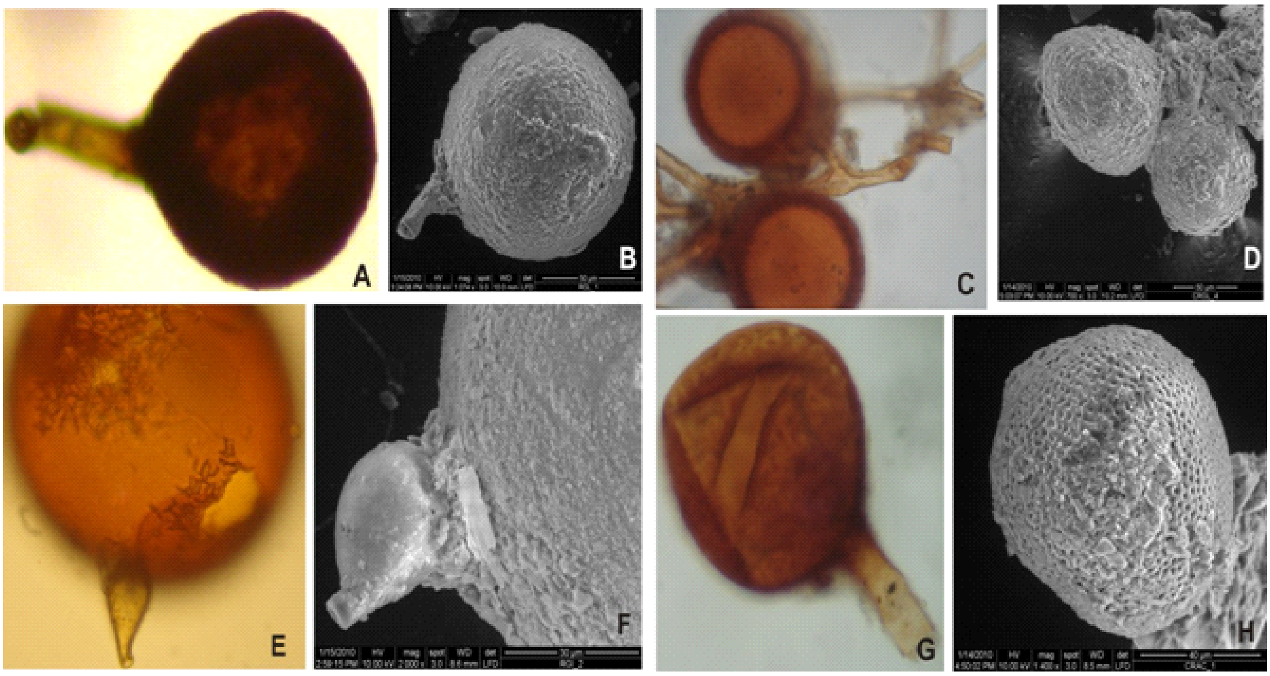

Fig 10 : Bright field compound microscopic views (A,C,E&G) and Scanning electron microscopic views (B,D,F&H) of AMF spores. (A&B) *Glomus mosseae* (C&D) *Rhizophagus fasciculatum* (E&F) *Gigaspora gigantea* (G&H) *Aculospora* sp.

