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Elicitation of antifungal compounds by *Glomerella cingulata* inducing defense against Brown Blight disease of tea

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Varietal resistance for *Glomerella cingulata* tested on ten different tea varieties under identical conditions confirmed TV-18 and TV-26 as susceptible and resistant ones respectively. Leaf diffusates collected, centrifuged and analyzed from the resistant varieties showed at least four to five times higher fungitoxicity than those from susceptible ones. Bioassay of mycelia wall extract of *G. cingulata* was performed to determine whether it elicited any antifungal compound. Out of four treatments applied on the leaves of resistant variety (TV-26)- distilled water, spore suspension, distilled water mycelial wall extract (MWE) and spore suspension with MWE, spore suspended in MWE revealed high inhibitory effect on spore germination and appressoria formation of *G. cingulata* indicating MWE may elicit antifungal compound production. Glycoprotein nature of cell wall extract was confirmed by staining for carbohydrate and protein with periodate-Schiff's and Coomassie blue R250 respectively following SDS-PAGE. Detection of strong fluorescence in hyphae and isolated cell walls of *G. cingulata* with FITC labeled concanavalin A also confirmed glycoprotein nature of the cell wall extract. An antifungal compound isolated from healthy, *G. cingulata* inoculated tea leaf (TV-26) as well as spore suspension of *G. cingulata* with MWE 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, which gave a maximum absorption peak at 274 nm which was identical to an authentic sample of pyrocatecol. It is interesting to note that higher accumulation (625µg/g fresh tissue) of pyrocatechol was evident in tea leaf tissue (TV-26) elicited by spore suspension and mycelial wall extract (MWE) of *G. cingulata* than spore suspension of the pathogen alone (540µg/g fresh tissue), while a very low concentration (96 µg/g fresh tissue) of this compound was found in healthy leaf tissues.

Key words: Elicitor, *Glomerella cingulata*, glycoprotein, mycelial wall extract, tea, pyrocatechol

INTRODUCTION

Tea is made from the young leaves and unopened leaf buds of the tea plant. A number of fungal pathogens cause foliar diseases of tea, which assume extreme importance economically, as even slight damages to the leaves reduces the quality and quantity of tea production throughout the world (Chakraborty and Chakraborty, 2018). A commonly occurring pathogen on tea leaves in all tea growing areas is *Glomerella cingulata* (Stoneman) Spauld. & Schrenk (=*Colletotrichum camelliae* Massee), causing brown blight disease (Fig.1A-C). The disease patches usually start on the margin of leaves and spread inwards and when two or more patches occur side by side whole leaf may be affected. The edge of patches are sharply defined and mostly marked with a delicate concentric zonation. The colour on the upper surface is chocolate brown at first gradually chage to grey from centre onwards. Polyclonal antibody based immunodiagnosis for early detection of this brown blight pathogen (*G. cingulata*) has been described (Chakraborty *et al*, 2002; 2008). Pathogen-induced protein of 24 KDa has also been reported in susceptible tea variety towards brown blight disease development (Som and Chakraborty, 2016).

A thorough understanding of the physiology and biochemistry of host-pathogen interaction has made it clear, in recent years, that very effective mechanisms of defense enable plants to ward off

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potential attacks by an enormous number of pathogens. Resistance of a plant against pathogen depends chiefly on the metabolic processes of its cells preceding or following infection or other stresses. It is well established that incompatible interactions between plant and microorganisms results from some early recognition event leading to the activation of plant defense mechanisms (Chakraborty, 2018).

