RESEARCH ARTICLE

A comparative virulence analysis of *Bipolaris sorokiniana* based on host pathogen interaction and its toxin

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ABSTRACT: *Bipolaris sorokiniana* is a hemibiotrophic fungus causing spot blotch of wheat and barley. The pathogen produces phytotoxins in culture that induces necrosis and play role in pathogenesis. Our results indicated the existence of variability based on host interactions with pathogen and toxin produced by the pathogen, separately. Twelve isolates of *B. sorokiniana* showed differences in disease reaction on wheat differential hosts. BS 25 was identified as most virulent and BS 7 as least virulent isolate. These two selected isolates further showed differences in the intensity of toxin band when resolved by thin layer chromatography (TLC). The grades of symptoms produced by the toxin of these isolates were similar to those produced by the respective pathogen isolates. Besides producing necrotic lesion on wheat the partially purified toxin caused necrotic lesions on barley, maize and sorghum studied using leaf infiltration bioassay.

Keywords: *Bipolaris sorokiniana*, toxin, virulence, wheat

Bipolaris sorokiniana [(Sacc.) Subram. & Jain] is one of the most important diseases of wheat causing up to 36 percent loss in favorable conditions. The disease is important in Africa, South America, Australia, Canada and Asia particularly to Indian sub-continent having warm and humid environments (Singh and Srivastava, 1997). In Indian sub-continent, such environments are encountered in the warmer parts of eastern India, most of Bangladesh and tarai region of Nepal. Recently, rice and wheat rotations have occupied larger areas in Punjab, Haryana, western U.P. and late sowing of wheat is a common practice. The delayed sowing experiences a warm and humid weather in February which is favorable to spot blotch. Therefore, spot blotch has emerged as a major disease in North-western as well as in the Peninsular India and disease acquired the status of national importance (Sharma *et al.*, 1998).

Although, spot blotch pathogen is capable of producing disease symptoms in all plant parts but leaf spot and spike blight are most common which result in the formation of long blotches with chlorotic margins ultimately coalescing covering 50-90 percent of area. The pathogen produces phytotoxins which play role in pathogenesis (Aggarwal *et al.*, 2008). The toxins may function as essential determinants of pathogenicity or virulence. Investigations into the molecular and biochemical responses to these disease determinants reveal responses typically associated with host

susceptibility and compatibility. Keeping this in view, present investigation was undertaken to assess comparative virulence of various isolates of *B. sorokiniana* on wheat and other hosts/weeds.

MATERIALS AND METHODS

Pathogenic behavior of *Bipolaris sorokiniana* isolates

Plant material and pathogen isolates: Fourteen wheat genotypes were obtained from Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi for studying pathogenic variability among the isolates of *B. sorokiniana*. This set of genotypes included some of the differential hosts used by Dr. Y.R.Mehta (Mehta, 1985). The experiment was conducted in the net house and glass house for two years. A mixture of sandy loam and farm yard manure (FYM) in the ratio of 1:1 was prepared for sowing of differentials. Twelve isolates of *B. sorokiniana* collected from different geographic areas were established in pure form on potato dextrose agar (PDA), details of which are mentioned in Table 1.

Pathogenic variability: After three weeks of sowing of seeds, five seedlings per pot were maintained for inoculation with different isolates of *B. sorokiniana*. The inoculum of each isolate was prepared from ten days old PDA grown culture in Petri dishes. Inoculation was done by atomizer on upper surface of leaves @10⁴ conidia/ml. Observations on disease severity were recorded after 10 days of inoculation and disease severity was measured in terms of lesion number per leaf and

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Table 1. Isolates of *Bipolaris sorokiniana* and their pathogenicity on susceptible wheat genotype, Agra local