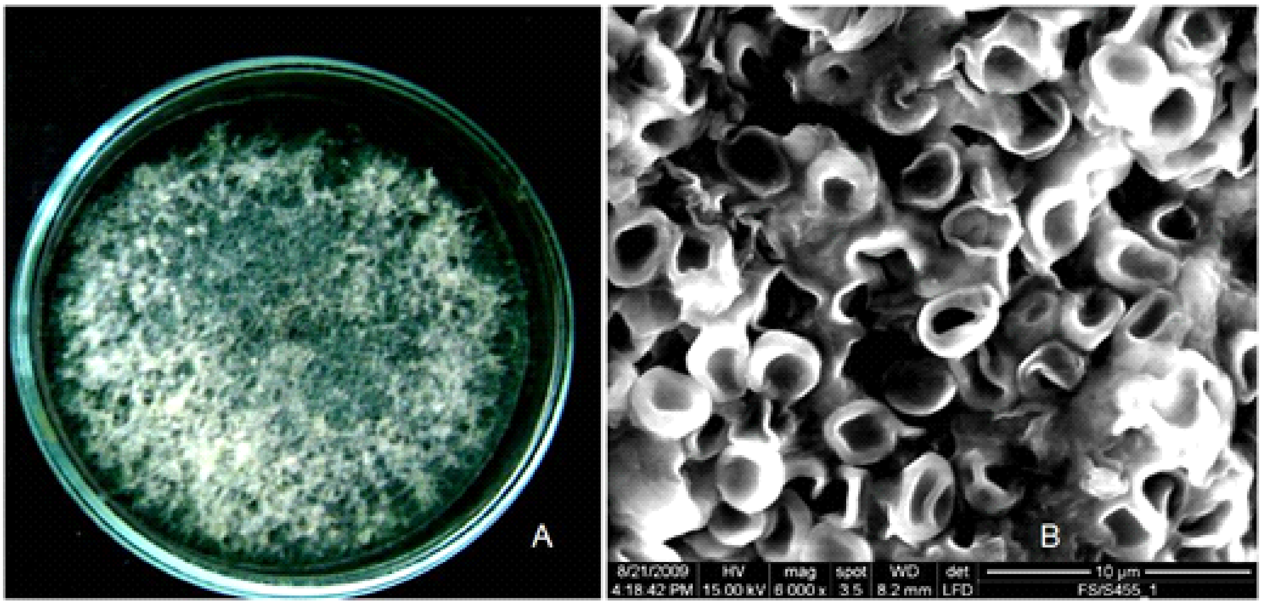

Fig 11: *Trichoderma harzianum* (A) mycelia growth with sporulation in PDA medium and (B) scanning electron microscopic view of spores of *T. harzianum*

Evaluation of bioinoculants for induction of resistance in tea root against S. rolfsii

Tea root colonization separately with *Rhizophagus fasciculatum* and *Gigaspora gigantea* as well as soil amendment with sand-maize meal culture of *Trichoderma harzianum* in management of sclerotial blight disease of tea were tested in pot as well as nursery conditions. Growth of the tea plants following colonization with *Rhizophagus fasciculatum* have been presented in Fig. 14. Under nursery condition, *R. fasciculatum* and *G. gigantea* alone could effectively reduce disease incidence. However, *R. fasciculatum* reduced sclerotial blight disease more than *G. gigantean* in all the tested four tea varieties. It was interesting to note that biocontrol agent (*T. harzianum*) markedly reduced disease incidence

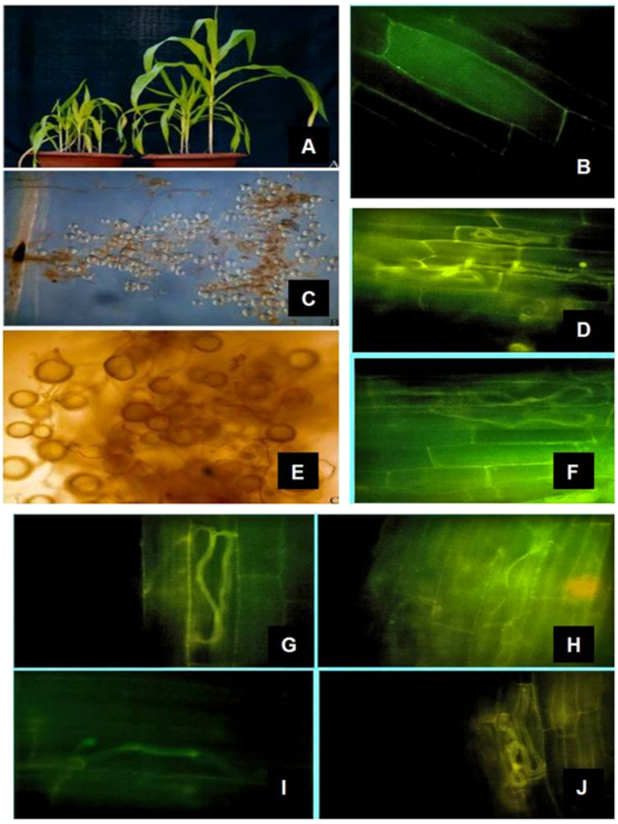

Fig 12: Mass multiplication of *Rhizophagus fasciculatum* in maize plant (A), microscopic view of successfully maize root colonization and multiplication of *R. fasciculatum* (C) and spore (E). Healthy tea root tissue showing its compatibility and recognition with PAb of AMF using FITC labeling showing fluorescence (B) Successful colonization of tea root tissue with *R. fasciculatum* as evident bright apple green fluorescence by indirect immunofluorescence reacting with PAb of AMF and labelled with FITC conjugates (D,F& G-J).

