

**PATHOGENIC VARIABILITY IN F₂ GENERATION OF
Cochliobolus sativus (S. Ito & Kurib.)**

**A THESIS
BY
Maliha Momtaz
STUDENT ID. MS-170801**



**A Thesis (Course No. PLP-6104) Submitted to Agrotechnology Discipline in Partial
Fulfillment of the Requirements for the Degree of**

**MASTER OF SCIENCE
IN
PLANT PATHOLOGY**



**AGROTECHNOLOGY DISCIPLINE
LIFE SCIENCE SCHOOL
KHULNA UNIVERSITY
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Abstract

A total of 24 isolates of F_2 generation of *Cochliobolussativus* (S. Ito & Kurib.) [anamorph, *Bipolarissorokiniana* (Sacc. In Sorok)] the causal pathogen of spot blotch of wheat were collected from Plant Pathology Laboratory, Agrotechnology discipline, Khulna University, Khulna; for determining virulence spectra following pathogenic variability test. A susceptible wheat cultivar "Kanchan" was used in pathogenic variability test of those isolates of F_2 generation of *C. sativus*. Seven components of disease development like infection frequency (IF), lesion size (LS), lesion cover (LC), necrosis (NEC), secondary spore production (SPD), disease severity (DS) and percent disease index (PDI) were studied. Wheat seedlings were inoculated and data on those seven disease development components were checked. After performing multivariate analysis a positive correlation was found among the seven components of disease development, where DS was strongly related with PDI. However, LS and IF did not show any significant relation to cause disease. After conducting a principal component analysis (PCA) infection frequency and lesion size were most promoting components to create variability and also in a Biplot, isolates with high infection frequency and lesion size were closely situated. Isolate CSF₂-32 was found to be most virulent and isolate CSF₂-58 was most virulent and aggressive ones by producing highest percent disease index (64.44) and secondary spore (2444.44), respectively. A cluster analysis indicated that the isolates exhibited distinct differential virulence patterns with five groups supported by a dendrogram figure. Cluster III contains 16.66% of total isolates with the most virulent isolates (CSF₂-32, CSF₂-27, CSF₂-67, and CSF₂-58). Cluster I (16.66%) contains least virulent isolates (CSF₂-10, CSF₂-16, CSF₂-61 and CSF₂-33). This result also reflected in mean cluster distance by showing maximum distance (2080.63) between cluster I and cluster III. Findings of the present study reveal that seven disease development components are significantly differed to each other showing difference among the isolates; positive correlation is found among the disease development components; in PCA analysis, cumulatively Infection frequency and lesion size are the most attributing components to create variability; and five major groups of F_2 generation of *C. sativus* isolates have been found through cluster analysis.

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The Author

**Dedicated
To
My Beloved Little Sister & Brother**

**Sabiha Mumtaz
&
Md. Sadikuzzaman**

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Chapter I

Introduction

Wheat is counted as the most important cereal crops, with over 600 million tons being harvested annually worldwide (Shewry *et al.*, 2015). Globally, total area under wheat production is 215.48 million hectare with production 670.87 million tons and productivity of 3117 kg ^{-ha} (Kumar *et al.*, 2019). Within a period of 30 years, wheat became as a secure crop in Bangladesh (Karim *et al.*, 2010). The total area under wheat crop has been estimated 3, 51, 213 hectares with obtaining yield of 3.130 metric tons per hectare (BBS, 2019).

Spot blotch of wheat popularly known as *Bipolaris* leaf blight, is one of the major constraints of wheat production in the world especially in south Asian countries including Bangladesh. The yield loss due to the disease is significant. Estimates of yield losses due to spot blotch were vary from 15.5 to 19.6% and up to 100% under severe infection conditions when pathogen attacks at the spike of wheat seedling (Kumar *et al.*, 2019). Besides pathogen can attack in its root, leaf, sheath and even in spikelet (Chand *et al.*, 2002).

Cochliobolus sativus (anamorph: *Bipolaris sorokiniana*) is the fungal pathogen responsible for spot blotch in wheat (*Triticum aestivum* L.) and occurs worldwide where warmer, humid growing conditions are prevailed (Gupta *et al.*, 2018). Over the last four decades, spot blotch has emerged as a serious problem 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).

Early symptoms are characterized by small, dark brown lesions ranging 1 to 2 millimeter long without chlorotic margin. In susceptible genotypes, these lesions extend very quickly in oval to elongated blotches, light brown to dark brown in color (Chowdhury, 2013).

C. sativus is a variable fungus due to hetrokaryosis, parasexual recombination (Tinline and Dickson, 1958; Burdon and Silk, 1997) and sexual reproduction (Lahr *et al.*, 2011). But sexual reproduction in *B. sorokiniana* is rare and reported only from Zambia (Raemaekers, 1987). The sexual stage (*C. sativus*) have been obtained in laboratory under controlled conditions with opposite mating types (Tinline, 1958 and Sultana *et al.*, 2018).

C. sativus is highly variable and there is several virulence types (Fetch and Steffenson, 1994). Variability in the isolates of *C. sativus* have been reported at morphological and pathological levels (Chauhan, 2017). Pathogenic variability has obtained through genetic variation (Andres *et al.*, 2001) and suitable environmental condition (Kumar *et al.*, 2019).

Pathogenic variability and aggressiveness of *B. sorokiniana* seemed to increase over time (Asad *et al.*, 2007 and Sultana *et al.*, 2017) and now its need to know the pathogenic variability of the fungus (*C. sativus*) at sexual stage. It is an obvious benefit in developing a better understanding of the host pathogen relationship. Pathogenic variability of a pathogen is crucial significance in their management (Joshi *et al.*, 2007).

