EVALUATION OF SELECTED CULTURE MEDIA FOR GROWTH AND SPORULATION OF *Alternaria* ISOLATE CAUSING GREY BLIGHT DISEASE OF MUSTARD AND ITS MOLECULAR CHARACTERIZATION

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CERTIFICATE

*This is to certify that the thesis entitled, "**EVALUATION OF SELECTED CULTURE MEDIA FOR GROWTH AND SPORULATION OF Alternaria ISOLATE CAUSING GREY BLIGHT DISEASE OF MUSTARD AND ITS MOLECULAR CHARACTERIZATION**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) in PLANT PATHOLOGY,** embodies the result of a piece of bona-fide research work carried out by **YOBAIDA AFRIN ARA ETI, REGISTRATION NO. 15-06630** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Date:

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Dedicated to

My

Beloved Parents

LIST OF ABBREVIATIONS AND ACRONYMS

Sl. No.	Abbreviation	Full meaning
1.	%	Percent
2.	AUGPC	Area Under Growth Progress Curve
3.	@	At the rate
4.	°C	Degree Centigrade
5.	µm	Micrometer
6.	AEZ	Agro-ecological Zone
7.	ANOVA	Analysis of Variance
8.	BARI	Bangladesh Agricultural Research Institute
9.	BBS	Bangladesh Bureau of Statistics
10.	bp	Base Pair
11.	BLAST	Basic Local Alignment Search Tool
12.	cm	Centimeter
13.	CV	Coefficient of Variation
14.	CoMA	Cornmeal Agar
15.	CPDA	Cabbage Potato Dextrose Agar
16.	CPSA	Cabbage Potato Sucrose Agar
17.	CPGA	Cabbage Potato Glucose Agar
18.	CRBD	Completely Randomized Block Design
19.	DAI	Days After Inoculation
20.	DNA	Deoxyribonucleic Acid
21.	D-PDA	Dehydrated Potato Dextrose Agar
22.	*et. al.*	And others
23.	g	Gram
24.	ha	Hectare
25.	ITS	Intelligent Transport System
26.	kb	Kilobase

27.	Kg	Kilogram
28.	L	Liter
29.	LSD	Least Significant Difference
30.	ml	Milliliter
31.	mm	Millimeter
32.	MPDA	Mustard Potato Dextrose Agar
33.	MPSA	Mustard Potato Sucrose Agar
34.	MPGA	Mustard Potato Glucose Agar
35.	NCBI	National Center for Biotechnology Information
36.	PCR	Polymerase Chain Reaction
37.	PDA	Potato Dextrose Agar
38.	PSA	Potato Sucrose Agar
40.	PGA	Potato Glucose Agar
41.	SAU	Sher-e-Bangla Agricultural University
42.	t	Ton

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SAU, Dhaka

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ABSTRACT

Mustard (*Brassica spp.*) is the most important oilseed crop in Bangladesh and Grey blight disease caused by *Alternaria sp.* is a major constraint causing severe yield loss every year. For the reason to study cultural, morphological and molecular characterization of the pathogen is very important. The experiment was conducted in the Molecular biology and plant virology laboratory, Department of plant pathology, Sher-e-Bangla Agricultural University, Dhaka-1207. For molecular characterization of the pathogen fungal DNA was extracted from the pure culture of *Alternaria* isolate and PCR amplification was done using the ITS primer which was designed to amplify at 700 bp. To study the growth and sporulation of *Alternaria*, 11 media were selected *viz.* T_1= Dehydrated Potato Dextrose Agar (D-PDA) media, T_2= Potato Dextrose Agar (PDA) media, T_3= Corn Meal Agar (CoMA) media, T_4= Potato Sucrose Agar (PSA) media, T_5= Potato Glucose Agar (PGA) media , T_6= Mustard Potato Dextrose Agar (MPDA) media, T_7= Mustard Potato Sucrose Agar (MPSA) media, T_8= Mustard Potato Glucose Agar (MPGA) media, T_9= Cabbage Potato Dextrose Agar (CPDA) media, T_{10}= Cabbage Potato Sucrose Agar (CPSA) media and T_{11}= Cabbage Potato Glucose Agar (CPGA) media. From the study of the cultural and morphological characteristics, it was found that the highest radial mycelial growth was observed in CoMA media but spread very thinly. The best media selected for radial mycelial growth was D-PDA media from this study. In case of sporulation, MPGA media gave the highest spore concentration. From the molecular characterization study, the nucleotide sequence of *Alternaria* isolate had showed 99% similarities with the existing nucleotide sequences and closely related to *Alternaria brassicicola* that globally found in GenBank database.

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Chapter I

INTRODUCTION

Mustard (*Brassica spp.*) is a leading oilseed crop covering about 80% of the total oilseed area and contributing to more than 60% of the total oilseed production in Bangladesh. It belongs to the family Brassicaceae (or Cruciferae) and is the second most important source of edible oil after Soybean all over the world. It is widely cultivated all over the world. China is the largest producer of mustard all over the world, including India and Pakistan covers 90% of the world mustard production.

The average production of mustard in the world is 0.87 ton/ha (FAO, 2020). It is grown in tropical as well as temperate agro-climatic zones and well adapted to areas having a relatively cool, moist climate during the growing season (Kumar *et al.*, 2014). It is a cold loving crop which is grown during Rabi season from the month of October to February in Bangladesh. At present about 0.46 million hectares of land are put to mustard cultivation in Bangladesh with yield of mustard seed in the order of 0.62 million tons per year (BBS, 2020). This quantity meets only a fraction of the country's cooking oil needs. For this large quantity of Soybean and Sunflower oil is to be imported. This popular oilseed crop has high economic value for its versatile uses. At seedling stage the plant can be consumed as green vegetable. The raw mustard seed can be used as condiment during cooking. The oilseed brassicas usually contain 38-57% of erucic acid, 4.7-13% linolenic acid and 27% of oleic and linoleic acid, which are of high nutritive value required for human health (Singh *et. al.*, 2012). Rapeseed and mustard are rich source of oil and contains 44% to 46% good quality oil respectively (Rashid, 2013). The different varieties of mustard seed contain 40-44% oil and mustard oil cake contains 40% protein (Chowdhury and Hassan, 2013). Mustard oil of plant origin constitute important component of human diet, ranking third after cereals and animal products and are nutritionally superior to animal oil (Singh, *et. al.*, 2002). It can be used for cooking and also as food preservative. It has antifungal effect for which it is also used for body massage. In the village the plant debris is popularly used as fodder and fuel.

Every year farmers face a great loss in mustard production in Bangladesh and the yield is also very low. There are many biotic and abiotic factors responsible for the poor yield of mustard in our country. Diseases have been identified as one of the major causes (Ahmed, 1992). Mustard suffers from about 14 diseases (fungus 9, virus 2, bacteria 1, nematode 1 and parasitic plant 1) in Bangladesh (Baker *et. al.,* 2009). Different diseases of mustard like *Alternaria* grey blight, Downy mildew, Powdery mildew etc. causes severe yield loss. *Alternaria* grey blight is a chronic disease of mustard reported in all over the world including Bangladesh. It is caused by the pathogen *Alternaria spp.* The pathogens are greatly influenced by weather with the highest disease incidence reported in wet seasons and areas with relatively heavy rainfall (Meena *et. al.*, 2010).

This disease attacks on the lower leaves and pods as small circular brown necrotic spots which slowly increase in size. Many concentric spots coalesce to cover large patches showing blighted area and defoliation in severe cases. Report on yield loss due to the disease is not available in Bangladesh but 30-40% loss has been reported in India (Kolte *et. al.*, 1985). In Bangladesh due to lack of proper concern mustard production has been overlooked for a long time. Recently it has become a burning issue to increase production of mustard. For this reason to study the *Alternaria* grey blight disease, it is very urgent to isolate the pathogen and determine the morphological and physiological variability. The primary goal of the proposed study is to find out the best media to observe the growth of mycelia and rate of sporulation of the pathogen. From this study we can find out the best media to isolate *Alternaria* pathogen.

The major aspects of biology of an organism is the cultural, morphological and physiological characters of an individual within a species (Kabir, M.H., 2021). Variability studies are important to document the changes occurring in populations and individuals as variability in morphological and physiological traits indicate the existence of different pathotypes (Meena, *et. al.*, 2016). Although, studies on pathogenic variability are important for the development of pre-breeding populations (Meena, *et. al.,* 2010). For this molecular characterization is very important to study the *Alternaria* pathogen causing grey blight

disease of mustard. This pathogen shows high variability in morphological, cultural and molecular characteristics. The main purpose of the study was to also observe the molecular characteristics of the pathogen. The output of the study will be helpful for the further research.

OBJECTIVES:

The present study was conducted to achieve the following specific objectives-

- To evaluate the effect of different media on the mycelial growth and sporulation of *Alternaria*
- To characterize the *Alternaria* isolate through morphological and molecular study
- To identify the *Alternaria* species associated with grey blight disease of mustard on the basis of morphological and molecular characterization.

Chapter II

Review of literature

Grey blight of mustard caused by *Alternaria sp.* is considered the most devastating one as it attacks almost every part of the plant. Severe yield loss due to this disease has been reported in Bangladesh and all over the world. Researchers of all over the world have conducted investigation on this disease. To control disease study on its causal organism is a must. Here in this chapter literature related to *Alternaria sp.*, its growth, development and life cycle, appropriate media, morphological, molecular and cultural characterization have been studied and presented.

2.1. Mustard as Oilseed Crop

According to Kumar, *et. al*., (2014), Mustard is grown in tropical as well as temperate agro-climatic zones and well adapted to areas having a relatively cool, moist climate during the growing season.

The average production of mustard in the world is 0.87 ton/ha (FAO, 2020).

Based on the report of BBS, (2020), at present about 0.46 million hectares of land are put to mustard cultivation in Bangladesh with yield of mustard seed in the order of 0.62 million tons per year.

Singh, *et. al.*, (2012) said that the oilseed brassicas usually contain 38-57% of erucic acid, 4.7-13% linolenic acid and 27% of oleic and linoleic acid, which are of high nutritive value required for human health.

Rashid, (2013) reported that Rapeseed and mustard are rich source of oil and contains 44% to 46% good quality oil.

Chowdhury and Hassan, (2013) conducted a research and found that the different varieties of mustard seed contain 40-44% oil and mustard oil cake contains 40% protein.

According to the report of Singh, *et. al.*, (2002), mustard oil of plant origin constitute important component of human diet, ranking third after cereals and animal products and are nutritionally superior to animal oil.

2.2. Mustard Disease

Ahmed, (1992) reported that diseases have been identified as one of the major causes.

