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Biochemical and Immunological characterization of isolated cell walls of *Glomerella cingulata* and evaluation of their interaction on tea leaf surface

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Disease of tea caused by *Glomerella cingulata* (Stoneman) Spauld. & Schrenk affects the quality of the harvested foliage in Terai and Dooars regions of North Bengal. The (polyclonal antibody PAbs raised against mycelial antigen of *G. cingulata* were used to characterize the isolated cell walls immunologically using RITC conjugates. The cell walls were further purified to get a single glycoprotein band of 66kDa. Tea leaf surfaces of two varieties – TV-30 and T-17/1/54 were used for evaluation of their interaction . The disease reaction elicited by the isolated cell walls and the spore suspension of *G.cingulata* were monitored. The diffusates were bioassayed and percentage of spore germination and appressoria formation were documented.Itwas found that the isolatedcell walls elicited more fungitoxic activity than the spore.

Key words: Brown blight,*Camellia sinensis*, *Glomerella cingulata*, cell wall ,antifungal compound

INTRODUCTION

Glomerella cingulata (Stoneman) Spauld. & Schrenk is a foliar fungal pathogen that affects the quality of the harvested tea leaves causing brown blight of tea(Fig.1). Eighteen tea varieties are reported to be screened against a virulent strain of *Glomerella cingulata* (Stoneman) Spauld & Schrenk (W7659)(Chakraborty and Som, 2010; Som Paul and Chakraborty, 2016). Two varieties- TV-30 and T-17/1/54, highly resistant and highly susceptible respectively are used for the present investigation.The analysis of the diffusates from leaf surfaces forms an important part in many investigations (Bhajbhuje, 2014). However, in order to determine the mechanism of resistance induction, an inducer of pathogen origin, may also be used. Fungal cell wall is an important participant of plant-pathogen interactions, since it comes directly in contact with the plant tissues right after spore germination. The involvement of cell wall glycoproteins as elicitors has been followed since 1975. Immunoassays for early detection of *G. cingulata* have already been standardized. Pathogen-induced protein of 24kDa has been detected in susceptible varieties in case of brown blight disease (Som and Chakraborty, 2016).However, no work has been done on the role and characterization of the isolated cell walls. The present investigation focuses on the role and analysis of cell extract of *G.cingulata* for better understanding of the fungal elicitor.

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MATERIALS AND METHODS

Plant material

The fully expanded young leaves (3rd and 4th leaves) were collected from the two varieties – TV-30 and T-17/1/54 maintained in tea germplasm bank, Department of Botany, University of North Bengal. The leaves were obtained afresh before artificial inoculation from the tea bushes during the rainy season.

Fungal culture

G. cingulata was isolated from naturally infected susceptible tea plants (TV-22) grown in Phytopathological experimental garden and subsequently the fungus was identified (W7659) from the Diagnostic and Advisory Service, CABI Bioscience UK Center, and routinely subcultured on Richard's medium agar grown under 12 h

photoperiod at 30°C. For preparation of mycelial protein and cell walls, the fungus was grown in liquid Richard's medium for 10 days.

Inoculation technique

Detatched leaf inoculation method was used for artificial inoculation of tea leaves.Percent drops that resulted in lesion production were calculated after 48h of inoculation, as described by Chakraborty and Saha (1994).

Extraction of fungal protein

Mycelial protein was extracted according to the method of Chakraborty and Saha (1994). Mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia were homogenized with 0.05 M sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$ and 0.05mM $MgCl_2$ at 4°C with sea sand in mortar and pestle. The homogenate was centrifuged at 10,000rpm for 15min. at 4°C and the supernatant obtained was used for protein analysis immediately and stored at -20°C for future use.

Isolation of cell walls

Cell wall was isolated from *G. cingulata* following the procedure of Keen and Legrand (1980). Mycelium of 10-day-old culture was collected and dried by vacuum filter. Fresh packed cells(20g) of were ground for 1 min in a high speed blendor with 80ml ice-cold water and disrupted in a homogenizer at 5°C for 1min. , then centrifuged for 1 min. at 1500g the supernatant fluids discarded, and the sedimented walls washed with 200ml ice-cold water and pelleted by centrifugation repeatedly and the isolated cell walls were frozen and kept at -20°C.

