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Analyses of soluble proteins and phenolics in susceptible and resistant wheat genotypes against *Bipolaris sorokiniana* causing spot blotch disease

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Wheat is one of the most important grain crops in India that plays a vital role in the national economy. *Bipolaris sorokiniana* Sacc. is one of the foliar blight pathogens of wheat causing spot blotch disease resulting in damaging the crop yield in India. One virulent isolate of *B. sorokiniana* (WH.PBW.IP.04-BS7) after molecular identification by 18 S rDNA sequencing (NCBI- KM066949) was selected for challenge inoculation of wheat plants. Among the 35 tested wheat genotypes, CWL-6702 was found to be highly susceptible whereas CWL-6726 and CWL-6715 showed resistance against spot blotch as evaluated on one month old field grown wheat plants on the basis of disease reactions. SDS PAGE analysis of soluble proteins in (susceptible genotype- CWL 6720 and resistant genotype- CWL 6726) revealed increased band number and intensity in pathogen inoculated plants in comparison to healthy control. Increased accumulation of phenolics in pathogen inoculated plants (resistant genotypes- CWL 6726, 6715) was observed when analysed by HPLC.

Key words : Wheat, *Bipolaris sorokiniana*, spot blotch, SDS-PAGE, HPLC

INTRODUCTION

Wheat (*Triticum aestivum* L.) is the most important cereal crop in India. Spot blotch caused by *Bipolaris sorokiniana* Sacc. (syn. *Helminthosporium sativum* Pamm., King & Bakke) is a major disease of wheat in warm and humid regions of the world including South East Asian countries. The fungus is most aggressive under high relative humidity and temperature associated with the low fertility of soils. Continuing rainfall for 5 to 6 days followed by daily average temperatures of 20 to 30 °C favours rapid development of spot blotch disease epidemics on wheat (Kumar *et al* 2002). Villareal *et al* (1995) reported yield losses of crops varied from (2.7- 100) % caused by *B. sorokiniana*. Random-amplified polymorphic DNA (RAPD) assay was used to investigate the genetic diversity of 20 isolates collected from different cultivars in wheat-producing regions in Brazil. Seventy primers, with random nucleotide sequences, were tested. After the RAPD analyses 19 isolates were closely grouped, having a similarity coefficient of 78%. Isolate I017 showed very low similarity coefficients, ranging between 38 and 46% (Muller *et al.,* 2005). Virulence and molecular diversity of *B. sorokiniana* isolates have also been studied using PCR assays, RAPD, AFLP analysis (Jahani *et al*., 2008). Molecular variability in *B.sorokiniana* using URP-PCR was studied by Aggarwal *et al.,* (2010) and they grouped the isolates according to their geographic origin. Quantitative trait loci for resistance to spot blotch caused by *B. sorokiniana* has been identified by Kumar *et al.,* (2010). In a study by Mann *et al.,* (2014), 19 polysporic and 57 monosporic isolates of *B. sorokiniana* were characterized using universal rice primers-URP-PCR. However, the similarity among the isolates was low where 37 and 26.3 % of the monosporic and polysporic isolates, respectively, showed similarity above 70 %. All primers amplified multiple DNA fragments of polysporic as well as the monosporic isolates. Serological and molecular detection of *B. sorokiniana* have been demonstrated by Chakraborty *et al*. (2016).

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A proteomics analysis of wheat seed germination also detected modifications in phosphorylation that can enhance environmental stress defenses in wheat (Dong *et al.,* 2015). Phenolic compounds in wheat grains are important biomolecules that have health benefits as they improve human health against diabetes, cardiovascular diseases, and associated diseases because of their high antioxidant properties (Nicoletti *et al.,* 2013). Keeping these in view, the present investigation was undertaken to screen for resistance in wheat germplasm against *B. sorokiniana* and to analyze changes in protein profile through SDS-PAGE and accumulation of phenolics by HPLC following challenge inoculation of selected resistant and susceptible wheat germplasm with fungal pathogen.