Baker, *et. al.*, (2009) found that mustard suffers from about 14 diseases (fungus 9, virus 2, bacteria 1, nematode 1 and parasitic plant 1) in Bangladesh.

According to Meena, *et. al*., (2010), the *Alternaria sp.* pathogens are greatly influenced by weather with the highest disease incidence reported in wet seasons and areas with relatively heavy rainfall.

Kolte, *et. al.*, (1985) reported that yield loss due to the disease is not available in Bangladesh but 30-40% loss has been reported in India.

2.3. Grey Blight in Mustard

Kabir, M.H., (2021) said that the major aspects of biology of an organism is the cultural, morphological and physiological characters of an individual within a species.

Karthikeyan, *et. al.*, (2021) studied that this pathogen can attack from seedling to adult stage at every part of the plant in leaves, stem, siliqua/pod.

Meena, *et. al*., (2016) reported that variability studies are important to document the changes occurring in populations and individuals as variability in morphological and physiological traits indicate the existence of different pathotypes.

Meena, *et. al.*, (2010) found that studies on pathogenic variability are important for the development of pre-breeding populations.

Among them grey blight of mustard caused by *Alternaria sp.* is widely distributed and the most serious disease of mustard (Fakir, 2008).

2.4. Media Preparation

According to the report of Darmady, *et. al.*, (1961), Needle, scalpel and inoculation loop were sterilized by spirit lamps flame.

Based on the research of Sahu, *et. al.*, (2018), the surface sterilization of the samples was done by dipping in 1000 times diluted (0.1%) mercuric chloride solution for 30 seconds and rinsed by sterilized water for 1 min and repeated the washing 3 times.

Sileshi, *et. al.*, (2016) found that wet paper supplied moisture for rapid sporulation on plant parts.

Ricker and Ricker, (1936) said that PDA media is a composition of 200g potato, 20g dextrose and 20g agar powder.

Afsana, (2018) found that an effective and comparatively cheap media can be helpful for the researchers.

2.5. Cultural and Morphological Characters of *Alternaria sp.*

Sreenivasprasad, *et. al.*, (2005) said that the diameters of the fungal mycelia growth were measured with a measuring scale in millimeter.

Mebratu, *et. al.*, (2015) reported that for conidia number of septation and color were also recorded.

Mycelial growth was measured according to the scale provided by Narendra (2006).

According to Padmanabhan, (1974), other cultural characters like mycelial growth rate, type of margin, colony color was also recorded.

Afsana, (2019) said that the marginal mycelial growth of fungus was picked-up aseptically with needle and inoculated in sterilized PDA media for sub- culturing in Laminar air flow cabinet.

Sharma, *et. al.*, (2013), Pramila, *et. al.*, (2014), Singh, *et. al.*, (2016), and Singh & Singh, (2018) mentioned that diversity in colony color, growth, its margin and topography were noticed among the isolates of *A. brassicae* by different workers.

Deep, *et. al.*, (2014) observed that thirty-two isolates of *A. brassicicola* for colony color and radial growth. Colony colour of *A. brassicicola* varied from olive green to dark olivacious black on PDA.

Yadav, *et. al.*, (2016) found that the conidia were obclavate to muriform, ovate elongated singly on conidiophore, sometimes in short acropetal chain.

2.6. Molecular study of *Alternaria sp.*

Jasalavich, *et. al.*,(1995) in his study found that *A. brassicicola* is actually more closely related to *A. raphani* than to *A.brassicae* based on the rDNA sequences data. *A. alternata*, *A. brassicae*, *A. brassicicola* and *A. raphani* formed clade of very closely related sister taxa.

Jung, *et. al.*, (2002) mentioned that due to the systematic and taxonomic usefulness the ITS region has been used for identification of the fungi, as it is suitable size for PCR amplification, restriction analysis and sequencing procedures, and because ITS regions are variable among species as well.

Michereff, *et. al.*, (2003) studied 38 isolates of *Alternaria brassicicola* and estimates variability based on disease development and pathogen physiology and found that *A. brassicicola* isolates were highly variable.

2.7. Spore count

Doullah and Okazaki, (2015) used the concentration of conidial suspension at 5×10^4 conidia /ml for inoculation of *Brassica rapa* leaf with *Alternaria brassicicola*.

The spots produced by the pathogen was >1cm in diameter and these findings are similar to Akhter, *et. al.* (2012) where eight mustard varieties (SAU-1, BINA-6, TORI-7, BARI-9, BARI-6, SOFOL, AGRANI and SS-75) were evaluated for their reaction against

Alternaria blight (*Alternaria brassicicola*) under natural condition at the experimental field of Sher-e-Bangla Agricultural University, Dhaka during the winter season from November 2007 to February 2008.

Based on the findings of Waghunde, *et.al.,* (2010), Spore produced by the microorganism acts as the reproductive unit and the spore count of biological control agent indicates its potency of antagonism against the pathogen.

Singh and Singh, (2007) showed that Several methods have been developed to quantify sporulation by hemacytometer.

Valdez and Piccalo, (2007) found that one of the most common ways used in plant pathology is the use of the haemocytometer.

Prasad, *et. al.,* (2005) reported that moistened condition inside the plastic bag should be maintained because nearly 95% humidity for 24 hours give better inoculation.

According to the findings of Prasad, *et. al.*, (2008), the increasing inoculum concentration significantly increased the infection of *Alternaria helianthi* in sunflower, whereas Doullah and Okazaki (2015) used the concentration of conidial suspension at 5×10^4 conidia /ml for inoculation of *Brassicae rapa* leaf with *Alternaria brassicicola*.

Chapter III

Materials and Methods

The present experiment was conducted in Molecular Biology and Plant Virology Laboratory under the Department of Plant Pathology, Sher-e-Bangla Agricultural University (SAU), Dhaka- 1207 during 2022 to 2023. The samples were collected from the field of SAU. The materials required and the methodology followed during the experiment are described in details in this chapter.

The experiment was set under three sections, they are-

a) Isolation of grey blight of mustard pathogen (*Alternaria sp.*) and its cultural and morphological study
b) Molecular characterization of *Alternaria* isolate
c) Evaluation of different selected media for its mycelial growth and sporulation of *Alternaria sp.*

3.1. Sample collection from diseased field for isolation

3.1.1. Location of the disease sampling field

The samples were collected from the field of Sher-e-Bangla Agricultural University in December, 2022. The collected sample field was located in Madhupur tract (AEZ 28). It occupies around 4244 km^2 area covering Dhaka, Gazipur, Narsingdi, Narayanganj, Tangail, Mymensingh and Kishoreganj. The soil is red or brown in color which contains 87% clay and 13% loam. The organic matter content and fertility level is low because of the soil of the tract have developed largely on Madhupur clays which are nutrient poor and somewhat acidic. (Appendix-I)

3.1.2. Climate

Dhaka is located in central Bangladesh at 23°42^{l}N 90°22^{l}E, on the eastern banks of the Buriganga River. The city lies on the lower reaches of the Ganges Delta and covers a total area of 306.38 square kilometers (118.29 sq. ml). The climate of Dhaka is tropical, influenced by the monsoon, with a dry season from November to March and a rainy season

from May to October. In winter, from December to February, the night temperature can drop to 11-12°C or even below.

3.1.3. Soil Characteristics

The soil texture of the central farm of Sher-e-Bangla Agricultural University (SAU) was silty loam, non-calcarious and dark grey in color. It belongs to the Tejgaon soil series with a P^H 6.7.

3.1.4. Sample collection

From the central farm of Sher-e-Bangla Agricultural University grey blight of mustard disease samples were collected through observing the spots. The disease spots were observed based on visual inspection. Infected leaves were collected from the field and kept in a zip lock plastic bag with required information in a small paper (Name of the sample crop, disease name and date of collection).

3.1.5. Crop stage in time of sampling

The sampling was done in the period of November, 2022. The samples were collected during the vegetative stage of the mustard crop (BARI sarisha 14) to observe the grey blight of mustard disease symptoms. The sampling was done in winter season characterized by low temperature and minimum rainfall.

3.2. Isolation of Pathogen from Collected Mustard Leaf Sample

3.2.1. Required equipment's

The following equipment were used for pathogen isolation

- Incubator- used for incubation
- Autoclave- used for media sterilization
- Oven- used for glassware sterilization and for melting media
- Thermometer- used for temperature reading
- Laminar air flow cabinet- used as working chamber
- Compound Microscope- used for pathogen study
- Weighing balance- used for weighing materials

- Refrigerator- used for sample and pure culture storage (4°C and -20°C)
- Spirit lamp- used for sterilizing needles
- Inoculation needle, forceps, knife, 9 cm petri dish, conical flask, scissor, scotch tape, pipette, p^H meter, blotter paper etc.

3.2.2. Cleaning and sterilization of the glassware

The glassware used for the experiment were cleaned with detergent and washed thoroughly using clean tap water. Then they were air dried and sterilized in the hot air oven at 160°C for an hour. Required working media and water were sterilized in the autoclave at 121°C and 15 psi pressure for 40 minutes. 70% ethyl alcohol was used to sterilize the laminar air flow cabinet and working bench. Needle, scalpel and inoculation loop were sterilized by spirit lamps flame (Darmady, *et. al.,* 1961).

3.2.3. Isolation of pathogen from the collected sample

3.2.3.1. Placement of disease sample on blotter paper

The infected parts of diseased leaves were cut into small pieces (0.5-1 cm) with diseased and healthy parts. The surface sterilization of the samples was done by dipping in 1000 times diluted (0.1%) mercuric chloride solution for 30 seconds and rinsed by sterilized water for 1 min and repeated the washing 3 times (Sahu, *et. al.,* 2018). To remove excess water the samples were placed and soaked in sterilized blotter paper. Then the small pieces were inoculated in another wet sterilized blotter paper.

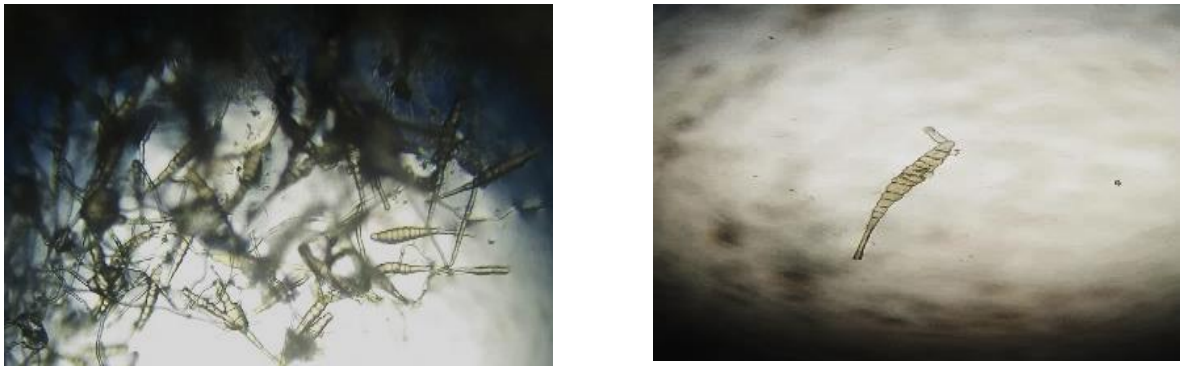
For blotter paper, small leaves were also inoculated in sterilized petri dish containing blotter paper which were wetted with distilled water. Wet paper supplied moisture for rapid sporulation on plant parts (Sileshi, *et. al.*, 2016).