These were extracted by the method of Chakraborty *et al* (1996). Isolated cell walls (40ml/g cell walls)were suspended in ice-cold 0.1 (N) NaOH by blending in a chilled mixer-cup at full speed for 20sec.and slowly stirred in an ice bath for 15h. Following centrifugation at 8,000g for 10min., the residue was washed with ice-cold water and the pooled supernatants were carefully neutralized to pH7.0 with 1(N) HCl and finally dialysed against double distilled water and concentrated with PEG. This was used as a mycelial wall extract (MWE) for elicitor studies and characterization.

Estimation of protein content

Soluble protein content for mycelia protein and MWE was estimated following the method as described by Lowry *et al* (1951).To 1ml of protein sample (taking 10^{-1} or 10^{-2} dilution) 5ml of alkaline reagent (0.5 ml of 1% $CuSO_4$ and 0.5 ml of 2% sodium potassium tartarate, dissolved in 50 ml of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 min. at 30^0+ 20^0C. and then 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for 30 min., following which optical density was measured at 700nm.Quantity of protein, was estimated from the standard curve made with bovine serum albumin (BSA).

Estimation of carbohydrate content

The carbohydrate content was estimated according to the method of Plummer (1978) with Anthrone Reagent. 8ml of MWE was pre-treated with 1ml of 0.3(M) $Ba(OH)_2$ and 1ml of 5%$ZnSO_4$. After centrifugation at 3000g for 10min., supernatant was collected. 1ml of supernatant was mixed with 4ml of Anthrone reagent (0.2g of Anthrone powder in 100ml of concentrated H_2SO_4). The mixture was kept in boiling water bath for 10min. After cooling, absorbancy was measured in a colorimeter at 620nm. The carbohydrate content was estimated using glucose as a standard.

Collection of leaf diffusates and bioassay

Diffusible compounds from tea leaves were collected following drop diffusate technique. Each leaf was slightly wounded with a sterile needle. 20ml droplets (2-4 per leaf) of sterile distilled water or conidial suspension or crude cell wall of *G.cingulata* (1.2 x 10^6 conidia ml^{-1}) prepared from 10 days old cultures with sterile distilled water were placed on the dorsal surface of each leaf. Finally, diffusates were passed through millipore filter and then used for experimental purpose.Method of Rouxel *et al* (1989)was followed for bioassay of diffusates. The spores were allowed to settle and adhere to the slide for at least 1h. The water was carefully removed with a blotting paper. Equal amount of test solution was added and incubated in humid chamber for 18h, after which the test solution was removed. Spores were stained with

cotton blue and the percentage of spore germination, germ tube length and appressoria formation was determined from at least 200 spores in each treatment under high power (x450).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE)

SDS-PAGE analysis of fungal protein and the extracted cell wall was carried out on 10% gel as suggested by Sambrook *et al.*(1989). Protein extracts (50 mg/ml) along with standard molecular weight markers were loaded on the gel and separated at 18mA for 3h. Half of the gel was fixed and stained for protein and the other half - an exact replicate was stained for carbohydrate.

Fixing and staining for protein

The gel was fixed overnight in glacial acetic acid : methanol : water (1:2:7) , stained in coomassie blue R250 (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 °C with constant shaking.

Fixing and staining for carbohydrate

After electrophoresis, gel with mycelial and cell wall protein with replica was fixed overnight in fixer solution (40% ethanol, 5% glacial acetic acid, aqueous). Staining was done with Periodic acid-Schiff's (PAS) regent as described by Segrest and Jackson (1972). The gel was treated with 0.7% Periodic acid solution (0.7g periodic acid in 100ml of 5% acetic acid) for 2-3h. The gel was then washed with 0.2% $Na_2S_2O_5$ solution (0.2g $Na_2S_2O_5$ in 100ml of 5% acetic acid) for 2-3 hours with change of 30min. each. After addition of Schiff 's reagent (10g of Basic Fuchsin dissolved in 2L of hot double distilled water, cooled and 200ml of 1N HCl and 17g of $Na_2S_2O_5$was mixed to the solution until it is decolorized), bands appear after 12-18 hours at 30±2⁰C. The gel was stored at 4°C.