Locality/ State	Isolate I.D.	Average disease index on Agra local
Jind (Haryana)	BS 5	29.3a
Karnal (do)	BS 7	28.3a
Jaipur (Rajasthan)	BS 9	55.4 ^a
Jaipur (do)	BS 18	45.0 ^d
Jaipur (do)	BS 25	61.6 ^f
Faridabad (Uttar Pradesh)	BS 27	59.1 ^f
Kalyani (West Bengal)	BS 47	35.1 ^b
Shillongami (Assam)	BS 48	30.9 ^{ab}
Malan (Himachal Pradesh)	BS 53	54.3 ^e
Almora (Uttarakhand)	BS 54	41.0 ^c
Almora (do)	BS 55	33.1 ^b
Powarkheda (Madhya Pradesh)	BS 64	42.0 ^c
CD (P=5%)		3.47

infection index was calculated as per the scale given by Adlakha *et al.* (1984). Disease index of each isolate with respect to differential hosts was calculated as: Immune (I): No infection; Resistant (R): Infection index 1.0-10.0; Moderately resistant (MR): Infection index 10.1-20.0; Moderately Susceptible (MS): Infection index 20.1-30.0; Susceptible (S): Infection index 30.1-50.0; Highly susceptible (HS): Infection index >50.0

Pathogenicity on other hosts / weeds

Pathogenicity of the two selected isolates of *B. sorokiniana* was tested on chickpea (*Cicer arietinum*), tomato (*Lycopersicon esculentum*), cotton (*Gossypium spp.*), Maize (*Zea mays*), Rice (*Oryza sativa*), barley (*Hordeum vulgare*) and sorghum (*Sorghum bicolor*) at seeding stage. The inoculum of each isolate was prepared as mentioned above. The experiment was repeated two times keeping three replications. The observations were recorded after ten days of inoculation and disease severity was measured in terms of lesion number per leaf and infection index was calculated as mentioned above.

Studies on toxin production by *Bipolaris sorokiniana*

Extraction of Crude toxin: Crude toxin was isolated from most virulent (Bs-25) and least virulent (Bs-7) isolates. Each isolate inoculated into three replicated flasks (1 L) containing 340 ml Fries modified medium and were incubated at $25 \pm 1^\circ\text{C}$ for 3 weeks. The culture filtrate was extracted with chloroform taken in a separating funnel. The collected chloroform layer was evaporated by rotary evaporator (55°C and 120 rpm) and the residue (crude toxin) dissolved in dichloromethane and was stored in small tubes at 4°C . This partially purified toxin was used for TLC and detached leaf bioassay.

Thin layer chromatography (TLC): 50 μl of partially purified toxin isolated from all isolates was loaded on Silica-gel TLC plates and developed in Benzene: Acetone in different combinations (4: 1, 1:1 and 3: 2), Benzene: Hexane (1:1) and Chloroform: Ethylacetate (19:1) solvent system. Air dried plates were visualized by iodine vapours in iodine chamber and under UV light (254 and 365 nm). Rf values of various compounds resolved on TLC plate were measured.

Detached wheat leaf bioassay with crude toxin: The biological activity of toxins extracted from BS 25 and BS 7 were determined by detached leaf method on 14 wheat genotypes by following the method given by Pillai and Wilcoxson (1970). Primary and secondary leaf segments from three week old seedlings of the fourteen wheat cultivars were collected and washed thoroughly with sterile distilled water. Two dilutions of toxin i.e. 1:1 and 1:10 were prepared. The purified toxin was infiltrated (10 μl) and in control treatment leaves were infiltrated with sterile water and chloroform. The infiltrated leaves were incubated in moist chamber at 25°C for 48 hours under light in Petri dishes. This *in vitro* bioassay was done to observe symptoms like chlorosis and necrosis. This experiment was repeated twice.

Testing the toxicity of crude toxin on other crops and weeds: Seven crop species tested against pathogen as mentioned earlier and a few weeds like *Phalaris minor*, *Avena sativa*, *Cynodon dactylon* (Bermuda grass) and *Amaranthus tricolor* (pigweed) were selected for toxin bioassay studies as described earlier.