For preparing the wet blotter paper, at first 9 cm radial blotter papers were sterilized in hot air oven for 10 minutes at 160°C temperature. Then two sterilized papers were placed in a sterilized petri dish. After that sterilized distilled water was added aseptically using dropper. When the whole paper became wet and no excess water was floated then it was

ready for the next step. Infected leaf samples were then placed on the wet blotter paper using forcep. Then it was covered with lid and transparent scotch tape was used to seal the petri dish so that it could be easily visible. The whole process was conducted in the Laminar air flow cabinet.

3.2.3.2. Identification and observation of pathogen under microscope

Before pathogen isolation confirmation of presence of pathogen into the sample was done. For this, at first a small portion of the sample was picked by the tip of a sterilized needle and placed onto a slide. Then a drop of glycerin was added and the sample was crushed properly. After that the pathogenic structure was observed under compound microscope (Figure 1).



200X magnification **400X magnification**

Figure 1: Pathogenic structure viewed under compound microscope

3.2.3.3. Preparation of PDA Media for pathogen isolation

Potato Dextrose Agar (PDA) media was used for the isolation of grey blight of mustard disease pathogen. This media was prepared with a composition of 200g potato, 20g dextrose and 20g agar powder (Ricker and Ricker, 1936).

At first disease free potatoes were peeled and cut into small pieces, then 200 gm was weighed. After that they were boiled in 500 ml distilled water for 15 to 20 minutes in a container. Then the potato extract was collected through filtration with muslin cloth filter. After that 20 gm dextrose was added and dissolved by stirring with glass rod. Then 20 gm agar powder was added and dissolved uniformly through shaking. The final volume was

made up to 1000 ml with distilled water. After that the open mouth of the conical flask was temporarily covered thoroughly by wrapping with aluminum foil paper. It was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.2.3.4. Preparation of the culture plates and inoculation of fungi

All the sterilized equipment for the study were opened only in the laminar air flow cabinet to avoid contamination. The autoclaved media was cooled to near 45°C so that it can be handled and the media can be poured in the sterilized petri dish properly. To avoid bacterial contamination and to facilitate fungal mycelial growth p^H should be maintained near 5 to 6. For this 10 ml lactic acid was added in 1 liter media before pouring in the petri dish in laminar air flow cabinet. The plates were marked distinctly and 18 to 20 ml liquid media was poured in each petri dish. Then it was kept until the media became solidified. Before inoculation the needle was sterilized through holding the tip into the flame of the spirit lamp till its became bright red in color. After that it was cooled for 5 to 6 seconds and the needle was re-sterilized before each inoculation. To avoid the contamination by air borne particles the lid of the petri dish should be opened just enough to insert the tip of the needle only. After inoculation the lids were closed carefully and sealed with scotch tape. Then it was incubated at 24°C for at least 7 days and examined. Three replications were made for the culture and it was stored in the room temperature. For later use, the prepared media can be stored in the refrigerator at 4°C temperature for maximum 7 days.

3.2.4. Identification of the pathogen

The pathogen was identified on PDA media based on their morphological growth pattern and spore characteristics which were examined studying mycelial size, shape and color through naked eye. Colony characteristics were observed at 48 hours interval from inoculation for 15 days. For conidia number of septation and color were also recorded (Mebratu, *et. al.,* 2015). Photographs were also taken to show the typical spore morphology and then they were identified as the isolates of *Alternaria sp.* The diameters of the fungal mycelia growth were measured with a measuring scale in millimeter (Prasad, *et. al.,* 2005). Two cross lines using a semi-permanent marker pen were drawn on the lower surface of

the plate maintaining a center (Figure 2). Mycelial growth was measured according to the scale provided by Narendra (2006). According to this scale very good mycelial growth gave 76-90 mm, moderate 56-75 mm and poor gave <56 mm mycelial diameter.

Each treatment was replicated for three times and the sporulation of *Alternaria sp.* was detected by compound microscope from 10 days after inoculation in PDA media. For observing number of spore per isolate Hemacytometer was used and to study septation per conidia the prepared slide was placed under microscope at 40X. Other cultural characters like mycelial growth rate, type of margin, colony color was also recorded (Padmanabhan, 1974).

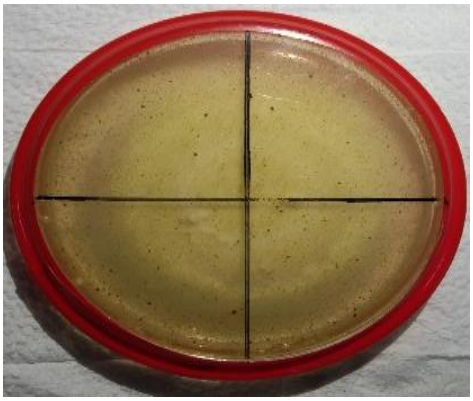


Figure 2: Radial mycelial growth measurement of *Alternaria sp.*

3.2.5. Purification and re culture of the *Alternaria sp.*

In case of contamination purification and re culture of culture media was done. The marginal mycelial growth of fungus was picked-up aseptically with needle and inoculated in prepared and sterilized PDA media for sub- culturing in Laminar air flow cabinet (Afsana, 2019). It was done at an interval of 3-5 days based on the condition of the pure culture.

3.2.6. Storage of fungal isolates

The fungus was grown on PDA media for 7 days at room temperature. For short term preservation the culture media was stored at 4°C and for long term preservation -20°C temperature was appropriate.

3.3. Molecular characterization of *Alternaria* isolate

3.3.1. Pure culture preparation

The pathogen was isolated from diseased sample on PDA media and pure culture for molecular characterization was prepared.

3.3.2. Reagents and Instruments used

Instruments Used

DNA Isolation

1. Homogenizer, Pro Scientific, USA

2. Centrifuge Machine: Model: Z-216 M, HERMLE Labortechnik GmbH; Origin: Germany

3. Heat Block: Model: My Block, Benchmark, Origin: USA

DNA Quantification

1. Nano Drop Spectrophotometer, Model: ND2000, Origin: Thermo Scientific, USA.

PCR:

1. Gene Atlas, Model: G2, Origin: Astec, Japan.

Gel Electrophoresis System

1. Horizontal, Model: Mini, Origin: CBS Scientific, USA.

Gel Documentation

1.Alpha Imager, Model: mini, Origin: Protein Simple, USA.

3.3.3. Reagents and Chemicals Used

DNA Isolation

1. Promega Wizard™ Genomic DNA Purification Kits, Catalog No: PR-A1120 Assecories, Mfr. No:A1120 , Origin: Promega, USA.

PCR

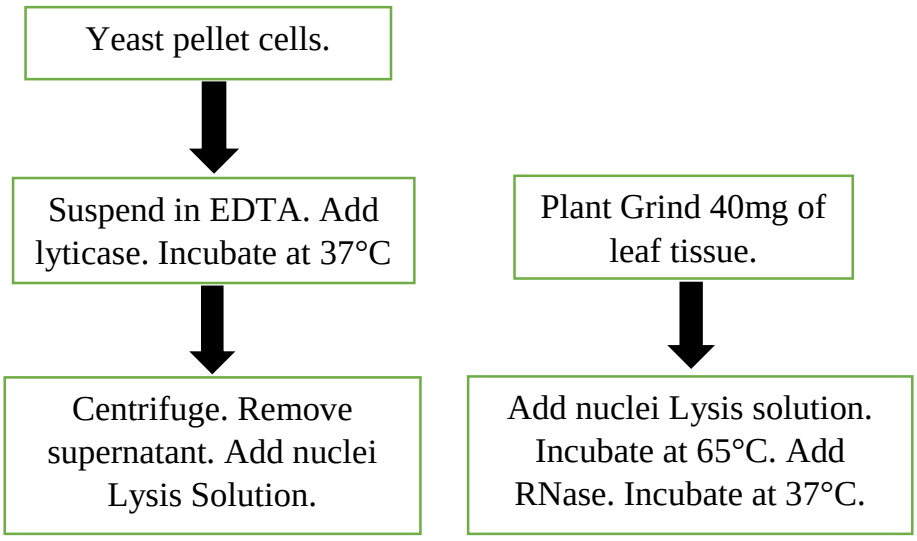
1. GoTaq® Green Master Mix (dNTPs, Buffer, MgCl2, Taq Pol), Cat: M782A, Origin: Promega, USA.

Gel

1. Agarose, Cat: V3125, Origin: Promega, USA.
2. Bench Top1kb DNA Ladder, Cat: G754B, Origin: Promega, USA.
3. Ethidium Bromide Solution, Cat: H5041, Origin: Promega, USA.
4. TAE Buffer: Cat: V4251, Origin: Promega, USA.

