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Evaluation of peroxidase isozymes and antifungal phenolics in tea plants triggered by *Sclerotium rolfsii*

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Peroxidase and antifungal phenolics were extracted separately from healthy and *S. rolfsii* inoculated tea roots of resistant (K 1/1 and TV-26) and susceptible (B-157 and UP-3) tea varieties that inhibited mycelial growth and scelrotial germination of *S. rolfsii.* PAGE analyses of isozymes of peroxidase from roots of healthy and inoculated tea plants were carried out. HPLC profile and UV-analysis of phenolics clearly showed the presence of antifungal compounds in infected tea roots. An antifungal compound isolated from healthy and *S.rolfsii* infected tea roots exhibited clear inhibition zones at R_F 0.58 in a chromatographic bioassay. On the basis of their color reaction on TLC and UV-spectra these were identified to be pyrocatechol. It is also interesting to that extracts from *S.rolfsii* inoculated root tissue gave a maximum absorption peak at 274 nm which was identical to an authentic sample of pyrocatecol. In resistant variety, there was higher accumulation 525-678µg/g fresh tissue of pyrocatechol 96h after inoculation with *S.rolfsii* while a very low concentration(60-93 µg/g fresh) of this compound was found in healthy root tissues.

Key words: *Sclerotium rolfsii*, *Camellia sinensis*, peroxizyme, pyrocatechol

INTRODUCTION

Sclerotial blight caused by Sclerotium rolfsii Sacc. 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 is a wide spread pathogen that affects at least 500 species in about 100 plant families. The interaction between plants and their pathogens is complex and may be very specific to a given combination of the plant and the fungus. Polyclonal antibody based serological formats such as PTA-ELISA, Dot immunobinding assay (DIBA) and indirect immunofluorescence was developed for Sclerotium rolfsii causing sclerotial blight of tea (Bhagat and Chakraborty, 2020).

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 *et al*., 2005). The responses include formation of lignin, the accumulation of cell-wall appositions such as papillae (Prats *et al*., 2005), and the early accumulation of phenols within host cell walls. Resistance in plants towards pathogens depends on a variety of factors. Several authors have demonstrated a distinct correlation between the degree of plant resistance and phenolics present in plant tissues. In the present study an attempts have been made to analyse peroxizyme and antifungal phenolics in tea plants triggered by *Sclerotium rolfsii* .

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MATERIAL AND METHODs

Plant samples

Two resistant varieties (K 1/1 and TV-26) and two susceptible varieties (B-157 and UP-3) of tea were selected for experimental purposes.

Fungal culture

Sclerotium rolfsii (RHS/T-381) was isolated from naturally infected root of susceptible tea plant and on the basis of morphological characters and

microscopic observations initially identified and further 18S rDNA sequence of the isolate RHS/T-381 was done. The BLAST query of this isolate against GenBank database confirmed the identity of the isolate as *Athelia rolfsii* (anamorph of *S. rolfsii*) and deposited in NCBI under the accession no. JQ429785. The fungus was maintained in the Laboratory with subsequent sub-culturing on potato-dextrose agar (PDA) medium.

Isozyme analysis of peroxidase

Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase. Extract for peroxizyme analysis was prepared by crushing 1 g of root tissue of healthy and artificially inoculated with *S. rolfsii* separately in a mortar and pestle in 2 M sodium phosphate buffer (pH 7.0) in ice cold condition and used immediately for the isozyme analysis. For PAGE analysis of peroxizyme, following the method as described by Davis (1964), mini slab gel was prepared and finally mounted in the electrophoretic apparatus. Tris-Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe. Sample (32 ìl) was prepared by mixing the sample enzyme (20 ìl) with gel loading dye (40 % sucrose and 1 % bromophenol blue in distilled water) in cyclomixture in ice cold condition. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe. Electrophoresis was performed at constant 15 mA current for a period of 3 – 4 h at 4°C until the dye front reached the bottom of the gel. 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 stained. Staining of the gel was performed following the method of Reddy and Gasber (1973). The gel was incubated in the aqueous (80 ml) solution of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 78 7 % Acetic acid. After the appearance of clear blue colored bands, analysis of isozyme was done immediately.

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 as suggested by Chakraborty and Saha (1995).. 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.

Chromatographic analysis

Ethyl acetate fractions of both healthy and infected tea roots were analyzed 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 (1994). 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.

Bioassay of antifungal phenolics
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+ 2^{0}C.

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 was 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 80 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.

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.

HPLC analysis

Analysis of antifungal phenolic 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 ìl/min, sensitivity 0.5 aufs, injection volume 20 ìl and monitored at 220 nm (Shimadzu, Japan).

