MULTIVARIATE ANALYSIS AND CLUSTERING OF MORPHOLOGICAL DIVERSITY IN THE POPULATION OF *BIPOLARIS SOROKINIANA* CAUSING LEAF BLOTCH OF WHEAT

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ABSTRACT

Sultana, S., Rahman, S. M. M., Islam, M. M. and Adhikary, S. K. 2019. Multivariate analysis and clustering of morphological diversity in the population of *Bipolaris sorokiniana* causing leaf blotch of wheat. Bangladesh J. Plant Pathol. 35(1&2):9-18

Morphological changes are required for eukaryotic pathogens to originate diseases and several virulence properties appeared with morphological shifts. The aim of this work was to enrich our knowledge determining morphological diversity in *Bipolaris sorokiniana* population by using multivariate analysis. For this, 169 isolates of *B. sorokiniana* collected from different agro-ecological zones of Bangladesh were evaluated for morphological variability considering mycelial and conidial characters at plant protection laboratory of Khulna University during 2016. In this study, thirteen morphological components like length, width, color, shape and septation of conidia, spore production, radial mycelial growth at different days of incubation, colony margin, colony growth behavior, colony color and number of colony zonation were taken into consideration. These characters contributed at various levels to the variations observed among the isolates studied. In descriptive analysis, colony zonation was found to have significant involvement in creating variation. In correlation matrix, some of the descriptors showed positive relation and some showed negative relations, while no correlation was observed among the other components. Principal component analysis disclosed that colony color, conidial color, colony margin, colony growth behavior and radial mycelial growth after 3 days of incubation generated 68.11% of the variation among the morphological components. Five distinct clusters were found from cluster analysis and the highest frequency (43.78%) of isolates belonged to cluster IV. Under cluster IV exhibited diverse isolates and most of them were from High and Low Ganges River Floodplain areas. The most dominant and variable isolates of the fungus displayed greenish black colony morphology with olive brown conidia production.

Key Words: Multivariate analysis; clustering; PCA; morphological diversity; *Bipolaris sorokiniana*

INTRODUCTION

Wheat production plays a significant role in ensuring food security in the population dense areas of the world, which mainly comprises Bangladesh, India, Pakistan, and Nepal (Basheer and Atawnah 2014). About 35% of the world population depends on wheat which signifies the importance of wheat as a staple food. Demand for wheat has been growing faster than for any other major cereal (Pingali 1999; Kumar *et al*. 2015), probably due to its satiety value and satiety index that is also true for Bangladesh. Wheat production was introduced in Bengal in 1930-31(Anonymous 2014). Now it occupied 444805 ha land area (BBS 2017). At present wheat meets about 12% of total cereal consumption in Bangladesh. It was realized that rice alone could not meet the food requirements of the burgeoning population of Bangladesh (Anonymous 2006) and simultaneously wheat can play a vital role as a good supplement to meet the food demand. In Bangladesh, 1250 thousand metric ton wheat was produced in 2016 (Anonymous 2017) with productivity approximately 0.17% lower than world wheat productivity (Anonymous 2018).

Leaf blotch or spot blotch caused by *Bipolaris sorokiniana* (Sacc.) Shoem. (syn. *Drechslera sorokiniana* (Sacc.) Subram. & Jain; syn. *Helminthosporium sativum* Pamm., King & Bakke, teleomorph: *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur) is one of the most important

diseases of wheat in the warm and humid regions of the world. The leaf blotch pathogen affects all above ground plant parts causing enormous losses, particularly in areas characterized by high temperature, high humidity, and low soil fertility with deficiency in potash and nitrogen, which accelerating the changes in growth and altered crop physiology, enhance the severity of leaf blotch (Ragiba *et al.* 2004; Poloni *et al.* 2009, Singh *et al*. 2016). The Eastern Gangetic Plains (EGP) of South Asia is characterized by the warm humid environment, thereby leaf blotch is highly prevalent and considered as the hot spot area (Gupta *et al.* 2018, Sharma and Duveiller 2006, Sharma *et al*. 2007). Wheat production becomes lessen due to several factors, such as reduction of soil fertility, rice-wheat cropping system and alteration of climatic condition with warm and humid winters, which also contribute a lot to the development of fungal diseases (Dubin and Rajaram 1996). Rice-wheat cropping pattern is the main practice in Bangladesh, so this situation provides a favorable environment for the survival and multiplication of leaf blotch pathogen and rice stubble may also serve as a substrate for the fungus after harvest (Iram and Ahmad 2005, Regmi *et al*. 2002). Global climate changes have a major impact on wheat production in the world and Bangladesh due to increasing environmental stress, rise in temperature and shortening winter, which also result in increased severity of diseases including leaf blotch (Poulton and Rawson 2011, Hossain and Teixeira da Silva 2013).