But a very few study on pathogenic variability of F₂ generation (*C. sativus*) has been conducted. As the pathogen is variable, so for clear understanding of the extent of variation in virulence of *C. sativus* would be helpful in developing wheat breeding line with stable resistance. Almost every time it's changing its virulence spectrum. That's why no sustainable resistant cultivars have made yet in Bangladesh (Sultana *et al.*, 2017).

Considering the facts, the experiment was undertaken to satisfy the following objective:

- To determine the pathogenic variability exists in the F₂ generation of *C. sativus*

Chapter II

Review of Literature

2.1 The Pathogen

Name of this fungus was first time given by (Pammel *et al.*, 1910) as *Helminthosporium sativum*. 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* as anamorphs and the teleomorphs of *C. sativus* (sexual stage) were only reported from Zambia (Raemaekers, 1991).

The fungus uses all plant organs as substrate. Conidia germinate completely in four hours followed by appressoria formation at the juncture of epidermal cell wall in eight hours. Hyphae from initially infected cells enter adjacent cells in 24 hours, which results in the granularisation of the host cytoplasm (Bisen and Channy, 1983). Under favorable conditions, hyphae produce conidiophores, which emerge out through stomata of the host tissue. The emerging conidiophores produce a succession of conidia, which produce germ tube.

The fungus is self sterile heterothallic (Tinline, 1951). It can reproduce through both asexually and sexually (Chand *et al.*, 2003). Asexual reproduction dominates worldwide whereas sexual reproduction confounding (Raper, 1966). Evolution in the pathogen population by sexual recombination is a distant possibility though already have found in Zambia (Raemaker, 1991).

Sexual stage of *Bipolaris sorokiniana* obtained through induction of sexual crosses in the laboratory (Shoemaker, 1955; Tinline, 1958 and Sultana *et al.*, 2018).

Tinline and Dixon (1958) suspected that both the mating types are not present in different parts of the world where *B. sorokiniana* is a trouble. Valjavec-Gratian and Steffenson (1997) also reported that the only sheer presence of the two mating types was not enough for sexual reproduction, if other factors were limiting (Chand *et al.*, 2003). Pathogen asexually reproduces through parasexuality and heterokaryosis (Tinline, 1962; Burdon and Silk, 1997).

It has a large host range among gramineous species. It also has been reported on dicotyledonous crops including beans, alfalfa, red and yellow clover but it is considered as less harmful pathogen on them. Among the cereals, wheat and barley are the most important hosts. The fungus can attack all parts of wheat plants causing common root rot in basal parts, spot blotch on the leaves, black nodes and breakage of stems, blighting of heads, and black point or smudge of seed (Manamgoda *et al.*, 2011).

2.2 Spot blotch disease of wheat

Cochliobolus sativus (anamorph *B. sorokiniana*) is the causal agent of common root rot, leaf spot disease, seedling blight, head blight, and black point of wheat and barley. High temperature and high relative humidity favor the outbreak of the disease, in particular in South Asia's intensive 'irrigated wheat-rice' production systems with the low fertility of soils (Jagdish *et al.*, 2002; Kumar *et al.*, 2019).

Spot blotch was reported first time by Mohy (Joshi, 1986) but got attention after green revolution (Saari *et al.*, 1998). Because of production of wheat has expended in newer growing areas

characterized by high temperature (coolest month greater than 17°C) and high humidity 95% (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).

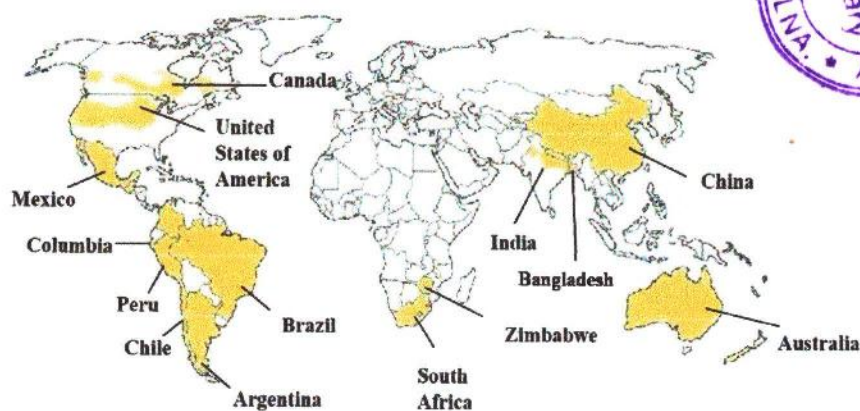
Spot blotch is a seed and soil borne disease and transmitted by seed themselves in nature (Neergaard, 1997). *C. sativus* (anamorph *B. sorokiniana*) usually induces symptoms on leaf, sheath and stem (Chand *et al.*, 2002).

The dark brown necrotic spots (boat shaped) occur on the coleoptiles, leaves, crowns, stems, and roots with or without yellow halo around these. Lesions on the leaves start as a few mm that extend as elongated dark brown spots greater than 1-2 cm (Chand *et al.*, 2002). A yellowing due to toxin production is sometimes observed extending from the lesion. Later such spots coalesce each other thus results blight on large leaf portion. As the disease progresses the spots join together forming large blotches that cover the leaves and eventually killing it or causing premature senescence (Minotto *et al.*, 2014).



