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Role of Melanin Production by *Helminthosporium* species on Pathogenicity in Graminaceous Hosts

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Abstract: Fifty foliar blight diseased samples collected from wheat, barley and oat growing areas of north-western India revealed that *Bipolaris sorokiniana* was present in all the isolates collected from barley, wheat and *Phalaris minor,* while *Drechslera avenae* was associated with isolates from oats. All the test isolates were pathogenic on their susceptible check *viz.,* PL426 (barley), HD-2329 (wheat), OL-9 (oats) and *Phalaris minor.* Minimum incubation period (2 days), maximum no. of lesions per leaf (9.29), maximum size of lesions (9.30 mm^2) and highest terminal disease severity score (89) were recorded with isolate Bsb1 from barley, followed by Bsw32 from wheat. The highest melanin production was recorded in isolate Bsb1 (2.58 µg g^{-1}) followed by 2.52 µg g^{-1} and 2.49 µg g^{-1} in isolate Bsp43 and Bsw32, respectively. Comparison of melanin production and pathogenic behaviour of different isolates revealed that more aggressive isolates on their respective hosts also produced higher melanin as compared to other isolates and strong positive correlation (r=0.749) existed between the pathogenicity and melanin production in different isolates of *Helminthosporium*.

Keywords: *Helminthosporium, Bipolaris, Drechslera*, Pathogenicity, Melanin, Correlation, Temperature regimes

Cereals are an important component of diet of the most of human beings throughout the world. The cereals crops are infected by a number of pathogenic microbes i.e. fungi, bacteria, viruses and insect pests in world and India and among the different fungal pathogens, *Helminthosporium* spp. are of major threat leading to huge economic yield losses in different cereal crops (Jayasena et al 2000, Vaish et al 2011, Singh et al 2021). *Helminthosporium* species parasitize on graminaceous hosts including barley, corn, rice, oat, wheat, sorghum and several weed hosts thus causing various types of foliar blights or leaf spot diseases and the type of lesions produced on these hosts depends upon the species involved. Different strains of *Helminthosporium* species may vary in their pathogenic or physiological behaviour. In nature or in axenic culture, sectoring usually give rise to new forms. Graminicolous *Helminthosporium* were categorised into several generas including *Bipolaris, Curvularia, Drechslera* and *Exserohilum* (Manamgoda et al 2014). The fungus *Bipolaris sorokiniana* cause spot blotch or foliar blight and is one of the most important fungal diseases of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) (Arabi et al 2011). This pathogen is favoured by warm and humid climate and produces necrotic lesions on leaf, sheath and stem and reproduces asexually by producing thick walled conidia (Aggarwal 2009). Continuing rainfall for 5 to 6 days followed by daily mean temperatures of 20- 30 ºC favours rapid development of the disease epidemics on wheat and barley (Kumar et al 2002) resulting in yield loses of upto 6-7% and 10 % in wheat and barley, respectively due to poor grain filling (Rioux et al 2016). *Drechslera avenae* (*Helminthosporium avenae;* teleomorph *Pyrenophora avenae*) causes necrotic dark brown lesions at seedling stage in oats causing mortality of the plants. The symptoms on young leaves appear as reddish to dark black coloured longitudinal stripes causing foliar blight disease (Kaur et al 2021). Physiological specialization at species level in *Helminthosporium* was first described by Christensen (1926) reporting varied virulence in fungal isolates to various graminaceous hosts and moreover, the isolates from the same geographical region and morphological group had different degrees of virulence (Ghazvini and Tekauz 2007, Polini et al 2009).

All loculoascomycetes, some pyrenomycetes, discomycetes and many deuteromycetes produce melanin, a polymer of 1,8 dihydroxynaphthalene (DHN) which is important for the survival of the fungal pathogens of these groups (Cordero and Cassadevall 2017). It has been implicated as a pathogenicity factor in *Magnaporthe* and *Colletotrichum* spp (Wang et al 2018, 2020, Gupta et al 2021). Melanin helps in the persistence of conidia and hyphae, and in the formation of appressoria and perithecia in many phytopathogenic fungi (Henson et al 1999). It provides protection against oxygen free radicals, cell wall degrading enzymes produced by microbial antagonists (Butler et al 2001), UV radiation (Cordero and Cassadevall 2017) and thus enabling fungal pathogens of plants and animals to

survive under adverse environmental conditions (Romero-Martinez et al 2000). Melanin synthesis in melanocytes was proved to be regulated by temperature (Kim et al 2003). However, information about its association with pathogenicity among different *Helminthosporium* spp. under Indian conditions is meagre, hence, the present study was undertaken to assess the association of melanin with the aggressiveness of *Helminthosporium* isolates infecting different graminaceous hosts from different north western regions of India and the effect of different temperature regimes on its production.