RESULTS AND DISCUSSION

Isozyme profile

Staining of native PAGE demonstrated the existence of isoforms. Analysis of peroxidase showed the existence of three isoforms in resistant variety (TV26) of tea. All the isoforms which were present in untreated control plants were also present in *S. rolfsii* inoculated ones. However, there was clear distinction between the treated and untreated control plants as far as intensities were considered. Maximum intensity of bands was noticed in the treated plants with Rm (Relative mobility) values of 0.93, 0.79, 0.562, 0.452 in TV-26 variety (Fig.1). Among these Rm 0.79 and 0.562 were maximum. PAGE analyses of peroxizymes of naturally blister infected tea leaf tissues also demonstrated the existence of isoforms, and a few of these were constitutively present in healthy leaves, but one of Rm 0.44 was induced after infection with *Exobasidium vexans* in resistant tea plants (Sharma and Chakraborty, 2004).

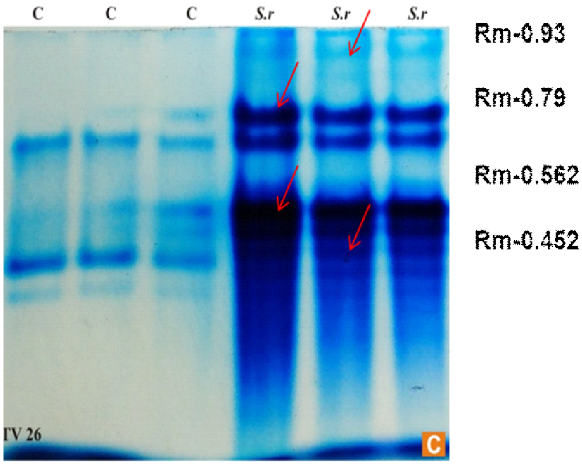

Fig 1. Peroxizyme analysis of Healthy (C) and artificially inoculated tea roots (TV-26) with *S. rolfsii* (*S.r*)

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 phenolic 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.

Bioassay

Crude extract (ethyl acetate fraction dissolved in methanol) prepared from healthy and *S. rolfsii* inoculated roots of four tea varieties (K 1/1, TV-26, B-157 and UP-3) were bio-assayed following radial growth inhibition assay. Results (Table 1) 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 petri dish (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 Petriplate bioassay in relation to distilled water control wherein full sclerotial germination was evident (Fig 2.).

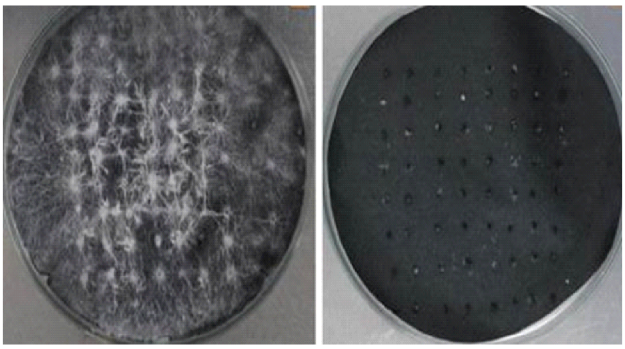

Fig 2. Germination of sclerotia of *Sclerotium rolfsii* in Petri plates on sterilized black paper soaked with sterile distilled water (left) and soaked with sample solution (right).

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. 3 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 (525-678µ g/g fresh wt) of antifungal compound (pyrocatechol) accumulated in resistant varieties than in the susceptible varieties (212-290µg/g fresh wt). Concentration of this compound in healthy root tissues were very low (60-93 µg/g fresh wt).

Table 1: 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	2.3
TV-26	14.2	1.5
Susceptible		
B- 157	19.8	11.6
UP-3	20.0	13.5
Distilled water control	30	

[a]Average of three experimental trial; [b] Inoculated 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. HPLC profile and UV-analysis clearly showed the presence of antifungal compounds in infected tea roots.

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. 4 (A&B). In both cases noticeable peaks were resolved (Table 2), however, in the inoculated sample one new peak was evident. There are several 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.