Tea plants exhibit a wide of range of defense strategies against pathogen attack. The resistance against pathogens is performed by both preexisting and induced defense systems. In general, inducible defense responses are triggered following recognition of a range of chemical factors termed 'elicitors' (Hammond-Kosack and Jones 2000). Elicitors are compounds stimulating any type of plant defense. This broader definition of elicitors includes both substances of pathogen origin i.e. exogenous elicitors and compounds released from plants by the action of the pathogen i.e. endogenous elicitors (Angelova *et al.* 2006) . These are natural biocompounds which modulate plant growth, trigger plant defense responses resulting in impaired pathogen development. Elicitors are classified as physical or chemical, biotic or abiotic, and complex or defined depending on their origin and molecular structure (Thakur and Sohal, 2013). Biotic elicitors are seen to trigger Phytoalexin accumulation or hypersensitive response which are molecules of either pathogen or host origin. Examples of such elicitors are yeast extract and microbial cell-wall preparations. In recent years, the exact molecular structure of an increasing number of elicitors has been elucidated, including various polysaccharides, oligosaccharides, proteins, glycoproteins, and fatty acids.

In the present investigaton attempts were made to isolate the antifungal compound (s) elicited in tea leaves by the mycelial cell wall extract of *Glomerella cingulata* inducing defense against brown blight disease.

MATERIALS AND METHODS

Plant Culture

The 18 month old plants of ten different tea varieties (TV-18, TV-20, TV-23, TV-25, TV-26, TV-27, TV-28 ,Teen Ali 17/1/54, CP-1 and TS-449) were selected for plantation in the Phytopathological Experimental Garden, Department of Botany, University of North Bengal, on basis of their growing stability as observed over the years by Bezbaruah and Singh (1988). Both the clonal and seed varieties were collected from the vegetative propagation nurseries of some renowned tea estates located in the Himalayan foothills of Darjeeling district.

Fungal Culture

A virulent strain of *Glomerella cingulata* (Stoneman) Spauld. & Schrenk.was originally isolated from naturally infected leaves of TV- 18 was identified by the Commonwealth Mycological Institute, Kew, Surrey,UK (IMI No.356806). The strain was used in all studies after completion of Koch's postulate.

Inoculation technique and assessment of disease intensity

Leaf diffusates were obtained following drop diffusate technique of Muller (1958) with modifications. Conidial suspension of *G. cingulata* ($1.2x10^6$ conidia/ml) prepared from 10-day old cultures with sterile distilled water. Similarly, the water drops were collected as exudates from the leaf surfaces, in both cases, diffusates and exudates were combined, centrifuged and the supernatants were passed through sintered glass filter and immediately assayed for their effect on spore germination of the pathogen.

Preparation of mycelial wall extract

Isolation of cell walls from *G. cingulata* was done following the method of Keen and Legrand (1980) with modification. Mycelium from 8-day old log phase fungus cultures was collected on fiter paper in a Buchner funnel, and 20g of fresh packed cells were ground for 15-30 sec. in a homogenizer at 50^0C with 80 ml distilled water. The slurry was centrifuged for 1 min at 1500xg, the supernatant fluids discarded sedimented walls washed with distilled water (10ml/g) and pelleted by centrifugation at least six times or until the supernatant fluids were visually clear. Finally the isolated cell walls were frozen and kept at -20^0C.

Extraction of isolated cell walls

Isolated cell walls were suspended in ice cold 0.1(N) NaOH (40ml/g) by blending in a chilled mixer at

full speed for 20 sec. Then the suspension was slowly stirred in an ice bath for 15h. Following centrifugation at 8000Xg. The residue was washed with ice cold water and the pooled supernatants were carefully neutralized to pH 7with 1N HCl at 4^0 C and finally dialyzed against water and concentrated, which were then used at crude mycelia wall extract (MWE). Cell walls were extracted following Falcone and Nickerson (1956) method with modifications.