MATERIAL AND METHODS

Fungal isolates, culture conditions, maintenance of pure culture and pathogenicity of isolates: Surveys of different barley, wheat and oats growing areas of Punjab and adjoining states were conducted during the year 2016-17. Fifty leaf samples showing typical symptoms of foliar blight/ spot blotch were collected from different infected hosts and locations (Table 1). The pathogen was isolated from single lesion from the infected leaf tissue following standard procedure and incubated at 25±2°C (Kolte and Vishunavat 2005). The purified monoconidial cultures were maintained on potato dextrose agar (PDA) slants at 4°C. The isolates were tested for their pathogenicity on susceptible variety of the respective hosts *viz.,* PL 426 (barley), HD 2329 (wheat), OL -9 (oats) and *Phalaris minor* (weed host) under pot-house maintained at temperature and relative humidity of 26±2°C and 80-95%, respectively. Plants were grown in 10 inches diameter pots filled with sand, FYM and field soil (1:1:2) using recommended package of practices (Anonymous 2019).

Preparation and spraying of inoculum: Mass inoculum of all the isolates were prepared separately in 100ml Erlenmeyer flasks on sorghum seed medium. The imbibed sorghum grains @ 20g flask^{-1} were autoclaved at 20psi for 20 minutes and were inoculated with 2-3 discs (10mm diameter) of 7 days old colony of each isolate separately under aseptic conditions. The flasks were incubated at 25±2°C for 15 days. The flasks were shaken daily to promote sporulation and prevent mycelium clumps and the grains were completely covered with fungal growth after 10-12 days. Inoculum was prepared in sterilized water by taking approx. 20 infected sorghum seeds and shaking those well to detach the spores from grains in water. 1-2 drops of Tween-80 were also added in spore suspension. The suspension thus obtained was sieved through the sterilized muslin cloth to remove the grains and mycelium. The spore concentration was adjusted to 5 x 10^4 spores/ ml with the help of haemocytometer.

One-month old seedlings of each host cultivar at 3-4 leaf stage were spray inoculated with conidial suspension of each of their respective isolates in isolation @ 5 x 10^4 spores ml^{-1} using hand atomizer. The inoculated plants were then allowed to dry for 2-3 hrs and were incubated in polyhouse having humidifiers for 12 h, to provide enough saturated moisture to enhance infection and were later incubated for 10 days.

Symptom development: Initially water-soaked spots developed within 2-3days which later turned into yellow and necrotic area on the inoculated leaves. Observations were recorded on incubation period (days after inoculation), number of lesions leafn^{-1} (10-15 days after inoculation on flag, first and second leaf), size of lesions (in mm^2, length x breadth of five biggest spots) for each isolate. The terminal disease severity was measured in double-digit figure using 0-9 scale (Saari and Presscott 1975).

Estimation of melanin content in *Helminthosporium* isolates: Melanin content was extracted and estimated in all the 50 isolates as per the method described by Gadd (1982).

Effect of different temperature regimes on melanin production by *Helminthosporium* isolates: Petri plates containing freshly prepared PDA medium were inoculated aseptically by placing 5 mm discs from 6 days old culture of each of the 50 isolates and then these plates were incubated at different temperature regimes viz; 15°C, 20°C, 25°C, 30°C and 35°C for the growth of the pathogen. The extraction and estimation of melanin in each isolate at different temperature regimes was undertaken according to the method as described above.

Statistical analysis: The data obtained from the pot house and laboratory experiments were analysed using completely randomized design (CRD) and dendrogram based on pathogenic variability among the different isolates was generated using the software PAST (version 2.6). Pearson's correlation coefficient was also calculated to analyse the correlation between aggressiveness and melanin production in different isolates.

RESULTS AND DISCUSSION

Pathogen associated with diseased samples: The isolated fungus and its species were identified based on the morphological characters as per the key of Manamgoda et al (2014). The test isolates were arbitrarily named as Bsb1 to Bsb29 (barley), Bsw30 to Bsw41 (wheat) Bsp42 to Bsp45 (*Phalaris minor*) and Da46 to Da50 (oats) (Table 1). Based on colony characteristics on PDA medium, the isolates were divided into three categories having black, greyish white and white coloured colonies (Table 2).

