

## Association of melanin content with conidiogenesis in *Bipolaris Sorokiniana* of barley (*Hordeum vulgare* L.)

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**Abstract** Sixty-seven isolates of *Bipolaris sorokiniana* of barley, belonging to three groups (black, white and mixed) were studied to find an association of melanin with the spore production of the fungus. Conidiogenesis in black, white and mixed subpopulation of *B. sorokiniana* was positively correlated with melanin content/g of mycelium. Primary hyphae of black and mixed subpopulation differentiated into secondary hyphal structures which subsequently produced conidiophores and conidia. Primary hyphae could not differentiate into secondary hyphae and subsequently conidiophores and conidia in white subpopulation. A melanin containing mutant developed from white subpopulation regained its ability to differentiate into secondary hyphae, conidiophores and conidia. Results showed that melanization of mycelia *B. sorokiniana* mycelia is an important factor for conidia production.

**Keywords** *Bipolaris sorokiniana* · Conidiogenesis · Melanin · Spot blotch · Barley

### Introduction

*Bipolaris sorokiniana* (Sacc.) Shoemaker (Syn. *Helminthosporium sativum* telomorph) is a well known cause of

spot blotch disease in barley (*Hordeum vulgare*) and wheat (*Triticum aestivum* L.). This pathogen induces visible necrotic symptoms on the leaf, sheath and stem (Kumar et al. 2007). In nature, *B. sorokiniana* reproduces asexually by producing thick walled conidia. Each conidium arises through distinct conidiogenous cells of conidiophores (Kendrick and Chang 1971). Both morphological and pathogenic variability in the population of *B. sorokiniana* had been reported (Mishra 1981; Nelson 1960; Jaiswal et al. 2007). Based on colony morphology and RAPD markers, isolates of *B. sorokiniana* of wheat were grouped in five groups namely black, brown/dull black, grey with white spots, dull white/greenish black and white (Chand et al. 2002, 2003). Of which black subpopulation occurred in nature at high frequency when compared to white. White subpopulation produced fewer conidia which subsequently lowered its fitness when compared to black subpopulation (Pandey et al. 2008; Jaiswal et al. 2007).

Dark pigmentation in fungi is mainly attributed to the presence of melanin that enables fungal pathogens of plants and animals to survive adverse environmental conditions by protecting them against oxygen free radicals (Romero-Martinez et al. 2000), UV radiation (Kawamura et al. 1997), and wall-degrading enzymes produced by microbial antagonists (Butler et al. 2001). 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). However, the importance of melanin in spot blotch pathogen *B. sorokiniana* and its associations with aggressiveness is limited. Therefore, studies were undertaken to find whether melanin content of the pathogen is related to conidiogenesis and subsequently in its growth, fitness and disease progression/aggressiveness in the plant host.

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## Materials and methods

### Fungal isolates and culture conditions

*B. sorokiniana* infected leaves characterized by small 1–4 mm long dark brown lesions with chlorotic margin were collected from different germplasm lines of barley. The infected lesions (1 mm<sup>2</sup>) were excised under sterilized conditions, surface sterilized with 10% NaOCl and transferred to Potato Dextrose Agar (PDA) medium under aseptic conditions for incubation at 25°C. Black, white and mixed colonies were obtained. Pure cultures of black and mixed isolates were established by single spore isolation (Kumar et al. 2007) using visible colonies that appeared on 4th day of incubation. White isolates were purified by mycelia tip culture as conidia were absent in these isolates. Mycelial mats of 4 days old culture was removed and washed in sterile distilled water at 10,000 rpm at the temperature of 25°C for 2 min in Waring blender (700S/700G of Waring Laboratory Science). The resulting suspension was filtered through fine wire gauge to remove large fragments and concentrated by centrifugation to make hyphal suspension. Aliquots of hyphal suspension (0.2 ml containing 100–200 fragments) were distributed on the surface of Potato Dextrose Agar in Petri plates and was incubated at 2°C for 4 days.