Figure 1. Symptoms of spot blotch disease in wheat caused by *C. sativus*

The fungus has a worldwide distribution but as a pathogen it is the most aggressive under the conditions of high relative humidity and temperature associated with the low fertility of soils is one of the most concerning disease in warm and humid regions of India and other South Asian countries due to its wide spread prevalence and increasing severity (Joshi *et al.*, 2002). According to Acharya *et al.* (2011), the specific area where it is most prevalent includes; African countries (Kenya, Malawi, Sudan, South Africa, Tanzania, Zimbabwe), Asia (China, Korea, India, Bangladesh, Pakistan, Indonesia, Taiwan, Thailand), Europe (Austria, Belgium, former Czechoslovakia, Denmark, Germany, Greece, Hungary, Italy, Poland, Scotland, United Kingdom), South America (Argentina, Brazil) and North America (Indiana, Kansas, Minnesota, Montana, North Dakota, Virginia). In Bangladesh, it occurs in south east part of country where prevails short winter (Alam *et al.*, 1998).



(Acharya *et al.*, 2011)

Figure 2. Global distribution of spot blotch disease caused by *C. sativus* in wheat

2.3 Loss of wheat production due to spot blotch disease

The destructive capacity of this pathogen is evident from the reports around the world. Indian subcontinent has 10 million ha of affected land most of which is in the rice wheat cropping system (Nagarajan and Kumar, 1998). It has been considered as a major constraint to wheat yields in South Asia due to reduction in 1000-grain weight and grain yield (Singh *et al.*, 2007 and Chowdhury *et al.*, 2013). Annual yield loss of wheat due to this disease in South Asia is estimated to 15-20% (Duveiller and Sharma, 2009 and Kumar *et al.*, 2019). It caused 15% grain yield reduction in Bangladesh (Alam *et al.*, 1998) and China (Xiao *et al.*, 1998). Due to this pathogen, the seed germination and seedling emergence significantly decreases with increasing of black pointed seeds, observed in studies conducted in Bangladesh (Hossain & Hossain, 2001) and in China (Song *et al.*, 2001). In Nepal, under rice-wheat cropping system, spot blight severity went up to 100% and 70% in 2004 and 2005 respectively (Sharma and Duveiller, 2007).

2.4 Epidemiology

Infected seeds and soil serve as primary inoculums in the field condition. Pathogen may attack at the first leaf stage but host susceptibility increases generally after flowering stage (Neupane *et al.*, 2010 and Kumar *et al.*, 2019). Spot blotch is seed transmitted disease and its conidia survive in the soil.

Acharya *et al.* (2011) found, spot blotch get serious concern for wheat cultivation in warmer and humid regions of world. Disease severity is directly related to humidity, temperature and soil nutrient condition. Ideal conditions for spot blotch development on the leaves are high relative humidity with high temperature and long periods (more than 12 to 18 hours) of leaf wetness caused by rainfall, irrigation, fog or dew. The greatest yield loss occurred when the flag leaf and

leaf below the flag leaf becomes infected before emergence of head (Kumar *et al.*, 2019). In cropping season secondary infection causes through wind and water drops. Epidemiological studies have revealed that judicious sowing avoids the physiological stress that often coincides with the flowering stage which in turn reduces spot blotch (Duveiller *et al.*, 2005).

2.5 Variability

Pathogen may show considerably variability in genetic diversity, morphologically and pathogenically (Minotto *et al.*, 2014). *Cochliobolus sativus* isolates from Nepal greatly varied in morphology and genetic structure (Mahto, 2012) based on aggressiveness.

Maraite (1998) and Poloni (2008) established morphological variability in the population of *B. sorokiniana* aiming that it could be useful in monitoring the pathogen population if correlated with pathological variability.

But all this variability is possesses due to some causes. According to Kumar *et al.* (2019), without the environmental support, pathogen can't create disease at a large scale though being aggressive. Besides, some genetical causes of the pathogen also help to create variability (Andres *et al.*, 2001; Michele *et al.*, 2014 and Sultana, 2019).

2.6 Pathogenic variability

Variability in the asexual (*B. sorokiniana*) populations of the pathogen was thought to be due to parasexual recombination (Tinline, 1962). However, these results were not verified and other means of variability were not explored (Chand *et al.*, 2002). Pathogenic variability is consequence in disease management. Spot blotch pathogen is a variable fungus with many morphological (Christensen, 1925) and physiological (Tinline, 1962) variants. Some variables

achieved through heterokaryosis and parasexual mechanism (Tinline, 1958). But variability and aggressiveness of this pathogen seems to increase over time (Hetzler *et al.*, 1991 and Jeger, 2004). Variability through pathological level was also observed by Nelson (1960); Jaiswal *et al.* (2007) and Chauhan *et al.* (2017). Kumar *et al.* (2007) studied the pathogenic variability and aggressiveness among the spot blotch pathogen groups characterized by Chand *et al.* (2002) and found that the black group is the most aggressive. *B. sorokiniana* isolates from different plant parts do not form different groups of aggressiveness in seedling tests (Duveiller *et al.*, 2000). Asad *et al.* (2007) stated that severity of isolates exhibiting aggressive reaction which was varied in *In-vitro* and field conditions. It reflects the variable conditions provided to the pathogen or for the success of the isolate there is a need of its interaction with the environment and the inoculum pressure.

As the teleomorph is not found in nature, except in Zambia (Raemaekers, 1991), variability may result from mutation or parasexuality. Because pathogenic specialization may change over time and across locations, pathogen monitoring at reliable key sites and global studies on pathogen populations will be needed (Duveillor *et al.*, 2000). Minnoto (2014) observed wide pathological variability as well as Sultana *et al.* (2017).

Chapter III

Materials and Methods

3.1 Experimental site and duration

The experiment was conducted in Plant Pathology Laboratory and net house under the Genetics and Plant Breeding laboratory of Agrotechnology Discipline, Khulna University, Khulna, Bangladesh during January to February 2019.