Pathogenic behavior of isolates: The isolates were pathogenic on their susceptible check i.e. PL426 (barley), HD

Table 1. Identification of pathogens associated with spot blotch/foliar blight disease on different hosts under north-western regions of India

Isolate No.	Host	Location	Pathogen involved
Bsb1	Barley	Ludhiana (Punjab) (30.9°N 75.85°E)	*Bipolaris sorokiniana*
Bsb2	Barley	Samrala (Punjab) (30.84°N 76.19°E)	*Bipolaris sorokiniana*
Bsb3	Barley	Abohar (Punjab) (30.1334°N 74.2001°E)	*Bipolaris sorokiniana*
Bsb4	Barley	Ganganagar (Rajasthan) (29.9094° N, 73.8800° E)	*Bipolaris sorokiniana*
Bsb5	Barley	Ganganagar (Rajasthan) (29.9094° N, 73.8800° E)	*Bipolaris sorokiniana*
Bsb6	Barley	Barnala (Punjab) (30.3819° N, 75.5468° E)	*Bipolaris sorokiniana*
Bsb7	Barley	Hisar (Haryana) (29.1492° N, 75.7217° E)	*Bipolaris sorokiniana*
Bsb8	Barley	Rohtak (Haryana) (28.8955° N, 76.6066° E)	*Bipolaris sorokiniana*
Bsb9	Barley	Jagraon (Punjab) (30.7923° N, 75.4670° E)	*Bipolaris sorokiniana*
Bsb10	Barley	Sangala, Sangrur (Punjab) (30.4646° N, 75.8875° E)	*Bipolaris sorokiniana*
Bsb11	Barley	Mastuana sahib (Punjab) (30.2662° N, 75.7752° E)	*Bipolaris sorokiniana*
Bsb12	Barley	Malerkotla (Punjab) (30.5246° N, 75.8783° E)	*Bipolaris sorokiniana*
Bsb13	Barley	Kheri, Sangrur (Punjab) (30.1905° N, 75.8811° E)	*Bipolaris sorokiniana*
Bsb14	Barley	Patiala (Punjab) (30.3398° N, 76.3869° E)	*Bipolaris sorokiniana*
Bsb15	Barley	Mansa (Punjab)(29.9995° N, 75.3937° E)	*Bipolaris sorokiniana*
Bsb16	Barley	Ahmadgarh (Punjab) (30.6796° N, 75.8243° E)	*Bipolaris sorokiniana*
Bsb17	Barley	Jalandhar (Punjab) (31.3260° N, 75.5762° E)	*Bipolaris sorokiniana*
Bsb18	Barley	Amritsar (Punjab) (31.6340° N, 74.8723° E)	*Bipolaris sorokiniana*
Bsb19	Barley	Sri Muktsar Sahib (Punjab) (30.4762° N, 74.5122° E)	*Bipolaris sorokiniana*
Bsb20	Barley	Fazilka (Punjab) (30.4036° N, 74.0280° E)	*Bipolaris sorokiniana*
Bsb21	Barley	Sidhpur (Himachal Pradesh) (32.1953° N, 76.3536° E)	*Bipolaris sorokiniana*
Bsb22	Barley	Malana (Himachal Pradesh) (32.0617° N, 77.2613° E)	*Bipolaris sorokiniana*
Bsb23	Barley	Bajaura (Himachal Pradesh) (31.8465° N, 77.1605° E)	*Bipolaris sorokiniana*