Polyclonal antibody (PAb)preparation

.Polyclonal antibodies were raised against the 8 days old fungal antigen (*G. cingulata*) in New Zaeland white rabbit according to the method of Chakraborty and Purkayastha (1983). Prior to immunization, normal serum was collected from the rabbit by marginal ear vein puncture. Antisera were sored at -20°C until required. Immunoglobulin G (IgG) was purified by ammonium sulphate precipitation and ion-exchange chromatography using diethylaminoethyl (DEAE) cellulose column. Concentration of IgG was calculated according to the method of Jayaraman (1996).

Immunofluorescence

Immunofluorescence of the mycelia and isolated cell walls was done as described by Chakraborty *et al* (1995). Mycelia or cell walls were washed with phosphate buffered saline (PBS) at pH 7.2 by centrifugation at low speed, treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1 h at room temperature. These were washed thrice with PBST (phosphate buffered saline-Tris) as mentioned above and treated with goat anti-rabbit immunoglobulin G (IgG) whole molecule conjugated with fluorescein isothicyanate (FITC) (Sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45 min at 30 ± 2⁰C, again washed in PBS and mounted in 10% glycerol. The observations were made under UV light using a Leica Leitz Biomed microscope with fluorescence optics suitably equipped with I3 filter. Photographs were taken using 800ASA film.

RESULTS AND DISCUSSION

Initially, progress of spore germination on different surfaces, that is, glass surface, dorsal and ventral surfaces of susceptible (T-17/1/54) and resistant (TV-30) varieties was assessed at 6h, 18h and 24h. after initiation. It was found that the spores germinated producing distinct germ tubes 6h after inoculation in case of glass slide as well as on ventral surface of T-17/1/54 leaves (Table 1). Germination was not initiated al all in case of resistant variety on ventral surface at this stage.High percentage of appressoria were produced 18 h after inoculation on glass slide(69.4%) as well as T-17/1/54 dorsal surface (67.7%), while on TV-30 leaf surface this percentage was very low (7.3%). At 24 h after inoculation the percentage of appressoria formation reached its peak. However, it was highest on ventral surface of T-17/1/54 (74.2%) and lowest on the dorsal surface of TV-30 (15.4%). Germination started earlier on the surface of the susceptible variety, which was highly conducive for appressoria formation. Penetration hyphae were produced 48 h after inoculation and germ

Table 1: Effect of incubation period on spore germination of *G. cingulata* on different surfaces

Surface	Incubation period(h)	% spore germination	% appressoria formation[a]	Germ tube length(μm)[b]
Glass				
	6	21.5 ± 2.50	0	21.1 ± 1.5
	18	63.6 ± 2.6	69.4 ± 2.9	63.2 ± 3.5
	24	75.7 ± 3.5	73.6 ± 2.5	77.3 ± 2.9
Leaf				
Dorsal surface (T-17/1/54)				
	6	24.3 ± 3.30		20.6 ± 3.5
	18	67.6 ± 2.2	60.9 ± 3.7	43.8 ± 3.4
	24	74.1 ± 2.7	70.8 ± 2.2	61.2 ± 2.8
Dorsal surface (TV-30)				
	6	05.8 ± 1.80		04.5 ± 2.4
	18	60.3 ± 2.0	07.2 ± 1.8	55.6 ± 4.7
	24	73.1 ± 1.9	15.4 ± 2.0	69.4 ± 2.3
Ventral surface (T-17/1/54)				
	6	10.5 ± 2.50		06.1 ± 2.8
	18	64.9 ± 3.6	67.7± 2.5	46.2 ± 2.6
	24	78.1 ± 1.5	74.2 ± 3.2	50.3 ± 2.7
Ventral surface (TV-30)				
	6	0	0	0
	18	59.2 ± 2.4	18.3 ± 2.1	83.3 ± 3.1
	24	72.6 ± 3.0	25.6 ± 3.5	96.1 ± 2.9