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

The pure culture 24 isolates of *Cochliobolus sativus* (F₂ generation) were collected from plant pathology laboratory, Khulna University, Khulna. The isolates were selected randomly from stock culture and further cultured on PDA medium for ten days. The parents of F₂ generation were BS-134 and BS-65 (Sultana *et al.*, 2019)

3.3 Selection of susceptible variety of wheat

The seed of wheat variety 'Kanchan' susceptible to spot blotch disease (Siddique *et al.*, 2006 and Sultana *et al.*, 2017) was collected from Regional Agricultural Research Station (RARS), BARI, Jashore and used for this study.

3.4 Pathogenicity test of F₂ generation of *Cochliobolus sativus*

In vitro pathogenicity test was conducted following the method of Sultana *et al.* (2017). The method is given bellow:

3.4.1 Materials

Wheat variety Kanchan and 24 isolates of F₂ generation of *Cochliobolus sativus* were used in this experiment.

3.4.2 Growing of Wheat Seedlings

Poly bags (13 cm × 10 cm) containing sandy loam soil of pH 7.4 were prepared. In each poly bag 5-6 seeds were sown. A conducive condition of poly bag soil was maintained for easy germination of seed. After a few days of emergence seedlings were thinned keeping three seedlings per poly bag. Poly bags were kept in a net house and watering was done until each seedling reaches three leaved stage.

3.4.3 Preparation of Suspension and Inoculation

Conidia were collected by flooding the petriplates with distilled water from ten days old culture of *C. sativus* (F₂ generation) grown on PDA. Conidia were scraped gently by a spatula and sieved in a 500 ml beaker with a cheese cloth to separate hyphae from the spores. In this way a spore suspension was produced. The concentration of conidia per ml was counted by using haemocytometer and adjusted to 10³ conidia/ml by adding distilled water. Twelve drops of Tween 20 (polyoxyethylene 20 sorbitanmonolaurate, 0.02%) was added to the suspension for dispersion of conidia. Nine seedlings of three poly bags were inoculated by spraying with the suspension of one isolate using an atomizer.

After air drying of the inoculated seedlings poly bags were placed in a polyethylene humid chamber with above 90% relative humidity for 30 hours. After 30 h poly bags were placed in an open environment beyond humid chamber for 4 days.

3.4.4 Pathogenicity Assessment

According to Adhikary and Mian (2005), seven components of disease development was recorded for each leaf of every seedling. Pathogenicity of a pathogen varies based on the following disease development parameters:

1. Infection frequency (IF-number of lesions per square centimeter of leaf area),
2. Lesion size (LS-mm²),
3. Lesion cover (LC-percent leaf showing by lesion),
4. Necrosis (NC-percent necrotic area of the leaf),
5. Secondary spore production/leaf (SP),
6. Disease severity (DI) and
7. Percent disease index (PDI).

Data on secondary spore production per leaf were recorded after seven days of inoculation.

Disease severity was measured on a standard scale of 0-5 as suggested by (Rahman *et al.*, 2011) where, 0: refers to no visible symptoms, 1: flecks or minute lesions, 2: small dark lesion, no distinct halo, less than 10% area affected, 3: typical leaf with yellow halo covering 10-50% leaf area affected, 4: small but coalescing lesion covering more Than 50% leaf area and 5: necrotic lesions and total leaf collapsed (Fig 3).



Figure 3. Disease severity measurement scale

$$DI = \frac{\text{Sum of all disease ratings}}{\text{total no of leaf}}$$

The percent disease index (PDI) was calculated following a standard formula (McKinney, 1923).

$$PDI = \frac{\text{Sum of all disease ratings}}{\text{Total number of ratings} \times \text{Maximum grade}} \times 100$$

3.5 Data analysis

Based on disease development components, pathogenic variability among the isolates was analyzed following multivariate analysis. In multivariate analysis two techniques viz: (a) Principal component analysis (PCA), (b) Cluster Analysis (CLSA) were used. Contributions of the components for the disease development were analyzed by Principal component Analysis using SPSS and by correlation matrices. Mean data were graphically computed by using MS-Excel (2010 version, Microsoft, WA, USA). Dendrogram was constructed following Squared Euclidean distance method. Cluster distance was analyzed by using KMEANS method. The multivariate analysis was performed by using SPSS statistics (version 20, IBM, NY, USA).

Chapter IV

Results and Discussion

4.1 Pathogenic variability in F₂ generation of *Cochliobolus sativus*

For determination of pathogenic variability among 24 isolates of F₂ generation of *Cochliobolus sativus* multivariate analysis was performed.

Seven component of disease developments like as infection frequency (IF), lesion size (LS), lesion cover (LC), necrosis (NEC), secondary spore production (SPD), disease severity (DI), percent disease index (PDI) were 5.05 leaf⁻¹, 1.54 mm², 9.22%, 64.85%, 1246.29 leaf⁻¹, 1.77, 35.37% respectively. Mean value of all seven disease development component was greater than standard error, which indicate a significance differences belong among the isolates based on seven disease development components (Table 1).

Table 1. Range and mean values of disease development components of *C. sativus* isolates

Components	Unit	Minimum	Maximum	Mean $\pm$ SE
Lesion cover	%	0.02	54.77	9.22 $\pm$ 2.39
Infection frequency	No. of lesion cm ⁻²	0.29	31.48	5.05 $\pm$ 1.30
Secondary spore production	Leaf ⁻¹	222.22	2444.44	1246.29 $\pm$ 129.45
Disease severity	Score	0.11	3.22	1.77 $\pm$ 0.18
Percent disease index	%	2.22	64.44	35.37 $\pm$ 3.62
Lesion size	mm ²	0.10	3.15	1.54 $\pm$ 0.16
Necrosis	%	23.19	128.81	64.85 $\pm$ 5.99

Correlation among the disease development components states direct relation between the components of disease development. Each component showed positive co-efficient with other disease development components (Table 2). Disease severity (DS) is strongly related ($r=1.00$)

with Percent disease index (PDI). Relation between Infection frequency (IF) and Lesion cover (LC) are also showing strong relationship ($r=0.963$) to cause disease. Insignificant relationships were observed between necrosis and secondary spore production, disease index and percent disease index and infection frequency and lesion size.