Grain yield losses due to wheat leaf blotch had been reported in South Asia, which ranged 4-38% in 2004 and 25-43% in 2005, with thousand-kernel weight reduced by 10-15% and 11-18%, respectively (Kumar *et al*. 2015). On-farm studies showed yield losses up to 16% in Nepal (Bhatta *et al*. 1997) and 15% in Bangladesh (Alam *et al*. 1998), and might go up to 100% in case of severe attack depending on variety (Rahman *et al*. 2009).

Bipolaris sorokiniana is a heterothallic fungus and exhibits variability, which is considered as heterokaryosis (Jaiswal *et al*. 2007; Aggarwal *et al.* 2009). Bangladeshi isolates of *B. sorokiniana* from different wheat growing regions showed morphological variability like radial mycelial growth, mycelia color and growth behavior (Hossain and Azad 1992, Ahmed *et al*. 1997, Sultana *et al*. 2005). South American isolates of *B. sorokiniana* also exhibited morphological variations based on mycelial color, sector formation, and growth rate (Poloni *et al.* 2009). High morphological variability was also observed among Brazilan isolates of *B. sorokiniana* by Valim *et al.* (1997). In present situation, detailed knowledge on morphological variability of *B. sorokiniana* is useful to observe taxonomic characters of the pathogen and epidemiological aspects of disease spread. Although conidial characters are known to have pathogenic impact during infection and disease progress (Wang and Lin 2012) but unfortunately such studies were not performed with Bangladeshi isolates and multivariate technique was seldom used to resolve the complex problems in morphological variability determination. This underlines the need for intensive research on different components of morphological variation in *B. sorokiniana* and their potential relationship with disease development. The main purpose of this research was to update our understanding through study of morphological diversity in *Bipolaris sorokiniana* population by using multivariate analysis.

MATERIALS AND METHODS

Sample collection, isolation and purification

Leaf blotch diseased leaf samples were collected at soft dough stage (Zadoks *et al*. 1974) from minimum one and maximum seven wheat fields selected randomly in each of the different wheat growing agro-ecological zones (AEZ) of Bangladesh during 2013 and 2014 (Table 1). The samples were put in paper envelopes with proper labeling, dried overnight at room temperature and brought to plant protection laboratory of Khulna University for pathogen isolation and further studies. The fungus was isolated following tissue planting method (Mian 1995) and purified using the procedures described by Mahto *et al.* (2012).

Morphological identification

Bipolar germination of a spore is the characteristic feature that confirms *Bipolaris* species. The fungus was identified by observing morphological characters using key described in Barnett and Hunter (1972). For confirmation, a microscopic slide with fungal spores was prepared using water as mounting fluid and kept at room temperature for 3 hours to allow germination. Then observed under Carl Zeiss compound microscope (40X) and found bipolar germination (Fig. 1).

Culture of *B. sorokiniana* isolates

The isolates of *B. sorokiniana* were grown separately on PDA at pH 6.5. For morphological characterization 5 mm blocks were cut from 5 days old culture plate by flamed cork-borer, placed at the center of PDA plates and incubated up to 12 days in a growth chamber with constant temperature (25ºC). The plates were arranged in the incubator (25ºC) following CRD with three replications.