Means ± SE, n=3
Incubation temperature ± 25°C. R.H. 90%
[a]Average of 300 spores per experiment
[b]Average of 60 germlings per experiment

Table 2: Comparison of lesion production by mycelial wall extract and spore suspension of *G. cingulata* on detached tea leaves of resistant and susceptible varieties

Variety	Treatment	Percentage lesion formation		
		Hours post inoculation		
		48	72	96
TV-30	Distilled water	0	0	0
	Mycelial wall extract	02.3 ± 1.45	03.8 ± 1.80	05.4 ± 1.74
	Spore suspension + Mycelial wall extract	10.1 ± 1.82	12.5 ± 2.44	14.6 ± 1.88
	Spore suspension	12.4 ± 2.13	19.3 ± 1.21	22.9 ± 0.94
T-17/1/54	Distilled water	0	0	0
	Mycelial wall extract	15.3 ± 3.01	18.5 ± 2.46	20.1 ± 2.77
	Spore suspension + Mycelial wall extract	52.8 ± 2.04	54.1 ± 2.11	55.8 ± 2.36
	Spore suspension	71.4 ± 3.25	94.3 ± 2.55	95.9 ± 1.47

Data are the mean of 200 inoculum droplets made on 50 leaves of each variety per experiment Values are means ± SE, n=3

tubes were no longer distinct , thus it was not possible to take any reading at this stage when the symptoms on the leaf were distinct.Next , the mycelia wall extract (MWE) of *G. cingulata* was prepared and disease reaction was assessed. The MWE itself did cause very mild symptoms. Nevertheless, it mimicked the symptoms elicited by the fungal spore suspension, developing higher

Table 3: Spore germination bioassay of diffusible compounds elicited by mycelial wall extract of *G. cingulata*

Treatment	Variety	Spore germination[a] %	Appressoria formation %
Distilled water	TV-30	55.9 ± 2.08	52.1 ± 2.11
	T-17/1/54	62.4 ± 1.09	60.4 ± 1.55
Mycelial wall extract	TV-30	09.1 ± 1.29	0.7 ± 0.09
	T-17/1/54	44.5 ± 1.22	39.2 ± 1.43
Spore suspension	TV-30	13.9 ± 2.08	12.1 ± 2.11
	T-17/1/54	66.4 ± 1.13	65.1 ± 1.55
Mycelial wall extract + Spore suspension	TV-30	11.5 ± 1.28	10.2 ± 1.88
	T-17/1/54	52.7 ± 1.24	50.9 ± 1.79
Slide control:			
Distilled water		73.4 ± 1.64	70.3 ± 2.42
Mycelial wall extract		70.1 ± 3.06	68.6 ± 1.09

[a] Average of 200 spores per experiment
[b] Average of 60 germlings per experiment; Values are means ± SE , n=3

Fig. 1 : Symptoms of brown blight disease in the field condition

percentage lesion formation in susceptible tea variety T-17/1/54 (20.1% at 96h after inoculation) and lesser percentage lesion formation in the resistant variety TV-30 (5.2% at 96h. after inoculation) as evident from Table 2. Similarly, cell wall fragment elicitors have been reported to elicit defence-related responses such as HR (hypersensitive response). It has been reported that the crude oligosaccharide extract was purified from mycelial mats of *Fusarium oxysporum* that induced HR in pearl millet seedlings against downy mildew disease.

In the present study, the diffusible compounds elicited by the MWE have been bioassayed and found to be fungitoxic especially in resistant variety (Table 3; Fig. 2 A & B). Enhanced accumulation of antifungal compounds in the resistant varieties of tea has also been reported in case of *Pestalotiopsis theae*. Leaf diffusates of untreated plants possessed a weak fungitoxicity that