Table 2. Pearson correlation among seven components of disease development with *C. sativus*

Components	IF	LS	LC	NEC	SP	DI	PDI
IF		0.28 ^{NS}	0.96**	0.47*	0.45*	0.70**	0.70**
LS			0.48*	0.70**	0.35 ^{NS}	0.47*	0.47*
LC				0.61**	0.46*	0.66**	0.66**
NEC					0.23 ^{NS}	0.33 ^{NS}	0.34 ^{NS}
SP						0.54**	0.54**
DI							1.00**
PDI							

IF-Infection frequency (No), LS-lesion size (mm²), LC-lesion cover (%), NEC-necrosis (%), SPD-secondary spore production leaf⁻¹, DI-disease severity, PDI-percent disease index (%). ** - significance @ 0.01, * - significance @ 0.05 and NS- Non-significance

Principal component analysis (PCA) entails a mathematical procedure that transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables (Chatfield *et al.*, 1980). *Eigenvalue* is the sum to the variables analyzed. In table 3, it has been observed that *eigenvalues* corresponding to infection frequency (IF) and lesion size (LS) are 4.33 and 1.13 respectively which are more than one and indicate their major contribution (probable) creating variability in pathogenicity among the isolates. Infection frequency (IF)

accounts more than half (61.87%) of the total variance and cumulatively 77.95% with lesion size (LS) (Table 3). Infection frequency and lesion size indicated as PC1 and PC2 (Figure 4) where the isolates with high infection frequency and lesion size were tend to lie close together proving that infection frequency and lesion size can influence each other (Esposito *et al.*, 2007 and Chang *et al.*, 2014).

Table 3. *Eigenvalue* and percentages of variation for seven components of disease development of wheat of *C. sativus*

Components of disease development	<i>Eigenvalue</i>	Variation (%)	Cumulative variation (%)
Infection frequency	4.33	61.87	61.87
Lesion size	1.13	16.08	77.95
Lesion cover	0.76	10.91	88.86
Necrosis	0.57	08.21	97.08
Secondary spore prod.	0.19	02.82	99.89
Disease severity	0.01	0.11	100.00
Percent disease index	0.00	00	100.00

Extraction Method: Principal Component Analysis

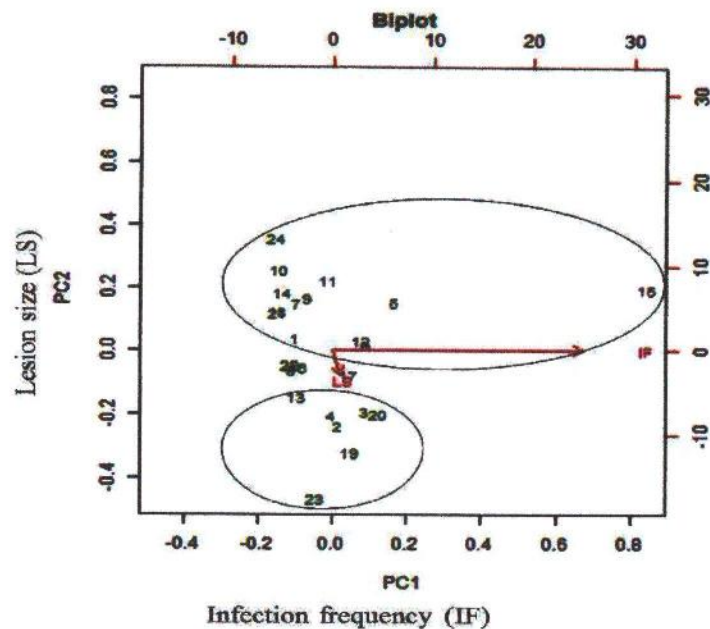


Figure 4. Pairs of datasets (Infection frequency and lesion size) of the of the disease development components are displayed on PC1 and PC2

Aggressiveness of a pathogen depends on its invasive capability and ability to produce secondary spores (Cumagun *et al.*, 2003). Maximum percent disease index was found in isolate CSF₂-32 (Figure 5) and maximum secondary spore leaf⁻¹ was found in isolate CSF₂-58 (Figure 6). The ability to produce maximum Secondary spore production leaf⁻¹ of a pathogen is the indicator to cause disease in an epidemic form (Sultana *et al.*, 2017). So isolate CSF₂-58 is the most virulent and aggressive one among 24 isolates. Maximum percent necrotic area of a leaf was found in isolate CSF₂-37 (Figure 7).