Bioassay of mycelial wall extract

Crude neutralized extracts were placed on the adaxial surface of tea leaves, kept in a humid chamber and collected for bioassay. The collected crude extracts were assayed for fungitoxicity by spore germination method (Rouxel *et.al.*1989).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS- PAGE was performed following the method of Marriott (1977) with 10% acrylamide for the separation gel and 5% for the stacking gel. Cell wall extracts were dissolved in a solution of 1% SDS and 1% mercaptoethanol and heated at 500 C for 2h. To dilute the sample by adding a volume of 4M urea containing 5% sucrose and 25-50 ml of samples were applied per gel tube. Electrophoresis was performed at 1.5mA per gel until the samples penetrated the separation gel and then at 2.5mA per gel. After run, the gels were removed from the tubes each was fixed by shaking for 20 h in 25% isopropanol in 10% acetic acid for protein staining, or in 40% ethanol in aqueous 5% acetic acid for carbohydrate staining. Replicate gels were then stained for protein with Coomassie Blue R 250 or for carbohydrate with periodate Schiff's reagent. The gels were finally destained until the background was clear. Molecular weights were determined by concomitant run of a mixture of 6 proteins (carbonic anhydrase, ovalbumin, bovine plasma, phosphorylase, ß-galactosidase and myosin) ranging from 29 to 205 KD molecular weight.

Binding of fluroscein labeled concanavalin A to G. cingulata

For Binding of fluroscein labeled concanavalin A to *G. cingulata,* the method of Keen and Legrand (1980) was followed. Mycelium or isolated cell walls were treated with buffered saline (0.85% NaCl in 0.01 M potassium phosphate, pH 7.4) containing 1mg/ml fluroscein isothiocyanate (FITC) labeled concanavalin A (Con A, sigma chemicals). The samples were washed thrice with the saline solution only by repeated low speed centrifugation and re- suspension. All preparations were viewed under Leica photomicroscope equipped with epifluorescence optics (BP 450-490 exciting filter, RKP 520 beam splitting mirror, 515 suppression filter).

Extraction of antifungal compounds from tea leaves triggered by G. cingulata

Antifungal compounds from tea leaf samples of resistant variety (TV-26) were extracted following the method as described by Daayf *et al.,* (1995). Leaf samples (500 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 (1994). 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 leaves were analyzed by thin layer chromatography (TLC) on silica gel G 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, 1999). Colour reactions and Rf values were noted.

Bioassay of antifungal compounds

Radial growth

Ethyl acetate fractions of healthy and infected leaf

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 5-day-old culture of *G. cingulata* grown in PDA and was placed in the center of each Petri plate. Radial growth of *G. cingulata* was recorded after 96h of incubation at 28+ 2^0C.

Spore germination

Sample solution was placed on a clean grease free slide, and it was dried. Conidial suspension of *G. cingulata* 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 spore suspension were placed on sterile distilled water. Slides were observed under the microscope and percentage of germination and appressoria formation was determined. Germination percentage was computed and photographs were taken.

UV- spectrophotometric analysis

For spectral analysis of antifungal compounds extracted from healthy and *G. cingulata* inoculated leaf tissue, 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 in spore 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.

RESULTS AND DISCUSSION

Varietal resistance test

Pathgenicity of *Glomerella cingulata* against ten varieties of tea was evaluated based on detached leaf inoculation technique. Among the ten tea varieties tested, TV-18 was found to be most susceptible with 95% lesion production (96h) while TV-26 was highly resistant with only 28% lesion production even after 96h of inoculation (Fig 2).

Studies on biological activities of exudates and diffusates of tea leaves

As results of pathogenicity test revealed that different varieties of tea showed differential response

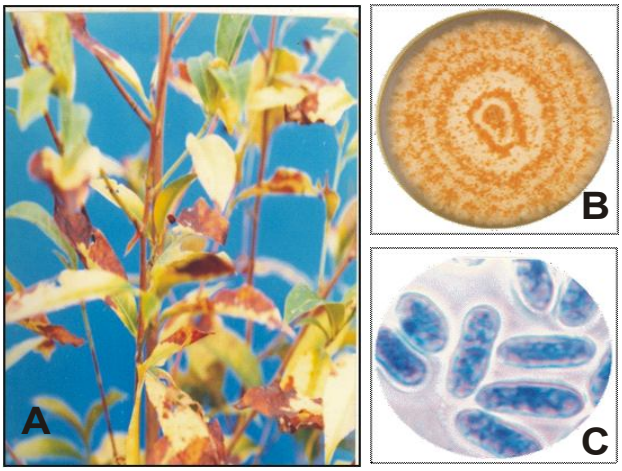

Fig.1 : (A-C) Natural symptom of brown blight disease of tea (A), Mycelial growth with sporulation on PDA medium (B) and conidia (C) of *Glomerella cingulata*