MATERIAL AND METHODS

Molecular Identification of fungal isolate

One isolate (WH.PBW.IP.04-BS29), obtained from the infected wheat leaves of PBW 343 was identified by 18 S rDNA sequencing and submitted to NCBI GENBANK(KM066949).

Wheat genotypes

Wheat genotypes- CWL 6701, CWL 6709, CWL (6719-20), CWL 6728, CWL (6732-33), CWL (6739-40), CWL 6742, CWL 6744, CWL 6703, CWL6707, CWL 6711, CWL6715, CWL6717, CWL 6722, CWL 6731, CWL6743, CWL6745, CWL 6746, CWL-6726(MUNAL 1), CWL-6702, CWL-6704, CWL-6705, CWL-6706, CWL-6708, CWL-6714, CWL- 6718, CWL-6723, CWL-6712, CWL-6734, CWL-6738(FRNCLN), CWL- 6047(CIANO T79) and CHIRYA 3 were obtained from Borlaug Institute for South Asia (BISA), PUSA, Bihar, International maize and wheat improvement center (CIMMYT) unit.

Artificial inoculation of wheat plant and Assessment of spot blotch disease severity index

Whole plant inoculation technique as described by Chakraborty *et al* (2016) was followed. Susceptible and resistant reactions were evaluated after 12, 24, 48, 72 and 96h of inoculation, on the basis of appearance of infection on leaves. The disease severity was measured in terms of lesion number per leaf and infection index was calculated by following the method (using 0-5 scale) of Adlakha *et al.,* (1984). Percentage disease index (PDI) was calculated- [(class rating x class frequency)/ (Total no of leaves x maximum rating)] x 100. The mean PDI was transformed into disease reaction (Adlakha *et al.,* 1984) as: 0%= no infection/ immune; 0-10%= resistant response (R); 10.1-20.0%= moderately resistant (MR); 20.1-30.0%=moderately susceptible (MS); 30.1-50.0%= susceptible (S) and >50.0%= highly susceptible (HS).

SDS-PAGE analysis of proteins

SDS PAGE analysis for observing the protein pattern of the healthy and artificially inoculated (with *B. sorokiniana*) wheat leaf sample was done following the methods as described by Sambrook *et al.,* (1989). The molecular weight of protein bands visualized after staining with coomassie blue in SDS-PAGE were determined from the known molecular weight marker. The bands in the gels were further analyzed by IMAGE-LAB Version 5.1 software.

HPLC analysis of phenolics
Sample preparation

Phenol extraction and preparation of the sample for HPLC was done by the method described by Pari and Latha (2004) in the dark. Fresh leaf tissues from healthy and artificially inoculated wheat plants were taken and separately chopped into small pieces and soaked overnight in absolute methanol at solid material to methanol ratio of 1:3 (w/v) in dark. The suspension was filtered and the filtrate was evaporated using a rotary evaporator. It was dissolved in 1mL of HPLC grade methanol and filtered through Millipore membrane (0.45μm) filter.

Total phenol analysis

For the analysis of total phenols in HPLC a method suggested by Pari *et al.,* (2007) was followed. For the HPLC analysis of phenolic compounds present in extracts a Shimadzu system (Shimadzu Corp., Kyoto, Japan) was used. A flow rate of 1 mL/min, and gradient elution of HPLC grade 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], a 0– 50 min solvent B from 0 to 100%; and injection volume of 20 μL were applied;

whereas the separation of compounds was monitored at 280 nm.