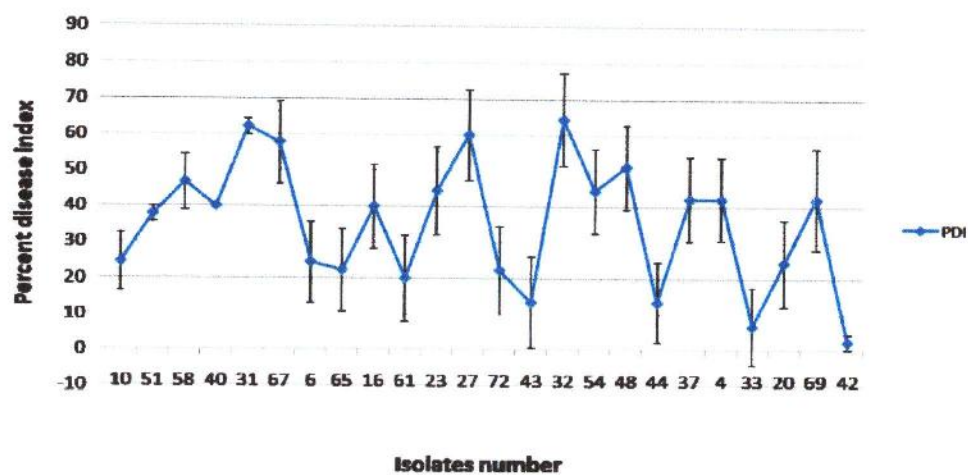


Figure 5. Percent disease index for 24 isolates of *C. sativus*

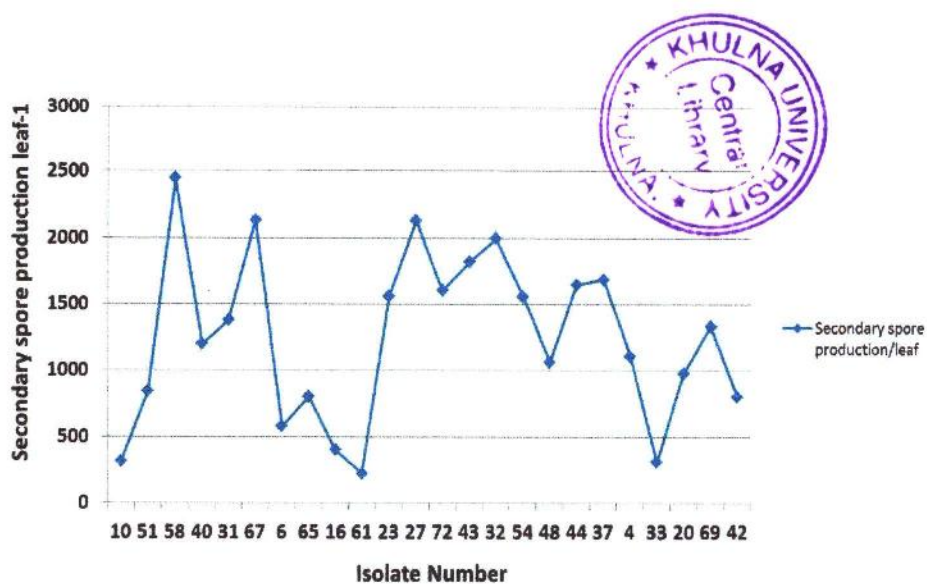


Figure 6. Secondary spore production leaf⁻¹ for 24 isolates of *C. sativus*

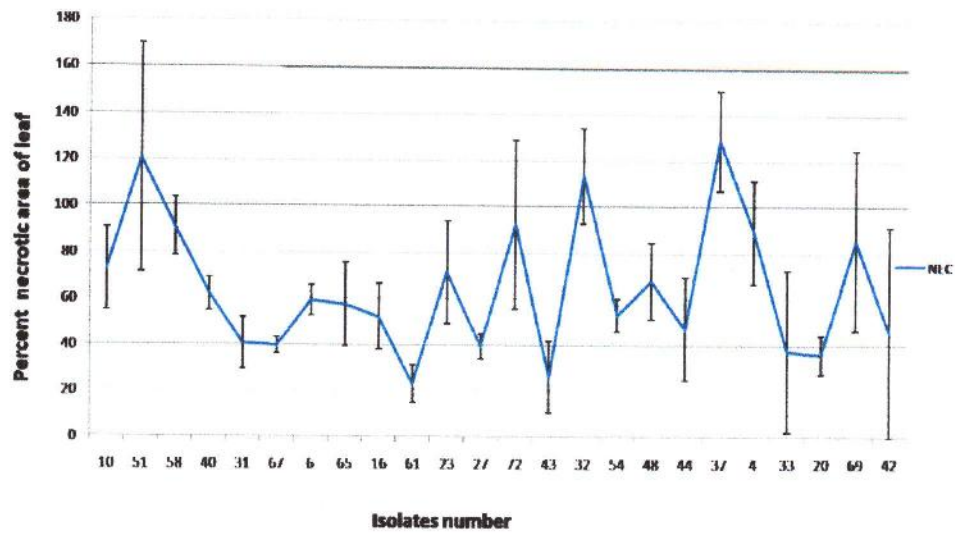


Figure 7. Percent necrotic area of leaf for 24 isolates of *C. sativus*

Five major clusters were found based on seven disease development components. Cluster I (16.66%) contains 4 isolates, cluster II (16.66%) contains 4 isolates, cluster III (16.66%) contains 4 isolates also, cluster IV (25%) contains 6 isolates and cluster V (25%) contains 6 isolates. Members of a cluster are more or less of similar characteristics in nature. This classification indicates differences among the twenty four isolates of *C. sativus* to cause disease on wheat (Table 4).

Table 4. Distribution of isolates of *C. sativus* under different cluster based on pathogenic components

Cluster No.	<i>Cochliobolus sativus</i> isolates	Number of isolates	Isolates (%)
I	CSF ₂ -10, CSF ₂ -16, CSF ₂ -61, CSF ₂ -33	4	16.66%
II	CSF ₂ -51, CSF ₂ -06, CSF ₂ -65, CSF ₂ -42	4	16.66%
III	CSF ₂ -32, CSF ₂ -27, CSF ₂ -67, CSF ₂ -58	4	16.66%
IV	CSF ₂ -40, CSF ₂ -31, CSF ₂ -48, CSF ₂ -04, CSF ₂ -20, CSF ₂ -69	6	25.00 %
V	CSF ₂ -23, CSF ₂ -72, CSF ₂ -43, CSF ₂ -54, CSF ₂ -44, CSF ₂ -37	6	25.00 %

From the hierarchical cluster analysis (Figure 8) five distinct cluster were found. It has also proved that isolates CSF₂-58 and CSF₂-32 belong to same cluster III which are the most aggressive ones.