Spore production was recorded by excising 5 mm<sup>2</sup> colony area using cork borer and was stirred in 1 ml water using magnetic stirrer to dislodge the conidia. Numbers of spores/cm<sup>2</sup> were counted by the haemocytometer (B.S 748) of Rohem, India.

### Area under disease progress curve

Black, mixed and white isolate of *B. Sorokiniana* were inoculated in susceptible check variety RD 2503 on three different growth stages viz. GS 57, 69 and 77 (Zadock et al. 1974) to know aggressiveness of isolates. Spot blotch severity was recorded using the double digit (DD, 00–99), modified Sarri and Prescott severity scale (Saari and Prescott 1975; Eyal et al. 1987) three times at an interval of 8 days. The AUDPC was calculated using the percent severity estimations corresponding to the disease ratings as outlined by Roelfs et al. (1992):

$$\text{AUDPC} = \sum_{i=1}^a \{(Y_i + Y(i+1))/2[t(i+1) - t_i]\}$$

where  $Y_i$  = disease level at time  $t_i$ ,  $T(i+1) - t_i$  = time (days) between two disease scores, and  $n$  = number of dates at which spot blotch was recorded.

### Morphogenetic studies in black and white isolates of *B. sorokiniana* in growth medium

Circular discs (5 mm<sup>2</sup>) were excised out of black and white colonies with the help of a flame-sterilized metal cork borer. So obtained discs were mounted on a glass slide, stained with cotton blue and lacto-phenol and monitored for mycelial differentiation and conidia development. Observations were recorded after 6 h after inoculation at every 2 h interval for development of secondary hyphae, conidiophores and conidia from each microscopic field (4.538 mm<sup>2</sup>). Long, thin, blue, interwoven hyphae were designated as primary hyphae; while, black, thickened, stumpy hyphae with close septa were the characteristic features of the secondary hyphae. Differences for septal distance in primary and secondary mycelia and other observations related to the development i.e. secondary hyphae, conidiophores and conidia were also recorded. The entire experiment was repeated ten times on Minimal medium (Leach et al. 1982). The 10 isolates from each subpopulation was observed and 10 replications were taken to record the entire process of conidiogenesis in *B. sorokiniana*.

### Extraction and purification of melanin

Extraction and estimation of melanin from the hyphae was performed following Gadd (1982). One gram mycelium was scraped from 6 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 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.

For spectrophotometric assay, dried pellet was solubilized in 1 ml of 1 N NaOH for 2 h at 80°C and centrifuged at 12,000g for 10 min. The supernatant was transferred to fresh tubes and the absorbance measured at 405 nm (Carzaniga et al. 2002). Melanin content (µg/g of mycelium) was determined using a standard curve generated from melanin. Standard melanin (Sigma Chemicals Co., St. Louis, USA) at a concentration of 0–20 µg/ml was dissolved in 1 ml of 1 N NaOH. It was centrifuged at 12,000g for 10 min and supernatant was transferred to fresh tubes and the absorbance measured at 405 nm to generate standard curve for melanin.

### Melanin containing mutant from white isolate

Methodology of Tinline (1961) was followed for the development of black mutant from white isolate. White

isolate of *B. sorokiniana* (23 W) was grown in Potato Dextrose Agar medium. Mycelial mat of 6 days old culture were removed, washed in sterile distilled water, and macerated in Waring Blender at 10,000 rpm with a temperature of 25°C for 2 min. The resulting suspension was filtered through fine wire gauge to remove large fragments and concentrated by centrifugation to make hyphal suspension. Aliquots of a hyphal suspension (0.2 ml containing 100–200 fragments) were distributed on the surface of Potato Dextrose Agar in Petri Plates. After they had been dried for about 2 h at room temperature, the lid of Petri dishes were removed and exposed to Ultraviolet (UV) radiation. The source of UV was Klenzoids medium pressure tube that emitted 95% radiation at 2,537 Å with an intensity of  $108 \mu\text{w}/\text{cm}^2$  at a distance of 20 cm for 30 min. Immediately after treatment the dishes were covered and placed in dark to prevent photo-reactivation of the propagules. Hyphal tips were grown in PDA and observations were made for colony color. Black colonies observed were further transferred in PDA for three times at an interval of 5 days to check the stability of the colony that was selected as mutant for further study.

