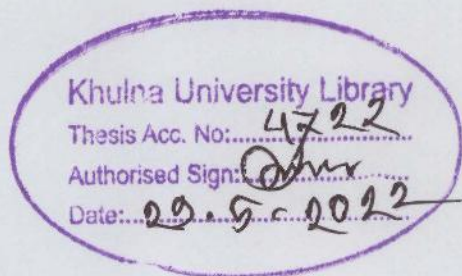


**VARIATION IN MORPHOLOGY OF *Cochliobolus sativus* (S. Ito & Kurib.)
IN F₂ GENERATION**



**A THESIS
BY
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DEDICATED TO



MY BELOVED PARENTS

ABSTRACT

Leaf blotch, caused by *Cochliobolus sativus*, is a devastating foliar disease of wheat in Bangladesh. To develop effective disease management strategy against *Cochliobolus sativus*, knowledge of morphological variability of the fungal pathogen is essential for breeding program. For considering this fact present experiment was carried out in the Plant Pathology Laboratory of Agrotechnology Discipline, Khulna University, Khulna during June to August, 2019 to determine the morphological variation among the isolates of *Cochliobolus sativus* in F₂ generation. Twenty five isolates of F₂ generation of *Cochliobolus sativus* were used for observation of morphological variation. The experiment was laid out Complete Randomized Design with three replications. In morphological study, eleven morphological components viz. colony color, colony margin, colony growth behavior, radial growth, number of zonation, conidial color, conidial shape, septation number, length and width of conidia and conidia production cm⁻² were considered. Highest coefficient of variation (114.77) was observed among the number of zonation formation among the F₂ generation of *Cochliobolus sativus*. Five distinct clusters were found from cluster analysis based on eleven morphological components. Four distinct colony colors (whitish, blackish grey, ash grey and greenish black), three types of colony margin (regular, irregular and wavy), three types of colony growth behavior (fluffy, suppressed and too much suppressed), three types of conidial color (olive, brown and dark brown) and three types of conidial shape (straight, slightly curved cylindrical) were observed. No sporulation was found in too much suppressed growth isolates. Based on mean of five cluster centers, maximum spore producing and higher radial growth isolates were belonged in cluster V. Between the clusters distance, maximum cluster distance was found between cluster IV and V (61.59) and minimum distance was observed between cluster II and IV (20.06). Higher correlation was observed among width of conidia, length of conidia, shape of conidia, conidial color and septation number. Principal component analysis (PCA) represented that colony color, colony margin and growth behavior contributed more than 80% of the variation among the eleven morphological components. PCA biplot showed that most of the highest sporulating isolates belonged to the same group and they produced olive colored conidia.

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CHAPTER I

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important cereal crop and major staple food of South Asian countries like as Bangladesh, India, Pakistan, and Nepal (Joshi et al., 2007). Wheat production played a key role in ensuring food security in the population dense region of the world (Joshi et al., 2007 and Basheer-Salimia and Atawnah, 2014). The world's population is increasing by one billion in every 11 years. In present, it is expected to be 8.4 billion by the year 2025 (Acharya et al., 2011 and Chowdhury et al., 2013). But wheat is providing nearly 20% of the total world food requirement (Uddin et al., 2006). In the past five years, developing countries have produced more than 45 percent of the world's wheat (USDA, 2019). However wheat production faces many constrains especially various fungal diseases, among them leaf blotch of wheat is very serious yield losing and most devastating diseases in wheat (*Triticum aestivum* L.) worldwide (Kumar et al., 2002).

Leaf blotch of wheat caused by *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana*) (Sacc.) Shoemaker *C. sativus* is extremely rare in nature and thus it is the asexual or anamorphic stage which causes diseases.

Detailed studies of morphological structure of *Cochliobolus sativus* are used to examine taxonomy of the pathogen and epidemiological aspects of disease spread, which may be useful in developing resistant wheat cultivars in breeding programs (Mahto et al., 2012). Morphological approach has been used to identify and investigate the variability among isolates of *C. sativus* (Jaiswal et al., 2007 and Poloni et al., 2009). Morphology of *Cochliobolus sativus* has been characterized on the basis of colony colour and growth patterns such as zonation and sporulation (Pandey et al., 2008). A high morphological

variability among isolates of *Cochliobolus sativus* has been reported from South America (Oliveira et al., 1998 and Poloni et al., 2009).

High temperature and high relative humidity favor the outbreak of the disease, in particular in South Asia's 'intensive irrigated wheat-rice production systems' (Kumar et al., 2002). Yield losses up to 80% in the warm and humid regions (Alam et al., 1998; Kumar et al., 2002 and Gupta et al., 2018) due to leaf blotch of wheat. In addition, it has expanded its area of colonization with the warming of the temperature between 22-25 °C during growing season (Kaur and Nanda, 2001). Thus, over the last four decades, spot blotch has been seen as a serious constraint in wheat production, not only in the Eastern Gangetic Plains (EGP) of north India, but also in Bangladesh, Nepal, Brazil and other countries (Gupta et al., 2018). In Bangladesh yield losses due to spot blotch were reported as high as 57.6% and 64.5% in Kanchan and Sonalika variety respectively (Rashid and Fakir, 1998). According to Mehta (1997) spot blotch of wheat caused by *Cochliobolus sativus* adversely destroy seed germination, development of the root system or kill the seedling within a few days and ultimately capable of causing up to 100% yield losses.

Cochliobolus sativus is a hemi-biotrophic pathogen and survives primarily as thick-walled conidia, but can also survive as mycelium in soil or crop debris. Primary inoculum includes mycelium from infected seeds, conidia in the soil and on the kernel surface and conidia from crop debris. Any movement of soil by wind, water or implements can move inoculum of the pathogen. Infested seeds can also serve as a means of dissemination of the pathogen over long distances (Mathre et al., 1987). *Cochliobolus sativus* is characterized by thick-walled, elliptical conidia with five to nine cells. In axenic culture, the mycelium is composed of hyphae interwoven as a loose cottony mass and appears white or light to dark grey depending on the isolates (Raza et al., 2014).

Tinline (1951) demonstrated that *Cochliobolus sativus* was a heterothallic fungus. Parasexual recombination, which plays a more important role in the fungus life cycle, has been demonstrated to occur in *Cochliobolus sativus* *In vitro* (Tinline, 1988). Raemaekers (1988) only found the sexual stage of *B. sorokiniana* in Zambia field and this sexual recombination used as another source of variability. The sexual state formed in laboratory under controlled condition by crossing opposite mating type and produces black, globose pseudothecia. Asci were clavate and ascospores were hyaline, uniformly filamentous, and spirally flexed within asci. *Cochliobolus sativus* exhibits cultural, morphological, physiological, pathogenic and genetic variability which is considered heterokaryosis (Jaiswal et al., 2007 and Aggarwal et al., 2010). It has high morphological variability, which makes it difficult to identify and also to determine the appropriate measures for disease control (Tinline, 1988).

Information is insufficient on the study of morphological characterization of *Cochliobolus sativus* in Bangladesh agricultural ecosystem.

In these circumstances, the present research was conducted with the following objective-

- to determine the morphological variation among the isolates of *Cochliobolus sativus* in F₂ generation



CHAPTER II

REVIEW OF LITERATURE

2.1 Wheat:

Wheat (*Triticum aestivum* L.) is one of the most important grain crops providing nearly 20% of the total world food requirement (Uddin et al., 2006). Green revolution played a key role in ensuring food security in this population dense region of the world, which mainly comprises of India, Pakistan, Nepal and Bangladesh (Joshi et al., 2007).

Recent yield trials conducted by different breeding centers around the world have shown that the production of bread wheat (*Triticum aestivum* L.) is being constrained by several biotic and abiotic stresses (Duveiller, 2004). The warmer parts of the world are mainly affected by many diseases and among these diseases, leaf blotch caused by *Cochliobolus sativus* (anamorph *Bipolaris sorokiniana*) (Chowdhury et al., 2013).