RESULTS

Pathogenic variability

All the twelve isolates were pathogenic on genotype Agralocal, although the infection index varied from 28.3 (BS 7) to 61.6 (BS 25) (Table 1). These isolates when inoculated on a set of 14 differential hosts, showed different disease responses. Wheat genotypes, IA 814 and CN 70/ PARULA showed resistance response to all the isolates, while Agralocal showed susceptible to highly susceptible response to all the isolates. Other genotypes showed different disease responses depending on the isolates (Table 2). Therefore, these twelve isolates were divided into two categories viz. highly virulent pathotypes (3 isolates) and least virulent pathotypes (9 isolates). Further two isolates of *B. sorokiniana* i.e BS 25 (highly virulent) and BS 7 (least virulent) were compared for their differential behaviour on a set of 14 wheat genotypes. The data indicated that these two isolates showed differences in the infection on differential hosts. Isolate BS 25 showed the highest average infection index (61.65) on Agralocal, followed by (52.5) on UP 262 which are highly susceptible and 48.66 on the HD 2329 which is a susceptible genotype. The lowest ADI (9.66) was observed on the Sanghai 4. In case of BS 7 isolate, the highest average disease index (28.3) was observed on Agra local genotype, followed by 27.99 on the UP 262 and 26.66 on YM # 6. The lowest ADI (4.99) was observed on Sanghai 4 (Table 3, Fig. 1).

Table 2. Pathogenic variability of isolates of *Bipolaris sorokiniana* on a set of wheat differentials

Wheat genotype differential	BS5	BS7	BS9	BS18	BS25	BS27	BS47	BS48	BS53	BS54	BS55	BS64
IA 814 (T.AEST/4/TPIICNO/NO/3/CNO/7C/5/J UP	R	R	R	R	MS	R	MR	R	R	R	MR	MR
YM #6	MR	R	R	MR	S	R	MR	R	R	R	MS	S
MO "S" CM 8288-A3-M-6Y-5M-1Y-OM	MR	R	MS	MR	S	R	R	R	R	R	MS	MS
8230-54/32* FR/KAD//CB/4PAT (B)/5/CKA "S"	R	R	R	MR	MS	R	MR	R	R	R	MS	MS
BON/YR/3/F 3570//KAL/ BB	R	R	MS	R	MS	MR	MR	R	R	R	MS	MS
BH1146(FRONTTEIRA/MENTANA//PG1)	R	R	MR	R	MS	MR	R	R	MR	R	MS	MR
Ning 8319	R	R	R	R	S	MS	MS	R	R	R	MR	MS'
CNT 8	R	R	R	R	MS	MR	R	R	R	R	S	MR
Sanghai 4	MR	R	MR	R	R	MR	MR	R	MR	R	S	MR
UP 262	R	MS	MS	R	HS	MR	R	R	R	R	S	MS
VL 616	R	R	R	R	S	MR	R	MR	R	R	MS	S
CN 70/PARULA	R	R	R	MR	MR	MR	MR	MR	MR	MR	MR	MR
HD 2329	R	MS	MS	R	S	MR	R	MR	MR	R	MR	MS
Agralocal	S	MS	S	MS	HS	HS	MS	S	HS	S	MS	S

R: Resistant; MR: Moderately resistant; MS: Moderately susceptible; S: Susceptible; HS: Highly susceptible

Table 3. Symptoms produced on wheat differentials by pathogen and crude toxin of isolate BS 25 and BS 7 of *B. sorokiniana*