#### Spectral values of isolates for melanin

Thirty different isolates that differed in numbers of conidia were grown in PDA and incubated at 25°C. After, 7 days growth were photographed using a Nikon digital Camera (Nikon E995, Japan) with 995 pixels and the spectral RGB (Red, Green and Blue) values of the individual isolates assessed using Adobe Photoshop (Chand et al. 2008). Vertical and horizontal grid lines were constructed at a distance of 0.5 cm and observations drawn from all areas of the growing mycelium. Nine rectangles were covered for one replication and seven such replications for every isolate were analyzed statistically.

#### Treatment with melanin synthesis inhibitor

Tricyclazole, an inhibitor of 1, 8-dihydroxynaphthalene (DHN) melanin synthesis (Sigma Chemical, St Louis, MO), was used.  $100 \mu\text{g ml}^{-1}$  of Tricyclazole was dissolved in ethanol and was added to cooled PDA medium (Elliott 1995). The medium was inoculated by placing a 5 mm diameter plug of mycelium cut from the margin of an actively growing colony of B5, B13 isolates of black and mixed subpopulation (Table 2) and a white isolate. Seven replicates were maintained for each isolate of black, mixed and white color. Plates were incubated for 10 days at 25°C and observed for growth, pigmentation, development of hypha, conidiophore and conidia.

#### DNA extraction and RAPD analysis

Ten isolates were selected randomly from each of the three subpopulation (black, white and mixed) of *B. sorokiniana* isolated from leaves and DNA was isolated using CTAB method with minor modifications in the protocol given by Saghai Maroof et al. (1984). DNA was quantified with a spectrophotometer and quality analysis done on 0.8% agarose gel. Twenty arbitrary decamer primers (operon USA) were selected to screen 30 isolates of *B. sorokiniana*. PCR amplification reaction was carried out in the final volume of 25  $\mu\text{L}$ , which consisted of  $1 \times$  PCR assay buffer, 200 mM dNTPs mixture, 20  $\mu\text{M}$  primer, 2 U Taq DNA Polymerase (Bangalore Genie, India), and 50 ng template DNA. All of the reactions were carried out in thermal cycler, PTC-200 (Techne, UK), under following thermal cycler parameter conditions: initial denaturation 94°C for 3 min, annealing at 36°C for 1 min, 72°C for 1 min 20 s, and final extension at 72°C for 5 min. Amplified product was visualized on 1.4% agarose gel.

#### Analysis of RAPD data

Statistical analysis of the data was performed using NTSYS-pc program. The degree of genetic relatedness or similarity was estimated using the Jaccard coefficient ( $a/(n-d)$ ) in which the data were defined in a two way contingency table such that for any pair wise combinations of isolates,  $a = (1,1)$ ,  $b = (0,0)$  and  $n = (a + b + c + d)$ . The RAPD Bands were scored as present (1) or absent (0) for each DNA sample. Clustering of similarity matrices was done by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and projection by TREE program of NTSYS-pc. Only amplification products that were reproducible over two amplifications were included.

## Results

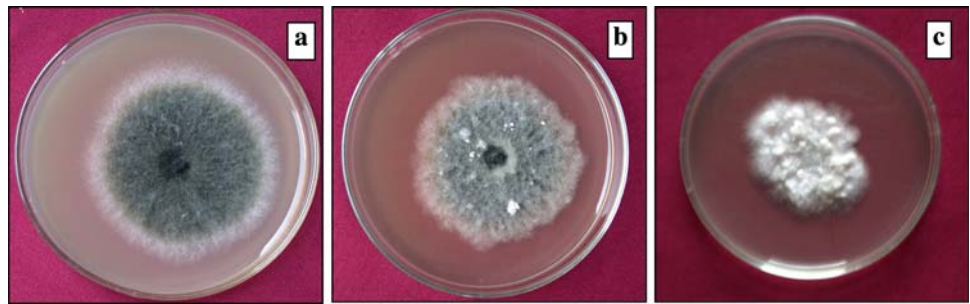
#### Frequency distribution of *B. sorokiniana* population

Three type of *B. sorokiniana* subpopulation i.e. black, white and mixed were obtained from the leaves of barley genotypes (Fig. 1). Maximum distribution was obtained for the mixed subpopulation (47.76%) while minimum (20.89%) for the white subpopulation (Table 1).