2.2 Pathogen

Name of this fungus was first time given by (Pammel et al., 1910) as *Helminthosporium sativum* without taking into account the earlier description of *Helminthosporium sorokiniana* Sacco in Sorokin, (Trans. Soc. Nat. Univ. Kazan 22: 15, 1890) (Sivanesan, 1987). The Ascigerous state (teleomorph) was first observed in the laboratory on natural media and described as *Ophiobolus sativus* Ito and Kurib which was later named as *Cochliobolus sativus* (Ito and Kurib) Drechsler Ex. (Dastur, 1942). *Cochliobolus drechsler* is characterized by globose ascomata usually with a long cylindrical neck, obclavate-cylindric asci, and helically coiled filiform ascospores. This genus is associated with *Bipolaris* and *Curvularia* anamorphs and the teleomorphs of *C. sativus* were only reported from Zambia (Raemaekers, 1991). Shoemaker (1959) proposed the generic name *Bipolaris* for the *Helminthosporium*

species with fusoid, straight or curved conidia, germinating by one germ tube from each end (bipolar germination), the leaf blotch pathogen was renamed *Bipolaris sorokiniana* (Sacc.) Shoem. This name is presently widely adopted. *Helminthosporium acrothecioides* Lindfors, *Helminthosporium californicum* Mackie and Paxton, and *Drechslera sorokiniana* (Sacc.) Subram and Jain are considered as its synonyms. The correct name for *Helminthosporium* leaf blight of wheat is accordingly proposed as: *Cochliobolus sativus* (Ito and Kurib.) Drechsler ex Dastur, anamorph *Bipolaris sorokiniana* (Sacc.) Shoem.

2.3 Leaf blotch disease of wheat

Leaf blotch of wheat, caused by *Cochliobolus sativus* (Sacc.) (anamorph *Bipolaris sorokiniana*) is an important disease in warm and humid wheat growing regions. The fungus *C. sativus* is the teleomorph (sexual stage) of *Bipolaris sorokiniana* (anamorph) which is the causal agent of wide variety of diseases. Recently, more attention was given to this disease due to its importance in various part of the world for its occurrence and yield losses. Although the pathogen was discovered in 1910 but causing the disease was reported four years later in 1914 by leaf blotch was first time reported by Mohy (Joshi, 1986) but attention was paid only after green revolution (Saari et al., 1998). As the production of wheat has expended in newer growing areas characterized by high temperature (coolest month greater than 17 °C) and high humidity (Van Ginkel and Rajaram, 1993). Increase in temperature coupled with high relative humidity and soil nutrient condition favors the outbreak of the disease (Ragiba et al., 2004). The pathogen affects almost all the crops belonging to *Gramineae* family, though the chances of migration of isolate of one crop to other crop are rare (Bakonyi et al., 1998)). Leaf blotch is seed & soil borne disease and transmitted by seed themselves in nature (Neergaard, 1997). Seed infection by *Cochliobolus sativus* can adversely affect germination and root system development and can kill seedlings within a few days (Mehta, 1993). Ideal conditions for leaf blotch development on the leaves are relative

humidity near to 100% caused by rainfall, irrigation or dew with high temperature. The disease becomes severe especially during grain filling stage (Joshi and Chand, 2002) and causes significant grain yield losses up to 100% under most severe conditions (Sharma et al., 1997). *Cochliobolus sativus* usually induces symptoms on leaf, sheath and stem (Chand et al., 2002). However, under severe conditions, it also infects spikelet's resulting in shrivelled grains, with black point at the embryo end of kernels (Kumar et al., 2001). It is generally believed that the level of resistance in high-yielding wheat genotypes is still unsatisfactory and needs to be improved significantly in warmer humid regions of South Asia (Sharma et al., 2006 and Joshi et al., 2007). Consequently, an integrated approach, with host resistance as a major component, is generally considered best for controlling the disease (Hetzler et al., 1991 and Mehta et al., 1992).

2.4 Distribution of leaf blotch disease

Leaf blotch of wheat is an important disease in warm and humid wheat growing regions. Leaf blotch is not only confined to certain parts of South Asia but it occurs in several parts of the world also, particularly in South East Asia and Latin American countries where nearly 12 million hectares of land under cultivation are affected (Nagarajan and Kumar, 1998 and Joshi et al., 2007). The specific area where it is most prevalent includes African countries (Kenya, Malawi, Sudan, South Africa, Tanzania, Zimbabwe), Asia (China, Korea, India, Indonesia, Taiwan, Thailand), Europe (Austria, Belgium, former Czechoslovakia, Denmark, Germany, Greece, Hungary, Italy, Poland, Scotland, United Kingdom, former USSR), South America (Argentina, Brazil) and North America (Indiana, Kansas, Minnesota, Montana, North Dakota, South Dakota, Utah, Virginia). In former USSR, Northern Kazakhstan was reported a zone of the highest severity of leaf blotch (Villareal et al., 1995). Figure 1 represents the world distribution of wheat growing areas affected by leaf blotch disease (Acharya et al., 2011 and Sultana, 2019).

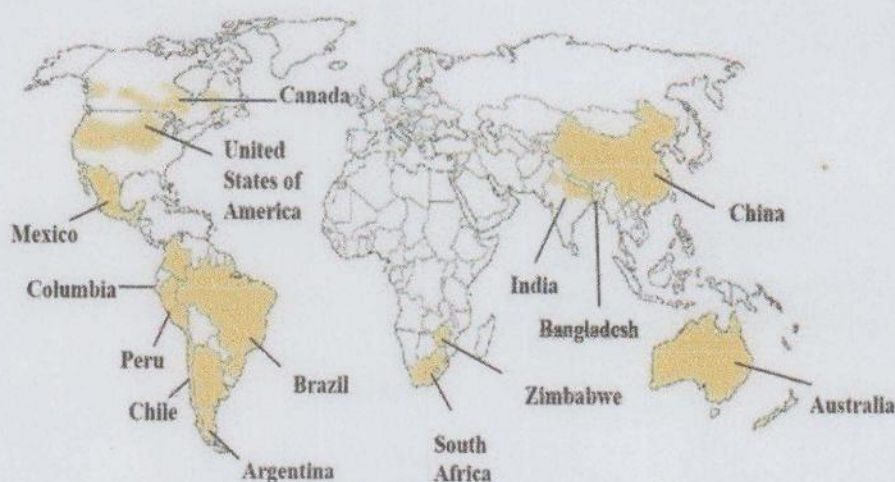


Figure 1. World distribution of leaf blotch disease of wheat caused by *Cochliobolus sativus*

2.5 Host range of *Cochliobolus sativus* (anamorph: *Bipolaris sorokiniana*)

Cochliobolus sativus (anamorph *Bipolaris sorokiniana*) is a pathogen of broad host range and able to infect a large number of grasses include: *Triticum aestivum* (bread wheat), *Triticum turgidum* (durum wheat), *Triticale*, *Secale cereale* (rye), *Zea Maize*, *Guzmania sp.*, *Lacromani anum*, *Phleum pratense*, *Setaria italica*, *T. durum*, *Hordeum vulgare* (barley) and wild grasses (Manamgoda et al., 2011 and Singh et al., 2016).

2.6 Yield loss of wheat due to leaf blotch disease

Leaf blotch is considered as one of the most damaging diseases of wheat, causing yield and quality losses as a consequence of shriveled grains and decreased tillering. Yield losses due to leaf blotch are reported to vary from 15.5 to 19.6% (Dubin and Ginkel, 1991), 20 to 80% (Duveiller and Gilchrist, 1994), and may be up to 100% under severe conditions (Mehta, 1997). It has been estimated that infected plots yields nearly 43% less as compared to the fungicide protected (Villareal et al., 1995). The on farm studies indicated on an average up to 15% yield losses in Bangladesh (Alam et al., 1994) and 16% in Nepal (Bhatta et al., 1997) for the crop season 1991-1994. Seed infection reduces seedling emergence by up to 38% (Clark,

1979). (Sharma et al., 2006) recorded the disease severity up to 100% and yield losses were as high as 43%. Grain yield loss due to spot blotch in South Asia ranged from 4% to 38% and 25% to 43% in the year 2004 and 2005 respectively and the number of kernels per spike as well as thousand-kernel weight were reduced respectively by 10% and 15% in 2004 and 11% and 18% in 2005 (Sharma et al., 2006).

