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Biochemical changes in *Persea bombycina* following infection with *Colletotrichum gloeosporioides*

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Colletotrichum gloeosporioides was isolated from the blight infected blighted leaves of *Persea bombycina* Kost which was confirmed by Koch's postulate as well as by bright field microscopy of the spores. Diffusates were collected from detached leaves inoculated with the pathogen and effect of these diffusates on spore germination and appressoria formation by the spore were observed. Quantification of total and O-dihydroxyphenol from healthy and infected leaves revealed that phenol contents were higher in the infected leaves. Activity of defense enzymes such as peroxidase (POX), phenylalanine ammonia lyase (PAL), chitinase (CHT) and glucanase (GLU) revealed that they were enhanced in infected leaves than in healthy leaves. TLC and HPLC analysis of phenolic compounds present in healthy and naturally infected leaves was also performed. HPLC analysis revealed the presence of Resorcinol, catechol and chlorogenic acid in both healthy and infected leaf samples but the height of these peaks increased in infected samples. In addition presence of two new peaks in infected sample could be identified as ferulic acid and salicylic acid. Results revealed that the pathogen triggered the production of resorcinol, catechol, chlorogenic acid, ferulic acid and salicylic acid in the muga host plant as biochemical defense strategy.

Key words: Leaf blight, *Persea bombycina*, *Colletotrichum gloeosporioides*, HPLC analysis

INTRODUCTION

Leaf blight disease is one of the major foliar fungal diseases of *Persea bombycina* Kost, commonly known as "som" plant. The plant is mainly infected with the disease during the rainy and humid months.The symptoms appear as brown spots in the tip which moves towards the base of the leaf. The leaf withers, dries and finally falls off. This causes an estimated leaf yield loss of about 2932 kg/ha/yr. Since the leaves of this plant are vital for the feeding and growth of the silkworms, they should be maintained properly and the disease should be irradicated. But management of disease is not easy without the proper knowledge of the biochemical constituents of the plant as well as the details of the causal organism. Proper knowledge will help in formulating the correct management practise. C*olletotrichum gloeosporioides* Penz can attack leaves, petioles and blooms during periodsof prolonged leaf moisture and high humidity. The ability to cause latent or quiescent infections has grouped *Colletotrichum* among the most important post-harvest pathogens (Chowdappa *et al*, 2012). In this study attempts have been made to determine biochemical changes in leaves of som plants following infection with *C. gloeosporioides* causing leaf blight disease.

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MATERIALS AND METHODS

Plant material

Eight morphotypes (S1 – S8) of Som plants (which are the genotypes of PB001-PB008) were collected from Central Muga Eri Research and Training Institute (CMER&TI),

Jorhat, Assam for our study which are being maintained under net house condition as well as in experimental field of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. These common genotypes are classified based on their leaf shape like Ampotia (Morphotypes S1 and S2), Nahorpotia (S3), Jampotia (S4), Belpotia (S5), Kothalpotia (S6) etc.as mentioned in their website (http://cmerti.res.in/faq.html).

Isolation and identification of pathogen

The pathogen was isolated from infected leaf of som plants showing typical symptoms of leaf blight in potato dextrose agar (PDA) medium and the fungal culture thus obtained was purified using hyphal tip method in PDA slants. These slants were maintained for further studies. For light microscopic studies, hyphae and spores were stained with lactophenol-cotton blue.

Preparation of spore suspension

The fungal pathogen was grown in PDA medium in 100 ml flask till sporulation occurred. The spores were then scrapped off using sterile distilled water from the surface of the medium, filtered through muslin cloth and finally spore concentration was measured by hemocytometer. The concentration was approximately $3x10^4$ spores/ml.

Completion of Koch's postulate

Koch postulate was confirmed following detached leaf inoculation technique as described by Chakraborty *et al,* (1995). The disease assessment of the leaves was carried out by the process described by Chakraborty and Saha (1994).

Assessment of disease incidence

The occurrence of blight disease was recorded in case of all the eight morphotypes under nursery condition and the disease incidence was calculated according to the method of Chakraborty *et al,* (2014).

Collection of leaf diffusates and their bioassay

Leaf diffusates were obtained by a modified drop diffusate technique of Muller (1958). Forty young leaves, each of 8 different morphotypes, were collected from net house, washed and placed on moist filter paper in separate trays. Wound (1mm dia scratch) was made on the adaxial surface of the leaves using needle and 20 µl droplets of spore suspension of *C. gloeosporioides* was placed on the scratch (4-6 per leaf) using Pasteur pipette, the trays were covered with glass plates properly sealed with petroleum jelly and incubated for 48 hours. In case of control, distilled water was placed on the scratch in place of spore suspension. Drops of spore suspension were collected from each leaf of the eight morphotypes separately, centrifuged and supernatants collected. These were passed through sintered glass filter and their biological activities were assayed on spore germination and appressoria formation (Chakraborty *et al*, 1995).