#### AUDPC

Among the different subpopulation white was most aggressive with 1,800 AUDPC units followed by mixed subpopulation with 1,600 AUDPC units. AUDPC of black

**Fig. 1** **a** Black isolate **b** mixed isolate **c** white isolate of *B. sorokiniana*



**Table 1** Characteristics of the three different groups of *Bipolaris sorokiniana* isolates

Isolate group		Characteristics				AUDPC
		Melanin content (µg/g)	No. of secondary mycelium <sup>a</sup>	Isolate population	Frequency distribution (%)	
I	Black suppressed growth	1.29–2.50	29.00–48.67	1, 2, 3, 4, 5, 6, 7, 8, 9, 41, 42, 43, 44, 57, 58, 59, 61, 63, 64, 65, 66	31.34	1,400
II	Black with white knots	1.17–0.13	27.66–11.67	10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 50, 51, 52, 53, 54, 55, 56, 60, 62, 74	47.76	1,600
III	White fluffy growth	0	0	22, 23, 24, 25, 26, 27, 28, 29, 30, 45, 46, 47, 48, 49	20.89	1,800

<sup>a</sup> Mean of 10 observations from 4.538 mm<sup>2</sup>

subpopulation of *B. sorokiniana* was 1,400 (Table 1) on susceptible check RD 2503.

#### Morphogenetic studies

Black and mixed subpopulation recovered from nature produced the first phenotypically distinct evidence of a secondary hyphal structure within the colony after 24 h. These secondary hyphae differentiated into conidiophores and finally into conidia, 28 h from spore germination. Fresh spores ceased to form after 28–32 h. The formation of conidiophores initiated in the centre of the colony and gradually spread outwards, towards the edge of the respective colonies.

On an average, the length and thickness of the primary hyphal cell was 36.99 and 5.63 µm, respectively. Secondary and aerial hyphae averaged 18.93 and 8.55 µm, respectively for the same characteristics. The white isolates of *B. sorokiniana* did not differentiate into any secondary structure. Conidiophores and conidia were totally absent in all the replications of the white isolates studied.

The black subpopulation of *B. sorokiniana* recorded a maximum number (48.67/4.538 mm<sup>2</sup>) of secondary mycelium which developed into conidiophores and subsequently produced conidia. Number of secondary hyphae

that developed in different isolates of mixed subpopulation of *B. sorokiniana* was significantly lesser (27.66/4.538 mm<sup>2</sup>) as compared to the black subpopulation (Table 1).

The number of conidia formation was positively correlated to the number of conidiophores present in different isolates of *B. sorokiniana* which in turn was further positively correlated with the number of aerial hyphae differentiation from the primary mycelium of the pathogen in growth medium. A positive correlation ( $r$ ) 0.98 was established between melanin content and spectral RGB value with respect to black and mixed subpopulation of *B. sorokiniana*. Significant differences was observed in the number of secondary hyphae, conidia formation, melanin content and spectral values for the black and mixed subpopulation of *B. Sorokiniana* (Table 2).

The mean spore numbers varied from 17.40 to 32.60/cm<sup>2</sup> in black subpopulations. In mixed subpopulation, it varied from 2.80 to 16.20/cm<sup>2</sup>. No spore production was observed in the white subpopulation. The black mutant developed from the white isolate of *B. sorokiniana* successfully induced morphogenetic differentiation of the primary hyphae into secondary hyphae. Conidiogenesis occurred at 32–52 h after formation of the aerial hyphae (Table 3).