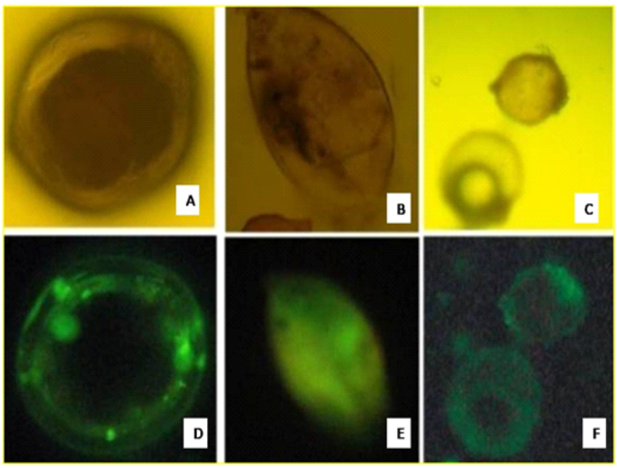

Fig.13: Bright field compound microscopic views (A,B&C) and indirect immunofluorescence of AMF spores (D,E&F) using PAb of *Rhizophagus fasciculatum* and labelled with FITC conjugates showing apple green fluorescence on the outer wall indicating cross reactive antigen shared between PAb of *R. fasciculatum* and cell surface antigen of *Glomus mosseae* (A&D), folded and partially broken spores of *Glomus* sp. (B&E) and *Glomus intraradisces* (C&F)

Fig 14: Plant growth status of nursery grown tea varieties (TV-25, TV-26, TV-20 and TV-09) following root colonization with AMF (*Rhizophagus fasciculatum*)

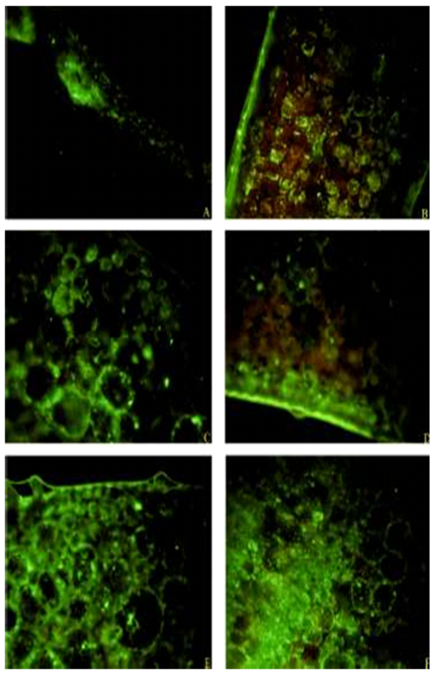

Fig 15: Tea leaf tissues treated with PAb of chitinase enzyme and labelling with FITC conjugates following treatment with *R. fasciculatum, G. gigantea* and *T. harzianum* and challenge inoculation with *S. Rolfsii*. (A) Untreated healthy control, and *S. rolfsii* inoculated : (B) Untreated healthy plants (C) *R. fasciculaum* treated (D) *G. Gigantean* treated (E & F), *T. harzianum* treated.