Analysis of defense enzymes

Four major defense enzymes of plants were assayed during infection with healthy and naturally infected leaf samples. The leaf samples of healthy and infected plants were crushed using different buffers specific for the different enzymes, centrifuged and the supernatant collected which was used as crude enzyme source. Phenylalanine Ammonia lyase (PAL) as well as Peroxidase (POX) was performed following the method of Chakraborty *et al,* (1993).Chitinase was assayed following the method Boller and Mauch (1988) and â-1,3Glucanase was assayed following the method of Pan *et al*, (1991).

Isozyme analysis of peroxidase by PAGE

The healthy and infected leaves were used to prepare the enzyme extract in 0.1 M sodium phosphate buffer (pH 7). Polyacrylamide gel electrophoresis was performed according to the method of Davis (1967), followed by staining of the gel using Benzidine dye and hydrogen peroxide.

Estimation of phenols

Total Phenol: For estimation of total phenol, Folin Ciocalteau's method as described by Bray and Thorpe (1954) was followed. One ml of alcohol extract was taken, 1ml of Folin Ciocalteau was added and 2ml of Na_2CO_3 was added. The tube was shaken and heated in a boiling water bath for 1min , cooled and finally the volume was raised to 25ml. Quantity of total phenol was measured at 515 nm and calculated using caffeic acid as standard.

Ortho-dihydroxy Phenol: This was estimated by

addition of 1ml of alcohol extract, 2ml of 0.05 N HCl, 1ml of Arnows reagent and 2ml of 1N NaOH. The final volume was raised to 25 ml and the absorbance was recorded at 515 nm.

Thin Layer Chromatography of phenolic extracts

Thin layer chromatography technique was used to separate the different phenols present in healthy and infected leaves. Extraction of the sample was done following the method described by Neog *et al.* (2011), where the sample was extracted twice using petroleum ether and dichloromethane. After concentrating, it was dissolved in methanol and run in TLC plates using two different solvent systems, Acetic-acid and chloroform (1:9) and Ethyl-acetate and benzene (9:11).

HPLC analysis of phenolic compounds

Fresh leaves of som plant were chopped into pieces and soaked overnight in methanol in the ratio 1:3 (w/v), filtered through Buckner's funnel and the solvent was evaporated using lyophilizer as described by Pari and Latha (2004). The dried powder was finally mixed in HPLC graded methanol and stored at 4°C for further analysis. HPLC analysis of phenolic compounds present in the extracts was done using SPD-10A VP Shimadzu UV-VIS Detector. A flow rate of 1 mL/min, and gradient elution of acetonitrile-water-acetic acid (5:93:2, v/v/v) [solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B], 0– 50 min solvent B from 0 to 100%; and injection volume of 20 ìl were applied; whereas the separation of compounds was monitored at 280 nm (Pari *et al*, 2007).

RESULTS AND DISCUSSION

Assessment of Disease incidence

Maximum blight infection was noted during the month of April-August (Figure 1A). The disease incidence was recorded in nursery condition for all the eight morphotypes. Results (Table 1) revealed that the percentage disease index was lowest in S7 morphotype and highest in S3 and S6morphotype.The pathogen isolated from infected som leaf was identified as *Colletotrichum gloeosporioides* based on the spore characters, germination behaviour, appressoria formation (Fig-1 C-D) followingcompletion of Koch postulate using detached leaf technique (Fig 1B) and finally confirmed by 18S rDNA sequencing which has been deposited in NCBI data base (KM491736).

Table 1 : Percentage disease index of leaf blight disease in different morphotypes of som plants under green house condition

Morphotype	Percent disease index
S1	48.1±0.02
S2	42.5±0.01
S3	60.3±0.008
S4	50.1±0.007
S5	52.5±0.01
S6	68.2±0.006
S7	35.32±0.02
S8	56.5±0.008

Effect of leaf diffusates on spore germination and appressoria formation

The diffusates were collected from leaves of all morphotypes of som plants showing variable degree of susceptibility towards the disease. Their effect on spore germination and appressoria formation was tested *in vitro*. The diffusates from all morphotypes were fungitoxic (Table 2) but the activity of the diffusates from S7was higher than those from S3 and S6.