Recording mycelial and conidial characters, and data collection

Radial diameter at 3, 5 and 7 days of incubation, colony margin, colony growth behavior, colony color, and number of colony zonation after 7 days of incubation were recorded. Number of spores produced per cm^2 surface area of a colony, conidial length and width, number of septation, shape and color were recorded after 12 days of incubation. Mycelial characters were recorded through visual observation and conidial characters were recorded under microscopic observation at 40X magnification. Conidial color, shape and septation number were seen under microscope and 36 conidia of each isolates were taken randomly for measuring size through Zeiss version 2.0 computer program at 20 µm scale. For confirmation of these data each isolate was examined three times.

Table 1. *Bipolaris sorokiniana* isolates collected from different wheat growing areas of Bangladesh for morphological characterization and diversity analysis

Sl. No.	AEZ		Isolate Designation
	ID	Zones	
1	1	Old Himalayan Piedmont Plain	BS-61, BS-62, BS-63, BS-64
2	2	Active Tista Floodplain	BS-48
3	3	Tista Meander Floodplain	BS-50, BS-51
4	9	Old Brahmaputra Floodplain	BS-65, BS-67
5	11	High Ganges River Floodplain	BS-1, BS-3, BS-4, BS-8, BS-9, BS-10, BS-11, BS-12, BS- 53, BS-14, BS-15, BS-16, BS-17, BS -18, BS-20, BS-21, BS-22, BS-23, BS-24, BS-28, BS-29, BS-30, BS-31, BS-32, BS-33, BS-34, BS-36, BS-37, BS-38, BS-39, BS-40, BS-41, BS-42, BS-43, BS-43, BS-58, BS-60,BS- 91, BS-109, BS-118, BS-129, BS-130, BS-131, BS-132, BS-134, BS 135, BS-136, BS-137, BS-138, BS-139, BS-140, BS-141, BS-142, BS-143, BS-144, BS-145, BS-146, BS-147, BS-148, BS-149, BS-150, BS-151, BS-152, BS-153, BS-154, BS-155, BS-156, BS-157, BS-158, BS-159, BS-160, BS-161, BS-162, BS-163, BS- 165, BS-166, BS-167, BS-168, BS-169
6	12	Lower Ganges River Floodplain	BS-68, BS-69, BS-70, BS-71, BS-72, BS-74, BS-75, BS-76, BS-77, BS-78, BS-79, BS-80, BS-81, BS-82, BS-83, BS-84, BS-85, BS-86, BS-87, BS-88, BS-89, BS-164
7	13	Ganges Tidal Floodplain	BS-2, BS-26, BS-35, BS-45
8	14	Gopalganj-Khulna Beels	BS-13, BS-19, BS-25, BS-27, BS-46, BS-54, BS-55, BS-56, BS-57
9	19	Old Meghna Estuarine Floodplain	BS-73
10	25	Level Barind Tract	BS-44, BS-45, BS- 66, BS-90, BS-92, BS-93, BS-94, BS-95, BS-96, BS-97, BS-98, BS-99 BS-100, BS-101, BS-102, BS-103, BS-104, BS-105, BS-106, BS-107, BS- 108, BS-110, BS-111, BS-112, BS-113, BS-114, BS-115, BS-116, BS-117, BS- 119, BS-120, BS-121, BS-122, BS-123, BS-124, BS-125, BS- 127, BS -133
11	27	North-eastern Barind Tract	BS-126, BS-128
12	28	Madhupur Tract	BS-5, BS-6, BS-7, BS-52, BS-59

Source: AEZ regions were specified by Banglapedia, 2014; BS = *Bipolaris sorokiniana*