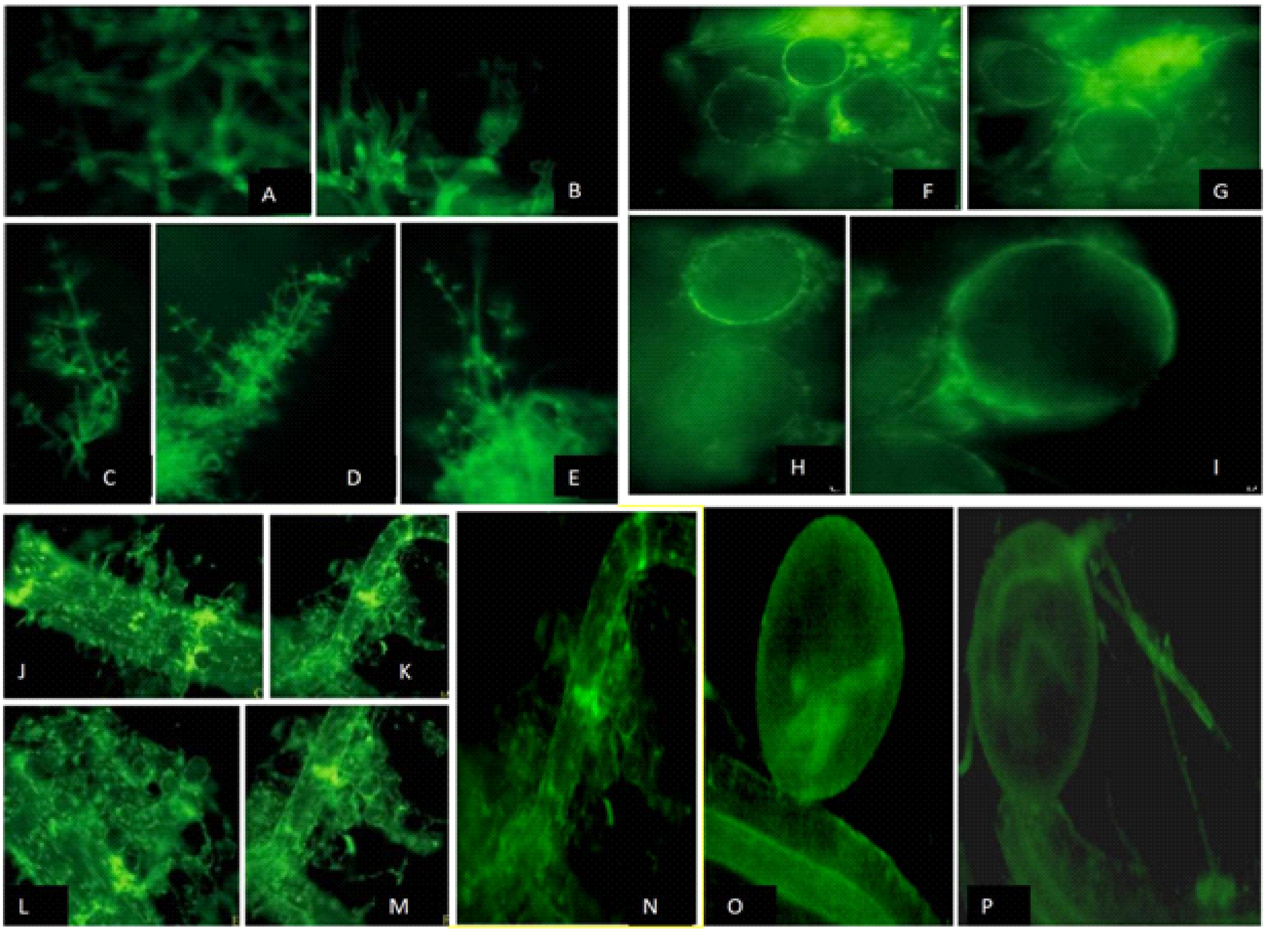

Fig 16: Indirect immunofluorescence of bioinoculants (BCA and AMF) using IgG of (A-E) *T. harzianum* , (F-N) *R. fasciculatum* and (O-P) *G. gigantea* labelled with FITC conjugates showing apple green bright fluorescence of hyphae and phialides of *T. harzianum* (A-E), Hyphae and AMF spores of AMFungi (E-I & O-P), tea root colonization with *R. fasciculatum* (J-N).

recorded after 45 days of pathogen challenge (Table 4).

Biochemical changes associated with induction of resistance in tea plants

Experiments were conducted to assess the effects of bioinoculants following challenge inoculation with pathogen on biochemical components of tea roots. Activities of some of the enzymes which are involved in phenol metabolism as well as in defense-i.e., peroxidase, phenylalanine ammonia lyase, chitinase, â-1, 3 glucanase were also determined. Multifold increase in activities of chitinase, â-1,3 glucanase, peroxidise and phenylalanine ammonia lyase in roots were observed on application of AMF and BCA to soil followed by inoculation with *S. rolfsii* (Table 5) which was significantly higher in those plants which were treated with biocontrol agent (*T. harzianum*).

Cellular location of chitinase in tea leaf tissues following induction of resistance by AMF and T. harzianum

Application of AMF and *T. harzianum* separately in the rhizosphere of tea saplings prior to pathogen challenge could successfully reduce sclerotial blight incidence. Disease reduction was found to be brought about by enhanced activities of key defense enzymes like chitinase, glucanase, phenylalanine ammonia lyase and peroxidase which increased significantly after pathogen challenge. Apart from assessment of defense enzymes, an attempt was also made to determine cellular localization of chitinase in tea leaf tissues following indirect immunofluorescence test using FITC binding and treatment with PAb raised against chitinase. For this most responsive tea variety, TV-20 was selected. Leaf sections from

