

Association of melanin content with conidiogenesis and virulence in *Bipolaris Sorokiniana*

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Abstract

B. sorokiniana pathogen has emerged as a serious threat for wheat cultivation in the developing world. Warm and humid environment is conducive for the development of the disease. The information on importance of melanin in spot blotch pathogen *B. sorokiniana* of wheat and its association with conidiogenesis and virulence is not available. Therefore, studies were undertaken to find whether melanin content of the pathogen is related to conidiogenesis and virulence.

Keywords: Virulence, melanin, *B. sorokiniana*

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Introduction

Bipolaris sorokiniana is the causal agent of spot blotch disease in wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). This pathogen produces visible necrotic lesions on leaf, sheath and stem. It reproduces asexually by producing thick walled conidia (Aggarwal, 2009). Each conidium arises through distinct conidiogenous cells of conidiophores (Kendrick and Chang, 1971). *B. sorokiniana* pathogen has emerged as a serious threat for wheat cultivation in the developing world. Warm and humid environment is conducive for the development of the disease. Disease occurs throughout the world, but severe losses on wheat occur in India, Bangladesh, Bolivia, Brazil, Paraguay, Zambia, Australia and Canada (Singh and Srivastava, 1997). 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 (Aggarwal *et al.*, 2010).

Many fungi produce melanins, which are dark brown and black pigments formed by oxidative polymerization of phenolic compounds. Melanin is known to contribute to the survival and longevity of fungal propagules (Wheeler and Bell, 1988) and has been implicated as a pathogenicity factor in *Magnaporthe* and *Colletotrichum* spp. (Woloshuk *et al.*, 1980; Kubo *et al.*, 1982; Wolkow *et al.*, 1983). However, using melanin-deficient (Mel⁻) mutants induced by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), Tanabe *et al.* (1995) showed that melanin is probably not relevant to pathogenicity of the Japanese pear pathotype of *Alternaria alternata*. In many phytopathogenic fungi melanin has been implicated in the persistence of conidia and hyphae, and in the formation of appressoria and perithecia (Butler and Day 1998; Henson *et al.*, 1999). The information on importance of melanin in spot blotch pathogen *B. sorokiniana* of wheat and its association with conidiogenesis and virulence is not available. Therefore, studies were undertaken to find

whether melanin content of the pathogen is related to conidiogenesis and virulence.

Materials and Methods

Fungal isolates, culture conditions and pathogenicity of monoconidial isolates

A total of 40 samples of wheat leaves infected with *Bipolaris sorokiniana* were collected from different geographical regions of India (Table 1). The fungus, *B. sorokiniana* was isolated from single discrete lesion from the infected leaf tissue following standard procedure and the purified monoconidial cultures were maintained on potato dextrose agar (PDA) slants at 4°C. These isolates were tested for their pathogenicity on susceptible variety Agra local at seedling stage under glass house conditions at 20-25 °C and approximately 78% RH. The fifteen days old seedlings were sprayed with conidial suspension of 2 weeks old culture of *B. sorokiniana* isolates @10⁴ conidia/ml using hand atomizer. The inoculated seedlings were kept in moist chamber for 48 hours and then incubated in alkathene house for 1 week. The disease severity was measured in terms of lesion number per leaf and infection index was calculated as per the scale given by Adlakha *et al.* (1984).

Colony growth and spore count

The colony morphology with respect to colour and growth behavior was studied among all the 40 isolates of *B. sorokiniana* on PDA medium. The observations on colony morphology were taken after 7 days of incubation at 25°C. After 15 days, spore count was taken using haemocytometer.

Spectral values of isolates for melanin

All the 40 isolates of *B. sorokiniana* were grown on PDA medium at 25°C. After 5 days of growth, the plates were photographed using a Sony digital camera (7.5mp). The spectral RGB (Red, Green, Blue) values of each isolate was calculated using Adobe Photoshop (Chand *et al.* 2008).