2.7 Reproduction

The pathogen reproduces through asexual means mainly through conidia, although occurrence of sexuality and a parasexual cycle have been reported (Tinline, 1962 and Chand et al., 2003). *B. sorokiniana* represents the asexual state of the pathogen, its sexual stage teleomorph being *C. sativus*. The perfect stage of *C. sativus* is extremely rare in nature (Tinline, 1988). It can, however, be produced in culture. Shoemaker (1955) disclosed sexual stage of *C. sativus* through induction of sexual crosses in the laboratory. Raemakers (1988) confirmed sexual reproduction in the field of Zambia. The cultural conditions that favor the hybridization between two opposite mating types of this heterothallic species were established by Tinline (1951) and Shoemaker (1955).

2.8 Causes of variability

Cochliobolus sativus has long been considered a highly variable phyto-pathogenic fungus (Tinline, 1962). Christensen and Davies (1937) reported that the variation was caused primarily by mutation rather than by heterokaryosis. Subsequently, Hrushovetz (1956) presented some cytological evidence for the perpetuation of heterokaryosis through the spores. The mechanisms involved in the variability include heterokaryosis, which occurs chiefly through the anastomosis between adjacent hyphae, enables the parasexual cycle, which is the main source of genetic diversity in fungi with asexual reproduction (Day, 1974). Variability of the sexual stage of *Cochliobolus sativus* in laboratory was greatly affected by environmental factors (Tinline and Dickson, 1958). The temperature and hydrogen-ion

concentration that were conducive to perithecial formation in laboratory cultures may be met with in nature.

2.9 Variability of *Cochliobolus sativus*

Cochliobolus sativus has been described as a variable fungus with many morphological (Christensen, 1925; Ahmed et al., 1997; Chand et al., 2003 and Thompson et al., 2011), physiological (Tinline, 1962), pathological (Adhikary and Mian, 2005 and Jaiswal et al., 2007) variants. Part of the variability has been attributed to heterokaryosis and parasexual mechanism (Tinline, 1958). The variability and aggressiveness of this pathogen seems to increase over time (Hetzler et al., 1991). Variability in cultural characteristics was observed in the morphology and growth rate between the original isolates and the re isolates (Oliveira et al., 1998). Chand et al. (2003) demonstrated that the five groups of the isolates of *Cochliobolus sativus* differed for their morphological appearance. Molecular characterization of pathogen variability in *Cochliobolus sativus* isolates causing spot blotch in wheat in India has also been reported (Saharan et al., 2008).

2.10 Morphological variability of *Cochliobolus sativus*

Variability among isolates of *Cochliobolus sativus* was determined based on morphological characters of conidia (Kumar et al., 2002). Rahman (2011) revealed that the collected 69 isolates differed significantly in respect of mycelia growth, conidia production ability, shape, color, size and septation of conidia. Poloni et al. (2009) reported that the 35 *Cochliobolus sativus* isolates were divided into five morphological groups on the basis of mycelial morphological variability and growth pattern. These groups were: (I) black fluffy growth with white sectors; (II) black fluffy growth; (III) gray cottony growth; (IV) white cottony growth; and (V) white suppressed growth.

Asad et al. (2009) studied that the isolates were grouped in 4 classes having black, grayish black, brown and albino (whitish) colony color with profusely sporulated and suppressed type of growth to fluffy and less sporulated type. The conidial average size ranged from 38.3–65.8 $\mu\text{m} \times 12.3\text{--}25 \mu\text{m}$ with slightly curved, brown to olivaceous brown with 2–13 septa. Some isolates had relatively long and broad slender conidia, while some were uniformly straight and cylindrical and light brown in color.

The dark colored colony showed a strong correlation with aggressiveness of the pathogen as Chand et al. (2003) studied the variability in natural populations of the leaf blotch pathogen (*Cochliobolus sativus*) and classified the isolates into 5 groups on the basis of colony morphology, he found that the majority (44.63%) of the isolates of black suppressed type in the natural population were of most aggressive and was identified as the epidemic population as compared to the lowest frequency of the isolates (4.96%) of white colored having very few conidia.

Singh et al. (2018) showed that cultural and morphological variability of *Cochliobolus sativus* isolates exhibited that the colonies were effuse grey-white to effuse black and velvety-white mycelial growth with regular to irregular margins. The color of colonies varied from grey to dark brown and white to light grey. The number of septation in isolates ranged from 3.9 to 6.3 and spore size ranged from 35.07 to 60.53 μm in length and 13.20 to 17.60 μm in breadth. The shape of the spore was elliptical, slight curved and straight with tapered end. Momtaz et al. (2019) reported that 150 isolates were characterized on the basis of colony color and texture. The results exhibited considerable variation of the isolates. Results indicated that the colony color varied from black, ash, brown, green to white and mycelial growth type was effuse (fluffy) to suppress (mat) with regular to irregular growth patterns.

CHAPTER III

MATERIALS AND METHODS

The research work was carried out to determine the morphological variation among the isolates of *Cochliobolus sativus* in F₂ generation. The materials used and methodologies followed in this experiment are discussed below.

3.1 Experimental site

The experiment was conducted in Plant Protection Laboratory of Agrotechnology Discipline, Khulna University, Khulna, Bangladesh.

3.2 Experimental period

The experiment was conducted during the period of June to August, 2019.

3.3 Collection of *Cochliobolus sativus* (F₂ generation) isolates

Isolates of *Cochliobolus sativus* (F₂ generation) were collected from Plant Protection Laboratory of Agrotechnology Discipline, Khulna University. Twenty five isolates were taken randomly and further cultured in PDA medium for study.

3.4 Procedure for obtaining *Cochliobolus sativus* (F₂ generation)

A general procedure for obtaining *Cochliobolus sativus* (F₂ generation) followed by Tinline and Dickson, 1958 and Sultana et al., 2018 is given below:

3.4.1 Induction of sexual crosses within the isolates of *B. sorokiniana*

5 isolates were randomly selected to test the sexual compatibility within the isolates originated from different geographical area. Sexual fusion was done on Sack's agar containing petri plates by inoculating amended barley (BARI barley-3) seeds with desired *B.*

sorokiniana isolates following the method proposed by Tinline and Dickson (1958) with all possible combinations.

3.4.2 Procedure of sexual crosses in laboratory

Each isolate was crossed with the other isolates on Sach's agar medium in a petri plate on susceptible barley seeds. The initial pH of the medium was 6.0 and seeds of the spot blotch susceptible barley cultivar were soaked in distilled water for 30 min, surface-sterilized with a solution of mercuric chloride (1g/1,000 ml) for 5 minutes and finally rinsed thrice with sterile distilled water. The seeds were boiled for 2 minutes to kill the embryos, drained out of excess water, and refrigerated at 4 °C in sterile petri plates until needed. Conidial suspensions of 12 days old cultures were prepared by adding 4 ml of sterile distilled water to each petri plate. Conidia were gently scraped from the surface of the cultures with a rubber spatula. Conidial suspensions of the parental isolates in equal volumes (4 ml) were then added to the sterilized seed. After about 30 min, seeds with the adsorbed conidia of both cultures were transferred to petri plates (about 3 kernels per plate). The plates were placed in a laminar flow hood to facilitate the evaporation of excess moisture on the seed and agar surface. This was done to reduce the amount of fungal vegetative growth that appeared to be favored by high moisture levels. Petri plates were sealed with laboratory film and placed in an incubator in the dark, first at 24 °C for a week, and then at 20 °C for next 14 days for obtaining F₁ generation.