Table 4 : Disease index of sclerotial blight incidence of tea saplings following root colonization with AM Fungi and soil ammended with *Trichoderma harzianum* and challenge inoculation with *Sclerotium rolfsii* in nursery condition

	Disease index (45 days after inoculation)[a] Pre treated with*			
Tea varieties	*Sclerotium rolfsii*[a]	**G. gigantea+ S.rolfsii*	**R. fasciculatum + S.rolfsii*	**T. harzianum*+ *S. rolfsii*
TV-25	4.18±0.83	2.15±0.55	1.60±0.47	1.30±0.36
TV-26	5.90±0.77	2.18±0.58	1.85±0.38	1.25±0.32
TV-9	4.55±0.72	2.50±0.43	1.75±0.33	1.15±0.43
TV-20	5.25±0.65	1.80±0.47	1.50±0.44	1.20±0.37

[a] Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-40% of roots turn brown; 3- leaves withered and 50% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6-whole plants die, with upper withered leaves Still remaining attached; roots fully rotted

Table 5 : Changes in the level of defense enzyme activities in tea root colonized with AMF and soil amended with BCA and challenge inoculation with *S. rolfsii*

Tea Varieties	Treatment	Enzyme activities			
		POX[a]	PAL[b]	CHT[c]	GLU[d]
TV-25	Control	3.8	62.0	12.5	320
	S. rolfsii	4.5	89.0	18.5	423
	G.gigantea +S.rolfsii	6.5	96.0	20.1	471
	R. fasciculatum +S.rolfsii	8.5	146.0	26.0	535
	T. harzianum +S.rolfsii	9.0	152.0	29.0	546
TV-26	Control	3.3	65.0	16.4	330
	S. rolfsii	4.6	75.0	17.8	482
	G.gigantea +S.rolfsii	5.0	88.0	22.5	549
	R. fasciculatum +S.rolfsii	6.7	132.0	31.5	614
	T. harzianum +S.rolfsii	8.9	149.0	34.0	619
TV-9	Control	4.5	84.0	18.3	450
	S. rolfsii	5.6	97.0	25.5	460
	G.gigantea +S.rolfsii	6.7	119.0	31.5	512
	R. fasciculatum +S.rolfsii	7.9	133.0	35.5	532
	T. harzianum +S.rolfsii	8.7	151.0	39.0	565
TV-20	Control	3.5	78.0	17.5	420
	S. rolfsii	6.6	98.0	27.5	470
	G.gigantea +S.rolfsii	7.7	131.0	27.0	522
	R. fasciculatum +S.rolfsii	8.0	142.0	33.3	537
	T. harzianum +S.rolfsii	9.0	154.0	39.8	543
CD	Treatments	0.983	13.80	2.90	56.92
(P=0.05)	Varieties	0.879	12.31	2.60	50.91

[a] POX activity assayed as " A 465 min^{-1} g $tissue^{-1}$;
[b] PAL activity assayed as µg N-Acetyl glucosamine released by enzyme from 1 g tissue min^{-1},
[c] Chitinase activity assayed as mg Glc-Nac / gm tissue/min and
[d] ß 1,3 GLU activity assayed as µg glucose released by enzyme from 1g tissue min^{-1}

untreated control, untreated pathogen (S. *rolfsii*) inoculated, *T. harzianum* treated and *S. rolfsii* inoculated, *R. fasciculaum* colonized and *S. rolfsii* inoculated as well as *G.gigantea* colonized and *S, rolfsii* inoculated plants were taken. Immunolocalization of chitinase in treated as well as pathogen inoculated tea leaves were observed using FITC labeling and treatment with PAb of chitinase. Both the bioinoculants (*T. harzianum*, *R. fasciculaum* and *G. gigantea*) following challenge inoculation with the pathogen (*S. rolfsii*) showed bright apple green fluorescence in the mesophyll tissues , but maximum fluorescence was evident in *T. harzianum* treated tea leaf tissues following challenge inoculation with the pathogen. (Fig. 15).

Immunodetection of bioinoculants using indirect immunofluorescence

By raising PAbs against bioinoculants used for induction of resistance , BCA (*Trichoderma harzianum*) , AMF (*Rhizophagus fasciculatum* , *Gigaspora gigantea*), confirmation were evident in tea leaf tissues following indirect immunofluorescence. In the present study, we have demonstrated first time immunodetection of bioinoculants using their respective PAb and FITC labeling. Bright apple green fluorescence were evident in hyphae and phialides of *T. harzianum* (Fig 16. A-E), AMF spores of *R. fasciculatum* with hyphal attachments (Fig 16. F-I), successful tea root colonization with *R. fasciculatum* (Fig 16. J-N), AMF Spores of *G. gigaspora* with hyphal attachments (Fig. 16. O-P), following induced resistance in tea plants against *S. rolfsii* .