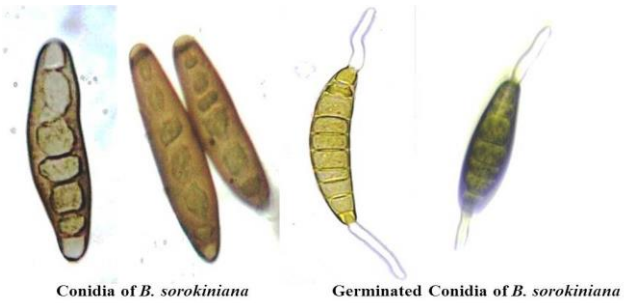


Figure 1. Conidia of *B. sorokiniana* and their bi-pole germination

Recording mycelial and conidial characters, and data collection

Radial diameter at 3, 5 and 7 days of incubation, colony margin, colony growth behavior, colony color, and number of colony zonation after 7 days of incubation were recorded. Number of spores produced per cm^2 surface area of a colony, conidial length and width, number of septation, shape and color were recorded after 12 days of incubation. Mycelial characters were recorded through visual observation and conidial characters were recorded under microscopic observation at 40X magnification. Conidial color, shape and septation number were seen under microscope and 36 conidia of each isolates were taken randomly for measuring size through Zeiss version 2.0 computer program at 20 µm scale. For confirmation of these data each isolate was examined three times.

Estimation of spore production

The number of spores produced per unit area of a colony was estimated using the formula (Chauhan and Panday 1995) as follows:

$$CP = \frac{NC \times VW}{TSA}$$

Where,

CP = Conidia produced cm^{-2}

NC = Number of $conidia^{-ml}$ of suspension

VW = Volume of water used to make suspension

TSA = Total surface area from which conidial suspension was derived

Statistical analysis for morphological variability

Data on thirteen components of morphological characters were used for statistical analysis. Variations among the isolates based on morphological (mycelial and conidial) characters were analyzed following multivariate analysis: descriptive statistics, correlation matrix, principal component analysis (PCA), and hierarchical cluster analysis (HCA) following Square Euclidean method between group linkage (Ward 1963) was performed by using SPSS statistics (version 20, IBM, NY, USA) program. Histogram and PCA was performed with the studied components of variability for their relative contribution to isolate variation.

RESULTS AND DISCUSSION

Characterization of the components of morphological variability in *B. sorokiniana* isolates

From the descriptive statistics mean values of thirteen components for morphological characterization like as radial growth after 3 days of incubation, radial growth after 5 days of incubation, radial growth after 7 days of incubation, spore production/cm^2 after 12 days of incubation, septation of conidia, length of conidia, width of conidia, number of zonation, growth behavior, colony margin, colony color, conidial color and conidial shape were 31.22 mm, 45.50 mm, 56.17 mm, 7316.12/cm^2, 5.86, 29.15 µm, 9.33 µm, 0.60, 1.48, 1.86, 2.61, 1.75 and 1.36 respectively (Table 2). Means for all the components of morphological characters were greater than the respective standard deviation except number of zonation. It indicates that all the morphological components except colony zonation were found important and played significant role to create diversity among the isolates.

Correlation between the components of morphological variation

The correlation between the thirteen components of morphological variability clearly represents some aspects of relationship (Table 3). Among the components some showed positive, some showed negative and some showed no correlation with some of the other components. High correlation was observed among the radial mycelia growth at 3, 5 and 7 days of incubation. Colony color showed non-significant correlation with the other components except spore production. Conidial color represents positive and significant correlation with radial growth at 5 and 7 days of incubation and also with colony color.