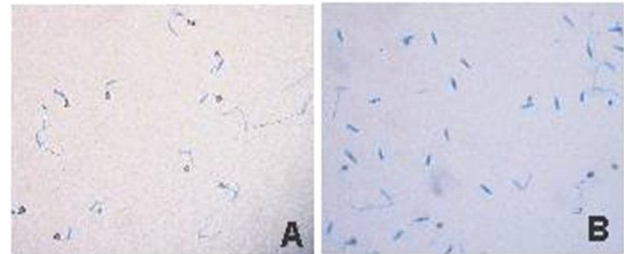

Fig. 2 : Bioassay of diffusates elicited by MWE (mycelia wall extract) on T-17/1/54 (A) and TV-30 (B)

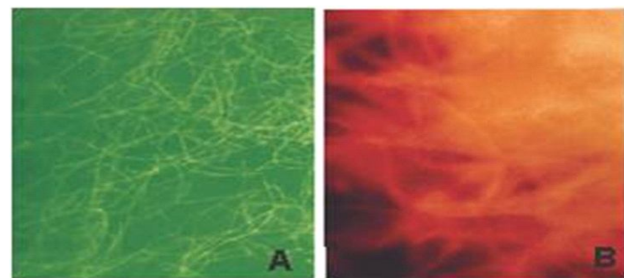

Fig. 3 : Indirect immunofluorescence of hyphae of *G. cingulata* treated with PAb of *G. cingulata* and labeled with FITC conjugates (A) and RITC conjugates (B).

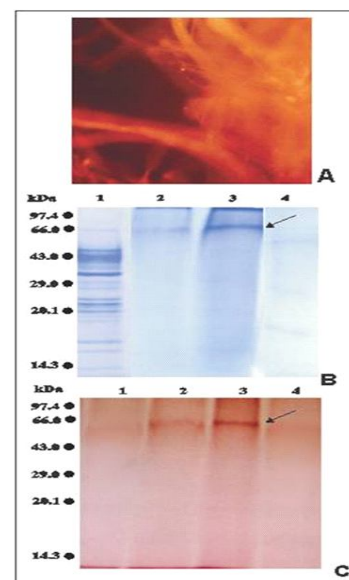

Fig. 4 : Indirect immunofluorescence of isolated cell walls of *G. cingulata* treated with PAb of *G. cingulata* and labeled with RITC conjugates (A); SDS-PAGE analysis of mycelia protein (Lane 1), undialyzed cell wall extract (Lane 2), cell wall extract after dialysis (Lane 3) and crude isolated cell walls (Lane 4) after staining with coomassie blue (B) and Periodic Schiff's reagent (C).

increased slightly after leaf infection in case of rice blast disease.Low fungitoxicity was found in the control sets.

The effectiveness of PAbs was checked with mycelia using FITC and RITC conjugates (Fig. 3 A & B). Both the conjugates were found to be effective – the former showing apple green fluorescence and the latter showing distinct red fluorescence on the hyphal strands.

These PAbs were also used to visualize the crude cell walls under fluorescence microscope using RITC conjugate (Fig. 4A).Quantitative and qualitative estimation of total protein and carbohydrate content was performed in order to confirm glycoprotein nature of MWE. The protein content in the MWE was estimated to be 10mg g^{-1} cell wall while,carbohydrate content was found to be 0.414 mg g^{-1} cell wall. This preparation was further analysed by SDS-PAGE. Mycelia, crude cell wall and cell wall extract were run on SDS-PAGE and replicates were stained for protein (Fig.4B) and carbohydrate (Fig. 4C) separately.

The results show that only a single high molecular weight protein band was visible in cell wall extract preparation as compared to the mycelial protein banding pattern. The same band was visible when stained for carbohydrate by PAS staining. The mycelial wall extract analysed before being concentrated by PEG also shows presence of the same band, but it was very faint. No visible carbohydrate moiety was detected in mycelial preparation and crude cell wall. The molecular weight of the single glycoprotein band was found to be of ca 66.0 kDa.Priviously electrophoretic analysis of cell wall protein of *Penicillium* sp. was performed and indicated that it contains proteins of approximately 50 kDa and 69 kDa. The glycoprotein nature of the mycelial cell wall has been reported by a number of workers which is confirmed in the present case of *G.cingulata*. This glycoprotein may act as a trigger in the elicitation of defense reaction pathway during pathogenesis.

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