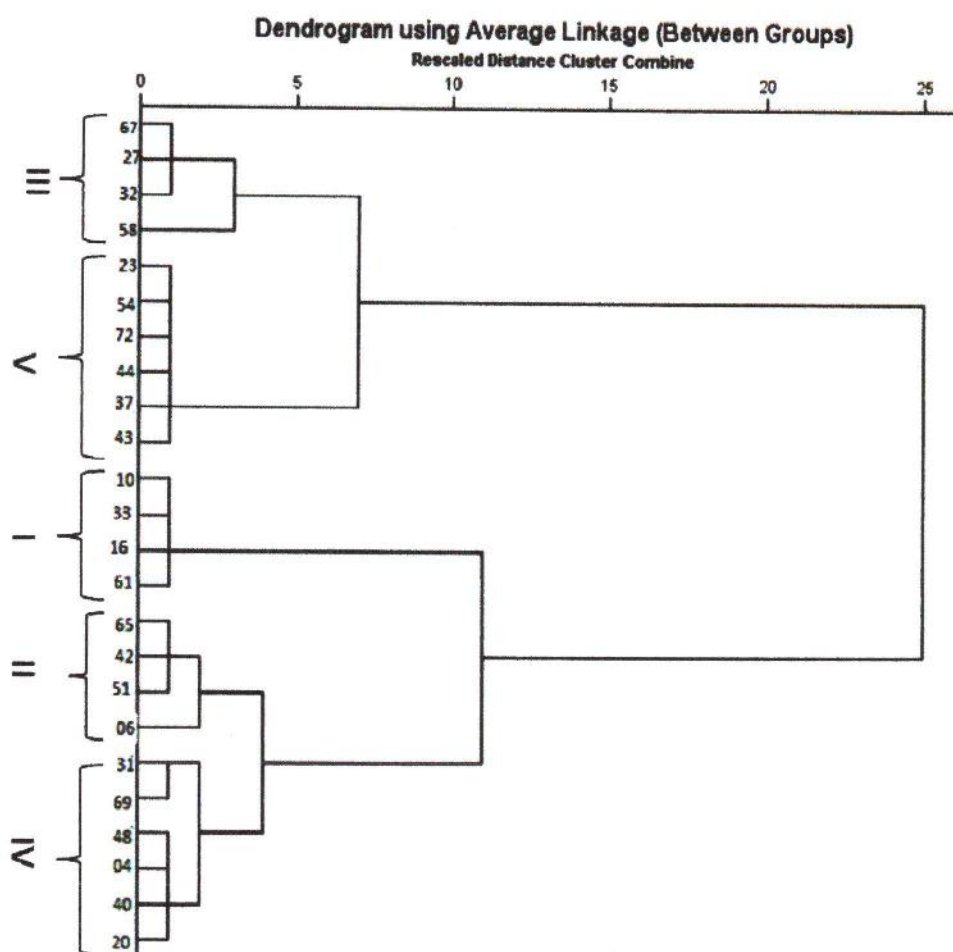


Figure 8. Dendrogram representing the similarities bases on seven component of disease development of 24 isolates of *C. sativus* generated by Squared Euclidean distance method

Through the analysis of KMEANS method differences between isolates have been found. It has shown that highest cluster distance exist between cluster I and cluster III (2080.63) and lowest distance is between cluster III and cluster V (423.81). Pathogenically most variable isolates belonging to cluster III (CSF₂-32, CSF₂-27, CSF₂-67 and CSF₂-58). So it can be said that pathogenic variability prevailed among the 24 isolates of F₂ generation of *C. sativus* (Table 5).

Table 5. Mean inter-cluster distance values of five clusters

Cluster	I	II	III	IV	V
I		607.41	2080.63	1172.46	1658.12
II			1473.29	565.16	1051.11
III				908.19	423.81
IV					486.43
V					

KMEANS method of analysis

4.2 Discussion:

Pathogenic variability has a good scope for effective management of *C. sativus* for ensuring profound yield of wheat (Chowdhury *et al.*, 2013). Seven disease development components of leaf blotch disease development are showing positive effectiveness to cause disease, similar result agreed with the Sultana *et al.* (2017).

In this study positive correlation was found between the seven components of disease development where infection frequency is strongly related with disease severity and percent disease index. Infection frequency is highly responsible to sporulation (Chauhan *et al.*, 2017). Chauhan *et al.* (2017) also stated that necrotic area of leaf didn't show any differences on spore production or disease severity so pathological traits; necrotic area and number of lesion are independent to each other which also support the present study. Duveiller *et al.* (2000) stated that pathogenicity of *B. sorokiniana*, obtained from roots, leaves and grains of spring wheat expressed no clear difference between groups of isolates on the basis of lesion density.

In the present study principal component analysis was done to determine which variable contributed more with its variance to total variance observed in disease development. As infection frequency again possessed a strong position containing more than half of total variation which also happened in case of Sultana *et al.* (2017). Sporulation and infection efficiency revealed their contributions to develop leaf blotch disease (Jegar *et al.*, 1983). Among the seven component of disease development lesion size also was a component of resistance (Hetzler *et al.*, 1991 and Arabi, 2002).

Present study stated that cluster III contained most virulent isolates (CSF₂-32 and CSF₂-58) and cluster I contained least virulent isolates (CSF₂-61, CSF₂-10) based on secondary spore production and percent disease index. Isolate CSF₂-58 has highest capacity to sporulation leaf⁻¹ which indicates it has more capability to cause disease in epidemic form (Sultana *et al.*, 2017).