Contribution of components to morphological variation

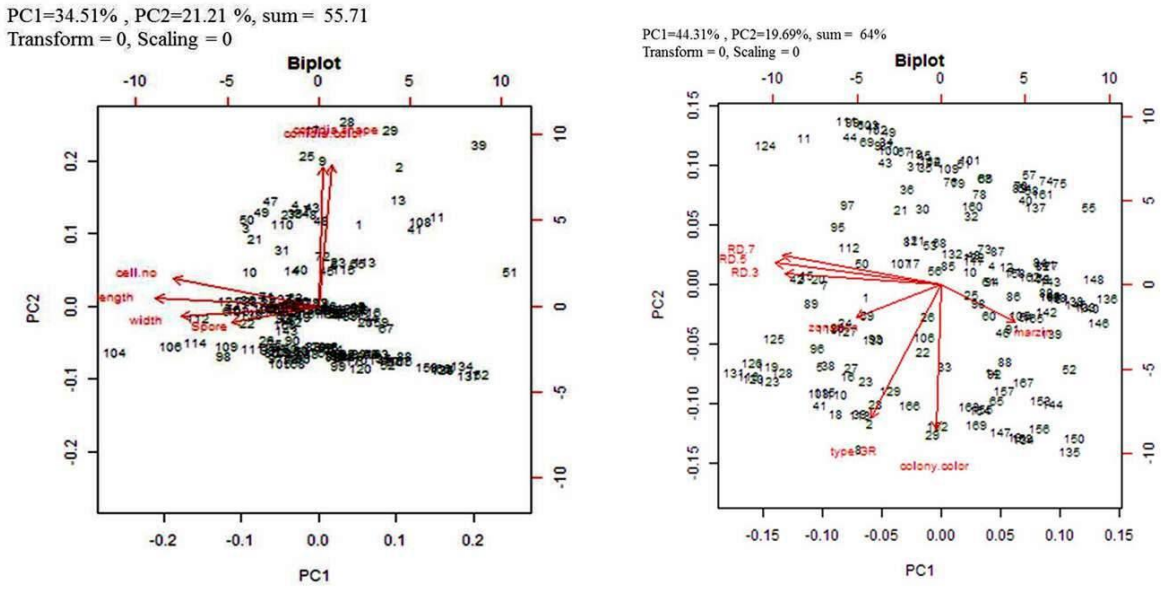
Each principal component is linked with an eigenvalue representing the amount of the total variation. In conidial character, conidial color and conidial shape accounted for more than half (55.71%) of the total variation. Conidial color and conidial shape were consistently closely correlated; if one of them was throwing down much information about morphological variation would have loosed. It implies that these two components gave the impression on morphological variation among the isolates. According to biplot graph, cosine of the angle between

the components represents approximate correlation between them (Fig. 2A). The presence of close association among the conidial components suggested the information about the conidial morphology and the potential to create isolate variation.

Colony color and radial mycelial growth after 7 days of incubation accounted for more than half (64%) of the total variation (Fig. 2B). It also suggests that these two components played a significant role in the development of morphological variability among the isolates

Table 2. Range and means of the components of morphological variation among 169 isolates of *Bipolaris sorokiniana*

Components of variability	Minimum	Maximum	Mean±SD
Radial growth after 3 days of incubation (mm)	11.33	57.66	31.22±10.90
Radial growth after 5 days of incubation (mm)	13.66	73.66	45.50±16.17
Radial growth after 7 days of incubation (mm)	17.66	83	56.17±19.28
Spore/cm^2 after 12 days of incubation	118.92	29578.66	7316.12±5600.02
Septation number of conidia	2.10	8.80	5.86±1.21
Length of conidia (μm)	11.30	44.80	29.15±5.90
Width of conidia (μm)	5.70	14.30	9.33±1.31
Number of zonation	0.00	4.00	0.60±1.08
Colony growth behavior	1.00	11.00	1.48±0.88
Colony margin	1.00	3.00	1.86±0.69
Colony color	1.00	5.00	2.61±1.30
Conidial color	1.00	5.00	1.75±0.99
Conidial shape	1.00	3.00	1.36±0.75



A: Conidial components of variation

B: Mycelial component s of variation

Figure 2. A and B: Dimensional relationship among the components of conidial and mycelial characters of *Bipolaris sorokiniana*

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Table 3. Correlation matrix among the thirteen morphological components of *Bipolaris sorokiniana*

Parameter	RG3	RG5	RG7	SP	SC	LC	WC	NZ	G B	C M	CC	Con C	CS
RG3													
RG5	0.843**												
RG7	0.758**	0.922**											
SP	-0.106^{NS}	-0.051^{NS}	-0.139^{NS}										
SC	0.092^{NS}	0.082^{NS}	0.043^{NS}	0.206**									
LC	0.043^{NS}	-0.012^{NS}	-0.042^{NS}	0.189*	0.602^{NS}								
WC	0.038^{NS}	0.014^{NS}	0.026^{NS}	0.232**	0.291^{NS}	0.511**							
NZ	0.304**	0.368**	0.280**	0.062^{NS}	-0.046^{NS}	-0.163*	-0.079^{NS}						
GB	0.237**	0.216**	0.206**	-0.169*	-0.137^{NS}	-0.138^{NS}	-0.142^{NS}	0.115^{NS}					
CM	-0.301**	-0.298**	-0.313**	0.112^{NS}	-0.027^{NS}	-0.061^{NS}	-0.057^{NS}	-0.101^{NS}	0.007^{NS}				
CC	0.002^{NS}	-0.047^{NS}	-0.056^{NS}	-0.157*	-0.247^{NS}	-0.075^{NS}	-0.011^{NS}	-0.003^{NS}	0.137^{NS}	-0.003^{NS}			
Con C	0.151^{NS}	0.229**	0.213**	-0.015^{NS}	-0.007^{NS}	-0.019^{NS}	-0.042^{NS}	0.035^{NS}	0.063^{NS}	-0.108^{NS}	0.304**		
CS	0.004^{NS}	0.190*	0.282^{NS}	-0.060^{NS}	0.028^{NS}	-0.023^{NS}	-0.058^{NS}	0.126^{NS}	-0.023^{NS}	-0.023^{NS}	-0.101^{NS}	0.177*	