Assay of defense enzymes in healthy and infected leaves

Levels of four major defense enzymes or PR-proteins were assayed in healthy and infected leaves where it was found that levels of these enzymes were significantly increased in infected leaves in comparison to healthy leaves. However the level was much higher in infected leaves of S7 morphotype and less in S6 leaves the results are shown in Figure 2.

Peroxyzyme analysis revealed the presence of a new band in infected leaf samples corresponding to Isozyme 1 being produced in infected leaves and not in healthy samples, shown in Figure 3. Isozyme 2 was produced in both healthy and infected samples.

Estimation of phenol content in healthy and infected leaves

Total and ortho-dihydroxyphenol content of healthy and infected leaves were estimated following the

Table 2: Effect of leaf diffusate of different sommorphotypes on spore germination and appressoria formation of *C. gloeosporioides*

Morphotype	Spore germination[a], %	Inhibition of spore germination, %	Appressoriaformation[a], %	Inhibition of appressoria formation, %
Control	83.5±1.2	-	65.0±1.8	-
S1	9.5±1.6	89	4.2±1.6	94
S2	6.5±1.3	92	0	100
S3	43.2±1.4	48	20.5±1.2	68
S4	24.2±1.8	73	8.3±1.1	87
S5	39.5±1.1	53	13.8±1.6	79
S6	45.0±1.5	46	24.6±1.8	62
S7	4.5±1.2	94	0	100
S8	35.2±1.6	58	10.2±1.1	84

[a]Average of 200 spores

above mentioned procedure and it was recorded that both the phenol content increased in infected leaves than their healthy counterparts but the level of phenol changes varied among the different morphotypes. It was observed that phenolics were highest in infected leaves of S7 morphotype and lowest in S6 morphotype (Table 3)

Analysis of phenols using thin layer chromatography

Analysis of phenols through TLC showed the presence of blue coloured spots with different Rf value when sprayed with Folin Ciocalteau's reagent as well as applying ammonia fumes. In infected samples two different spots were prominent at Rf 0.85 and 0.21 whereas two spots at Rf 0.72 and 0.15 were evident in healthy leaf samples.

Analysis of phenolic acids using HPLC

Phenolic acids present in healthy and infected leaves were further analysed using HPLC. Peak 1,2 and 3 are present both in healthy and infected leaves but the height of these peaks increased markedly in infected leaves in comparison with healthy leaves indicating an increase of these phenolic acids following infection. Peak 4 is present in healthy sample but disappeared in infected leaf samples. It is interesting to note that five new peaks viz 5,6,7,8 and 9 were evident in infected samples but all these peaks were absent in healthy leaf samples indicating that these phenolic acids might play an important role in defense mechanism of the plant against infection. When compared to standard phenolic acid it was confirmed that Peak 1, 2, 3 and 4 represented Resorcinol, Catechol, Morin and Chlorogenic acid respectively whereas peak 5 and 7 represented ferulic acid and peak 6 represented salicylic acid. (Table 4, Figure 4). Minor Peaks 8 and 9 were present only in infected samples and not in healthy samples; when compared with authentic phenolic acids, these two minor peaks could not be identified.

In the present study it was seen that almost all the morphotypes of som plants were infected with leaf blight under nursery condition. However morphotype S7 was least infected and morphotype S6 and S3 were highly infected. After isolation and microscopic study, *Colletotrichum gloeosporioides* was found to be the causal agent. Completion of Koch postulate also validated our findings. Das *et al.* (2005) reported the occurrence of *Colletotrichum* infection in Assam.

Table 3 : Total and Ortho-phenol content in healthy and infected leaves

Morphotype	Total Phenol Content (mg/g tissue)		Ortho-phenol Content (mg/gtissue)	
	Healthy	Infected	Healthy	Infected
S1	3.90±0.12	5.60±0.15	3.35±0.05	7.25±0.03
S2	2.50±0.36	5.20±0.11	4.80±0.03	8.50±0.03
S3	2.90±0.22	3.20±0.11	2.25±0.05	3.25±0.02
S4	3.60±0.16	4.50±0.12	4.25±0.04	6.25±0.05
S5	2.80±0.19	4.20±0.16	3.25±0.03	5.25±0.01
S6	2.20±0.11	2.50±0.14	3.36±0.02	2.75±0.04
S7	3.25±0.12	6.65±0.13	6.00±0.04	10.50±0.01
S8	3.29±0.14	4.80±0.11	4.63±0.05	5.25±0.01

Diffusates collected from all morphotypes of som plants inhibited spore germination but the diffusates from less infected morphotypes were much more effective than highly infected morphotypes. Earlier Chakraborty *et al,* (1995) also reported that diffusates of phenolic nature collected from susceptible and resistant varieties of *Camellia sinensis* to *Glomerella cingulata* inhibited spore germination and appressoria formation. These findings suggest the presence of some metabolites in the diffusates that is fungitoxic in nature and can be used by the plants for their innate immunity against the fungal pathogen.