RESULTS AND DISCUSSION

Assessment of spot blotch in wheat genotypes on the basis of percent disease index

Higher percent disease index (PDI) values was found in susceptible genotypes in comparison to resistant genotypes (Fig. 1). CWL-6726(MUNAL1) was found to be resistant, whereas, CWL (1-15), CWL22, CWL56, CWL57, CWL (63-69), CWL95,CWL96 were moderately resistant, CWL(16-21),CWL(23-43), CWL(44-50), CWL(51-55), CWL(58-62), CWL(70-89), CWL(90-92), CWL(97-100) were moderately susceptible and CWL-6702 was highly susceptible. CWL-6720, CWL-6726(MUNAL 1) and CWL-6715 were selected for further study on the basis of susceptible/resistance reactions against spot

Table 1: SDS PAGE analysis of leaf proteins of resistant (CWL 6726, CWL6715) and susceptible (CWL 6720) wheat genotypes. Lane and Band analysis are performed by Image-Lab version 5.1 software.

Lane and Band analysis (Resistant CWL6726)				Lane and Band analysis (Susceptible CWL6720)			
Band No.	Band Label	Mol. Wt. (KDa)	Band %	Band No.	Band Label	Mol. Wt. (KDa)	Band %
Lane 1- Untreated Healthy (UH)				Lane 4- Untreated Healthy (UH)			
1	Unknown	99.4	2.8	1	Unknown	99.8	2.1
2	Unknown	73.6	2.8	2	Unknown	95.5	1.1
3	Unknown	61.8	51.1	3	Unknown	73.2	1.9
4	Unknown	52.2	3.6	4	Unknown	58.9	36.8
5	Unknown	36.5	6.2	5	Unknown	51.3	1.8
6	Unknown	19.9	3.9	6	Unknown	33.1	6.1
7	Unknown	18.8	6.2	7	Unknown	18.5	20.3
8	Unknown	16.0	1.4	8	Unknown	15.8	1.5
9	Unknown	6.5	18.1	9	Unknown	6.5	23.2
Lane 2- *B. sorokiniana* inoculated (UI)				Lane 5- *B. sorokiniana* inoculated (UI)			
1	Unknown	113.0	1.6	1	Unknown	98.0	0.8
2	Unknown	109.5	0.5	2	Unknown	84.2	0.1
3	Unknown	99.2	3.4	3	Unknown	80.9	0.2
4	Unknown	72.8	2.0	4	Unknown	63.6	49.2
5	Unknown	59.3	36.8	5	Unknown	33.5	4.9
6	Unknown	34.6	6.1	6	Unknown	22.4	1.5
7	Unknown	18.9	21.7	7	Unknown	21.3	0.7
8	Unknown	16.0	1.5	8	Unknown	18.5	10.4
9	Unknown	6.5	24.9	9	Unknown	6.5	26.8
Lane 3- *B. sorokiniana* inoculated (UI)				Lane 6- *B. sorokiniana* inoculated (UI)			
1	Unknown	112.8	2.2	1	Unknown	116.2	1.9
2	Unknown	110.1	0.4	2	Unknown	113.0	0.5
3	Unknown	99.4	2.2	3	Unknown	102.9	2.1
4	Unknown	94.7	1.8	4	Unknown	99.5	1.0
5	Unknown	74.7	0.5	5	Unknown	77.9	1.9
6	Unknown	58.9	44.7	6	Unknown	63.3	37.7
7	Unknown	40.3	9.7	7	Unknown	45.3	6.3
8	Unknown	18.7	13.4	8	Unknown	19.4	20.0
9	Unknown	15.8	2.1	9	Unknown	17.3	0.1
10	Unknown	6.5	21.2	10	Unknown	10.7	26.1
Lane and Band analysis (Resistant CWL6715)							
Band No.	Band Label	Mol. Wt. (KDa)	Band %	Band No.	Band Label	Mol. Wt. (KDa)	Band %
Lane 7- Untreated Healthy (UH)				Lane 8- *B. sorokiniana* inoculated (UI)			
1	Unknown	200.0	2.2	1	Unknown	200.0	3.1
2	Unknown	200.0	0.5	2	Unknown	200.0	4.3
3	Unknown	200.0	7.2	3	Unknown	200.0	2.8
4	Unknown	163.2	59.8	4	Unknown	200.0	67.3
5	Unknown	92.2	6.4	5	Unknown	91.5	9.9
6	Unknown	84.2	2.8	6	Unknown	71.0	6.1
7	Unknown	70.4	9.3	7	Unknown	35.0	0.3
8	Unknown	24.6	4.0	8	Unknown	26.3	3.1
9	Unknown	11.5	4.6	9	Unknown	13.3	3.1