RG3 - Radial growth after 3 days of incubation, RG5 - Radial growth after 5 days of incubation, RG7 - Radial growth after 7 days of incubation, SP - Spore/cm^2 after 12 days of incubation, SC - Septation number of conidia, LC - Length of conidia, WC - Width of conidia, NZ - Number of zonation, GB - Growth behavior, CM - Colony margin, CC - Colony color, Con. C - Conidial color, CS - Conidial shape.

Morphological variability and distribution frequency of *Bipolaris sorokiniana* isolates

Cluster analysis revealed five distinct groups based on thirteen components of morphological characters (Fig. 3). Among these groups, cluster III and IV displayed the maximum number of isolates that accounted for 79.28% of the total number of isolates (134 among 169 isolates) and majority of these isolates were from High and Lower Ganges River Floodplain areas (Table 1). Group I, II and V included 11.83, 3.55 and 5.32% of the total isolates respectively. According to histogram, the isolates included in cluster IV showed higher distribution frequency for eleven tested morphological components except spore production and colony zonation that means cluster IV comprised morphologically most variable isolates (Fig. 4).

Considering the cultural variability five distinct colony color and conidial color were observed and simultaneously two types of growth pattern were found. These five distinct cultural variations were: (A, a) Black colony with suppressed growth, dark brown conidia with straight shape; (B, b) greenish black colony with suppressed growth, olive brown conidia with straight shape; (C, c) olive brown colony with suppressed growth, light olive green conidia with cylindrical shape; (D, d) ash gray colony with fluffy growth, light brown conidia with slightly curved shape and (E, e) whitish colony with fluffy growth, too light brown conidia with cylindrical shape (Fig.5).