Extraction, purification and quantification of melanin

Extraction and estimation of melanin from the hyphae was performed following the methodology of Gadd (1982). Mycelium was scraped from 5 days old colonies, boiled for 5 min in 5 ml distilled water and centrifuged (5,000g, 5 min). The mycelial pellet was then washed, centrifuged again and the pigment was extracted by autoclaving the pellet with 3 ml (1 M) NaOH for 20 min at 120°C. This was followed by acidification (pH 2) of the alkaline pigment extract with concentrated HCl to precipitate the melanin. The precipitate was washed thrice in distilled water and dried overnight at 20°C in a dehumidified condition for further analysis. Melanin content was measured spectrophotometrically at 405nm and melanin content ($\mu\text{g/g}$ of mycelium) was determined using a standard curve generated from pure melanin.

Results and Discussion

Based on the colony morphology with respect to colour and growth pattern of 40 isolates of *B. sorokiniana*, five different groups were identified. These groups were: (I) black suppressed growth; (II) brown/dull black suppressed growth; (III) gray with white spots cottony growth; (IV) dull white and/greenish black fluffy growth and (V) white fluffy growth (Table 2). Spectral RGB values of colonies differed among the isolates. Isolates belonging to group I had lesser RGB values and isolates of group V had more spectral values (Table 3). Observations on sporulation were taken after 15 days of incubation. The isolates differed significantly in spore production. BS-75 showed maximum spore production (7×10^7) followed by BS-65 (6.4×10^7) while BS 41 showed minimum spore production (0.8×10^7).

Forty different isolates of *B. sorokiniana* were tested on Agra local for their pathogenicity. All the isolates were pathogenic although the variations in no. of lesions/leaf and infection index were observed. The average number of lesions per leaf ranged between 1.6 (BS-41, BS-60, BS-92) and 15 (BS-1, BS-3). The infection index ranged between 4.5 (BS- 49) to 63.4 (BS-75). Based on pathogenicity studies, isolate BS-49 from Assam was found to be least virulent and BS-75 from Wellington was the most virulent (Table 3). Melanin content of all forty isolates ranged from 0.042mg/g (BS-41) to 3.2 mg/g (BS-75). No significant correlation ($r=0.28$) was obtained between pathogenicity and melanin content in the forty isolates of *B. sorokiniana*, but a significant positive correlation ($r= 0.7$) was obtained between sporulation and melanin content in *B.sorokiniana*.

Bipolaris sorokiniana is a hemibiotrophic pathogen which infects both wheat and barley. Host pathogen interaction studies have shown that the pathogen is initially biotrophic subsequently turning into necrotrophic phase (Aggarwal *et al.*, 2008). Our present work on colony morphology, pathogenicity and variations in melanin production has been done taking a large number of isolates from different agro-ecological zones of India. Five groups based on colony characteristics have been defined. The frequency of the dull white/greenish black colony type was maximum, while both black, suppressed type and white fluffy type colonies showed minimum frequency in the population studied. Our data on pathogenicity of these groups on susceptible genotype, Agra local indicated variations in terms of infection index. Earlier studies have also indicated a high level of morpho-pathological variability in the pathogen (Chand *et al.*, 2003; Mishra, 1981; Nelson, 1960).

Table 1 Isolates of *Bipolaris sorokiniana* collected from different zones

S. No.	Zone	No. of Isolates	Isolate Designation
1.	NWPZ (North-western plain zone)	12	BS 1, 3, 5, 6, 9, 11, 14, 88, 91, 92, 94, 95
2.	NEPZ (North-eastern plain zone)	13	BS27, 28, 29, 30, 31, 32, 34, 41, 42, 45, 48, 49, 52
3.	NHZ (Northern hill zone)	5	BS53, 54, 55, 59, 60
4.	CZ (Central zone)	1	BS63
5.	PZ (Peninsular zone)	5	BS65, 66, 68, 69, 72
6.	SHZ (Southern hill zone)	4	BS74, 75, 77, 79