3.3.4. Extraction of fungal genomic DNA

1. Cells were pelleted from 1ml of culture by centrifugation at 13000-16000 × g* for 2 minutes.
2. The cell pellet was suspended in 293µl of 50 mM EDTA.
3. 7.5µl of 75 units/µl lyticase was added and mixed gently.
4. Suspension was incubated for 30-60 minutes at 37°C and cooled to room temperature.
5. Suspension was centrifuged as Figure 3 and discarded the supernatant.
6. 300µl of Nuclei Lysis Solution was added and proceeded to protein precipitation and DNA rehydration. (Figure 3)



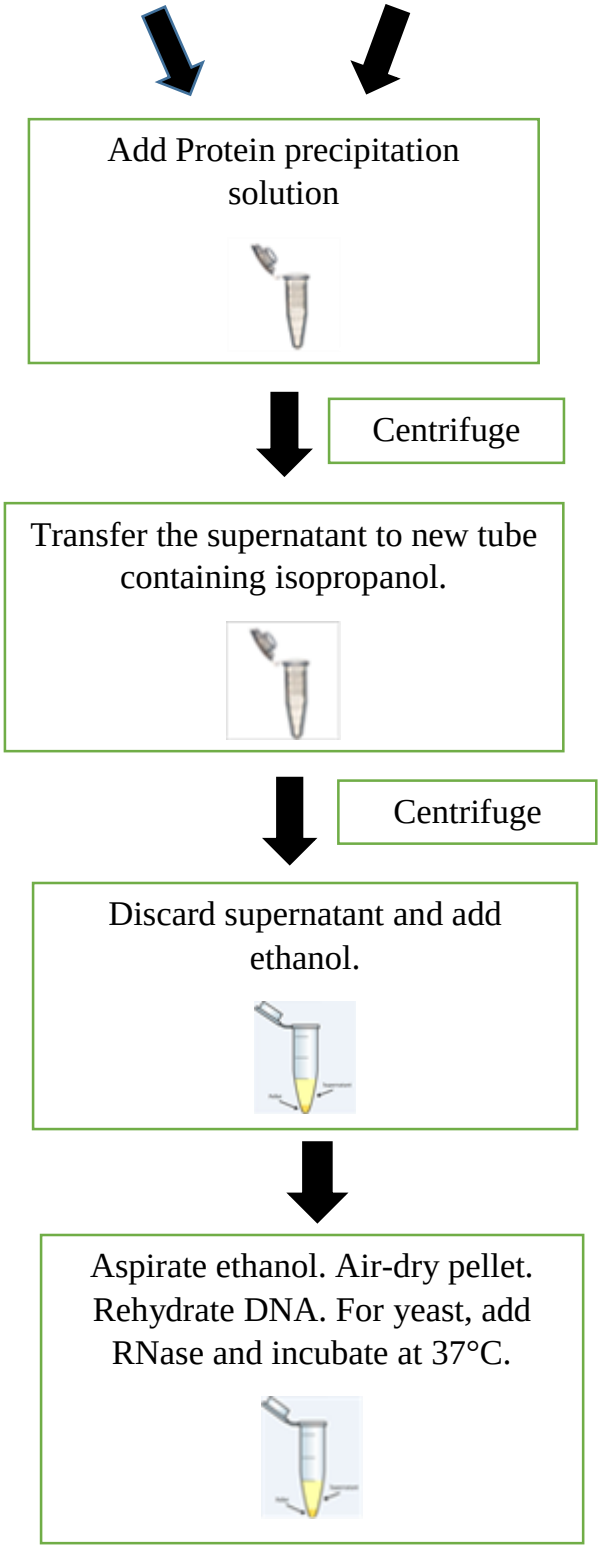


Figure 3: Flow chart of DNA extraction procedure

3.3.5. Prepared Plant Lysate

1. Approximately 40 mg of leaf tissue was grinded in liquid nitrogen.
2. 600µl of Nuclei Lysis Solution was added and incubated at 65°C for 15 minutes.
3. 3µl of RNAse Solution was added and incubated at 37°C for 15 minutes. Sample was cooled to room temperature for minutes and proceeded to Protein Precipitation and DNA Rehydration. Table 1 (below).

Table 1: Protein Precipitation and DNA Rehydration

		Yeast	**Plant**
1	Protein precipitation solution was added and Vortexed for Yeast only. It was incubated 5 minutes on ice.	100µl	200µl
2	It was centrifuged at 13000-16000 × g*	3 minutes	3 minutes
3	Supernatant was transferred to clean tube containing room temperature Isopropanol.	300µl	600µl
4	It was mixed by invasion and centrifuged at 13000-16000 × g*	2 minutes	1 minute
5	Supernatant was decanted and added room temperature 70% Ethanol	300µl	600µl
6	It was centrifuged at 13000-16000 × g*	2 minutes	1 minute
7	The Ethanol was aspirated and air- dried the pellet		
8	DNA rehydrated solution was added	50µl	100µl
9	For Yeast only: RNAse Solution was added and incubated at 37°C for 15 minutes	1.5 µl	
10	It was rehydrated at 65°C for 1 hour or overnight at 4°C		

***Maximum speed on a centrifuge**

3.3.6. Test protocol for PCR for fungal DNA

Reaction profile

Reagents	Volume per reaction
GoTaq™ G2 hot start master mix	12.5 µl
T DNA (concentration 25-65 ng/ul)	1 µl
Primer 27 F (concentration 10-20 pMol)	1 µl
Primer 1492 R (concentration 10-20 pMol)	1 µl
Nuclease free water	9.5 µl
Total reaction volume	25 µl

Thermal profile

Number of cycle	Step name	Temperature	Time
1 Cycle	Pre heat	- 95°C	3 min
35 Cycle	Denaturation	- 95°C	30 sec
	Annealing	- 49°C	30 sec
	Extension	- 72°C	90 Sec
	Final extension	-72°C	5 min
	Hold	- 4°C	Overnight

Phylotype identification of each isolate was done by PCR. Phylotype-specific PCR was carried out in 25µl final volume of the reaction mixture. 5µl aliquot of each amplified PCR product was subjected to electrophoresis on 1% agarose gel, Cat: V3125, Origin: Promega, USA. The PCR amplification was targeted at 1465-bp. The PCR product was stored in a refrigerator at -20°C.

3.3.7. Protocol for Electrophoresis and Gel Documentation

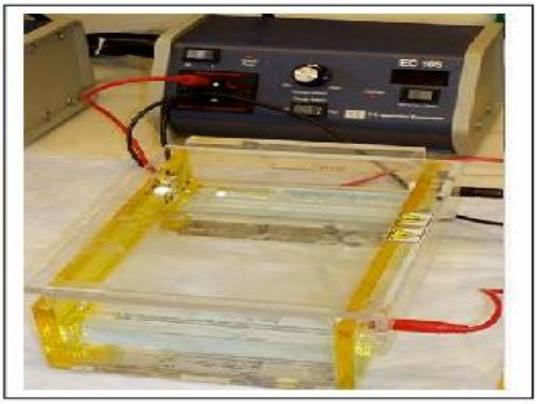


Figure 4: Gel electrophoresis apparatus

Gel electrophoresis is a laboratory method used to separate mixtures of DNA, RNA, or proteins according to molecular size. In gel electrophoresis, the molecules to be separated are pushed by an electrical field to move the negatively charged molecules through a matrix of agarose gel that contains small pores. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. Gel electrophoresis can also be used for the separation of nanoparticles.

Procedure Used

1. 1g agarose powder was taken into 100 mL Tris Borate EDTA (TBE) buffer in 500 mL beaker with a magnet.
2. It was heated at 80° C for 5 minutes on a magnetic stirrer.
3. Then it was cooled the gel for 2 minutes and added 6 µl ethidium bromide in it.
4. The gel was poured on the tray, combed of 20 teethes is placed on the gel
5. After 20 minutes samples were loaded into wells (indentations) at one end of a gel with 1kb ladder after placing the gel tray on electrophoresis.
6. Running buffer TBE was added on to the chamber of electrophoresis until the gel tray undergoing the buffer

7. The electrophoresis was run at 90 volt for 30 minutes.

8. After 30 minutes the electrophoresis was stopped and the gel was transferred to the Alpha imager MINI gel documentation system and the image was visualized.

3.3.8. Protocol for Purification of PCR product

Figure 5: Centrifuge machine

The PCR purification protocol achieves rapid and efficient removal of short primers, dNTPs, enzymes, short-failed PCR products, and salts from PCR fragments >100 bp, typically in less than 10 minutes. There were several methods for PCR cleanup such as ethanol precipitation, bead or column based purification, and Enzymatic approaches. Ethanol precipitation was most cost effective but was also the most labor intensive.

Procedure used

1. Following electrophoresis, DNA band from gel was excised and placed gel slice in a 1.5ml micro centrifuge tube.

2. 10µl Membrane Binding Solution per 10mg of gel slice was added, vortexed and incubated at 50–65°C until gel slice was completely dissolved.

3. Processing PCR Amplifications- An equal volume of Membrane Binding Solution was added to the PCR amplification Binding of DNA

4. SV Mini column was inserted into Collection Tube

5. Dissolved gel mixture was transferred or prepared PCR product to the Mini column assembly. It was Incubated at room temperature for 1 minute.

6. Suspension was centrifuged at 16,000 × g* for 1 minute, discarded flow through and reinserted Mini column into Collection Tube.

7. Washing-700µl Membrane Wash Solution (ethanol added) was added. It was centrifuged at 16,000 × g* for 1 minute and discarded Flow through and reinserted Mini column into Collection Tube.

8. Step 4 was repeated with 500µl Membrane Wash Solution. It was centrifuged at 16,000 × g* for 5 minutes

9. The Collection Tube was emptied and re-centrifuged the column assembly for 1 minute with the micro centrifuge lid open (or off) to allow evaporation of any residual ethanol.

10. Elution - Mini column to a clean 1.5ml micro centrifuge tube was transferred carefully.

11. 50µl of Nuclease-Free Water was added to the Mini column and incubated at room temperature for 1 minute. It was centrifuged at 16,000 × g* for 1 minute.

3.4. Evaluation of different selected media for mycelial growth and sporulation

Mycelial growth and sporulation of *Alternaria sp.* was studied on eleven different selected culture media.

3.4.1. Composition of different media and their preparation

The eleven selected culture media were prepared according to standard composition and maintaining appropriate protocol.

3.4.1.1. Composition and Preparation of Dehydrated Potato Dextrose Agar (D-PDA) media

Components	Amount
Dehydrated PDA powder	39g
Distilled water	1000 ml

Composition of Dehydrated Potato Dextrose Agar (D-PDA) media

Components	Amount
Dextrose	20 gm
Agar	15 gm
Potato starch	4 gm

At first 39 gm Dehydrated Potato Dextrose Agar (D-PDA) powder was weighed using electric balance. After that it was added into 500 ml distilled water in a conical flask and mixed up properly though shaking. The volume was made up to 1000 ml and then it was autoclaved at 121°C under 15 psi pressure for 40 minutes.

3.4.1.2. Composition and preparation of Potato Dextrose Agar (PDA) media

The potato dextrose agar media was prepared according to standard procedure which was mentioned earlier in section 3.2.3.3.

3.4.1.3. Composition and preparation of Corn Meal Agar (CoMA) media

Components	Amount
Corn meal agar powder	17 gm
Distilled water	1000 ml

At first 39 gm Corn Meal Agar (CoMA) powder was weighed using electric balance. After that it was added into 500 ml distilled water in a conical flask and mixed up properly though shaking. The volume was made up to 1000 ml and then it was autoclaved at 121°C under 15 psi pressure for 40 minutes.