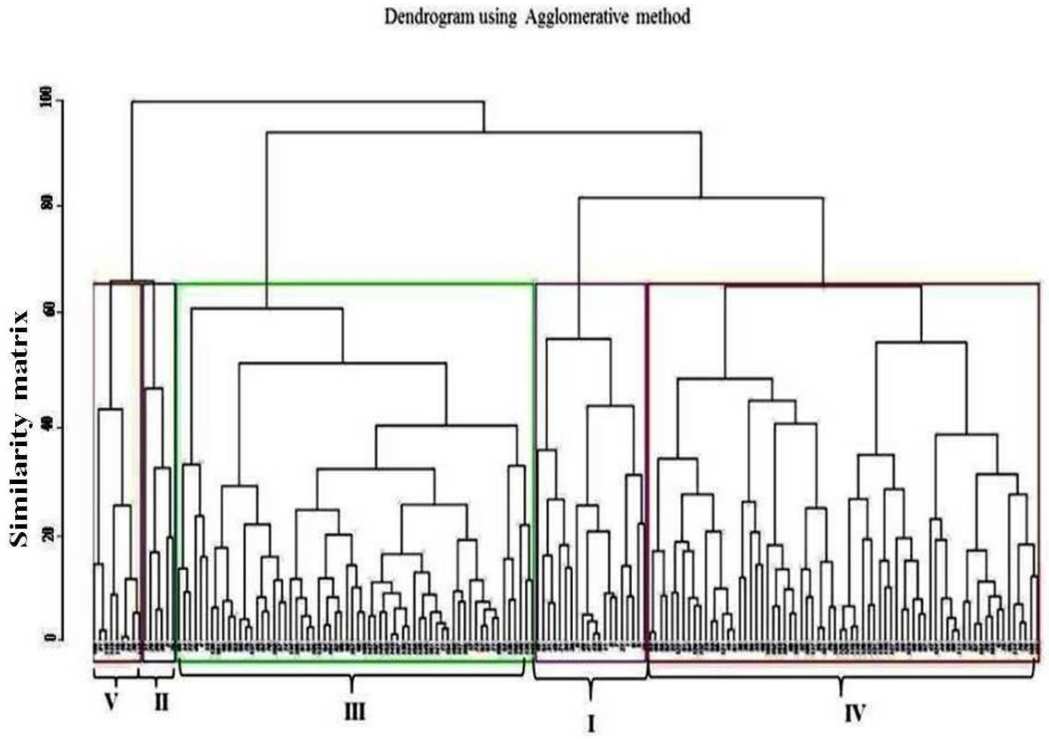

Figure 3. Dendrogram constructed with the data obtained from thirteen morphological components of *Bipolaris sorokiniana*

Now-a-days, multivariate techniques are applied to understand the various aspects of morphological variability (Intrieri *et al.* 2001, Borges *et al.* 2008). In this experiment multivariate analysis was used to estimate the morphological diversity as well as contributing components among and within the population of *B. sorokiniana* and its heterogeneity. Principal component analysis (PCA) and Hierarchical cluster analysis (HCA) were used to categorize the isolates and discriminate the components of morphological variability. In this study higher variability was observed in conidial (size, color, shape, septation number) and mycelial (spore production cm^{-2}, radial growth, colony margin, colony growth behavior, colony color and number of zonation) characters of *B. sorokiniana*. Here, all conidial and mycelial characters contribute to isolate variability except number of zonation. The exception might be due to media composition, age of culture and environmental conditions such as light and temperature (Mahto *et al.* 2012). Principal component analysis disclosed that among the thirteen studied components colony color, conidial color, colony margin, colony growth behavior and radial mycelial growth after 3 days of incubation generated more than 50% (68.11%) of the variation.

Phenotype is the expression of genotype; each phenotypic character is the expression of a single major or more minor genes. Changes in color are a visual manifestation of alterations in cell properties that means, alterations in cell surface molecules during morphogenesis help fungi to adapt in host conditions and to avoid or defend against host immunity. So, conidial color, shape and size likely underpin the link between morphogenesis and fungal virulence (Wang and Lin 2012). These morphological changes are important among many mechanisms that are known to play a role in virulence. Some researchers found a relationship between aggressiveness and colony color and conidial color; they also suggested that isolates with black mycelia are most aggressive (Jaiswal *et al*. 2007).