DISCUSSION

In the present study, initially morphological and cultural characteristics as well as molecular analysis of the pathogen (*S. rolfsii*), scanning electron microscopic observation of bioinoculants (*T. harziaum*, *R. fasciculatum* and *G. gigantea*) was done. Screening for resistance of ten tea varieties against *S. rolfsii* was carried out in sick plot developed specifically for this pathogen. Among the tested ten varieties , four varieties (TV-25, TV-26, TV-9 and TV-20) were found to be less susceptible in comparison with other tea varieties which were selected for evaluation of induced resistance against the pathogen using bioinoculants (BCA and AMF). Induced immunity developed in plants against phytopathogens using bioinoculants have been presented by Chakraborty (2020).

Detection and diagnosis serve related but for distinct purposes. Detection is about confirming whether a specific pathogen is present in a sample—often before any visible symptoms appear, which is especially important for asymptomatic plant material like seeds or propagules. In contrast, diagnosis comes into play once symptoms are visible, aiming to identify the causal agent and understand the nature and origin of the disease. For any detection system to be reliable in routine use, three qualities are critical: specificity, sensitivity, and rapidity. Specificity refers to the ability of a test to correctly identify only the target organism, avoiding false positives and false negatives. Sensitivity is the lowest amount of pathogen that can be consistently detected—essentially, how small a presence the test can pick up. High sensitivity is crucial when pathogen levels are very low, as in early infections or asymptomatic carriers. Rapidity (speed) matters because delays can allow pathogens to spread, especially in agricultural systems.

In the present study, serological detection of tea root pathogen (*S. rolfsii*) has been demonstrated using immuodiffusion, dot immunobinding assay, western blot analysis and indirect immunofluorescence of sclerotia of the pathogen. Serodiagnosis of *Bipolaris sorokiniana* causing spot blotch disease of wheat using PTA-ELISA, DIBA, Western Blot analysis, immuno fluorescence and immunogold localization of pathogen in leaf tissue has also been demonstrated (Chakraborty *et al*., 2016a). For the first time, immunogold localization of cross reactive antigen (CRA) shared by *Camellia sinensis* and *Exobasidium vexans*, causing blister blight disease of tea was demonstrated by Chakraborty and Sharma (2007). Immunological formats used for detection and *in situ* analyses of pathogenic fungi in various economically important crops have been elucidated (Chakraborty and Chakraborty, 2021).

For the first time we have developed immunodetection of bioinoculants, BCA (*T. harzianum*), AMF (*R. fasciculatum* and *G. gigantea*) by raising PAbs against them separately and demonstrated using indirect immunofluorescence labeling with FITC and confirming by bright apple green fluorescence in hyphae, phialides, spores of *T. harziamun* , spores and hyphal attachment of AMF (*R. fasciculatum* and *G. gigantea*) and tea root colonization with *R. fasciculatum.* Further, these bioinoculants on tea plants have been evaluated for the management of sclerotial blight disease. Induced resistance following application of AMF and BCA have been documented. Earlier dual application of AMF (*Rhizophagus fasciculatus*) and PGPR (*Bacillus pumilus*) on tea plants have been demonstrated for induction of resistance against *S. rolfsii* (Chakraborty *et.al.* 2016b). In the present study, enhanced accumulation of defense enzymes following application of bioinoculants (AMF and BCA) separately and challenge inoculation with *S. rolfsii* have been presented. Among these, *T. harzianum* was most effective than *R. fasciculaum* and *G. gigantea*. Using PAbs of these bioinoculants indirect immunofluoresence analyses also confirmed the induced resistance in BCA treated tea plants. Induced immunity developed by *Trichoderma* spp. in plants have also been illustrated (Chakraborty *et.al.,* 2020). Immunodetection of *Rhizophagus fasciculatus* and *Gigaspora gigantea* in soil and root tissues in mandarin (*Citrus reticulate*) plants, their exploitation as bioinoculants and cellular localization of defense enzymes following induced immunity developed against *Fusarium solani* have also been elucidated (Chakraborty and Allay, 2022). Recently, a review on Sero-diagnosis: a novel tool in precise detection of pathogens, bio inoculants andinduced immunity in plants has been published (Chakraborty and Chakraborty, 2025).

DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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