3.4.1.4. Composition and preparation of Potato Sucrose Agar (PSA) media

Components	Amount
Potato	200 g
Sucrose	20 g
Agar	20 g
Distilled water	1000 ml

At first disease free potatoes were peeled and cut into small pieces, then 200 gm was weighed. After that they were boiled in 500 ml distilled water for 15 to 20 minutes. Then the potato extract was collected through filtration with muslin cloth filter. After that 20 gm sucrose was added and dissolved by stirring with glass rod. Then 20 gm agar powder was added and dissolved uniformly through shaking. The final volume was made up to 1000 ml with distilled water. After that the open mouth of the conical flask was temporarily covered thoroughly by wrapping with aluminum foil paper. It was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.4.1.5. Composition and preparation of Potato Glucose Agar (PGA) media

Components	Amount
Potato	200 g
Glucose	20 g
Agar	20 g
Distilled water	1000 ml

At first disease free potatoes were peeled and cut into small pieces, then 200 gm was weighed. After that they were boiled in 500 ml distilled water for 15 to 20 minutes. Then the potato extract was collected through filtration with muslin cloth filter. After that 20 gm glucose was added and dissolved by stirring with glass rod. Then 20 gm agar powder was added and dissolved uniformly through shaking. The final volume was made up to 1000

ml with distilled water. After that the open mouth of the conical flask was temporarily covered thoroughly by wrapping with aluminum foil paper. It was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.4.1.6. Composition and preparation of Mustard Potato Dextrose Agar (MPDA) media

Components	Amount
Mustard leaf	100 gm
Potato	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1000 ml

At first 100 gm disease free fresh mustard leaf was washed thoroughly and chopped into small pieces. Then they were mashed properly with morter and pastel. Then 100 ml fresh leaf extract was collected through filtration. After that the extract was added into 500 ml boiled potato extract and mixed thoroughly. Then 20 gm dextrose and 20 gm agar powder were added into that and after dissolving the components properly the volume was made up to 1000 ml. After that it was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.4.1.7. Composition and preparation of Mustard Potato Sucrose Agar (MPSA) media

Components	Amount
Mustard leaf	100 gm
Potato	200 gm
Sucrose	20 gm
Agar	20 gm
Distilled water	1000 ml

At first 100 gm disease free fresh mustard leaf was washed thoroughly and chopped into small pieces. Then they were mashed properly with morter and pastel. Then 100 ml fresh leaf extract was collected through filtration. After that the extract was added into 500 ml boiled potato extract and mixed thoroughly. Then 20 gm sucrose and 20 gm agar powder were added into that and after dissolving the components properly the volume was made up to 1000 ml. After that it was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.4.1.8. Composition and preparation of Mustard Potato Glucose Agar (MPGA) media

Components	Amount
Mustard leaf	100 gm
Potato	200 gm
Glucose	20 gm
Agar	20 gm
Distilled water	1000 ml

At first 100 gm disease free fresh mustard leaf was washed thoroughly and chopped into small pieces. Then they were mashed properly with morter and pastel. Then 100 ml fresh leaf extract was collected through filtration. After that the extract was added into 500 ml boiled potato extract and mixed thoroughly. Then 20 gm glucose and 20 gm agar powder were added into that and after dissolving the components properly the volume was made up to 1000 ml. After that it was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.4.1.9. Composition and preparation of Cabbage Potato Dextrose Agar (CPDA) media

Components	Amount
Cabbage leaf	100 gm
Potato	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1000 ml

At first 100 gm disease free fresh cabbage leaf was washed thoroughly and chopped into small pieces. Then they were mashed properly with morter and pastel. Then 100 ml fresh leaf extract was collected through filtration. After that the extract was added into 500 ml boiled potato extract and mixed thoroughly. Then 20 gm dextrose and 20 gm agar powder were added into that and after dissolving the components properly the volume was made up to 1000 ml. After that it was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.4.1.10. Composition and preparation of Cabbage Potato Sucrose Agar (CPSA) media

Components	Amount
Cabbage leaf	100 gm
Potato	200 gm
Sucrose	20 gm
Agar	20 gm
Distilled water	1000 ml

At first 100 gm disease free fresh cabbage leaf was washed thoroughly and chopped into small pieces. Then they were mashed properly with morter and pastel. Then 100 ml fresh leaf extract was collected through filtration. After that the extract was added into 500 ml boiled potato extract and mixed thoroughly. Then 20 gm sucrose and 20 gm agar powder were added into that and after dissolving the components properly the volume was made up to 1000 ml. After that it was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.4.1.11. Composition and preparation of Cabbage Potato Glucose Agar (CPGA) media

Components	Amount
Cabbage leaf	100 gm
Potato	200 gm
Glucose	20 gm
Agar	20 gm
Distilled water	1000 ml

At first 100 gm disease free fresh cabbage leaf was washed thoroughly and chopped into small pieces. Then they were mashed properly with morter and pastel. Then 100 ml fresh leaf extract was collected through filtration. After that the extract was added into 500 ml boiled potato extract and mixed thoroughly. Then 20 gm glucose and 20 gm agar powder were added into that and after dissolving the components properly the volume was made up to 1000 ml. After that it was sterilized in the autoclave at 121°C under 15 psi pressure for 40 minutes.

3.4.2. Preparation of plates and inoculation from stock culture

Avoiding contamination is a primary concern in case of preparation of plates and inoculation. For this all the equipment required for the study were opened only in the laminar air flow cabinet and there the sterilized media were cooled to near 45°C so that it

can be handled and poured into sterilized glass petri dish with bare hand. Then 10 ml lactic acid was added in 1 L media just before pouring it into the glass petri dish to maintain the p^H near 5 to 6. The plates were labelled with semi-permanent marker with required information. After that 18 to 20 ml liquid media was poured in each plate and it was kept untouched until the media got solidified. To sterilize the needle the tip was hold into the flame until it became bright red in color and then it was allowed to cool. To avoid contamination from air borne pathogens the lid of the petri dish was opened enough just to insert the tip of the needle. A small portion of the mycelia was picked by the needle and placed carefully into the center of the solidified media. Then the petri dish was sealed with scotch tape and incubated at 28±1°C. Each selected treatment was replicated for three times. For later use the solidified media was stored in refrigerator at 4°C temperature for highest 7 days until inoculation.

3.4.3. Measurement of Mycelial Growth

After that observation was done after 48 hours of inoculation at 3 days interval. The radial growth of mycelia was measured in mm and two cross lines with semi-permanent black marker pen were drawn on the lower surface of the petri dish taking a center. Then radial growth was measured by taking the intersect point as the center and it was done until the petri plates were fully covered with mycelia. Colony characteristics were observed by visual observation through naked eye. Area Under Growth Progress Curve (AUGPC) was calculated by following formula:

$$AUGPC=\sum\{\frac{X1+X2}{2}\times(T2-T1)\}$$

Where,

X1= The growth of the pathogen on 1st date

X2= The growth of the pathogen on 2nd date

T1= The time when X1 measured

T2= The time when X2 measured

3.4.4. Spore count

Spore count is an essential quality parameter for bioagent efficacy. Spore produced by the microorganism acts as the reproductive unit and the spore count of biological control agent indicates its potency of antagonism against the pathogen. (Waghunde, R.R., *et.al.*, 2010). Several methods have been developed to quantify sporulation by haemocytometer (Singh and Singh, 2007). But one of the most common ways used in plant pathology is the use of the haemocytometer (Valdez and Piccalo, 2007). For this study, after sporulation the spore was scraped by scalpel and weighed. Then they were diluted and appropriate dilution was performed for spore count. Then it was placed on hemacytometer by micropipette and observed under microscope. The spore count was done by counting the spores of five small squares and made an average (Figure 6). The formula that was used for calculating spore concentration is:

C x D = B x E

Here, C= The final concentration that is needed/ml

D= The final volume that is needed (ml)

B= The total number of cells in 1 ml

E= The amount of suspension that is needed to be taken

Figure 6: Counting spore by using Hemacytometer

3.5. Statistical Analysis

In the laboratory the trials were conducted following Completely Randomized Design (CRD) method. The replicated data generated from different experiments were tabulated and analyzed by using STATISTIX 10 software. Treatment means were compared by using Least Significant Difference (LSD) range at 5% level of significance.

Chapter IV

Results

This chapter provided results about isolation of pathogen, cultural, morphological and molecular characterization and evaluation of different culture media for mycelial growth and sporulation. The results are presented in tables, graphs, diagrams and photographs.

4.1. Symptomology

The collected samples had typical grey blight symptoms on the leaves, stems and pods. The leaves contained disease symptoms circular brown spots with concentric ring. The spots mainly occurred on matured leaves which had a yellow halo and a crack through the middle of the spot. In advance stage, the leaves developed enough spots that they began to coincide together to create large necrotic areas on infected leaves. In advance stage, blighted symptom appeared on the diseased leaves which found into greyish in color as are shown in figure 7.

Figure 7: Characteristic symptoms of grey blight disease in Mustard

4.2. Cultural and morphological study of *Alternaria* isolate

From the cultural and morphological study, the isolate was identified as *Alternaria brassicicola* based on the morphological and colonial morphology (Section 3.2.5.). The mycelial growth was initially irregular to circular in shape and whitish in color at primary stage but it turned into greyish to blackish in color by time. The texture was cottony velvetty and the margin was undulated. The isolate was flat with slightly raised middle (Figure 8). From the microscopic study, the morphological characteristics of *Alternaria* isolate was the mycelia were brown in color, branched and septate. The conidiophores are septate and simple. The conidia were muriform and light brown in color (Figure 9). The morphology of fungi confirmed with the study of Yadav, *et. al*., 2016.

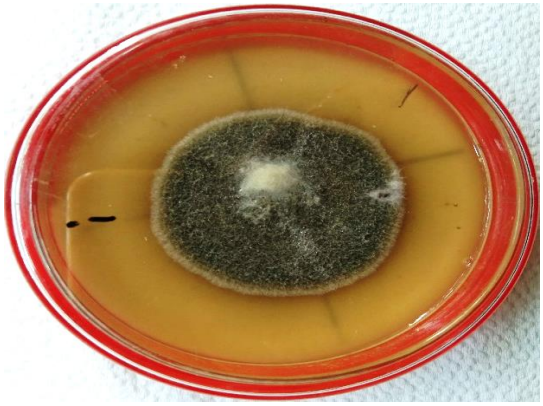
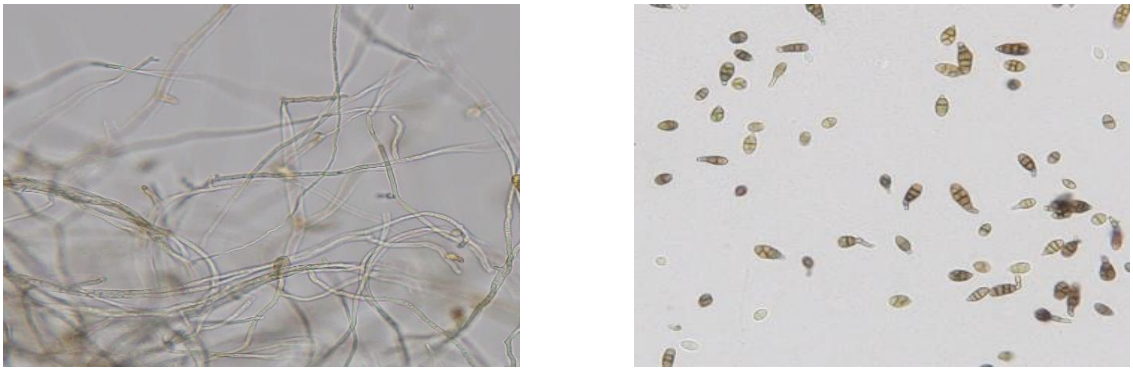


Figure 8: Pure culture of *Alternaria* isolate on PDA media



A **B**

Figure 9: Microscopic view of Mycelia, conidiophores (A) and Conidia (B) of *Alternaria* isolate

4.3. Molecular characterization of *Alternaria* isolate

4.3.1. Isolate of *Alternaria sp.*

The isolate of *Alternaria sp.* was used as pure culture to extract the genomic DNA for molecular characterization (Figure 10).