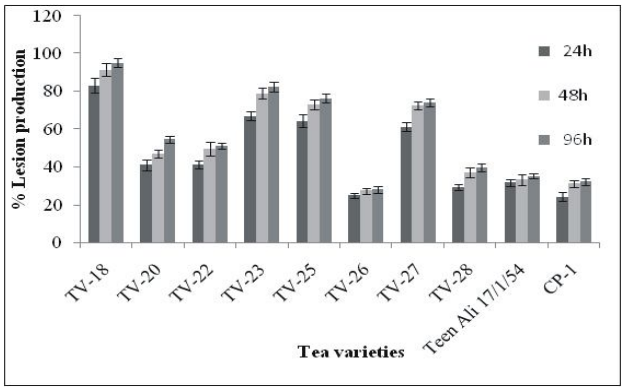

Fig. 2: Varietal resistance test of different tea varieties against *G. cingulata*

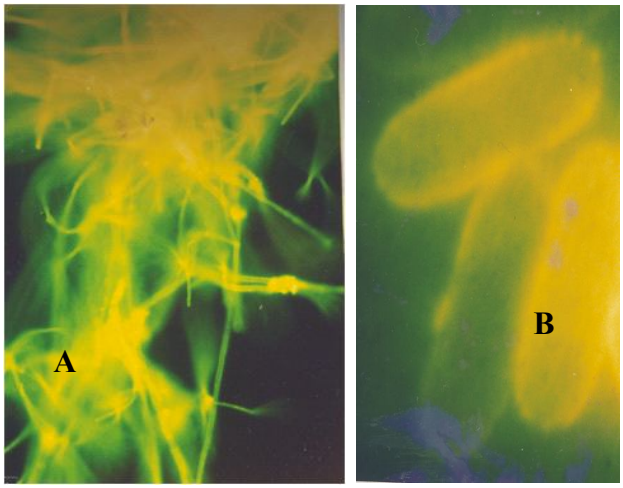

Fig.3: (A&B) Cell wall (A) and condia (B) of *Glomerella cingulata* treated with FITC labeled concanavalin A showing bright apple green fluorescence

Table 1: Effect of leaf exudates of different tea varieties on spore germination and appressoria formation of *G. cingulata*

Exudates collected from	Spore germination(%)*	Inhibition in Spore germination(%)+	Appressoria formation(%)*	Inhibition in appressoria germination(%)+
TV-23	36.8±1.4[a]	53.78	13.0±1.2[a]	81.66
TV-25	19.7±10.5[a]	75.26	10.0±0.87[a]	85.89
TV-26	33.3±1,2[a]	58.21	16.9±1.3[a]	76.05
CP-1	35.7±2.3[a]	35.17	6.2±1.2[a]	91.21
Teen Ali 17/1/54	61.1±3.4[a]	23.35	2.4±0.9[a]	96.54
TV-18	62.6±3.6[a]	21.44	31.3±2.1[a]	55.83
Control	79.7±2.8	-	70.8±2.5[a]	-

* Average of 200 spores; +Inhibition in relation to control; a Difference between control significant at P=0.05 & 0.01.

Table 2: Effect of leaf diffusates of different tea varieties on spore germination and appressoria formation

Diffusate collected from	Spore germination(%)*	Inhibition in Spore germination(%)+	Appressoria formation (%)*	Inhibition in appressoria germination(%)+
TV-23	24.1±1.3[a]	78.39	13.6±1.3[a]	71.79
TV-25	16.5±1.1[a]	89.24	09.0±0.9[a]	87.30
TV-26	5.8±2.3[a]	92.62	0	100.00
CP-1	5.3±1.1[a]	93.29	3.4±0.89[a]	95.18
Teen Ali 17/1/54	3.8±0.9[a]	95.19	0	100.00
TV-18	32.3±1.6[a]	59.48	24.6±1.5[a]	62.73
Control	79.7±1.8	-	70.4±2.5	-

* Average of 200 spores; +Inhibition in relation to control; a Difference between control significant at P=0.05 & 0.01.