Table 1. Composition of Sack's Agar medium

Elements	Quantity (g)
Ca(NO ₃) ₂	1.00
KCl	0.25
K ₂ HPO ₄	0.25
MgSO ₄ .7H ₂ O	0.25
FeCl ₃	trace
CaCO ₃	3.00 to 4.00
Agar	20.00

Source: (Tinline and Dickson, 1958)

3.4.3 Check for sexual compatibility

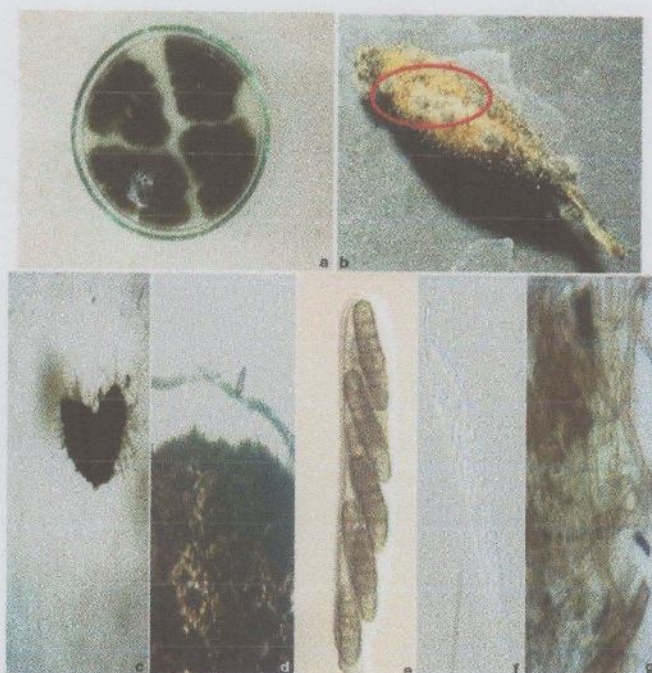
The occurrence of the sexual reproduction was considered successful when pseudothecia formed from each crosses after 3 weeks of incubation. Most fertile or highly compatible isolates (as expressed by the mature pseudothecia produced in crosses) were selected as highly fertile combination. Isolates that produced pseudothecia containing eight mature ascospores were considered highly fertile and crosses that produced numerous pseudothecia without ascospore were classified as low fertile after 21 days (3 week) of incubation.

3.4.4 Ascospores observation and isolation

For visualization, ascocarps and ascospores were stained with lactophenol (Sigma-Aldrich) as suggested by the manufacturer and observed in a Carl Zeiss (Germany) stereo microscope with 25×. For isolation of ascospores Valjavec-Gratian and Steffenson (1997) method was followed.

Pseudothecia that were freed of debris and adhering spores by rolling them across an agar surface were crushed in a water droplet; if examination showed abundant ascospores the material was washed from the slide to an agar surface in a petri plate; an ascus containing spores was selected and moved apart from the perithecial material; the ascus wall was

ruptured with sharp steel needles; the spores were withdrawn from the ascus case and distributed singly on a free agar surface in the dish; after spore germination (4 to 12 hours) blocks of agar that contained individual spores were transferred to fresh medium in petri plates.



Source: Sultana et al., 2018

Figure 2. Sexual structures (pseudothecia) over the barley seed (BARI Barley-3) after 21 days of incubation. a. Crosses opposite mating type isolates on barley seeds; b. Developed pseudothecia on barley seed; c. Ruptured pseudothecia; d. Pseudothecial wall, e. ascus with ascospores; f. Barren ascus; g. Paraphysis

3.4.5 Preparation of F₂ generation of *Cochliobolus sativus*

F₂ generations were obtained by selfing F₁

3.5 Morphological characterization of *Cochliobolus sativus* (F₂ generation)

3.5.1 Culture of *Cochliobolus sativus* isolates

Twenty five isolates of *Cochliobolus sativus* were grown separately on PDA at pH 6.5. For morphological characterization, 5mm block cut from 10 days old culture by flamed cork

borer was placed at the center of PDA plates and incubated up to 12 days in growth chamber with constant temperature (25 °C).

3.5.2 Experimental design

The plates were arranged in growth chamber following complete randomized design (CRD) with three replications.

3.5.3 Observation of mycelial characters

To observe mycelial characters, regular observation was done after incubation. The observed characters were:

- ✓ Radial growth (mm)
- ✓ Colony color
- ✓ Colony margin
- ✓ Colony growth behavior and
- ✓ Number of zonation

3.5.4 Observation of conidial characters

Conidial characters were observed after 12 days of incubation. The characters were:

- ✓ Conidia production cm^{-2} surface area of a colony
- ✓ Conidial color
- ✓ Conidia shape
- ✓ Length & width (μm) of conidia and
- ✓ Conidial septation number

3.5.5 Data collection

Mycelial characters were recorded through visual observation after 7 days of incubation and conidial characters were recorded under microscopic observation at 40× magnification after 12 days of incubation. Radial growth of colony were recorded using scale. Conidial color, shape and septation number were seen under microscope and size was measured using Zeiss version 2.0 computer program at 10 µm unit scale. The spores produced per unit area were estimated using the formula (Chauhan and Panday, 1995) as follows:

Conidia production cm^{-2} =

$$\frac{\text{Number of conidia/ml of suspension unit surface area} \times \text{volume of water used to make suspension}}{\text{Total surface area from which conidial suspension was derived}}$$

3.6 Statistical analysis

The experiment was conducted following CRD with three replications. Variations among the isolates based on morphological characters were analyzed following multivariate analysis: descriptive statistics, correlation matrix, principal component analysis (PCA), and cluster analysis was performed by using STAR (Statistical Tool for Agricultural Research), version-2, IRRI, Losbanos, Philippines. Cluster analysis and PCA was performed to identify accession groups and studied components of variability which may contribute in variation.



CHAPTER IV

RESULTS AND DISCUSSION

In the present study, morphological variation has been governed on the basis of mycelial and conidial characteristics of all 25 isolates of *Cochliobolus sativus* (F₂ generation).

4.1 Morphological variation of *Cochliobolus sativus*

4.1.1 Descriptive statistics

From the descriptive analysis, mean value of eleven components of morphological variability as colony color, colony margin, growth behavior, radial growth (mm), number of zonation, conidial color, conidial shape, septation number, length of conidia (μm), width of conidia (μm) and conidia production cm^{-2} were 2.32, 2.12, 1.44, 60.40, 0.88, 1.36, 1.52, 4.56, 13.30, 4.45 and 13730, respectively (Table 2). From the tabulated value, mean all of the components was greater than the standard error. The data also revealed that highest coefficient of variation was observed among the number of zonation and lowest coefficient of variation was observed among the growth behavior. That means number of zonation created more variability among the *Cochliobolus sativus* (F₂ generation) isolates.

Table 2. Range, mean, standard error and coefficient of variation of the components for morphological variability among the *Cochliobolus sativus* (F₂ generation) isolates

Variables	Minimum	Maximum	Mean $\pm$ SE	CV (%)
Number of zonation	0.00	3.00	0.88 $\pm$ 0.20	114.77
Conidia production cm ⁻²	0.00	36.80	13730 $\pm$ 2300.92	83.76
Conidial shape	0.00	3.00	1.52 $\pm$ 0.18	60.53
Conidial color	0.00	3.00	1.36 $\pm$ 0.16	59.56
Length of conidia (μ m)	0.00	30.74	13.30 $\pm$ 1.46	54.81
Septation number	0.00	7.33	4.56 $\pm$ 0.45	49.12
Width of conidia (μ m)	0.00	6.32	4.45 $\pm$ 0.41	46.07
Colony margin	1.00	3.00	2.12 $\pm$ 0.17	39.15
Colony color	1.00	4.00	2.32 $\pm$ 0.49	36.64
Radial growth (mm)	32.13	88.00	60.40 $\pm$ 4.41	36.52
Growth behavior	1.00	2.00	1.44 $\pm$ 0.10	35.42

SE – Standard error and CV – Coefficient of variation

4.1.2 Different morphological groups among the isolates of *Cochliobolus sativus* (F₂ generation)

For morphological grouping, eleven morphological components were considered. Cluster analysis showed five distinct groups based on morphological characteristics (Figure 3). Considering the cultural variability four distinct colony colors were observed. The colors were: a) Whitish, b) Blackish grey, c) Ash grey and d) Greenish black (Figure 4). Three types of colony margin were found. They were: a) Regular b) irregular and c) wavy. Three types of growth behavior were also observed. They were: a) Fluffy b) Suppressed and c) Too much suppressed. In case of too much suppressed growth, no sporulation was found (Figure 5). In case of conidial characters, three types of conidial color and three types of conidial shape were found. The conidial colors were: a) olive, b) brown and c) dark brown. The conidial shapes were: a) Straight, b) slightly curved and c) cylindrical (Figure 6).