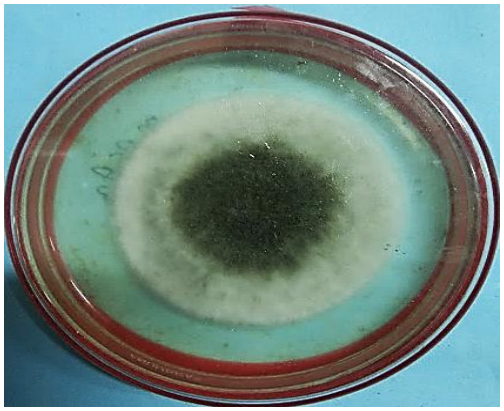


Figure 10: Pure culture of *Alternaria* isolate for molecular characterization

4.3.2. PCR amplification

From the mycelia of the pure culture of *Alternaria* isolate, total DNA was extracted and after DNA extraction, the fungal DNA was used as a template in PCR amplification using universal forward primer ITS4: TCCTCCGCTTATTGATATGC and reverse primer ITS5: GCA AGT AAA AGT CGT AAC AAG G which was designed to amplify fungal gene fragment to 700 bp. *Alternaria* fungal gene of 700 bp fragment was amplified and analyzed on 1.0% agarose gel along with 1 kb DNA ladder. In electrophoresis analysis, the PCR product showed a single amplified fragment at around ~ 700 bp that had been clearly shown in figure 11.

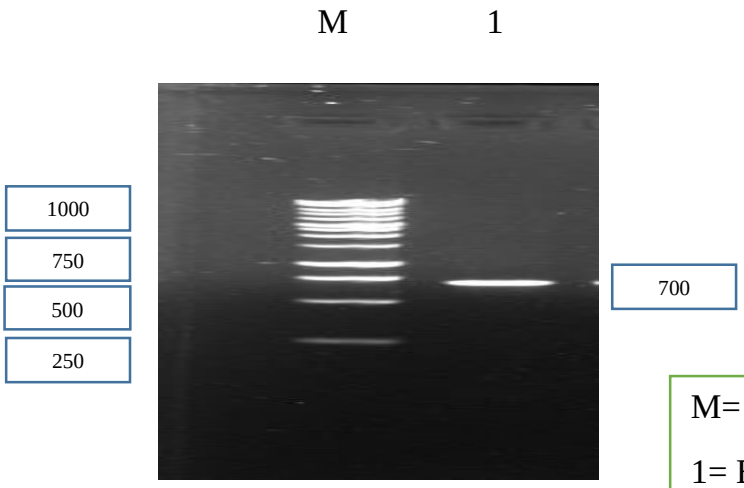


Figure 11: PCR amplification

4.3.3. Sequencing and analysis

For performing sequencing the PCR product was carried out in an automated sequencer and the sequencing was conducted with reverse primer ITS5: GCAAGTAAAAGTCGT AACAAGG .

Sanger Detoxi Sequence of *Alternaria brassicicola* (FASTA Form)

CTACCTGATCCGAGGTCAAAGTTGAAAAAAAGGCTTAATGGATGCTAGACCT
TTGCTGATAGAGAGTGCGACTTGTGCTGCGCTCCGAAACCAGTAGGCCGGCT
GCCAATTACTTTAAGGCGAGTCTCCAGCAAAGCTAGAGACAAGACGCCCAAC
ACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCTTTGG
AATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCT
GCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACC
AAGAGATCCGTTGTTGAAAGTTGTAATTATTAATTTGTTACTGACGCTGATTA
ATTACAAAAGGTTTATGTTTGTCCTAGTGGTGGGCGAACCCACCAAGGAAAC
AAGAAGTACGCAAAAGACAAGGGTGAATAATTCAGCAAGGCTGTAACCCCG
AGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCCTCCGCAGGTTCACC
TACGGAGACCTTGTTACGACTTTTACTTCCTCTAATTGACCAAGA

4.3.4. Confirmation of *Alternaria brassicicola*

The searches for similarities of nucleotide sequences were performed using the BLASTn on the NCBI website (https:// www.ncbi.nlm.nih.gov) for finding matches with the existing DNA sequences of the *Alternaria sp.* in the NCBI genbank to identify the fungal isolate. The nucleotide sequences found from the present study showed 99% similarity with the existing nucleotide sequences of genbank database.

4.3.5. Development of phylogenetic tree

From the phylogenetic tree, it had been observed that *Alternaria* isolate from the present study was closely related to *Alternaria sp.* isolates *Alternaria longipes, Alternaria tenuissima, Alternaria alternata, Alternaria arborescens, Alternaria brassicicola and Alternaria infectoria* found globally in the Genbank database. It was also revealed that from the phylogenetic tree of *Alternaria* local isolate (DHA) showed close relationship with *Alternaria brsssicicola* (Figure 12).

Phylogenetic Tree

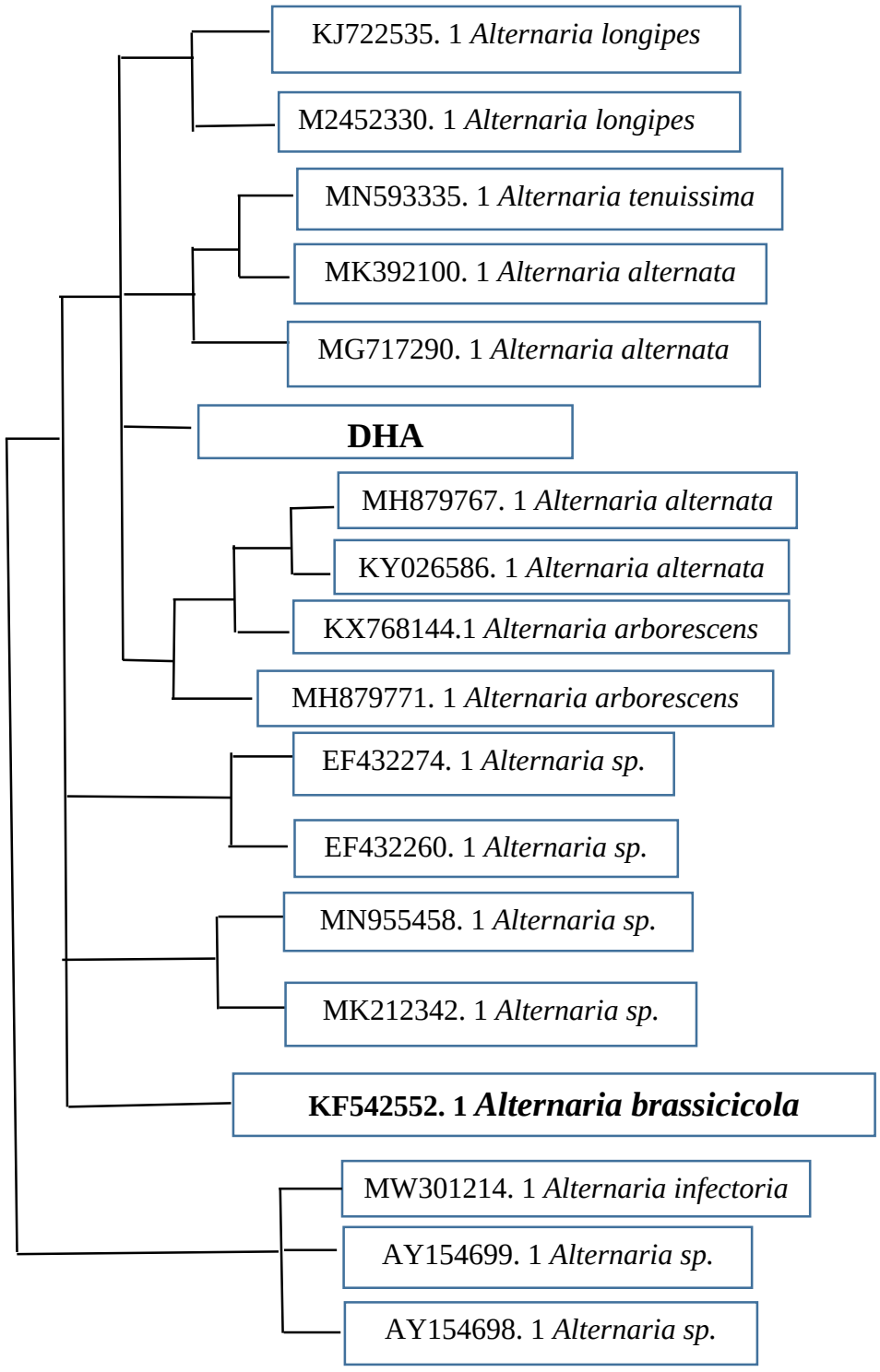


Figure 12: Phylogenetic tree of *Alternaria* local isolate (DHA) showed close relationship with globally found *Alternaria* isolates.

4.4. Evaluation of different selected media for mycelial growth and sporulation of *Alternaria brassicicola*

The *Alternaria brassicicola* pathogen was grown in 11 selected media and the radial mycelial growth was measured at 3, 6 and 9 Days After Inoculation (DAI). Area Under Growth Progressive Curve (AUGPC) was also calculated following the formula mentioned in Section 3.3.3. In the tabulated data presented in Table 3 measured the variation of mycelial growth on the selected culture media.

At 3 days after inoculation, the highest mycelial growth (13.33 mm) was estimated in Dehydrated Potato Dextrose Agar (D-PDA) media and the lowest mycelial growth (5.33 mm) was found in Potato Dextrose Agar (PDA) media.