Bsb24	Barley	Bhuntar (Himachal Pradesh) (31.8862° N, 77.1455° E)	*Bipolaris sorokiniana*
Bsb25	Barley	Shamshi (Himachal Pradesh) (31.8933° N, 77.1384° E)	*Bipolaris sorokiniana*
Bsb26	Barley	Ner chowk (Himachal Pradesh) (31.6085° N, 76.9153° E)	*Bipolaris sorokiniana*
Bsb27	Barley	Berthin (Himachal Pradesh) (31.4188° N, 76.6427° E)	*Bipolaris sorokiniana*
Bsb28	Barley	Andreta (Himachal Pradesh) (32.0401° N, 76.5676° E)	*Bipolaris sorokiniana*
Bsb29	Barley	Palampur (Himachal Pradesh) (32.12°N 76.53°E)	*Bipolaris sorokiniana*
Bsw30	Wheat	Mansa (Punjab) (29.9995° N, 75.3937° E)	*Bipolaris sorokiniana*
Bsw31	Wheat	Kheri, Sangrur (Punjab) (30.1905° N, 75.8811° E)	*Bipolaris sorokiniana*
Bsw32	Wheat	Ludhiana (Punjab) (30.9010° N, 75.8573° E)	*Bipolaris sorokiniana*
Bsw33	Wheat	Jagraon (Punjab) (30.7923° N, 75.4670° E)	*Bipolaris sorokiniana*
Bsw34	Wheat	Jalandhar (Punjab) (31.3260° N, 75.5762° E)	*Bipolaris sorokiniana*
Bsw35	Wheat	Ganganagar (Rajasthan) (29.9094° N, 73.8800° E)	*Bipolaris sorokiniana*
Bsw36	Wheat	Ganganagar (Rajasthan) (29.9094° N, 73.8800° E)	*Bipolaris sorokiniana*
Bsw37	Wheat	Hisar (Haryana) (29.1492° N, 75.7217° E)	*Bipolaris sorokiniana*
Bsw38	Wheat	Rohtak (Haryana) (28.8955° N, 76.6066° E)	*Bipolaris sorokiniana*
Bsw39	Wheat	Jagraon (Punjab) (30.7923° N, 75.4670° E)	*Bipolaris sorokiniana*
Bsw40	Wheat	Malan (H.P.) (32.1134° N, 76.4202° E)	*Bipolaris sorokiniana*
Bsw41	Wheat	Bajaura (H.P.) (31.8465° N, 77.1605° E)	*Bipolaris sorokiniana*
Bsp42	*Phalaris minor*	Abohar (Punjab) (30.1469° N, 74.2008° E)	*Bipolaris sorokiniana*
Bsp43	*Phalaris minor*	Sri Muktsar sahib (Punjab) (30.4762° N, 74.5122° E)	*Bipolaris sorokiniana*
Bsp44	*Phalaris minor*	Hisar (Haryana) (29.1492° N, 75.7217° E)	*Bipolaris sorokiniana*
Bsp45	*Phalaris minor*	Hoshiarpur (Punjab) (31.5143° N, 75.9115° E)	*Bipolaris sorokiniana*
Da46	Oats	Sangrur (Punjab) (30.2458° N, 75.8421° E)	*Drechslera avenae*
Da47	Oats	Malerkotla (Punjab) (30.5246° N, 75.8783° E)	*Drechslera avenae*
Da48	Oats	Jalandhar (Punjab) (31.3260° N, 75.5762° E)	*Drechslera avenae*
Da49	Oats	Amritsar (Punjab) (31.6340° N, 74.8723° E)	*Drechslera avenae*
Da50	Oats	Fazilka (Punjab) (30.4036° N, 74.0280° E)	*Drechslera avenae*