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

Treatment	Spore germination(%)[a]	Appressoria formation(%)[b]
Leaf exudate (distilled water)	35.5±3.9	14.2±3.9
Leaf diffusate (spore suspension)	9.0±3.7	1.5±1.1
Leaf exudates + Mycelial wall extract (MWE)	24.1±3.3	11.4±2.3
Leaf diffusates + Mycelial wall extract (MWE)	9.7 ±2.2	1.6 ±1.9
Mycelial wall extract (MWE)	87.5±3.1	83.2±3.9
Control	89.2±3.7	94.4±34.1

[a] Based on 200 spores ; [b] Average of 50 germlings

towards *G. cingulata,* the role of preformed and post-infectionally formed compounds, if any, in the differential resistance was investigated. Leaf exudates and diffusates collected from resistant and susceptible varieties were collected by drop diffusate method as described and assayed for fungitoxicity against *G. cingulata,* inhibition of germination as well as inhibition in appressoria formation was observed in all cases.

However, the degree of inhibition percentage ranged from 21.4% to 75.0% (Table 1). Diffusates from all varieties were highly fungitoxic but the activity of the diffusates collected from the resistant varieties (TV-26, CP-1 and Teen Ali 17/1/54) were at least four to five times higher than those from the susceptible ones (TV-18, TV-25 and TV-23) (Table 2).

Studies on elicitors of G. cingulata

It is known that many plants accumulate antifungal compounds or phytoalexins as a part of inducible defense mechanism in response to pathogen invasion or treatment with biotic or abiotic elicitors. Since results of the present study showed enhancement in accumulation of antifungal compounds in

the resistant varieties of tea following infection by *G. cingulata*, it was decided to study the role of elicitors of the pathogen in this mechanism. For this, mycelial wall extracts were prepared, their chemical nature determined and their role in elicitation of antifungal compounds studied.

Bioassay of mycelial wall extract of G. cingulata

The extract form isolated cell walls bio-assayed to determine whether it elicited any antifungal compound. For this, the following four treatments were applied separately on the adaxial surface of the leaves of resistant variety (TV-26): distilled water, spore suspension, distilled water mycelial wall extract and spore suspension with mycelial wall extract. After 48h of incubation, the drops of each treatment were collected, centrifuged and assayed. It was found that fungitoxic compound was present in all cases. The mycelial wall extract was almost as effective as the sore suspension in eliciting antifungal compound (Table 3).

In absence of spores, the mycelial wall extract also elicited the antifungal compound. When the spores were suspended in mycelial wall extract and allowed to germinate on glass slide, 87% germination was observed with 83% appressoria formation. The mycelial wall extract as such was therefore, not fungitoxic, but it induced the formation of antifungal compound.

Determination of chemical nature of the cell walls

For chemical characterization, cell walls isolated from *G. cingulata* were analyzed by SDS-polyacrylamide gel electrophoresis and stained for protein and carbohydrate to determine the presence of glycoproteins in the wall extracts. Staining of gels with Coomassie blue R250 exhibited eight protein bands. Carbohydrate staining on the other hand showed two bands at molecular weights 10 and 50KD. These two bands coincided with two of the bands of proteins. This indicates the presence of two glycoproteins of molecular weights of 10 and 50 KD in the mycelial wall extract of *G. cingulata*.To confirm the glycoprotein nature of the mycelial wall extract as well as conidia of *G. cingulata*, these were treated with FITC labeled concanavalin A and compared. Isolated cell wall and conidia exhibited strong florescence indicating the presence of con A binding material in the cell wall, as well as on the surface of the mycelia and conidial wall which is a glycoprotein (Fig. 3, A and B)