At 6 days after inoculation, the highest mycelial growth (22.67 mm) was estimated in Cornmeal Agar (CoMA) media and the lowest mycelial growth (10.33 mm) was found in Potato Dextrose Agar (PDA) media.

At 9 days after inoculation, the highest mycelial growth (32.33 mm) was estimated in Cornmeal Agar (CoMA) media and the lowest mycelial growth (14.00 mm) was found in Potato Dextrose Agar (PDA) media.

The most uniform, dense and proper growth of mycelia was observed in Dehydrated Potato Dextrose Agar (D-PDA) media. Based on the study, the best media selected for radial mycelial growth was Dehydrated Potato Dextrose Agar (D-PDA) media.

From the table, the highest AUGPC value 135.00 was calculated for Cornmeal Agar (CoMA) media and the lowest AUGPC value 59.99 was calculated for Potato Dextrose Agar (PDA) media. Results are presented in Table 2.

Table 2: Mycelial growth of *Alternaria brassicicola* at different selected media

Mycelia	Mycelial growth (mm)			AUGPC value
	3 DAI	6 DAI	9 DAI	
D-PDA	13.33 a	22.33 a	28.00 ab	128.99
CoMA	12.33 ab	22.67 a	32.33 a	135.00
PDA	5.33 d	10.33 d	14.00 e	59.99
PSA	7.33 cd	13.33 bcd	18.33 de	78.48
PGA	7.33 cd	14.00 bcd	20.00 b-e	83.00
MPDA	9.33 bc	17.33 abc	25.00 a-d	103.50
MPSA	9.67 bc	17.33 abc	27.33 abc	107.49
MPGA	8.00 cd	14.33 bcd	19.67 cde	84.50
CPDA	9.67 cd	17.67 ab	25.00 a-d	105.02
CPSA	6.67 cd	11.33 d	15.67 e	67.50
CPGA	7.00 cd	12.00 cd	18.00 de	73.50
CV (%)	20.20	20.94	21.61	

Note: DAI- Days After Inoculation, AUGPC- Area Under Growth Progress Curve

[In a column those having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of significance.]

4.5. Comparative analysis of different selected media for mycelial growth and sporulation of *Alternaria brassicicola*

From this study, it was observed that in the maximum selected media the mycelial color was whitish to greyish white, texture cottony to velvety, colony form circular to slightly irregular, margin entire to slightly undulated and elevation was flattened. The maximum mycelial growth was found in Dehydrated Potato Dextrose Agar (D-PDA) media but no spore was found in 7 days after inoculation and the minimum mycelial growth was observed in Cornmeal Agar (CoMA) media with no spore in 7 days after inoculation. The rest of the media showed good mycelial growth and

sporulation occurred within 7 days after inoculation. The results are presented in Table 3 and Plate 1.

Table 3: Comparative analysis of different selected media for colony and spore morphology of *Alternaria brassicicola*

Media	**Color**	**Texture**	**Colony form**	**Margin**	**Elevation**	**Mycelial growth**	**Spore (within 7 DAI)**
D-PDA	Greyish White	Cottony	Circular	Entire	Flat	Dense and Fluffy	No
CoMA	Whitish	Poorly cottony	Circular	Entire	Flat	Sparse	No
PDA	Greyish	Velvety	Circular	Entire	Flat	Dense	Yes
PSA	Greyish	Velvety	Circular	Entire	Flat	Dense	Yes
PGA	Greyish	Velvety	Slightly irregular	Slightly undulated	Flat	Dense	Yes
MPDA	Greyish White	Cottony	Slightly irregular	Slightly undulated	Flat	Dense	Yes
MPSA	Greyish	Cottony	Circular	Entire	Flat	Dense	Yes
MPGA	Greyish	Velvety	Circular	Entire	Flat	Dense	Yes
CPDA	Greyish White	Cottony	Slightly irregular	Slightly undulated	Flat	Dense	Yes
CPSA	Greyish	Velvety	Slightly irregular	Entire	Flat	Dense	Yes
CPGA	Greyish White	Velvety	Circular	Entire	Flat	Dense	Yes

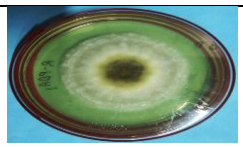
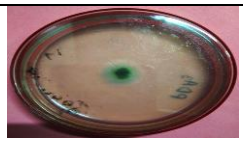
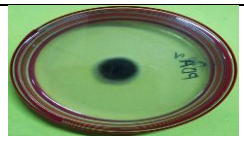
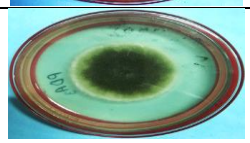
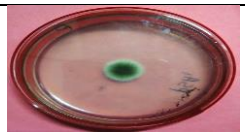
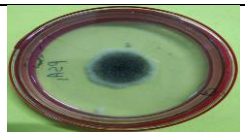
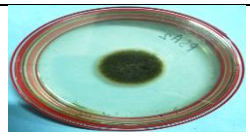
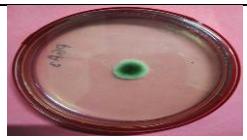
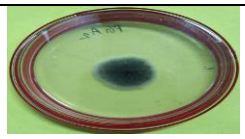
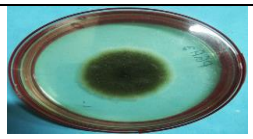
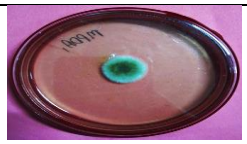
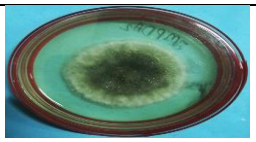
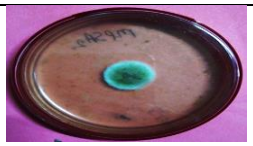
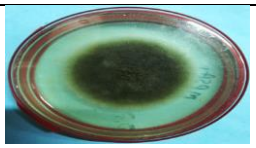
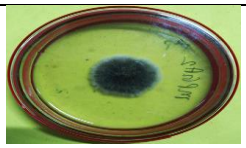
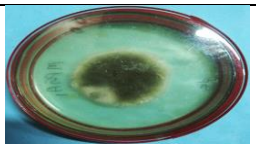
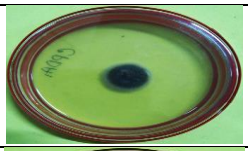
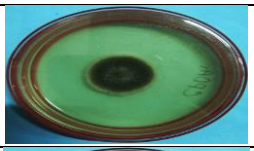
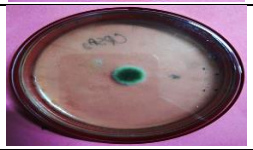
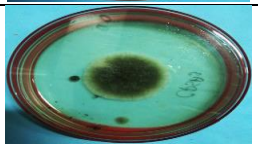
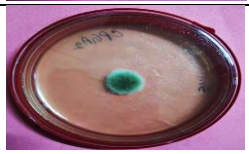
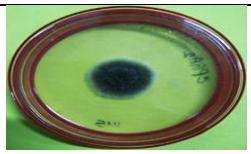
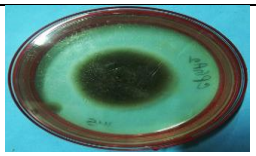
Media	3 DAI	6 DAI	9 DAI
D-PDA			
CoMA			
PDA			
PSA			
PGA			
MPDA			
MPSA			
MPGA			
CPDA			
CPSA			
CPGA			

Plate 1: Mycelial growth of *Alternaria brassicicola* on different selected media at 3, 6 and 9 consecutive DAI

4.6. Comparative study of conidia structure of *Alternaria brassicicola* growing on different selected media

In this study, the highest number of spore (9.33) was found in Mustard Potato Glucose Agar (MPGA) media which showed similarities to the Cabbage Potato Glucose Agar (CPGA) media and the lowest number of spore (2.67) was found in Cornmeal Agar (CoMA) media.

Based on septation, the highest longitudinal septation (6.33) was observed in Mustard Potato Sucrose Agar (MPSA) media which showed similarities to the Potato Sucrose Agar (PSA) media (5.67), Potato Glucose Agar (PGA) media (5.33) and Mustard Potato Glucose Agar (MPGA) media (5.67). The lowest longitudinal septation (2.67) was found on Cabbage Potato Dextrose Agar (CPDA) media which showed similarities to the Dehydrated Potato Dextrose Agar (D-PDA) media (3.33), Cornmeal Agar (CoMA) media (3.00) and Mustard Potato Dextrose Agar (MPDA) media (3.33).

The highest transverse septation (2.67) was observed on Mustard Potato Glucose Agar (MPGA) media which showed similarities to the Cabbage Potato Sucrose Agar (CPSA) media (2.33). The lowest transverse septation (1.00) was observed Potato Dextrose Agar (PDA) media which showed similarities to the Mustard Potato Dextrose Agar (MPDA) media (1.00) and Cabbage Potato Dextrose Agar (CPDA) media (1.00).

From the study, it was revealed that for better sporulation among the selected media the best performance was found in Mustard Potato Glucose Agar (MPGA) media. Results are presented in Table 4.

Table 4: Comparative study of conidia structure of *Alternaria brassicicola* on different selected media

Treatment	Number of Spore mm^2	Longitudinal Septation (No.)	Transverse Septation (No.)
D-PDA	4.33 e	3.33 d	1.00 c
CoMA	2.67 f	3.00 d	1.33 bc
PDA	4.67 e	3.67 cd	1.00 c
PSA	6.33 cd	5.67 ab	1.67 b
PGA	5.67 cd	5.33 ab	1.67 b
MPDA	4.33 e	3.33 d	1.00 c
MPSA	8.33 b	6.33 a	1.33 bc
MPGA	9.33 a	5.67 ab	2.67 a
CPDA	6.33 cd	2.67 d	1.00 c
CPSA	6.67 c	4.67 bc	2.33 a
CPGA	9.33 a	3.67 cd	1.33 bc
CV (%)	8.90	16.18	25.95

[In a column those having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of significance.]

4.7. Comparative study of spore concentration of *Alternaria brassicicola* growing in different selected media

In this study, the highest spore concentration 2.2×10^7 spore/10ml was estimated in Mustard Potato Glucose Agar (MPGA) media which showed similarities to the spore concentration of Cabbage Potato Glucose Agar (CPGA) media (2.1×10^7 spore/10ml) and the lowest spore concentration 6.5×10^6 spore/10ml was estimated in Cornmeal Agar (CoMA) media. Results are presented in Table 5 and pie chart in figure 13. Pictorial presentation of spores in selected media are presented in Plate 2.