**Table 2** Secondary mycelium, conidiophore and conidia development in isolates of black and mixed (natural) population of *B. sorokiniana*

Isolates <sup>a</sup>	Secondary hyphae <sup>a</sup>	Conidiophore <sup>a</sup>	Conidia <sup>a</sup>	Spectral value			Melanin content (µg/g)
				R value	G value	B value	
B1	29.00	22.33	17.40	45.11	51.67	50.00	1.29
B2	31.00	23.80	19.20	48.00	55.33	52.33	1.40
B3	33.33	25.87	20.40	50.00	56.33	52.00	1.50
B4	35.33	27.60	22.40	52.13	58.67	55.00	1.58
B5	37.33	29.47	23.20	54.17	59.67	56.00	1.61
B6	39.00	31.87	25.40	59.21	67.00	65.00	1.72
B7	41.00	34.80	26.80	61.14	69.00	63.33	1.91
B8	44.00	36.06	29.87	75.04	82.67	79.33	2.1
B9	45.67	38.40	32.60	79.24	85.33	78.67	2.4
B10	48.67	40.20	34.20	86.14	92.33	95.00	2.50
M1	27.66	21.20	16.20	94.17	101.00	107.00	1.17
M2	26.33	19.87	15.33	107.14	114.00	121.00	1.05
M3	24.00	18.40	13.06	122.19	127.67	132.00	0.94
M4	22.00	17.47	11.80	131.07	138.00	140.33	0.85
M5	20.33	15.13	9.60	132.17	140.00	143.00	0.75
M6	19.00	13.60	7.73	136.17	142.33	147.00	0.62
M7	18.67	12.27	6.20	140.20	146.33	152.00	0.51
M8	16.67	11.13	5.60	152.13	160.33	166.00	0.41
M9	13.67	9.80	4.00	160.00	166.33	172.00	0.29
M10	11.67	7.80	2.80	167.93	174.67	185.67	0.13
LSD (0.01)	1.35	0.62	0.36	0.94	1.20	1.40	0.12

*B* black isolate, *M* mixed isolate

<sup>a</sup> Mean of 10 observations from 4.538 mm<sup>2</sup>

**Table 3** Conidia development, melanin content and spectral (RGB) values of mutant and black isolate of *B. sorokiniana*

Isolates	Secondary hyphae (no.) <sup>a</sup>	Conidiophores (no.) <sup>a</sup>	Conidia (no.) <sup>a</sup>	Time for conidia development (h)	Melanin content µg/g of Mycelium	Spectral Value			AUDPC
						R value	G value	B value	
Black	34.0	28.85	22.14	28.32	1.50	48.28	54.71	55.14	1423.26
Mutant (black)	22.4	16.71	12.57	32.52	0.60	128.28	135.42	135.85	1657.14
White	0	0	0	–	0	182.42	186.14	186.14	1822.22
LSD (0.01)	0.53	0.53	0.45	–	0.023	12.07	12.59	13.23	88.81

<sup>a</sup> Mean of 10 observations from 4.538 mm<sup>2</sup>

### Effect of melanin synthesis inhibitor

Black and mixed isolates of *B. sorokiniana* produced less secondary mycelium, conidiophore and conidia (Table 4). There was no difference in radial growth of tricyclazole treated black, mixed and white isolates from the control. There was production of reddish brown colorations in black and mixed isolates at low concentrations of tricyclazole. Further conidia production was inhibited in black and mixed isolates at these concentrations of tricyclazole.