Results of this study revealed that the mycelia wall extract of *G. cingulata* , which elicits the formation of an antifungal substances contains glycoprotein. Several investigators have demonstrated that glycoproteins function as phytoalexin elicitors. Glycoproteins have been shown to elicit phytoalexins in plant cell cultures in a number of cases in recent past. The native conformation of a glycoprotein extracted from cell suspensions of fungus has been seen to causes an increase in secondary metabolite concentration in cell cultures . Glycoprotein preparations from bakers' yeast elicit the formation of the benzophenantridine alkaloids in cultured cells of *Eschscholzia californica* (Färber *et. al.,*2003). In case of the red rot pathogen, *Colletotrichum falcatum*, a high molecular weight elicitor was isolated from the mycelial walls and the elicitor appears to be a glycoprotein and the activity of elicitor resides in the carbohydrate moiety. The partially purified elicitor induced the accumulation of phenolics and the activities of phenylalanine ammonia-lyase (PAL) and peroxidase (PO) in sugarcane leaves and suspension-cultured cells. Phenolic content and enzyme activities consistently increased and the maximum increase was recorded 96 h after elicitor treatment. (Rameshsundar *et.al*., 2002).

Analysis of antifungal compound in tea leaf tissue following inoculation with G. cingulata

In the present investigation further experiments were carried out following facilitated diffusion technique for the detection of antimicrobial compounds from relatively large samples of freshly harvested healthy tea leaf, *G. cingulata* inoculated as well as mycelial wall extract and conidial suspension inoculated tissue of one of the highly resistant variety (TV-26) after 96 h of inoculation. Ethyl acetate fractions from all these three treatments 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 in all the treatment. Crude extract (ethyl acetate fraction dissolved in methanol) prepared from all these three treatments were bio-assayed following radial growth inhibition assay. Results revealed that mycelial growth of *G. cingulata* was inhibited markedly in the medium supplemented with the extracts from mycelial wall extracts and pathogen inoculated

tea leaf (TV-26) than those of pathogen inoculated leaf tissue in relation to their respective control (media supplemented with healthy leaf extract).

Partially purified compound (Rf 0.58) from extracts of healthy and inoculated tea leaf variety (TV-26) were examined in a UV-spectrophotometer. It is interesting to note that extracts from *G. cingulata* inoculated leaf 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. It is interesting to note that higher accumulation (625µg/g fresh tissue) of pyrocatechol was evident in tea leaf tissue (TV-26) elicited by spore suspension and mycelial wall extract (MWE) of *G. cingulata* than spore suspension of the pathogen alone (540µg/g fresh tissue), while a very low concentration (96 µg/g fresh tissue) of this compound was found in healthy leaf tissues.

It is known that catechin is oxidatively cleaved to some simpler phenols and phenolic acids like catechol, phloroglucinol and protocatechuic acid. In the present study antifungal compound cleaved to some simpler phenols. Accumulation of pyrocatechol in resistant variety increased after 48 h of inoculation with *G. cingulata*. However, 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 (*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). Similarly, Faria *et al* (2006) have reported the antifungal activity of eugenol isolated from *O. gratissimum* against *Alternaria* and *Penicillium chrysogenum*. Antifungal compounds in tea leaf tissues following challenge inoculation with a foliar pathogen (*Alternaria alternata*) have been elucidated (Das Biawas and Chakraborty, 2020). Antifungal phenolics (pyrocatechol) in tea plants triggered by *Sclerotium rolfsii* has also been demonstrated by Bhagat and Chakraborty (2021). Surface to surface cellular recognition is the first reaction of host pathogen interaction and determines the nature of further events (Chakraborty,2018). Defense mechanisms may be induced by the expression of genes resulting from the recognition of a particular microbe by a host. Accordingly, an understanding of the defense mechanism of tea against *G. cingulata* will be complete only if the fungal elicitor is fully characterized and its mechanism of action is worked out.

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