Table 4: Retention time and peak heights of phenolics from healthy and infected leaf sample of S7 morphotype analyzed by HPLC

Sample	Peak no	Retention time	Height
Healthy	1	9.960	79.284
	2	13.310	190.922
	3	14.890	508.657
	4	21.400	42.346
Infected	1	9.990	339.562
	2	13.250	698.064
	3	14.720	945.181
	5	25.650	237.487
	6	26.680	491.245
	7	29.340	89.340
	8	30.820	68.346
	9	34.890	46.420

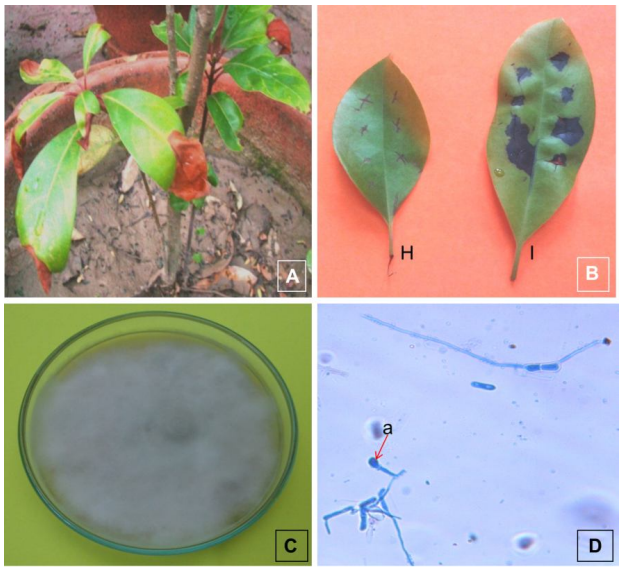

Fig 1: (A) – Leaf blight of Som plant, (B) – Untreated Healthy (H) and artificially inoculated leaf (I), (C) Mycelial growth of *Colletotrichum gloeosporioides* on PDA medium, (D) – Spore germination and appressoria formation(a) of *C. gloeosporioides*

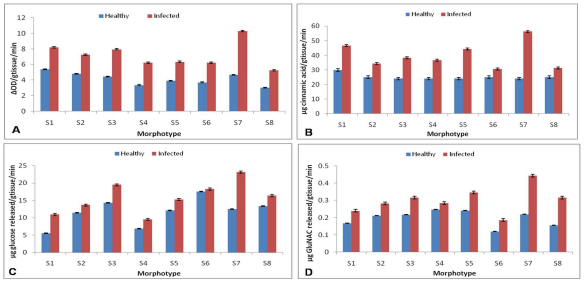

Fig 2: Activities of defense enzymes in healthy and blight infected leaves; A – Peroxidase, B – Phenylalanine ammonia Lyase, C – Chitinase, D – â-1,3 Glucanase

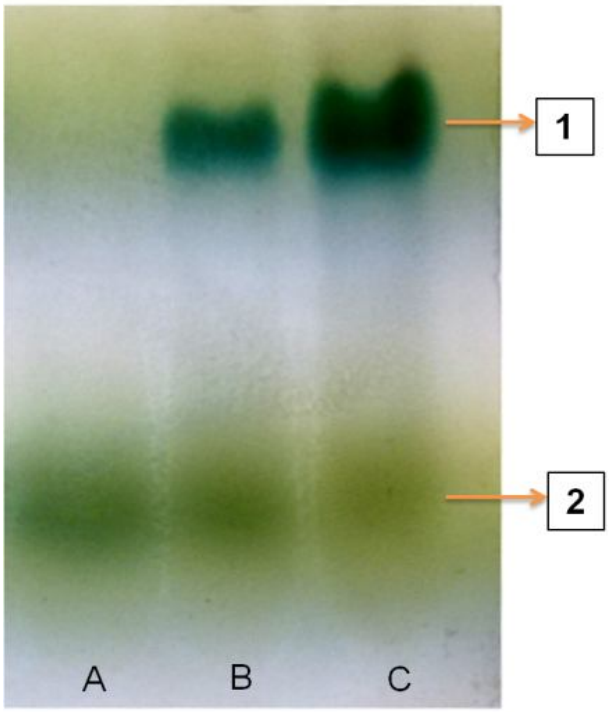

Fig 3: Peroxyzyme analysis of healthy and infected leaves of S7 morphotype in native PAGE. Lane A- Healthy, Lane B-C – Infected