**Marker lane and Band Label (KDa) : Myosin-200, â-galactosidase-116.2, Phosphorylase b- 97.4, BSA- 66.2, Ovalbumin- 45.0, Carbonic anhydrase- 31.0, Trypsin inhibitor- 21.5, Lysozyme- 14.4 & Aprotinin- 6.5. Molecular Weight (MW) expressed in Kilo Dalton (KDa) unit.

blotch disease. Chand *et al.,* (2008) studied resistance to spot blotch disease caused by *B. sorokiniana in vitro* for screening resistance of barley cultivars.

Analysis of protein profiles in B. sorokiniana inoculated susceptible and resistant wheat genotypes

SDS PAGE analysis of soluble proteins (in susceptible genotype- CWL 6720 and resistant genotype- CWL 6726 and CWL-6715) revealed new band appearance having molecular weight in the range of 97-43 KDa and increased band intensity having molecular weight of 43KDa in *B. sorokiniana* inoculated CWL6726 genotype in comparison to healthy control (Fig. 2; Table 1). Protein band patterns in all lanes were similar except the differences in band intensities. Similarly in CWL-6726 (MUNAL 1) and CWL-6715 resistant genotype, band intensity was comparatively higher in inoculated plants than healthy plants. Bach and

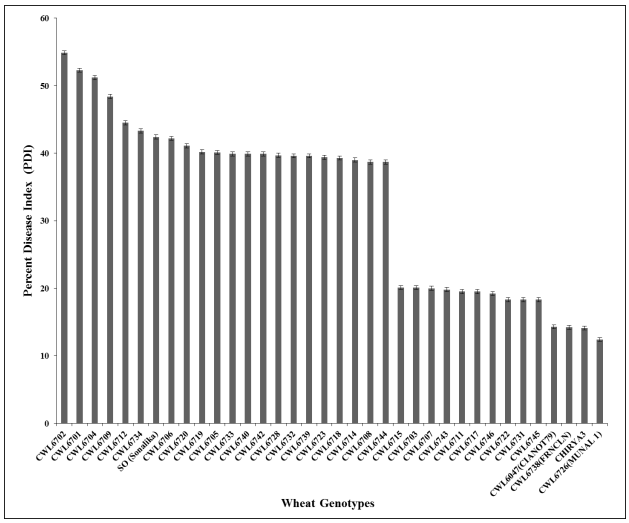

Fig. 1 : Percent Disease Index of susceptible and resistant wheat genotypes against *B. sorokiniana*

Kimati (1999) isolated, characterized and purified low molecular weight protein toxins and analysed SDS PAGE of those protein toxins from wheat isolates of *Drechslera tritici-repentis, Bipolaris bicolor* and *Bipolaris sorokiniana*.

Analysis of phenolics in B. sorokiniana inoculated resistant and Susceptible wheat genotypes by HPLC

The profile of total phenolics in the leaf of *B. sorokiniana* inoculated resistant wheat genotypes (CWL 6715, CWL 6726) and susceptible wheat genotype (CWL 6720) along with the healthy leaf samples were analysed by HPLC in order to determine and identify the nature of changes in phenols. It was evident that the content of total phenols during resistant reactions was enhanced