Dendrogram using Agglomerative Clustering Method

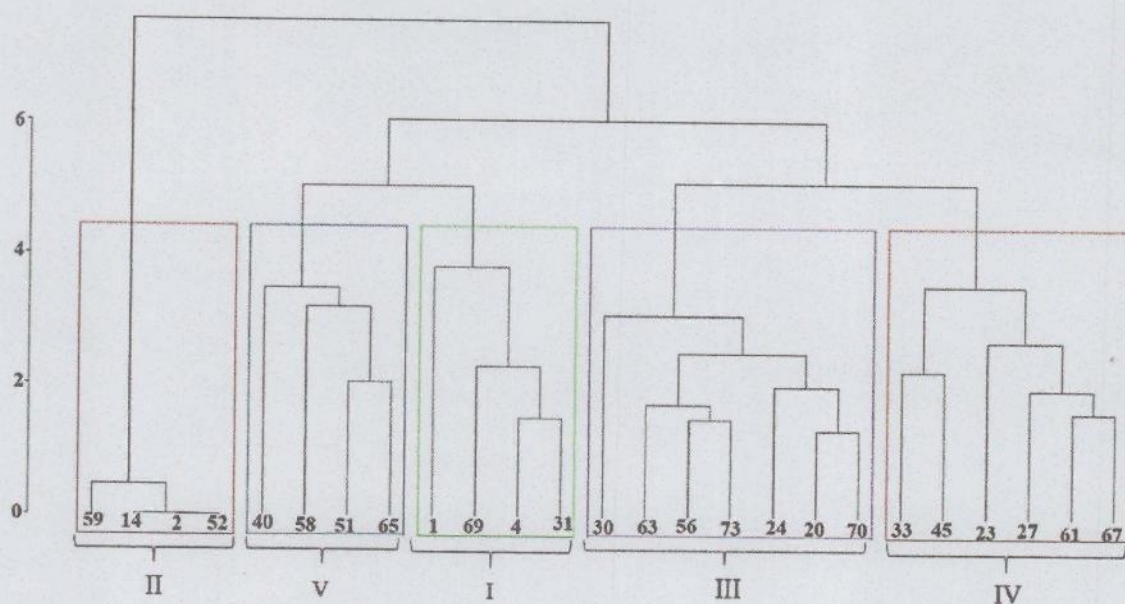


Figure 3. Dendrogram constructed with the data obtained from studied eleven morphological components of *Cochliobolus sativus* (F₂ generation)



Figure 4. Colony characteristics of *Cochliobolus sativus* (F₂ generation) isolates

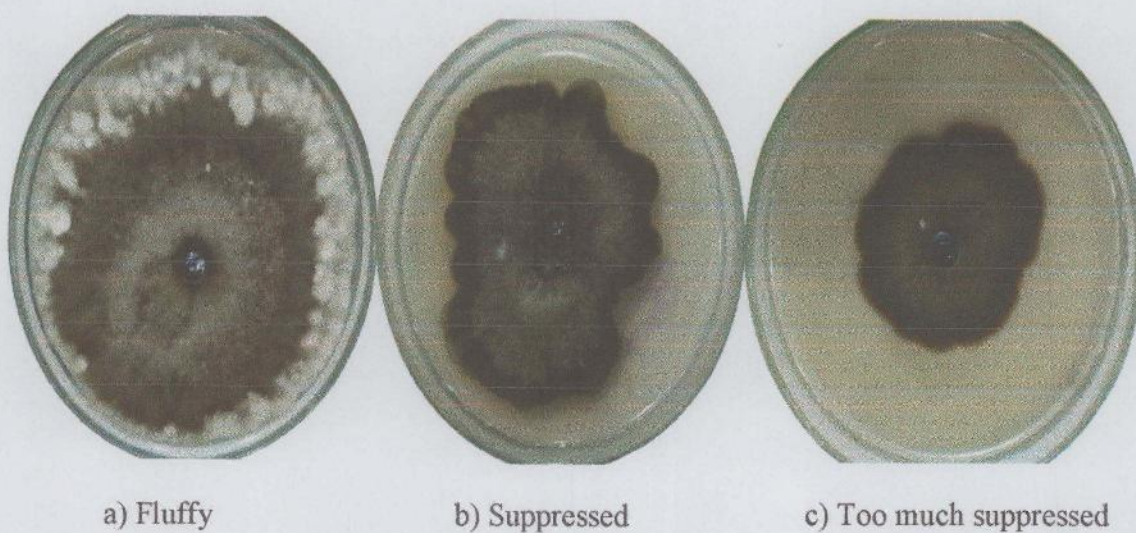


Figure 5. Colony growth behavior of *Cochliobolus sativus* (F₂ generation) isolates

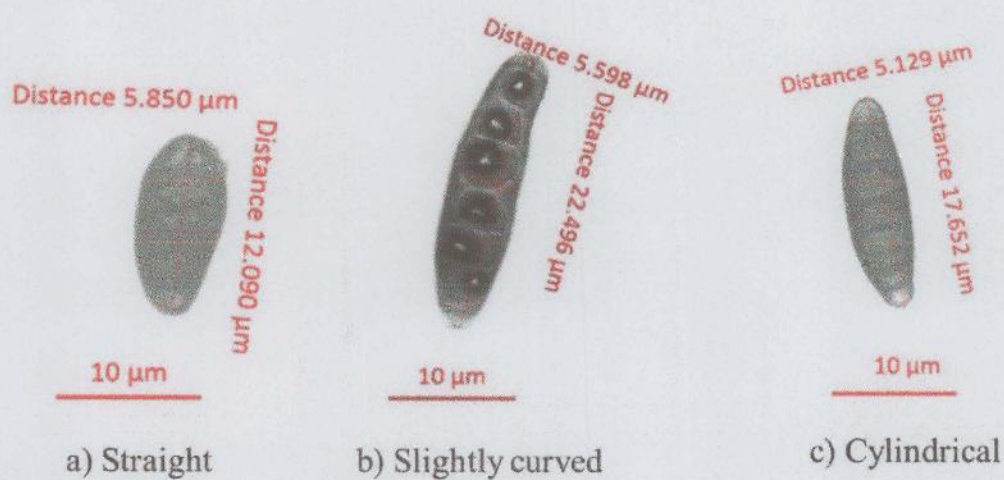


Figure 6. Conidial characteristics of *Cochliobolus sativus* (F₂ generation) isolates

Table 3. Grouping of twenty five isolates of *Cochliobolus sativus* (F₂ generation) under different cluster based on eleven morphological components

Cluster No.	<i>Cochliobolus sativus</i> isolates	Isolate No.
I	CSF ₂ 1, CSF ₂ 4, CSF ₂ 31, CSF ₂ 69	04
II	CSF ₂ 2, CSF ₂ 14, CSF ₂ 52, CSF ₂ 59	04
III	CSF ₂ 20, CSF ₂ 24, CSF ₂ 30, CSF ₂ 56, CSF ₂ 63, CSF ₂ 70, CSF ₂ 73	07
IV	CSF ₂ 23, CSF ₂ 27, CSF ₂ 33, CSF ₂ 45, CSF ₂ 61, CSF ₂ 67	06
V	CSF ₂ 40, CSF ₂ 51, CSF ₂ 58, CSF ₂ 65	04

The data showed that maximum spore producing isolates belonged in cluster V with fast radial growth (Table 4). The maximum mean of conidia production cm⁻² was 36800 and the highest mean of colony radial growth was 88.00 mm.

Table 4. Mean of five cluster centers

Components	Final Cluster Centers				
	Cluster				
	I	II	III	IV	V
CC	3.00	2.20	2.67	2.00	3.00
CM	2.67	1.40	2.00	3.00	1.00
GB	1.33	1.00	1.67	2.00	1.00
RG	42.57	83.44	52.83	37.69	88.00
NZ	1.33	1.50	0.33	0.00	2.00
Con. C	1.33	1.70	2.00	0.75	1.00
CS	1.67	1.90	1.67	0.75	3.00
S	5.67	5.46	5.55	2.69	4.25
L	14.22	17.28	15.51	7.05	14.15
W	5.09	5.49	5.28	2.57	4.70
CP	30670	17640	6530	2300	36800

CC – Colony color, CM – Colony margin, GB – Growth behavior, RG – Radial growth (mm), NZ – Number of zonation, Con. C – Conidial color, CS – Conidial shape, SN – Septation number, L – Length of conidia (µm), W – Width of conidia (µm) and CP – Conidia production cm⁻²

Between the clusters, maximum cluster distance was found between cluster IV and V (61.59) and minimum distance was observed between cluster II and IV (20.06). Maximum cluster distance indicates that highest morphological variability exists between the cluster IV and V isolates (Table 5).