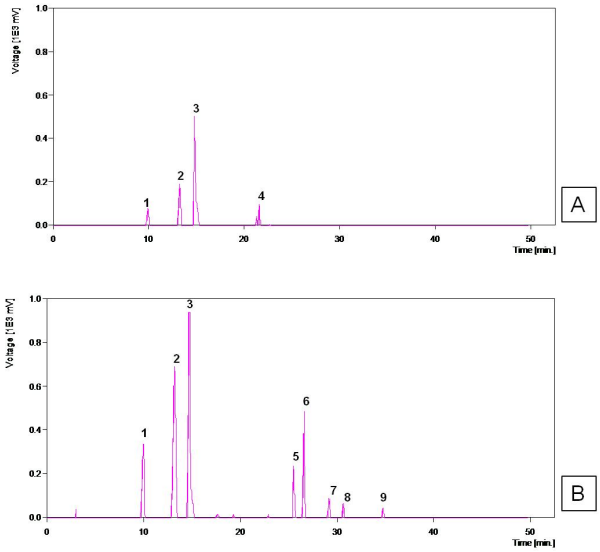

Fig 4: HPLC analysis of phenolic acids in leaves of S7 morphotype (A – Healthy, B – Infected leaf; 1 – Resorcinol, 2 – Catechol, 3 – Chlorogenic acid, 4 – Morin, 5 and 7 – Ferulic acid, 6 – Salicylic acid).

In connection with the disease establishment in the plants, different defense enzymes were also analysed and it was recorded that defense enzymes like PAL, POX, CHT and GLU showed an increased activity in infected samples. Moreover their activity was much higher in plants showing less disease index. Correlation of this result was also made with the study undertaken by Parihar *et al.* (2012) where it was seen that biochemical analysis of genotypes of *Brassica juncea* infected with *Alternaria* blight revealed an increase in PAL, PPO and peroxidase activity. Singh *et al.* (2014) reported that preformed phenolic compounds as well as Peroxidase enzyme play important role in resistance of Chili against *Colletotrichum capsici.*Analysis of peroxidase isozymes by polyacrylamide gel electrophoresis showed four isozymes in healthy tea leaf samples and five in tea leaves infected with *Exobasidium vexans*. They suggested that the appearance of new bands following infection can be correlated with the induction of the catalytic activity of more isozymes, leading also to an overall increase in peroxidase activity (Chakraborty *et al*, 2002). In the present study it was observed that total as well as ortho-dihyroxy phenol content increased in infected plants, more in plants with less infection. Infection by *Venturia inequalis* in apple caused an accumulation of phenolic compounds wherein Folin-Ciocalteu values increased by 1.4 to 2.4 fold (Petkovsek *et al*., 2008).Taware *et al,* (2010) reported that there was significant increase in total phenolic content of grape leaves due to foliar powdery mildew infection. These results are in accordance with the result obtained in the present study.Thin layer chromatography has been used by various workers to study the phenol profile of different plants like tea (Chakraborty and Saha, 1994), som (Neog *et al*, 2011), different medicinal plants (Maobe *et al*, 2012). Studies on secondary metabolites of som plant by Neog *et al.* (2011) revealed the presence of four major phenolic acids – chlorogenic acid, catechol, morin and gallic Acid. In the present study also HPLC analysis of healthy and blight infected som plants revealed the presence of Chlorogenic acid and catechol which was highly increase in infected leaf samples, indicating the function of this metabolite in defense mechanism of plant. Presence of chlorogenic acid as part of defense system has been studied by different workers in different crops, such as Potato tubers (Malamberg and Theander, 1985), apples (Petkovsek, *et al,* 2003), coffee (Rodrigues, *et al,* 2011) and tomato (Lopez-Gresa*et al*., 2011). Presence of salicylic acid and ferulic acid in infected leaves and not in healthy leaves indicate the role of this phenolic acid in defense against pathogen. When biochemical charecterization of maize plants infected with *Drechslera dactylidis* was done, it was found that salicylic acid increased 2-fold in infected leaf samples. (Abdel Ghany 2012).

Thus in conclusion it can be said that, studies on effect of diffusates on spore germination and appressoria formation of *C. gloeosporioides* as well as HPLC profile of the phenolic acids in healthy and infected leaf samples shows presence of some metabolites in less infected plants that might have certain activity that helps the plant in resisting the disease establishment and progression. This might be the key in analysing the phytoalexin component of the som plant against various fungal pathogens which will help in strategising for integrated management of the disease.

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