### Polymorphism in the isolates

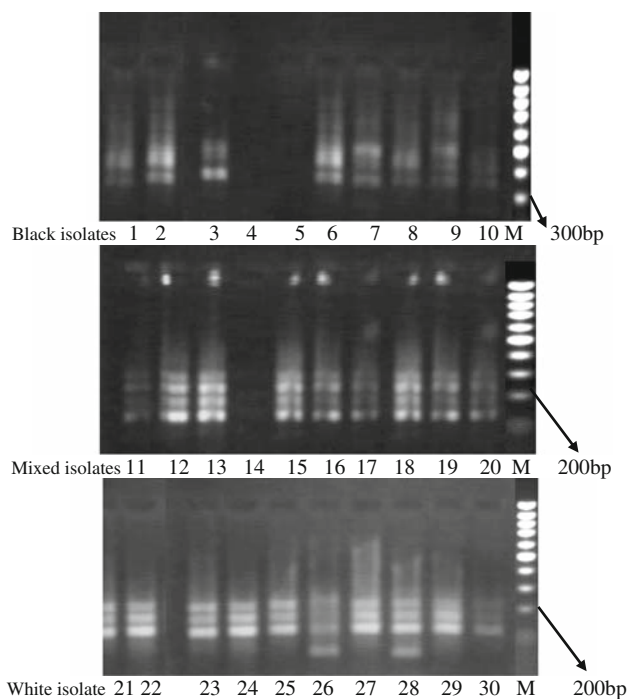
Out of 20 arbitrary primers used for the RAPD, five primers (OPY-01, OPY-c, OPY-05, OPY-06 and OPY-20) showed polymorphism. Primer OPY-06 showed maximum polymorphism (Fig. 2). Unique fragments of 150 and 200 base pairs were observed in mixed isolates and common fragments of 400 bp were observed in both black and mixed isolates. However, no such fragments were observed in case of white isolates.



**Table 4** Effect of melanin synthesis inhibitor (Tricyclazole) on different isolates of *B. sorokiniana*

Isolates	Secondary hyphae (no.) <sup>a</sup>	Conidiophores (no.) <sup>a</sup>	Conidia (no.) <sup>a</sup>	Melanin content µg/g of Mycelium	Spectral value		
					R value	G value	B value
Black (control)	48.67	40.20	34.20	2.50	86.14	92.33	95.00
Black (treated)	30.50	23.33	18.00	0.70	88.50	96.83	106.83
Mixed (control)	27.66	21.20	16.20	1.17	94.17	101.00	107.00
Mixed (treated)	17.67	12.33	5.83	0.48	110.83	122.16	141.66
White (control)	0.00	0.00	0.00	0.00	210.83	224.83	235.33
White (treated)	0.00	0.00	0.00	0.00	210.83	224.83	235.33
LSD (0.01)	3.19	2.62	1.84	0.03	5.084	3.90	2.77

<sup>a</sup> Mean of 10 observations from 4.538 mm<sup>2</sup>



**Fig. 2** Random amplified polymorphic DNA analysis of isolates belonging to the three group of *Bipolaris sorokiniana*, on the basis of morpho- biochemical characteristics, with primer OPY-06. Molecular ladder was a 100 bp ladder (Generalar<sup>TM</sup> 100 bp ladder, MBI Fermentas)

The dendrogram (Fig. 3) using polymorphic DNA bands of 30 isolates belonging to the three groups of *B. sorokiniana* formed three clusters. Out of these, 10 black isolates (B1–B10) formed one cluster, while 11 of mixed isolates (B11–21) formed the second cluster. Among them, isolates B14 and B15 showed maximum similarity. Interestingly, the 9 white isolates (B22–B30) formed the third cluster with isolates B25 and B26 showing maximum similarity.