Table 5. Distances between final cluster centers

Cluster	I	II	III	IV	V
I		43.04	26.30	45.93	29.99
II				20.06	49.57
III				46.52	18.40
IV					61.59
V					

Extraction method: Principal Component Analysis

4.1.3 Correlation among the morphological components

The correlation among the components of morphological variability represents the relationship among the components (Table 6). Among the components some of them showed positive, some of them showed negative and some of them showed non-significant relationship between the components. High correlation was observed among width of conidia, length of conidia, shape of conidia, conidial color and septation number. Colony color represented non-significant correlation among the all components. In case of colony margin, it showed significant and negative correlation among all components except colony growth behavior and colony color. Growth behavior also showed significant and negative correlation among the all components except colony margin and colony color. Radial growth represented significant and positive correlation among number of zonation, conidial shape, length of conidia, width of conidia and conidia produced cm^{-2} . On the other hand, non-significant correlation was found among radial growth, colony color, conidial color and septation number. Number of zonation showed significant correlation among colony margin, growth behavior, radial growth and conidia production cm^{-2} . On the other hand, conidial color represented significant correlation among colony margin, growth behavior, conidial

shape, length of conidia, width of conidia and septation number. Conidial shape showed significant correlation among the all components except colony color, radial growth and number of zonation. Length of conidia also showed significant correlation among the all components except colony color and number of zonation. Width of conidia represented significant correlation among the all components except colony color. Conidia production cm^{-2} showed significant correlation among the all components except colony color and conidial color.

Table 6. Pearson correlation between the eleven morphological components of *Cochliobolus sativus* (F₂ generation)

Components	CC	CM	GB	RG	NZ	Con. C	CS	SN	L	W	CP
CC											
CM	-0.291 ^{NS}										
GB	-0.340 ^{NS}	0.759**									
RG	-0.013 ^{NS}	-0.852**	-0.788**								
NZ	0.336 ^{NS}	-0.575**	-0.786**	0.591**							
Con. C	0.370 ^{NS}	-0.437*	-0.503*	0.356 ^{NS}	0.207 ^{NS}						
CS	0.204 ^{NS}	-0.412*	-0.602**	0.526**	0.383 ^{NS}	0.690**					
SN	0.073 ^{NS}	-0.403*	-0.416*	0.345 ^{NS}	0.218 ^{NS}	0.641**	0.614**				
L	-0.007 ^{NS}	-0.547**	-0.574**	0.472*	0.309 ^{NS}	0.635**	0.556**	0.864**			
W	0.138 ^{NS}	-0.484*	-0.561**	0.455*	0.320 ^{NS}	0.819**	0.768**	0.901**	0.865**		
CP	0.312 ^{NS}	-0.405*	-0.679**	0.485*	0.692**	0.286 ^{NS}	0.591**	0.442*	0.410*	0.482*	

CC – Colony color, CM – Colony margin, GB – Growth behavior, RG – Radial growth (mm), NZ – Number of zonation, Con. C – Conidial color,

CS – Conidial shape, SN – Septation number, L – Length of conidia (μm), W – Width of conidia (μm) and CP – Conidia production cm⁻²

** - Significance @ 0.01, * - Significance @ 0.05 and NS – Non-significance

4.1.4 Contribution of components in morphological variation

Principal component analysis (PCA) was done to identify the extent of contribution of each of the components to observe variations among the isolates. Each component is linked with an *eigenvalue* which represents the amount of variation. Colony color, colony margin and growth behavior accounted for more than 80% of the total variation and along with radial growth and number of zonation contributed more than 90% role in generating variation. These five components greatly added variation in morphology. Contribution of other components are presented in Table 7.

Table 7. Estimation of percentages of variation of eleven morphological components of *Cochliobolus sativus* (F₂ generation)

Components	<i>Eigenvalue</i>	Variation (%)	Cumulative variation (%)
Colony color	6.06	55.10	55.10
Colony margin	1.79	16.25	71.35
Growth behavior	1.14	10.39	81.74
Radial growth (mm)	0.77	7.02	88.76
Number of zonation	0.52	4.71	93.47
Conidial color	0.28	2.53	96.00
Conidial shape	0.16	1.47	97.46
Septation number	0.13	1.21	98.68
Length of conidia (μm)	0.08	0.72	99.40
Width of conidia (μm)	0.04	0.36	99.76
Conidia production cm ⁻²	0.03	0.24	100.00

Extraction method: Principal Component Analysis



Conidial color and conidia production cm^{-2} as PC1 and PC2. Dataset labels were indicated in the positive and negative relation among the isolates based on PC1 and PC2. From the figure 7, two distinct groups were observed based on datasets of PC1 and PC2. Most of the highest spore producing isolates belonged in the same group and they produced olive colored conidia.

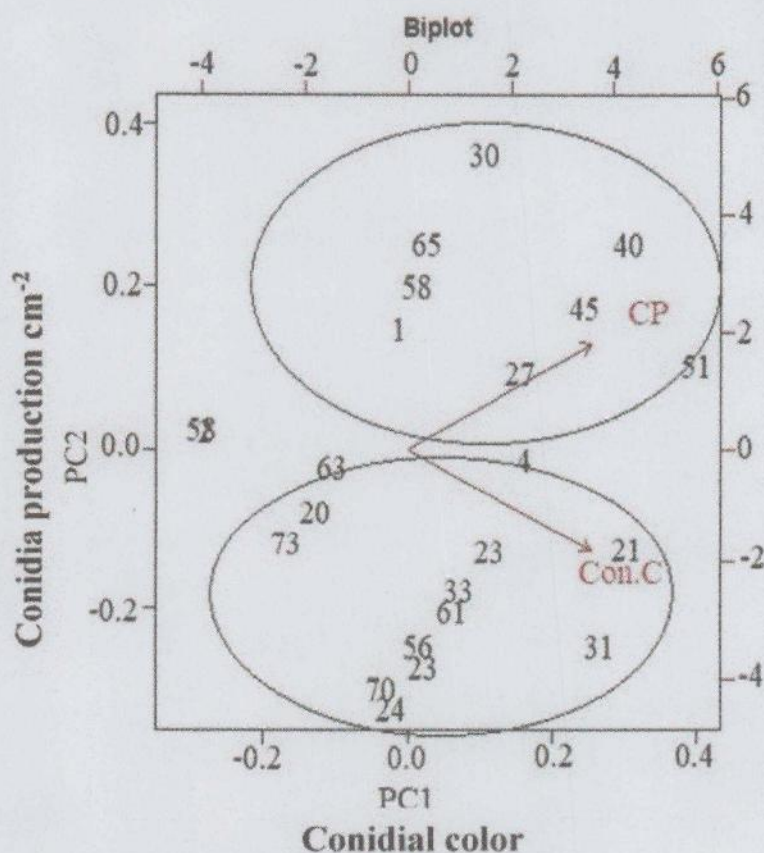


Figure 7. Pairs of data sets (Conidial color and Conidia production cm^{-2}) of the morphological variability components are displayed on PC1 and PC2

4.2 Discussion

In this experiment, multivariate analysis was performed for observing morphological variability based on eleven morphological components. After 12 days of incubation on potato dextrose agar, isolates of *C. sativus* showed great diversity in their growth (Figure 3, Figure 4 and Figure 5).

Some researcher found that virulence and aggressiveness is strongly correlated with morphological traits like as colony color, conidial color and spore production (Jaiswal et al., 2007 and Thompson et al., 2011). Considering the cultural variability four distinct colony color were observed like as whitish, ash grey, blackish grey and greenish black (Figure 3). Asad et al. (2009) also showed that four different types of colony colors on PDA medium among the thirty seven isolates, among them thirteen isolates exhibited black colony color, 19 grayish black, 2 brownish while 3 showed albino color colony. Mahto et al., (2012) observed three categories of colony color among the forty eight isolates of *C. sativus* (white, light grey, and dark grey).