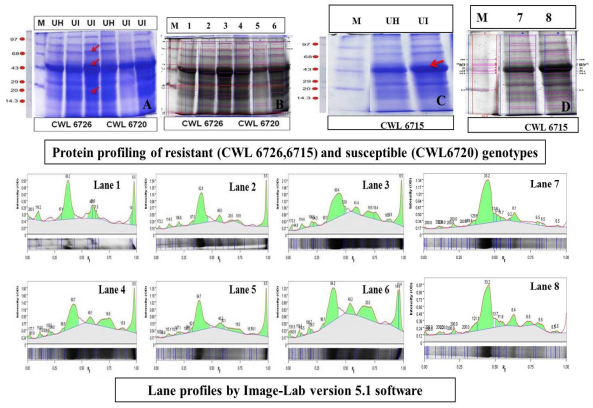

Fig.2: SDS PAGE analysis (A&C) and gel image scanning through Image-Lab version 5.1 software (B&D) of leaf proteins of resistant (CWL 6726 Lanes 1,2,3, CWL6715 Lanes 7,8) and susceptible (CWL 6720 Lanes 4,5,6) wheat genotypes. UH= Untreated Healthy (Lanes 1,4,7); UI= Untreated inoculated with *B. sorokiniana* (Lanes 2,3,5,6,8). (M)- Marker lane (97-14.3 KDa); Individual lane profile (1-8) and band analysis by Image-Lab version 5.1 software are presented in graph mode as Relative front (Rf) value versus Intensity (OD).

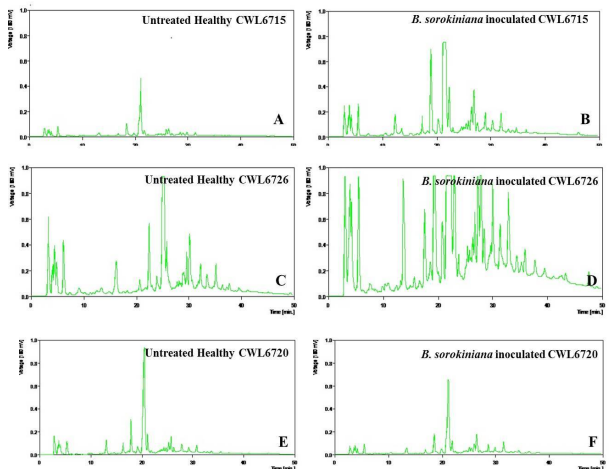

Fig. 3 : HPLC analysis of total phenolics in healthy (A,C,E) and *B. sorokiniana* inoculated (B,D.F) wheat genotypes. Resistant genotypes CWL 6715 (A&B), CWL 6726 (C&D) and susceptible wheat genotype-CWL 6720 (E&F)

in case of all the tested wheat genotypes with the highest content recorded in case of *B. sorokiniana* inoculated CWL 6726 and comparatively least in case of *B. sorokiniana* inoculated CWL 6715 in comparison to healthy leaf samples. Number, height of the peaks obtained in HPLC study in case of CWL 6726 were much more than that of CWL 6715 (Fig.3 A-F). Content of total phenols during susceptible reactions reduced in *B. sorokiniana* inoculated CWL 6720 genotype in comparison to

healthy one. A study indicated that total phenol contents were significantly higher in resistant wheat varieties compared to the susceptible ones to *Alternaria triticina* (Mishra *et al.,* 2011). Gupta *et al.,* (2012) determined standard HPLC chromatograms of twelve prominent phenolic compounds found in medicinal plants using four mobile phases having different elution gradients and run times. In a study by Sharma *et al.,* (2016), the identification and quantification of phenolic compounds using UPLC-QTOF-MS, MS/MS and functional genomics techniques such as microarrays and qRT-PCR of their biosynthesis genes had been studied in a good chapatti variety- "C 306" and a poor chapatti variety- "Sonalika." Nine phenolic compounds (hinokinin, coutaric acid, fertaric acid, p-coumaroylqunic acid, kaempferide, isorhamnetin, epigallocatechin gallate, methyl isoorientin-22 -O-rhamnoside, and cyanidin-3-rutinoside) were identified only in the good chapatti variety and four phenolic compounds (tricin, apigenindin, quercetin-3-O-glucuronide, and myricetin-3-glucoside) in the poor chapatti variety. The overall results suggest that the entire research work will pave the way for better understanding the changes in proteins and phenolics during biotic stress leading to resistant/susceptible reactions in wheat plants.

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