2329 (wheat) OL- 9 (oats) and *Phalaris minor* (Table 3). The barley isolates, Bsb1, Bsb2, Bsb9, Bsb12, Bsb13, Bsb19 and Bsb26 were fast in developing the initial symptoms and the minimum incubation period was recorded in Bsb1 (2 days) and in rest of the isolates the disease development took 3-5 days. Similar trend was also observed with isolates from wheat, weed host and oats. Isolate Bsw32 was comparatively fast in developing foliar blight symptoms on the susceptible host (3 days) while rest of the isolates it ranged from 4-6 days. Similarly, in case of weed host and oats, isolate Bsp43 and Da46 were faster in developing the initial symptoms in weed host (3 days) and oats (4 days), respectively as compared to other isolates. In barley, maximum average number of lesions (9.20) was recorded in isolate Bsb1 followed by Bsb26 and isolate Bsb16 produced least number of lesions (3.80). The average number of lesions ranged from 4.60 to 8.70 in rest of the isolates.

In wheat, isolate Bsw32 recorded maximum mean number of lesions (8.20) followed by Bsw33. Among weed host and oats, the maximum number of lesions were recorded with isolate Bsp43 (6.70) and Da46 (6.40). The development of necrotic area also varied significantly in different isolates. The maximum size of necrotic area in barley (9.30 mm^2) was in isolate Bsb1 followed by 9.20mm^2 in isolate Bsb26 and was minimum (2.8mm^2) in isolate Bsb22. Lesion size varied from 3.00 to 8.85mm^2 in rest of the isolates. Among wheat isolates, lesion size was maximum in Bsw32 (7.80mm^2) followed by 7.00mm^2 in isolate Bsw39 and was least in Bsw41 (2.6mm^2). In weed host and oats, the maximum size of lesions was recorded in isolates Bsp43 (7.60mm^2) and Da46 (6.60 mm^2), respectively. In comparison of all the *Helminthosporium* isolates, isolate Bsb1 from barley was the fastest in developing the initial symptoms with maximum number and size of the necrotic area and was highly aggressive on its susceptible check with maximum disease severity score of 89 as compared to all other isolates. Among barley and wheat isolates, the highest terminal disease severity (TDS) of 89 and 78 was in isolate Bsb1 and Bsw32, respectively, while isolates Bsp43 and Da46 from weed host and oats were more aggressive on their susceptible checks with maximum TDS of 68. All the isolates were differentiated by comparing all the different parameters using software PAST (version 3.26) and a dendrogram was generated (Fig. 1). All the isolates converged into two groups i.e. 1 and 2 (Table 4). Group 1 consisted of single isolate Bsb1 and group 2 was further divided into subgroups i.e. 2a and 2b. Group 2a further converged into 2a (i) with ten isolates, while 22 isolates comprised grouped 2A were clustered in group 2b (i), while 2b (ii) consist of 13 isolates. Based on their pathogenic behaviour, it was observed that isolate Bsb1 was distinctly variable in pathogenic behaviour as compared to all other isolates.

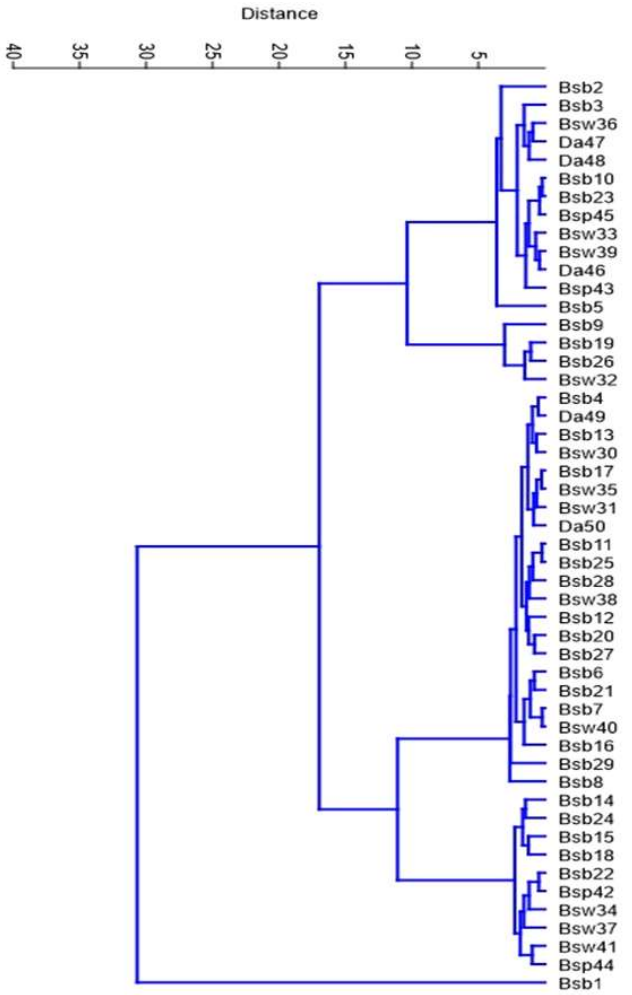

Fig. 1. Dendrogram generated based on the pathological variation among different isolates

Table 2. Grouping of isolates according to colony colour

Group	Colony colour	Isolates	Total no of isolates
1	Black	Bsb1, Bsb9, Bsb19, Bsb21, Bsb23, Bsb26, Bsw32, Bsw36, Bsw39, Bsp43, Da46, Da47, Da48	13
2	Greyish white	Bsb2, Bsb3, Bsb4, Bsb5, Bsb6, Bsb7, Bsb8, Bsb10, Bsb11, Bsb12, Bsb13, Bsb14, Bsb15, Bsb16, Bsb17, Bsb20, Bsb22, Bsb24, Bsb25, Bsb27, Bsb28, Bsb29, Bsw30, Bsw31, Bsw33, Bsw34, Bsw35, Bsw37, Bsw41, Bsp44, Bsp45, Da49, Da450	33
3	White	Bsb18, Bsw38, Bsw40, Bsp42	4

Table 3. Pathogenic behaviour of different *Helminthosporium* isolates as depicted by incubation period, no. of lesions/leaf, size of lesions and terminal disease severity