Differential hosts	BS 25				BS 7			
	Pathogen		Toxin		Pathogen		Toxin	
	Average disease index	Water Soaking (2days)	Chlorosis (7 days)	Necrosis (7 days)	Average disease index	Water Soaking (2days)	Chlorosis (7 days)	Necrosis (7 days)
IA 814 (T.AEST/4/ TPIICNO/NO/3/CNO/7C/5/JUP	28.3	+++	—	+++	23.3	++	—	+
YM #6	32.3	+++	—	+++	26.3	++	—	+
MO "S" CM 8288-A3-M-6Y-5M-1Y-OM	45.9	+++	—	+++	20.1	++	—	+
8230-54/32* FR/KAD//CB/4PAT (B)/5/CKA "S"	21.0	+	+	+	20.0	+	+	+
BON/YR/3/F 3570//KAL/ BB	28.3	++	+	++	15.0	++	+	++
BH1146(FRONTTEIRA/MENTANA//PG1)	19.6	+	+	+	11.3	+	+	+
Ning 8319	13.3	+	+	+	8.3	+	+	+
CNT 8	14.9	+	+	+	8.9	+	+	+
Sanghai 4	9.6	+	+	+	4.3	+	+	+
UP 262	52.5	+++	—	+++	29.3	++	—	+
VL 616	17.3	+	+	+	7.3	+	+	+
CN 70/PAURAL	17.3	+	+	+	5.6	+	+	+
HD 2329	48.6	+++	—	+++	23.6	++	—	++
Agralocal	61.0	+++	—	+++	28.3	+++	—	++

+++ Severe; ++ Moderate; + Mild

Pathogenicity on other hosts

Pathogenicity of these two isolates of *B. sorokiniana* when tested on various crops at seedling stage showed that no symptoms were observed on tomato, sorghum and chickpea, while only barley showed symptoms. The average infection index of two isolates, BS 25 and BS 7 on barley were observed to be 36.66 and 11.66 respectively.

Studies on toxin production by *B. sorokiniana*

Toxin profiling by TLC: TLC profile showed that solvent system, Benzene: Acetone (3: 2) was found to be the best giving a single major band of high resolution. Toxin from most virulent isolate BS 25 showed bigger, thicker and darker band of R_f value 0.47, while toxin from least virulent isolate BS 7 showed small, thin and light band of R_f value 0.36 (Fig. 2).

Fig. 1. Pathogenicity of *B. sorokiniana* isolates BS 25 and BS 7 on differential hosts

Fig. 2. Thin layer Chromatograph showing toxin profile from BS 25 and BS 7 isolates of *B. sorokiniana*

Bioassay of wheat cultivars with crude toxin of *B. sorokiniana*: The bioassay with crude toxin developed symptoms like water soaking, chlorosis and necrosis on the leaf at 1:1 dilution. In case of highly virulent isolate BS 25, all the cultivars showed severe to mild water soaking of the infiltrated leaf tissue. Severe water soaking was observed on IA 814 (T.AEST/4/TPIICNO/NO/3/CNO/7C/5/JUP), YM#6, MO "S" CM 8288-A3-6Y-5M-IY-OM, UP 262, HD 2329 and Agralocal. Moderate water soaking was observed only on BON/YR/F3570//KAL/BB and rest of the cultivars showed mild water soaking symptoms. The necrosis of the leaf tissue on cultivars also varied from severe to mild (Table 3). In case of less virulent isolate BS 7, all genotypes showed moderate to mild water soaking symptoms in the infiltrated leaf tissue except Agralocal, which showed severe water soaking. A characteristic chlorosis symptom was observed on 8230-54/32* FR/KAD/IICB/4PAT (B) 5/CKA "S" BON/YR/3/3570//KAL/BB, BH1146 (FRONTEIRA/MENTANA/

PG1), Ning 8319, CNT 78, Sanghai 4, VL 616 AND CN 70/PARULA (Table 3). The results showed that the symptoms produced by the toxin were similar to that by the pathogen i.e. toxin extracted from the most virulent and the least virulent isolate showed the same grades of disease as exhibited by pathogen inoculation.

Testing the toxicity of crude toxin on other crops and weeds: *In vitro* bioassay with the partially purified toxin of BS 25 and BS 7 showed positive reaction on wheat, barley, maize and sorghum. Toxicity of the toxin was also observed in some weeds like *Pharalis minor*, *Avena sativa* and *Cynodon dactylon*.