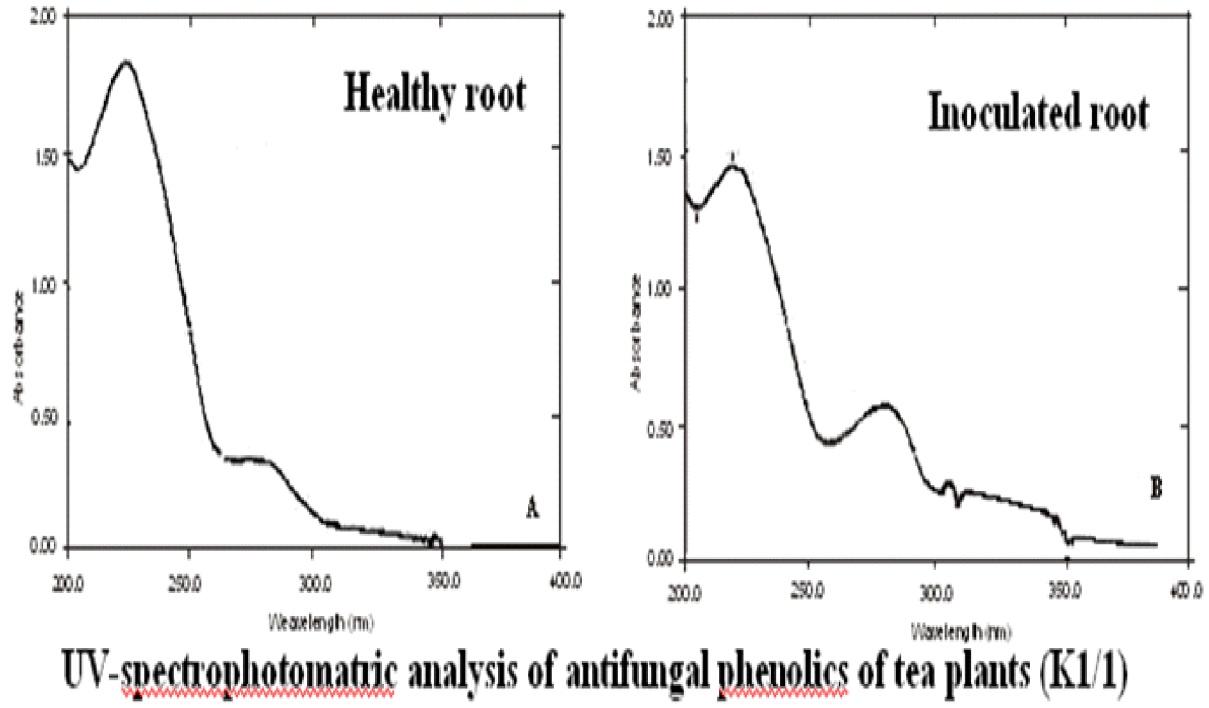

Fig.3 [A] Healthy tea root sample [B] *S.rolfsii* inoculated tea root sample

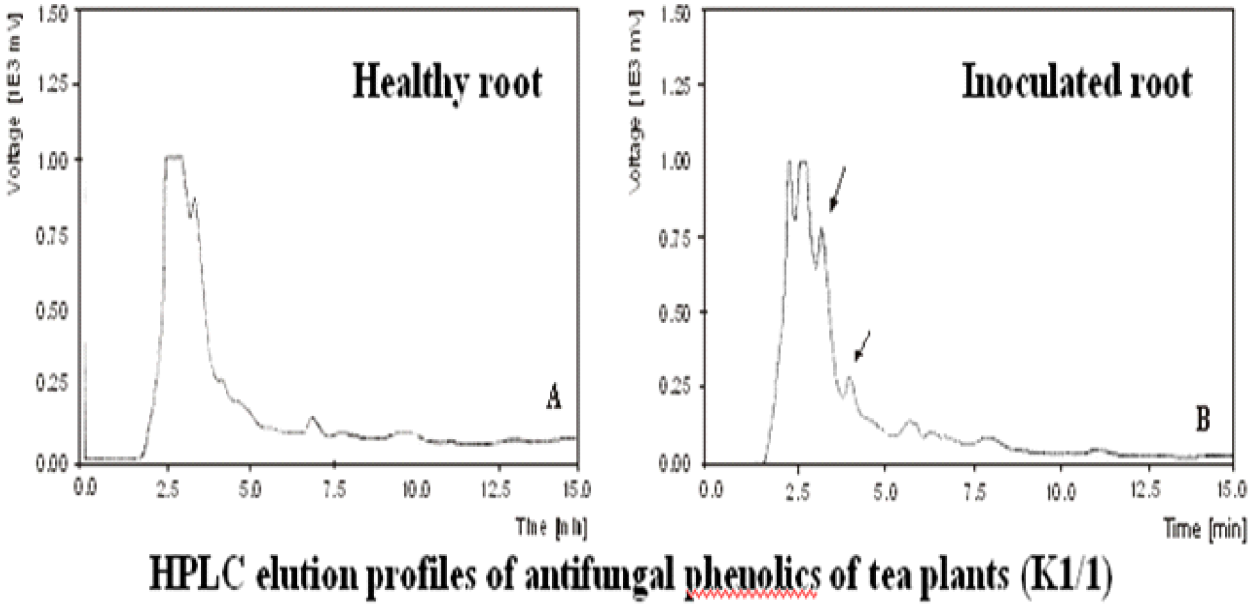

Fig.4. (A) Healthy root sample (B) *S. rolfsii* inoculated tea root sample

It is known that catechin is oxidatively cleaved to some simpler phenols and phenolic acids like catechol, phloroglucinol and protocatechuic acid. An enzyme catechin 2-3 dioxygensis was isolated 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. Chakraborty et al (2004) reported the production of antifungal compounds in tea leaves following infection with blister pathogen

Table 2: 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 with *S.rolfsii*	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

(*Exobasidium vexans*). HPLC analysis of the catechins from healthy and blister infected tea leaves showed marked differences and some quantitative changes (Chakraborty *et al*., 2002). Jham *et al* (2005) have communicated that the antifungal activity of cinnamon bark oil against *A. flavus* and *A. niger*. Similarly, Faria *et al* (2006) have reported the antifungal activity of eugenol isolated from *O.gratissimum* against *Alternaria* and *Penicillium chrysogenum*. Reddy *et al* (2007) have discussed the antifungal activity of isolated from clove that inhibits *Aspergillus* spp. colonizing rice grain grains.

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