Sr. No.	Isolate	Incubation period (days)	No. of lesions per leaf	Size of lesions (mm^2)	Terminal disease severity
1	Bsb1	2	9.20	9.30	89
2	Bsb2	3	8.66	8.50	68
3	Bsb3	4	7.33	5.00	67
4	Bsb4	5	5.00	4.10	56
5	Bsb5	4	6.00	3.20	67
6	Bsb6	4	4.80	3.60	57
7	Bsb7	4	5.00	3.10	56
8	Bsb8	4	6.80	4.00	58
9	Bsb9	3	6.50	7.30	76
10	Bsb10	4	6.66	7.20	67
11	Bsb11	4	5.60	5.10	56
12	Bsb12	4	5.70	6.10	57
13	Bsb13	5	4.40	5.20	56
14	Bsb14	4	5.40	5.00	46
15	Bsb15	4	5.20	4.00	47
16	Bsb16	4	3.80	3.70	56
17	Bsb17	5	5.00	4.20	57
18	Bsb18	4	4.60	3.60	46
19	Bsb19	3	8.70	8.85	77
20	Bsb20	4	5.60	5.10	57
21	Bsb21	4	5.33	3.00	57
22	Bsb22	5	4.80	2.80	46
23	Bsb23	4	6.60	7.00	67
24	Bsb24	4	5.75	4.00	45
25	Bsb25	4	5.40	5.20	56
26	Bsb26	3	8.75	9.20	78
27	Bsb27	4	6.33	4.80	57
28	Bsb28	4	5.20	6.00	56
29	Bsb29	5	5.33	6.20	58
30	Bsw30	5	4.25	4.60	56
31	Bsw31	5	4.80	4.70	57
32	Bsw32	3	8.20	7.80	78
33	Bsw33	4	7.00	6.50	68
34	Bsw34	6	4.80	3.60	46
35	Bsw35	5	5.25	4.20	57
36	Bsw36	4	6.25	5.10	67
37	Bsw37	6	5.50	3.80	45
38	Bsw38	5	5.80	5.20	56
39	Bsw39	4	6.40	7.00	68
40	Bsw40	4	5.20	3.10	56
41	Bsw41	5	3.33	2.60	45
42	Bsp42	5	5.00	3.20	46
43	Bsp43	3	6.70	7.60	68
44	Bsp44	5	4.20	3.00	45
45	Bsp45	4	6.60	7.50	67
46	Da46	4	6.40	6.60	68
47	Da47	4	6.33	6.00	67
48	Da48	4	5.20	5.40	67
49	Da49	5	5.00	4.60	56
50	Da50	5	5.50	5.00	57
	CD (p=0.05)	-	0.10	0.14	-

Melanin content in different *Helminthosporium* isolates: The highest melanin production was in isolate Bsb1 (2.58 µg g^{-1}) followed by Bsp43 and Bsw32 (Table 5). The lowest melanin production was recorded in the isolates Bsb18, Bsw38, Bsw40, Bsp42, respectively. Pearson's correlation coefficient (r) between melanin production and TDS among different isolates was 0.749, thus showing a strong correlation between the two variables (Fig. 2).

Effect of different temperature regimes on melanin production: In some isolates maximum melanin production was recorded at 25°C while in others at 30°C implying that overall maximum production of melanin was recorded in temperature range of 25-30°C with mean melanin production of 1.50µg g^{-1} among all the test isolates (Table 5). The growth of the isolates was scarce at minimum temperature of 15°C i.e. from zero to very less (0.05 µg g^{-1} in Bsb1), while was slightly high at temperature of 20°C with mean melanin production of 0.0046 and 0.69 µg g^{-1} at 15° and 20°C, respectively. Melanin production showed declining trend at higher temperature of 35°C and average melanin production among different isolates was recorded to be 0.64µg g^{-1}. A large amount of pathogenic diversity existed among *Helminthosporium* and variation in aggressiveness was observed among the isolates from different geographical regions. In the present study, all the test isolates were pathogenic on their susceptible check PL 426 (barley), HD 2329 (wheat) OL- 9 (oats) and *Phalaris minor* to varying degree of virulence. Based on the cluster analysis, all these isolates converged into two groups i.e. 1 and 2. The first group consist of only single isolate Bsb1 while group 2 was further divided into two subgroups i.e. 2a and 2b. Isolate Bsb1 was distinctly variable in its pathogenic behaviour with maximum number and size of the necrotic area developed and was highly virulent on its susceptible check with maximum disease severity score of 89. The results obtained in the present study corroborated with Ghazvini and Tekauz (2007) who evaluated virulence diversity of 127 *B. sorokiniana* isolates on 12 barley genotypes and identified eight virulent groups. The lesion number/leaf was significantly different depending on the isolate, irrespective of the plant part and infection by *B. sorokiniana* was highly variable, and very sensitive to environmental conditions. Similarly, Polini et al (2009) also observed that 35 *B. sorokiniana* isolates in Brazil and other countries showed different degree of virulence despite being from the same geographical region and morphological group. The169 virulent isolates of *B. sorokiniana* isolated from different wheat growing areas of Bangladesh by Sultana et al (2018) reported a clear evidence of positive relationship among the components. Further, hierarchical cluster analysis revealed that all the isolates converged into five groups and variation in aggressiveness was observed among the isolates from different wheat growing areas.