At the morphological level, virulence is correlated with morphological pattern of isolates (Chand et al., 2003 and Pandey et al., 2008). Pandey et al. (2007) reported morphological characters were able to correlate with variability and virulence (Kumar et al., 2002 and Biswas and Das, 2018). In Pearson correlation, it was observed that eleven morphological components some of them showed positive, some of them showed negative and some of them showed non-significant relationship between the components. Higher correlation was observed between width of conidia and septation number (Table 6). Jaiswal et al. (2007) and Maraite et al. (1998) also demonstrated higher correlation between the virulence and the morphological characters.

Here, three types of colony margin (regular, irregular and wavy) and three types of colony growth behavior (fluffy, suppressed and too much suppressed) were found. No sporulation was found in too much suppressed growth isolates. In case of conidial characters, three types of conidial color (olive, brown and dark brown) and three types of conidial shape (Straight, slightly curved and cylindrical) were found. Singh et al. (2018) also found that morphological variability among the anamorphic stage of *C. sativus* isolates. They were exhibited that the colonies with effuse grey-white to effuse black and velvety-white mycelial growth with regular to irregular margins. The color of colonies varied from grey to dark brown and white to light grey. The shape of the spore was elliptical, slight curved and straight with tapered end. Momtaz et al. (2019) reported that 150 isolates were characterized on the basis of colony color and texture. The results exhibited considerable variation of the isolates. Results indicated that the colony color varied from black, ash, brown, green to white and mycelial growth type was effuse (fluffy) to suppress (mat) with regular to irregular growth patterns.

In this study, Principal component analysis (PCA) revealed that mycelial and conidial characters contributed in variability. Among the components colony color, colony margin and colony growth behavior played for more than 80% role in generating variation. Sultana (2019) also found that colony color, conidial color, colony margin, colony growth behavior and radial mycelium growth after 3 days of incubation accounted for more than half (68.11%) of the variation and along with after 5 days of incubation contributed more than 75% role in creating variation.

Here, maximum cluster distance was found between cluster IV and V (61.59) and minimum distance was observed between cluster II and IV (20.06). Maximum cluster distance indicates that highest morphological variability exists between the cluster IV and V isolates. Sultana (2019) confirmed that maximum distance between cluster III and IV and isolates of these clusters created maximum diversity.

In this experiment, cluster analysis showed five distinct groups (Cluster I, II, III, IV and V) based on eleven morphological characteristics. The cluster I, II, III, IV and V consisted of 4, 4, 7, 6 and 4 isolates. Chand et al. (2003) also used cluster analysis for morphological grouping and found five distinct groups. Cluster analysis confirmed this classification. Mahto et al., (2012) reported that forty eight isolates of *C. sativus* isolates were grouped into three groups on the basis of their colony texture and mycelia color. Group I consisted of two isolates with white color mycelium. Group II contained nine isolates with light grey color mycelium and the remaining 37 isolates had dark grey mycelia and belonged to group III. Pandey et al. (2005) grouped the collected isolates in five different morphological groups and test the virulence of isolates under the different groups. Poloni et al. (2009) revealed that the 35 *C. sativus* isolates were divided into five morphological groups on the basis of mycelial morphological variability and growth pattern. These groups were (I) black fluffy growth with white sectors; (II) black fluffy growth; (III) gray cottony growth; (IV) white cottony growth; and (V) white suppressed growth.

In recent years, the incidence of leaf blotch disease in rice-wheat growing regions in Bangladesh has increased through the use of cropping system and increasing temperature and humidity in winter season. Such farming practices, together with pathogen's semi-biotrophic nature and wide host range, and the planting of high yielding but susceptible varieties have led to the dissemination, establishment and persistence of the fungus in all rice producing areas (Gupta et al., 2018). In the present investigation, morphological variations were observed among the isolates due to continuous genetic recombination is occur, so far, all aspect of morphological variability was found in *C. sativus* (Tinline, 1962 and Chand et al., 2003).

CHAPTER V

SUMMARY AND CONCLUSION

5.1 Summary

Leaf blotch of wheat caused by *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana*) (Sacc.) Shoemaker, is a serious disease in Bangladesh. The experiment was conducted in the Plant Pathology Laboratory of Agrotechnology Discipline, Khulna University, Khulna during the period of June to August, 2019 to determine the morphological variation among the isolates of *Cochliobolus sativus* in F₂ generation. Twenty five isolates of *Cochliobolus sativus* (F₂ generation) were collected from Plant Pathology Laboratory of Agrotechnology Discipline, Khulna University. Experiment on morphological variability was conducted following Complete Randomized Design (CRD) with three replications.

Morphological variability among *Cochliobolus sativus* (F₂ generation) isolates were observed by considering colony color, colony margin, colony growth behavior, radial growth, number of zonation, conidial color, conidial shape, septation number, length and width of conidia and conidia produced cm⁻². Multivariate analysis was done to observe morphological variation.

Mean all of the components were greater than the standard error. Highest coefficient of variation percentages (114.77) was observed among the number of zonation formation and lowest coefficient of variation percentages was found among the growth behavior (35.42). That means number of zonation formation help to create more variability within the F₂ generation of *Cochliobolus sativus*.

Five distinct clusters were found from cluster analysis based on morphological characters. The cluster I, II, III, IV and V consisted of 4, 4, 7, 6 and 4 number of isolates. Three types of

conidial color, conidial shape, colony margin and colony growth behavior were found. No sporulation was found in too much suppressed growth isolates. Four types of colony color were also found.

The maximum mean of conidia production cm^{-2} was 36800 and the highest mean of colony radial growth was 88.00 mm. The data showed that maximum spore producing and radial growth isolates were belonged in cluster V.

Between the cluster distances, maximum cluster distance was found between cluster IV and V (61.59) and minimum distance was observed between cluster II and IV (20.06). Maximum cluster distance indicates that highest morphological variability exists between the cluster IV and V isolates.

Principal component analysis (PCA) and correlation matrix revealed that mycelial and conidial characters contributed in variability. Width of conidia, length of conidia, shape of conidia, conidial color and septation number showed higher correlation among themselves. Among the components colony color, colony margin and colony growth behavior played for more than 80% role in generating variation. PCA biplot showed that most of the highest spore producing isolates belonged in the same group and they produced olive colored conidia.

5.2 Conclusion

From the result of this study it can be concluded that *Cochliobolus sativus* is highly variable in morphologically. The following conclusions are drawn:

- (i) Highest coefficient of variation (114.77%) is observed among the number of zonation formation and lowest coefficient of variation is observed among the growth behavior (35.42%).
- (ii) Five distinct clusters are found from cluster analysis based on morphological characters.
- (iii) Three types of conidial color, conidial shape, colony margin and colony growth behavior are found. In case of too much suppressed growth behavior isolates, no sporulation was found. Four types of colony color are also found.
- (iv) Maximum spore producing and radial growth isolates belong in cluster V.
- (v) In distance between final clusters centers, maximum cluster distance is found between cluster IV and V (61.59) and minimum distance between cluster II and IV (20.06).
- (vi) Width of conidia, length of conidia, shape of conidia, conidial color and septation number show higher correlation among themselves.
- (vii) Among the eleven morphological components, colony color, colony margin and colony growth behavior play for more than 80% role in generating variation.
- (viii) PCA biplot shows that most of the highest spore producing isolates belong to the same group and they produce olive colored conidia.