DISCUSSION

Bipolaris sorokiniana is a hemibiotrophic pathogen which infects both wheat and barley. Morphological, pathogenic and molecular variability has been studied in Bangladesh isolates (Ahmed *et al.* 1997), Brazilian isolates (Oliveira *et al.*, 1998; Muller *et al.*, 2005) and Indian isolates (Jaiswal *et al.*, 2007; Aggarwal *et al.*, 2010). In the present paper pathogenic variability and toxin production by different isolates collected from various geographical regions of India have been reported. Based on the reactions on differential hosts, these twelve isolates were divided into two categories viz. highly virulent pathotypes (3 isolates) and least virulent pathotypes (9 pathotypes) of which Bs 25 was highly virulent and Bs7 was found to be least virulent. Maraite *et al.* (1998) after inoculation of Ciano, Kanchan, BH 1146 and Mayoora with selected strain of pathogen also revealed variation in aggressiveness, with some specific and non-specific interactions. Mahto *et al.* (2002) observed that *B. sorokiniana* isolate of Pantnagar (Uttarakhand) was highly virulent. In our studies, isolate BS 25 collected from Jaipur was characterized as highly virulent which produced 'R' type disease response on only Sanghai 4 genotype, 'MR' type response on CN70/PARULA. Isolate BS 7 produced R type disease response on most of the genotypes except HD 2329, UP 262 and Agralocal where it produced MS type disease reaction. The study of virulence of the isolates on different genotypes indicated that the isolates may act differently with genotypes as shown by different levels of disease on wheat genotypes.

Toxins are known to be significant causal factors in the development of a number of destructive diseases of plants. The most terrible famine of all time in India during 1943 was caused in rice, induced by *Helminthosporium oryzae*. *B. maydis* produces ophiobolins which have role in pathogenesis (Sugawara *et al.*, 1987). There are a few earlier reports on production of phytotoxic substances which are sesquiterpenes and non-host specific and not directly associated with aggressiveness (Apoga *et al.*, 2002; Kumar *et al.*, 2002). In our present study, toxin was isolated from BS 25 and BS 7 isolates and their profiling on TLC plate indicated that there may be some quantitative and qualitative differences in the two isolates which are responsible for the virulence of the pathogen.

Aggarwal *et al.* (2008) through histopathological studies have reported that pathogen and the toxin brings

about similar histological changes in the leaf tissues. Rasmussen and Scheffer (1988) reported that *H. carbonum*, the causal agent of leaf spot of maize, produces host selective toxin that is required for pathogenicity, but in our studies, the toxin produced symptoms on other hosts also like barley, sorghum and maize, therefore, it is not a host specific toxin. We also observed toxicity of the toxin on some weeds like *Phalaris minor*, *Avena sativa* and *Cynodon dactylon*.

In our studies, bioassay with crude toxin developed symptoms like water-soaking, chlorosis and necrosis of the leaf. In the case of highly virulent isolate (BS 25) all the cultivars bioassayed, showed severe to mild water soaking and necrosis of the infiltrated leaf tissue. In case of least virulent isolate (BS 7), all genotypes showed mild to moderate water soaking and also mild necrotic symptoms except Agra local which showed severe necrosis. Brown and Hunger (1993) reported that chlorosis can be induced by highly selective toxin (victorin), those that are species selective (ten toxin) and that are non-selective (tab toxin). Lamari and Bernier (1989) also observed chlorosis in resistant genotypes in response to toxins.

We also observed during our investigations that reactions of differential hosts to pathogen inoculation and toxin infiltration were similar. Thus, by this method we can easily cross check the reactions of a number of wheat cultivars quickly and can differentiate them a resistant or susceptible variety saving lot of time and energy during isolations and culturing of the pathogen. Our results would be useful in characterizing isolates of spot blotch pathogen and in the resistance breeding programs of wheat and barley that are highly prone to this disease in warm and humid environments. The information regarding the presence and involvement of toxigenic strains with varying degree of toxin could be helpful in developing plants resistant to toxin, which is one of the important strategies to confer resistance against the disease.

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