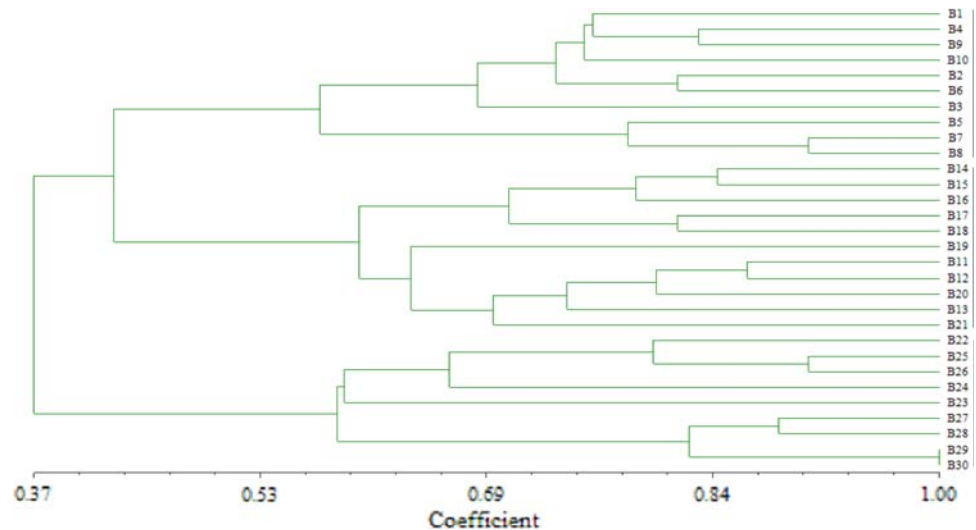
## Discussion

Melanin was present in the black and mixed subpopulations of *B. sorokiniana* however, white subpopulation was found to be melanin deficient. Sussman (1968) reported that melanin is important for survival of all loculoascomyces, some pyrenomyces and many deuteromyces. Bell and Wheeler (1986) also observed the significance of melanin pigments in spore dormancy survival and in protection in microbial lysis in soil. The fact that the melanin deficient white subpopulation is present in lower frequencies in natural population was probably due to their reduced fitness for survival.

Morphogenetic studies indicated that primary hyphae of black and mixed subpopulation which were hyaline, thin and showed large septal distances differentiated into secondary hyphae which were dark, thick and showed close septation. Absence of secondary hyphae in white subpopulation indicated the association of melanin in differentiation of secondary hyphae, conidiophores and conidia in *B. sorokiniana*. Fungal melanin has been reported to promote development of aerial hyphae and conidia that can disperse to more favourable environments (Frederick et al. 1999; Butler and Day 1998; Henson et al. 1999). The melanin containing mutant derived from white isolate showed differentiation of secondary hyphae, and subsequently conidiophores and conidia. This further established the association of melanin in morphogenesis of secondary hyphae and conidiogenesis. Similarly, melanization of the wild type colonies in *C. heterostrophus* lead to production of abundant conidia in contrast to albino mutant which was shown to be sterile (Eliahu et al. 2007).

Melanin content was negatively correlated with aggressiveness as white isolates were found to be more aggressive. Melanin did not show association with pathogenicity in *Verticillium dahliae*, *Alternaria alternata* and *Cochliobolus heterostrophus* (Bell and Wheeler 1986;

**Fig. 3** Dendrogram of 30 isolates of *Bipolaris sorokiniana* of barley as revealed by unweighted pair group method with arithmetic mean cluster analysis of genetic similarities based on random amplified polymorphic DNA data of 70 fragments with 5 primers



Tanabe et al. 1995; Kawamura et al. 1999). It has been proposed that constitutive melanization limits secretion of lytic enzymes necessary for host tissue degradation and subsequent pathogenesis (Frederick et al. 1999). Unmelanized mutant produced more extracellular enzyme activity than the wild melanized type in *G. graminis* var. *graminis* (Henson et al. 1999). Therefore, constitutive melanization of black and mixed subpopulations lowers the aggressiveness of the infection but at the same time provides better fitness for survival which could explain the predominance of these subpopulation in nature.

In the present study, appearance of reddish brown coloration in black and mixed isolates of *B. sorokiniana* when exposed to tricyclazole indicates that DHN melanin is synthesized in *B. sorokiniana* of barley. Tricyclazole has extensively been used to detect DHN melanin in fungi since it blocks melanin synthesis by inhibiting reductase enzymes (Bell and Wheeler 1986; Rizner and Wheeler 2003). Reduction in number of secondary hyphae, conidiophores and number of conidia in tricyclazole treated black and mixed isolates confirms the association of melanin in conidiogenesis.

Morpho-pathological characterization of isolates of three groups was largely confirmed by RAPD analysis where three distinct groups were spotted based on colony color. *Brn 1* reductase melanin biosynthesis gene has also been identified in *Bipolaris* spp (Shimizu et al. 1997). These findings also confirm that these primers may be used to group the isolates based on its melanin content.

Therefore, our results demonstrate that melanin has a major role in differentiation of hyphae and conidial development in *B. sorokiniana* and could account for increased fitness in nature.

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