CHAPTER VI

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APPENDICES

Appendix 1. Mean mycelial characteristics of *Cochliobolus sativus* (F₂ generation) isolates

Isolate No.	Colony color	Colony margin	Growth behavior	Radial growth	Number of zonation
CS 1	Whitish	Regular	Fluffy	82.83	2
CS 2	Blackish grey	Wavy	Too much suppressed	42	0
CS 4	Whitish	Irregular	Fluffy	85	1
CS 14	Blackish grey	Wavy	Too much suppressed	42.4	0
CS 20	Blackish grey	Wavy	Suppressed	34	0
CS 23	Ash grey	Regular	Fluffy	85.13	0
CS 24	Blackish grey	Irregular	Suppressed	59.3	0
CS 27	Ash grey	Regular	Fluffy	87.8	1
CS 30	Blackish grey	Wavy	Suppressed	43.4	0
CS 31	Whitish	Irregular	Fluffy	84.3	1
CS 33	Greenish black	Irregular	Fluffy	50.3	1
CS 40	Ash grey	Wavy	Fluffy	47.3	3
CS 45	Greenish black	Irregular	Fluffy	37	1
CS 51	Ash grey	Regular	Fluffy	88	2
CS 52	Blackish grey	Wavy	Too much suppressed	42.1	0
CS 56	Blackish grey	Wavy	Suppressed	40.8	0
CS 58	Ash grey	Regular	Fluffy	80.6	3
CS 59	Blackish grey	Wavy	Too much suppressed	32.13	0
CS 61	Ash grey	Regular	Fluffy	76	2
CS 63	Blackish grey	Irregular	Suppressed	48.9	0
CS 65	Ash grey	Irregular	Fluffy	85.2	2
CS 67	Ash grey	Regular	Fluffy	86.1	2
CS 69	Whitish	Irregular	Fluffy	81.4	1
CS 70	Blackish grey	Wavy	Suppressed	34.7	0
CS 73	Blackish grey	Wavy	Suppressed	33.4	0

Appendix 2. Mean conidial characteristics of *Cochliobolus sativus* (F₂ generation) isolates

Isolate No.	Conidial color	Conidial shape	Septation	Conidial size		Conidia produced/cm ² (10 ³)
				Length(μ)	Width(μ)	
CS 1	Olive	Straight	6	30.74	5.31	16.8
CS 2	No conidia	No conidia	No conidia	No conidia	No conidia	No conidia
CS 4	Brown	Slightly curved	5.67	20.62	6.19	19.6
CS 14	No conidia	No conidia	No conidia	No conidia	No conidia	No conidia
CS 20	Brown	Straight	5.33	14.3	5.26	4.8
CS 23	Brown	Slightly curved	5.25	18.96	6.32	6.8
CS 24	Brown	Slightly curved	5.33	12.92	5.25	3.2
CS 27	Brown	Slightly curved	7.33	17.74	5.33	24.4
CS 30	Olive	Straight	5.67	11.19	4.56	28
CS 31	Brown	Cylindrical	4.33	14.5	6.31	20.4
CS 33	Dark brown	Slightly curved	5	18.65	5.97	10
CS 40	Olive	Slightly curved	5	12.29	4.74	34.8
CS 45	Brown	Slightly curved	6.33	19.18	5.97	29.2
CS 51	Olive	Cylindrical	4.25	14.15	4.7	36.8
CS 52	No conidia	No conidia	No conidia	No conidia	No conidia	No conidia
CS 56	Olive	Slightly curved	5.5	13.31	4.89	7.6
CS 58	Olive	Straight	3.33	8.4	4.58	18.4
CS 59	No conidia	No conidia	No conidia	No conidia	No conidia	No conidia
CS 61	Brown	Slightly curved	5.67	14.61	5.02	9.2
CS 63	Olive	Straight	6.33	14.97	4.62	6.4
CS 65	Brown	Cylindrical	5	13.3	5.18	25.2
CS 67	Brown	Straight	4.67	15.05	5.05	20.8
CS 69	Olive	Slightly curved	7.33	18.86	5.62	14.8
CS 70	Brown	Slightly curved	4	11.19	4.88	4
CS 73	Olive	Straight	6.67	17.59	5.51	2

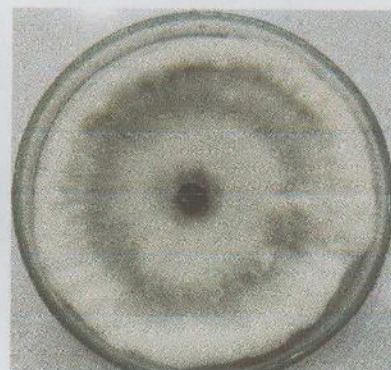
Appendix 3. Colony color of twenty five isolates of *Cochliobolus sativus* (F₂ generation)



CSF₂ 1



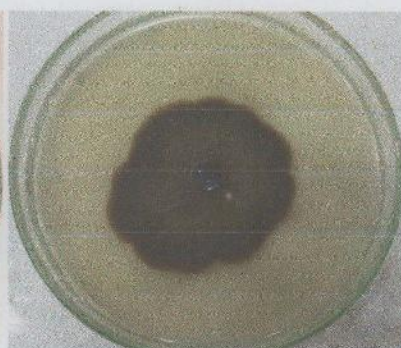
CSF₂ 2



CSF₂ 4



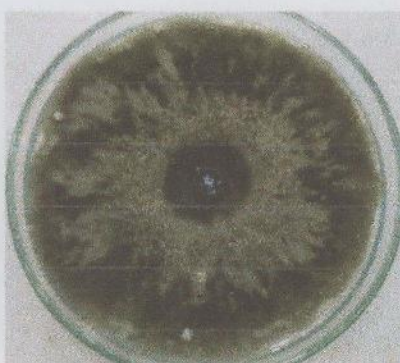
CSF₂ 14



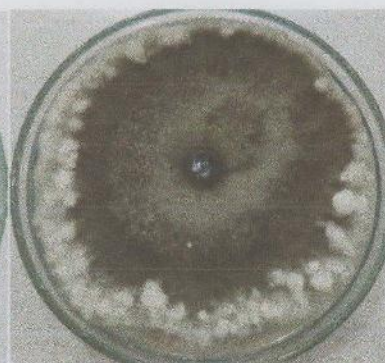
CSF₂ 20



CSF₂ 23



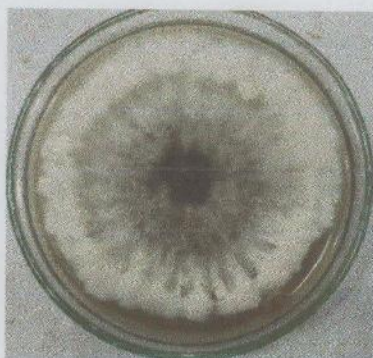
CSF₂ 24



CSF₂ 27



CSF₂ 30



CSF₂ 31



CSF₂ 33



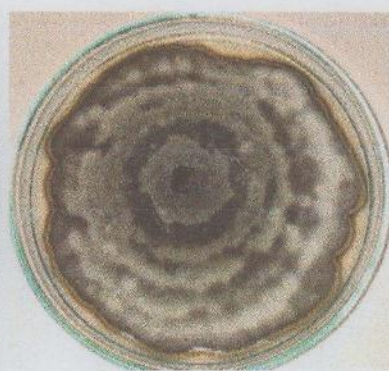
CSF₂ 40



CSF₂ 45



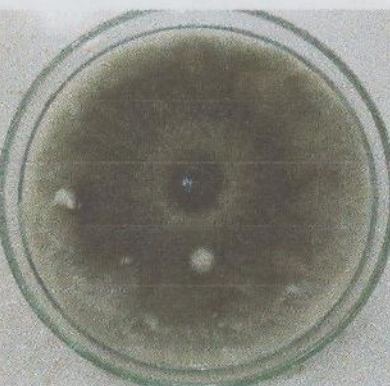
CSF₂ 51



CSF₂ 52



CSF₂ 56



CSF₂ 58



CSF₂ 59



CSF₂ 61



CSF₂ 63



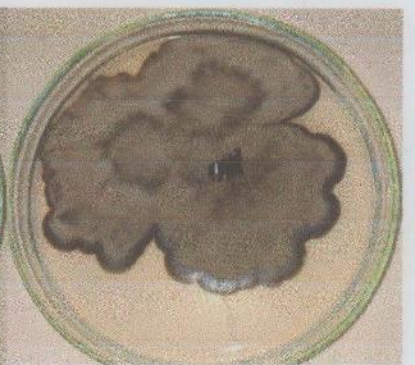
CSF₂ 65



CSF₂ 67



CSF₂ 69



CSF₂ 70



CSF₂ 73