- 2

In this study, five major cluster groups were found by constructing a dendrogram based on seven disease development components. However, clustering was independent, but it reflected the virulence spectra of the isolates. Chand *et al.* (2003) and Asad *et al.* (2007) also found five and three clusters based on aggressiveness. Arabi, (2002) also disclosed the virulence patterns through cluster analysis. Sultana *et al* (2017) has found five cluster groups of *B. sorokiniana* (teleomorph *C. sativus*) where the present study has also found same cluster number with F₂ generation of *C. sativus* (anamorph *B. sorokiniana*). That's create a possibility of occurrence of sexual reproduction in nature. So it can be said that no mutation might be occurred in nature that means pathogenic variability of leaf blotch causing fungi followed Hardy-Weinberg law.

X

Cluster distance strongly showing the differences of virulence among the isolates of *C. sativus*. Jaiswal *et al.* (2007) stated that the pathogenic variability and aggressiveness of *C. sativus* seems to increase over time. Similar observation was found in Asad *et al.* (2007) that *B. sorokiniana* forms a scale of strains differing in aggressiveness.

+

Bouajila *et al.* (2012) suggested that degree of pathogenic variability of all the isolates within the identified groups was equally important and strongly recommended in a screening program. Moreover, knowledge of *C. sativus* virulence spectrum may assist in designing proper breeding strategies.

Chapter V

Summary and conclusion

5.1 Summary

Cochliobolus sativus (anamorph *Bipolaris sorokiniana*) is the causal agent of common root rot, leaf spot blotch, seedling blight, head blight, and black point of wheat supported by high temperature and high relative humidity. A pathogenic variability study of F₂ generation of *C. sativus* was done in Plant Pathology Laboratory and net house of Plant Breeding and Biotechnology laboratory, Khulna University, Khulna.

Twenty four isolates of F₂ generation of *C. sativus* were collected from the Plant Pathology Laboratory, Khulna University, to evaluate the pathogenic variability. Isolates of F₂ generation of *C. sativus* were inoculated on a susceptible cultivar of wheat (Kanchan). From the descriptive analysis a significance differences were found among the seven disease development components. A positive correlation was found among the seven components of disease development where disease severity (DS) is strongly related ($r=1.00$) with percent disease index (PDI). Infection frequency (IF) also showed strong relation with lesion cover (LC) but no significant relation with lesion size (LS).

Principal components (PCA) reflected maximum variation (61.87%) in infection frequency (IF) i.e., infection frequency had highest probability to create pathogenic variability. Lesion size (LS) also had the possibility to make a mark on variability. From a Biplot, it had been proved that Infection frequency and lesion size influenced each other to cause variability.

Isolate number CSF₂-32 and CSF₂-58 were appeared as most virulent ones by obtaining maximum percent disease index (64.44) and highest secondary spore (2444.44) production respectively. After cluster analysis a dendrogram was constructed to obtain distinct groups among the isolates. Five distinct groups were found whereas, cluster IV and cluster V contain

25% isolates and rest of the clusters contains 16.66% isolates respectively. Cluster III (16.66%) contained the most virulent isolates as they provide highest percent disease index and secondary spore production

Maximum cluster distance (2080.63) was found between Cluster I and Cluster III that proved the differences among the isolates based on seven disease development components. Infection frequency and lesion size attributed most to create variability among the twenty four isolates. In this experiment, pathogenic variability of *C. sativus* existed which led the pathogen to cause disease in wheat.

5.2 Conclusion

After conducting the experiment following things are concluded:

- I. Seven disease development components are significantly differed to each other and showing difference among the isolates.
- II. Positive correlation has found among the disease development components.
- III. Cumulatively Infection frequency and lesion size are the most attributing components to create variability.
- IV. Isolate CSF₂-32 and isolate CSF₂-58 produce maximum percent disease index and highest secondary spore respectively.
- V. Five major groups of F₂ generation of *Cochliobolus sativus* isolates have been found through cluster analysis.
- VI. Maximum cluster distance has found between cluster I and cluster III.



5.3 Recommendation

- Further pathogenic variability study of *Cohliobolus sativus* is needed on a large scale in the field of hot spot of Bangladesh, which will consequently help in developing breeding line for the management of spot blotch disease.

Pictorial view of the experiment



Plate 1. 10 days old pure culture of *Cochliobolus sativus* (F₂ generation)

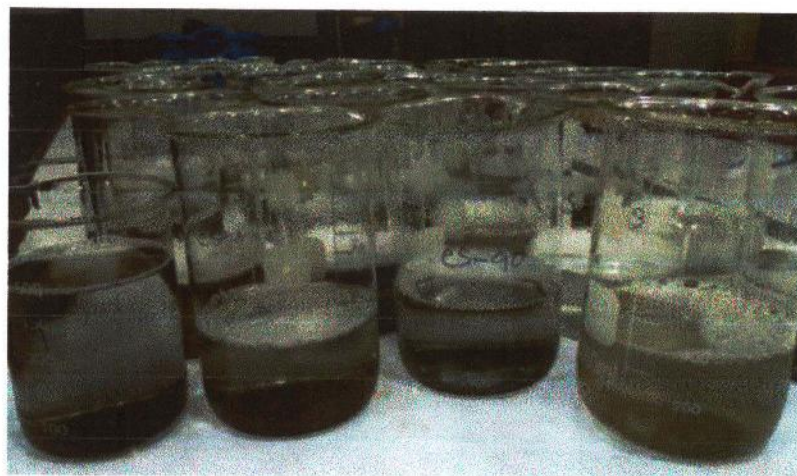


Plate 2. Suspension of pure culture (*C. sativus*)



Plate 3. Wheat seedlings at three-leaved stage



Plate 4. Seedlings after inoculation of suspension



Plate 5. Artificial inoculation chamber



Plate 6. First appearance of spot blotch symptom



Plate 7. Disease progress by symptom development



Plate 8. Typical symptom of spot blotch disease

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