Table 2 Morphological characteristics of 40 isolates of *B. sorokiniana*

S. No.	Colony Characteristics	Isolates No.	Group
1.	Black suppressed growth	9,45,59,60,63,88	I
2.	Brown /dull black suppressed growth	5,6,11,14,28,31,42,53,66,68,75,94,95	II
3.	Gray with white spots cottony growth	1,3,29,41,48,52,72,	III
4.	Dull white/greenish black fluffy growth	27,30,31,32,49,54,55,74,77,91,92	IV
5.	White fluffy growth	34,65,69	V

Table 3 Colony spectral values, sporulation, pathogenicity and melanin content in isolates of *Bipolaris sorokiniana*

Isolate	Pathogenicity	Sporulation/colony (1x10 ⁷)	Spectral values			Melanin concentration (mg/g)
			R value	G value	B value	
BS-1	34.5	1.4	111	174	208	0.10
BS-3	27.3	2.4	103	90	66	1.30
BS-5	29.3	3.5	58	55	55	1.72
BS-6	07.1	4.1	57	46	43	2.25
BS-9	35.7	2.6	52	47	45	0.80
BS-11	06.7	1.2	113	94	67	0.28
BS-14	25.0	2.0	107	174	208	0.19
BS-27	59.1	2.5	124	107	74	0.90
BS-28	34.1	1.4	145	148	154	0.09
BS-29	23.3	3.5	153	148	127	1.60
BS-30	23.5	1.5	130	192	221	0.10
BS-31	31.7	2.3	95	145	170	0.13
BS-32	30.2	2.4	43	48	37	0.85
BS-34	07.9	2.2	76	76	68	0.48
BS-41	05.0	0.8	175	186	222	0.04
BS-42	12.3	3.6	127	116	84	1.71
BS-45	16.7	2.2	85	148	182	0.16
BS-48	31.0	0.1	71	75	66	0.30
BS-49	04.5	2.1	108	172	206	0.62
BS-52	15.2	3.2	79	84	60	1.02
BS-53	54.3	2.4	85	148	133	0.61
BS-54	41.0	2.3	100	155	185	0.19
BS-55	22.7	6.1	53	51	38	1.35
BS-59	28.5	4.8	103	170	201	0.08
BS-60	35.7	3.5	48	41	60	1.98
BS-63	33.3	6.0	41	44	30	2.00
BS-65	29.1	6.4	40	42	38	2.75
BS-66	23.1	3.7	66	57	83	1.21
BS-68	21.3	2.2	100	162	194	0.18
BS-69	26.3	1.0	100	97	98	0.71
BS-72	36.3	6.1	124	114	102	0.22
BS-74	49.9	5.9	49	45	41	2.24
BS-75	63.4	7.0	38	29	40	3.20
BS-77	36.6	3.1	100	147	167	0.56
BS-79	21.5	1.9	98	149	171	1.10
BS-88	31.9	3.2	96	122	100	1.20
BS-91	38.0	1.9	102	108	144	0.99
BS-92	26.3	0.9	144	137	184	0.14
BS-94	38.9	1.1	81	96	132	0.81
BS-95	25.3	1.0	76	104	113	0.44

In many phytopathogenic fungi, melanin has been implicated in the persistence of conidia and hyphae and in the formation of appressoria and perithecia (Butler and Day, 1998; Henson *et al.*, 1999). In our studies, we observed positive significant correlation between sporulation and melanin content in *B. sorokiniana* infecting wheat. Similarly, Eliahu *et al.* (2007) observed production of abundant conidia in *Cochliobolus heterostrophus* isolates which produced melanin but albino mutants were sterile. Recently, Bashyal *et al.* (2010) reported association of melanin content with conidiogenesis in *B. sorokiniana* infecting barley. Further, there is a need to explore the role of melanin in host pathogen interaction and to identify genes involved in its biosynthetic pathway.

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