Melanins alone cannot be responsible for causing disease in the host but rather it is implicated to provide protection against oxygen free radicals, cell wall degrading enzymes produced by microbial antagonists, UV radiation and thus enabling fungal spores to survive under adverse environmental conditions. In the present study, the estimation of melanin content was recorded in the test isolates at standard temperature of 25 °C and also at different temperature regimes of 15° C to 35°C. The maximum melanin production was in the isolated Bsb1 (2.58 µg/g) followed by Bsp43 and Bsw32 at 25°C. Melanin production in some of the isolates was recorded maximum at 25°C while in others at 30°C and thereafter it decreased. Temperature

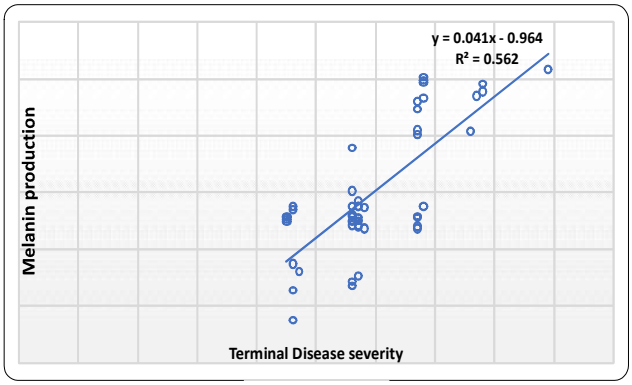

Fig. 2. Correlation between melanin production and virulence in different *Helminthosporium* isolates

Table 4. Grouping of the isolates based on their pathogenic behaviour

Group	Sub-group	Isolate (s)	Total number of isolate(s)
1	-	Bsb1	1
2	2a (i)	Bsb14, Bsb15, Bsb18, Bsb22, Bsb24, Bsw34, Bsw37, Bsw41, Bsp42, Bsp44	32
	2a (ii)	Bsb4, Bsb6, Bsb7, Bsb8, Bsb11, Bsb12, Bsb13, Bsb16, Bsb17, Bsb20, Bsb21, Bsb25, Bsb27, Bsb28, Bsb29, Bsw30, Bsw31, Bsw35, Bsw38, Bsw40, Da49, Da50.	
	2b (i)	Bsb9, Bsb19, Bsb26, Bsw32	17
	2b (ii)	Bsb2, Bsb3, Bsb5, Bsb10, Bsb23, Bsw33, Bsw36, Bsw39, Bsp43, Bsp45, Da46, Da47, Da48.	