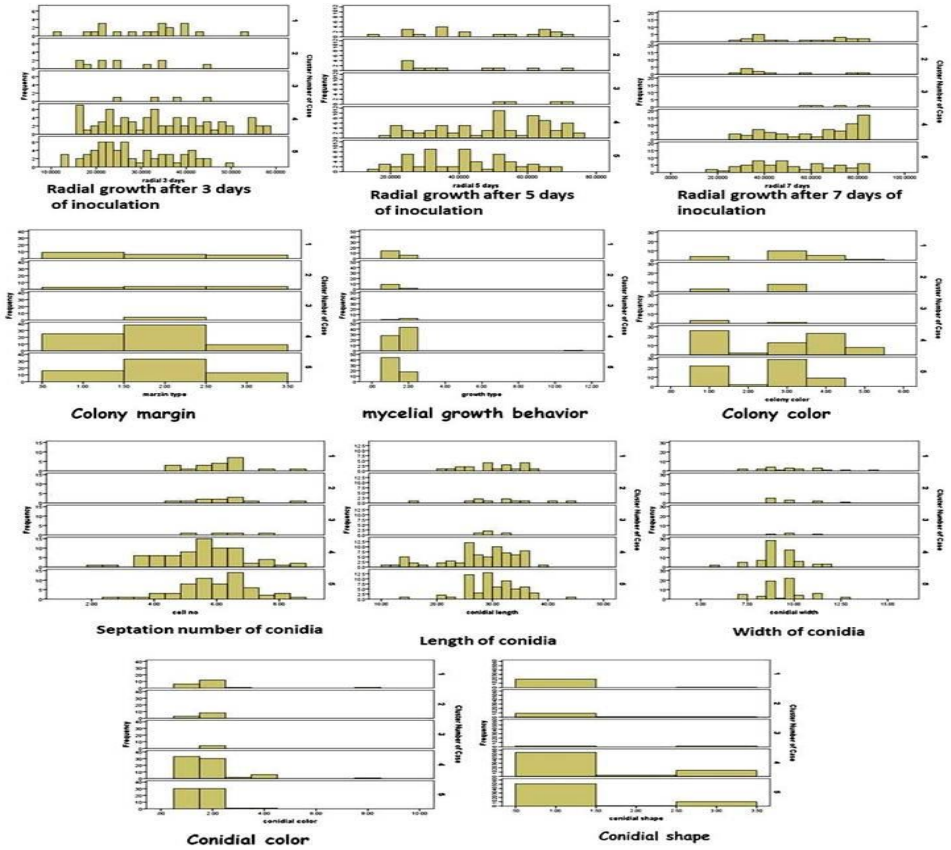

Figure 4. Histogram showing the distribution frequency of morphological components of *Bipolaris sorokiniana*

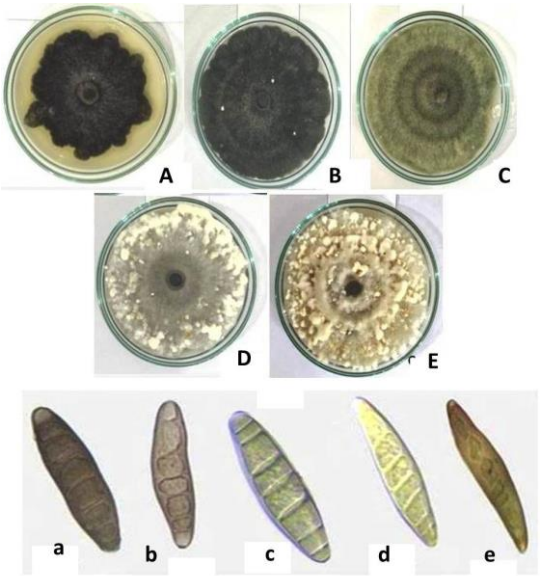

Figure 5. Colony characteristics with corresponding conidial characteristics of five different morphological groups of *Bipolaris sorokiniana*

Several virulence properties appear to have coevolved with morphological shifts (Thompson *et al*. 2011). In our study, two types of colony texture, colony margin and growth pattern and five types of colony and conidia color were found with the isolates collected from different wheat growing areas. However, there was no specific relationship observed among the morphological characters and agro-ecological zones of isolate collection. Among the five distinct morphological clusters realized in the present study cluster IV was comprising the highest frequency (43.78%) of the isolates. Poloni *et al*. (2009) also found five morphological groups with no relationship to the geographical regions.

Cluster analysis and intra cluster distance reveal that most dominant and maximum diverse isolates were belonging to cluster IV bearing greenish black colony with olive brown conidia. Most of the isolates of cluster IV were from High and Lower Ganges River Floodplain areas, a hot spot for leaf blotch disease in South Asia (Gupta *et al*. 2018). Several plant pathologists working in Eastern Gangetic Plains of India found similar results and pointed out that isolates with black mycelia were most virulent (Jaiswal *et al*. 2007, Pandey *et al*. 2008). This variability could be accredited to the interactions between genetic composition of the isolates and edaphoclimatic conditions in the areas from where the isolates are obtained (Poloni *et al*. 2009). Multivariate analyses of pathogen isolates would provide useful information to improve resistance in breeding populations by selecting distinct isolates and specific geographical regions for screening.

ACKNOWLEDGEMENT

The authors are thankful to the Ministry of Education, Government of the People's Republic of Bangladesh for financial support.

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