Table 5. Melanin production by different *Helminthosporium* isolates

Sr. No.	Isolate	Melanin (µg g^{-1}) production at different temperature regimes.				
		15°C	20°C	25°C	30°C	35°C
1	Bsb1	0.05	1.67	2.58	2.77	1.74
2	Bsb2	0.00	0.70	1.38	1.17	0.69
3	Bsb3	0.00	0.58	1.30	1.20	0.79
4	Bsb4	0.01	1.15	1.90	2.01	1.12
5	Bsb5	0.00	0.41	1.19	1.24	0.52
6	Bsb6	0.00	0.47	1.26	1.31	0.53
7	Bsb7	0.00	0.66	1.32	1.26	0.48
8	Bsb8	0.00	0.63	1.37	1.35	0.70
9	Bsb9	0.03	1.11	2.04	1.39	0.92
10	Bsb10	0.00	0.58	1.29	1.35	0.61
11	Bsb11	0.00	0.52	1.22	1.16	0.64
12	Bsb12	0.00	0.69	1.21	1.28	0.54
13	Bsb13	0.00	0.58	1.38	1.43	0.42
14	Bsb14	0.00	0.19	0.88	0.79	0.19
15	Bsb15	0.00	0.17	0.81	0.95	0.21
16	Bsb16	0.00	0.54	1.26	1.24	0.62
17	Bsb17	0.00	0.47	1.38	1.27	0.64
18	Bsb18	0.00	0.04	0.39	0.53	0.02
19	Bsb19	0.01	0.79	2.35	2.51	1.15
20	Bsb20	0.00	0.19	0.77	0.76	0.21
21	Bsb21	0.00	0.88	1.43	1.48	0.65
22	Bsb22	0.00	0.70	1.35	1.30	0.53
23	Bsb23	0.00	1.20	2.01	2.09	0.74
24	Bsb24	0.00	0.06	1.27	1.00	0.08
25	Bsb25	0.00	0.09	1.31	1.12	0.09
26	Bsb26	0.04	1.18	2.46	2.54	0.62
27	Bsb27	0.00	0.88	1.38	1.43	0.73
28	Bsb28	0.00	0.63	1.25	1.26	0.48
29	Bsb29	0.00	0.68	1.19	1.35	0.41
30	Bsw30	0.00	0.53	1.30	1.22	0.58
31	Bsw31	0.00	0.76	1.26	1.34	0.52
32	Bsw32	0.02	1.24	2.49	2.52	0.98
33	Bsw33	0.01	1.22	2.33	2.22	1.09
34	Bsw34	0.00	0.54	1.38	1.45	0.60
35	Bsw35	0.00	0.88	1.22	1.37	0.71
36	Bsw36	0.00	1.40	2.05	2.22	1.07
37	Bsw37	0.00	0.56	1.25	1.27	0.43
38	Bsw38	0.00	0.05	0.69	0.65	0.02
39	Bsw39	0.00	1.21	2.39	2.41	1.10
40	Bsw40	0.00	0.02	0.73	0.78	0.05
41	Bsw41	0.00	0.58	1.29	1.34	0.62
42	Bsp42	0.00	0.05	0.65	0.58	0.22
43	Bsp43	0.01	1.26	2.52	2.46	1.16
44	Bsp44	0.00	0.60	1.30	1.30	0.45
45	Bsp45	0.00	0.58	1.21	1.30	0.40
46	Da46	0.03	1.19	2.47	2.40	1.08
47	Da47	0.00	1.09	2.30	2.36	0.98
48	Da48	0.02	1.04	2.24	2.45	1.87
49	Da49	0.00	0.73	1.52	1.46	0.62
50	Da50	0.00	0.34	1.29	1.35	0.24
	Mean	0.0046	0.69	1.50	1.50	0.64
	CD (p=0.05)	0.0038	0.01	0.02	0.02	0.02

regulation of melanin synthesis in the present studies is in accordance with the results of Kim et al (2003). Similarly, Suwannarach et al (2019) also reported that melanin pigment production by an endophytic fungus, *Spissiomyces endophytica* SDBR-CMU319 was maximum at 25°C in glucose yeast extract peptone medium at an initial pH value of 6.0 over three weeks of cultivation. Further, it was observed that the isolates which were more aggressive on their respective hosts (Bsb1, Bsb9, Bsb26, Bsw32, Bsp43 and Da46) were also reported to be higher melanin producers as compared to other isolates. Hence, melanin production in the fungus was positively corelated (r= 0.749) with the pathogenicity of the fungal isolates. Black coloured isolates recorded more melanin content as compared to mixed and white isolates. The association of melanin content with pathogenicity and virulence in *Bipolaris oryzae* was also reported by Singh et al (2016). Melanization of *B. sorokiniana* mycelia was an important factor for conidia production was also proved by Bashyal et al (2010) who revealed that conidiogenesis in black, white and mixed subpopulation of *B. sorokiniana* was positively correlated with melanin content/g of mycelium. Similarly, positive significant correlation between sporulation and melanin content in *B. sorokiniana* infecting wheat has also been proved by Aggarwal et al (2011).

CONCLUSION

All the test isolates were pathogenic on their susceptible check. The isolate Bsb1 from barley recorded the highest melanin content and was also most aggressive on its susceptible check followed by Bsw32 from wheat. Comparing the melanin production and pathogenic behaviour of different isolates revealed that the isolates which were more aggressive on their respective hosts also produced higher melanin as compared to other isolates and this study proved that a strong positive correlation existed between the pathogenicity and melanin production in different isolates of *Helminthosporium*.

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