Table 5: Spore concentration of *Alternaria brassicicola* in different selected culture media

Treatment	Spore Concentration (Spore/10 ml)
D-PDA	1×10^7
CoMA	6.5×10^6
PDA	1.2×10^7
PSA	1.4×10^7
PGA	1.4×10^7
MPDA	1×10^7
MPSA	2×10^7
MPGA	2.2×10^7
CPDA	1.5×10^7
CPSA	1.4×10^7
CPGA	2.1×10^7

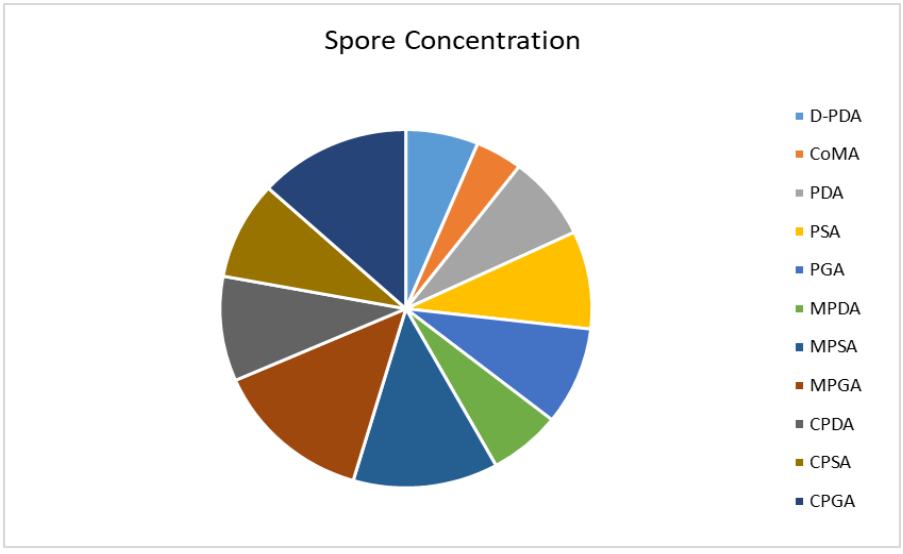
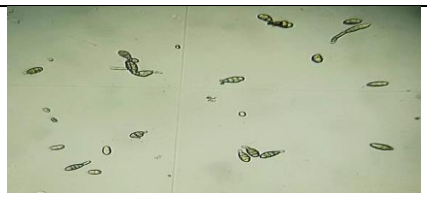
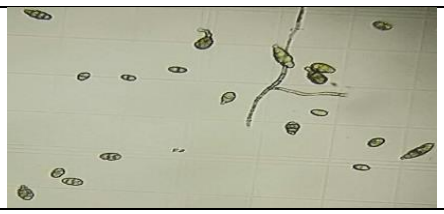


Figure 13: Spore concentration of *Alternaria brassicicola* on different selected culture media

Media	Spore
D-PDA	
CoMA	
PDA	
PSA	
PGA	
MPDA	
MPSA	
MPGA	

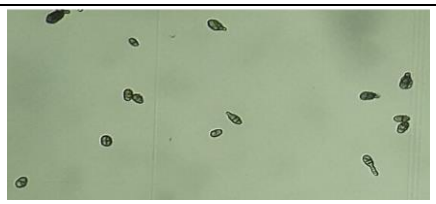
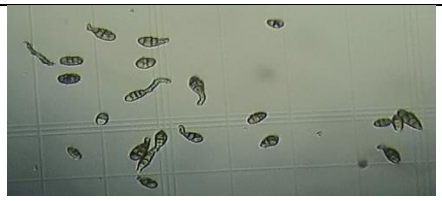
CPDA	
CPSA	
CPGA	

Plate 2: Conidia structure of *Alternaria brassicicola* in different selected media (200X)

Discussion

Mustard are rich source of oil and contains 44% to 46% good quality oil. It is widely cultivated in winter season from the month of October to February which covers 70% of the total oilseed production in Bangladesh (Rashid, 2013). This economically important crop is very prone to different diseases which deteriorates its quality. Among them grey blight of mustard caused by *Alternaria sp.* is widely distributed and the most serious disease of mustard (Fakir, 2008). This pathogen can attack from seedling to adult stage at every part of the plant in leaves, stem, siliqua/pod (Karthikeyan, *et. al.*, 2021). To manage the disease effectively, the morphological and molecular characterization of the pathogen is important. To understand the overall characters of the pathogen, and for confirmation and more authentication molecular characterization of pathogen is needed. (Afsana, 2018). In the present study, emphasizes are given to isolate and study the morphological characters in terms of mycelial growth and sporulation of *Alternaria sp.* causes grey blight disease in mustard, evaluation of effective and cheap media for isolation of this pathogen and its molecular characterization.

For isolation and morphological study of *Alternaria sp.* disease infected leaves were collected. The colony was moderately fast growing, fluffy and circular. Sporulation was good and occurred after 10 days of inoculation and it was observed under the microscope for confirmation. Based on the colony morphology and conidial characters, the isolate was identified as *Alternaria brassicicola.* The mycelia were brown in color, branched and septate, the conidia was oblong, dark in color, muriform (dictyopsporous with transverse or longitudinal) shaped with reduced beaked, multicellular which match with the findings of Yadav, *et. al.*, (2016).

Due to the systematic and taxonomic usefulness the ITS region has been used for identification of the fungi, as it is suitable size for PCR amplification, restriction analysis and sequencing procedures, and because ITS regions are variable among species as well (Jung, *et. al.*, 2002).

Molecular relationships amongst *Alternaria* species based on nucleotide sequence was performed using the BLASTn on the NCBI website for matching with existing nucleotide sequences in the NCBI genbank to identify the fungal isolate or with other related fungi have been analyzed. Searches for similarity of the nucleotide sequence of this *Alternaria* isolate from the present study was showed 99% similarity with the existing nucleotide sequences of GenBank data base. Variation in nucleotide sequences among *Alternaria* species pathogenic to crucifers has been found from this isolate each of *Alternaria longipes, A. tenuissima, A. alternate, A. arborescens, A. infectoria* and *A. brassicicola*. This molecular variation also match with one previous report, Jasalavich, *et. al.*, (1995) found that *A. brassicicola* is actually more closely related to *A. raphani* than to *A.brassicae* basedon the rDNA sequences data. *A. alternata, A. brassicae, A. brassicicola* and *A. raphani* formed clade of very closely related sister taxa. A similar study was also conducted by Michereff, *et. al.*, (2003) who studied 38 isolates of *Alternaria brassicicola* and estimates variability based on disease development and pathogen physiology and found that *A. brassicicola* isolates were highly variable. However, the study indicates that *Alternaria longipes, A. tenuissima, A. alternate, A. arborescens, A. infectoria* and *A. brassicicola*. encompasses less genetic variability. This study also indicates less genetic diversity in *Alternaria* species causing *Alternaria* leaf blight on rapeseed- mustard.

The *Alternaria isolate* was grown in 11 different selected solid media and the growth was noted at 3, 6 and 9 days after inoculation (DAI). Area Under Growth Progress Curve (AUGPC) was also calculated. It was found that at 3 DAI the highest radial mycelial growth was recorded in D-PDA media (13.33 mm) and the lowest was recorded in PDA media (5.33 mm). At 6 and 9 DAI it was found that the highest radial mycelial growth was recorded in Cornmeal Agar (CoMA) media which was 22.67 mm and 32.33 mm consecutively, the lowest was recorded in PDA media which was 10.33 mm and 14.00 mm consecutively. Though the texture of mycelia was very poor in CoMA media. The AUGPC value of Cornmeal Agar (CoMA) media was maximum (135.00) which is very close to the AUGPC value of Dehydrated Potato Dextrose Agar (D- PDA) media (128.99) and the

Potato Dextrose Agar (PDA) had the minimum AUGPC value (59.99). So based on the study, the best media selected for radial mycelial growth was Dehydrated Potato Dextrose Agar (D-PDA) media.

According to the study, based on spore count done by Hemacytometer maximum sporulation was found in Mustard Potato Glucose Agar (MPGA) media (9.33) which was similar to the result of Cabbage Potato Glucose Agar (CPGA) media (9.33) and the minimum sporulation was found in Cornmeal Agar (CoMA) media (2.67). The highest spore concentration was calculated in Mustard Potato Glucose Agar (MPGA) media (2.2×10^7 spore/L) which showed similarities to the result of Cabbage Potato Glucose Agar (CPGA) media (2.1×10^7 spore/L) and the lowest spore concentration was calculated in Cornmeal Agar (CoMA) media (6.5×10^6 spore/L). Deep *et al.* (2014) observed thirty-two isolates of *A. brassicicola* for colony color and radial growth. Colony color of *A. brassicicola* varied from olive green to dark olivacious black on PDA. Colony color, appearance, growth, margin and zonation were noted on 3 different media (Potato dextrose agar, Oatmeal agar, Host leaf extract agar) these characters are one of the important parameter deciding variability among the species. The differences in color of the colony were observed among all the ten isolates of *A. brassicae* showed variations among different media. The color of colonies varies between light brown to black on PDA, Oatmeal agar. Whereas, Host leaf extract agar showed white brown to black colony color of different isolate. The colony appearance and margin was recorded compressed to fluffy and smooth to wavy among different media for all isolates. The colony growth of *A. brassicae* was recorded medium to fast in PDA, Oatmeal agar and Host leaf extract agar. Diversity in colony color, growth, its margin and topography were noticed among the isolates of *A. brassicae* by different workers (Sharma, *et. al.*, 2013, Pramila, *et. al.*, 2014, Singh, *et. al.*, 2016, and Singh & Singh, 2018).

The isolate obtained from the collected sample was highly pathogenic in nature which was demonstrate by Pathogenicity test. The spots produced by the pathogen was >1cm in diameter and these findings are similar to Akhter, *et. al.*, (2012) where eight mustard

varieties (SAU-1, BINA-6, TORI-7, BARI-9, BARI-6, SOFOL, AGRANI and SS-75) were evaluated for their reaction against *Alternaria* blight (*Alternaria brassicicola*) under natural condition at the experimental field of Sher-e-Bangla Agricultural University, Dhaka during the winter season from November 2007 to February 2008. According to the findings of Prasad, *et. al.*, (2008) the increasing inoculum concentration significantly increased the infection of *Alternaria helianthi* in sunflower, whereas Doullah and Okazaki ,(2015) used the concentration of conidial suspension at 5×10^4 conidia /ml for inoculation of *Brassicae rapa* leaf with *Alternaria brassicicola*. In the present study, for pathogenicity test 2.2×10^7 conidia/L concentration of conidial suspension of *Alternaria brassicicola* was used and observed the effectiveness of inoculum pressure.

Chapter V

SUMMARY AND CONCLUSION

In the present study four steps were maintained sequentially to conduct the experiment. Grey blight is a highly reported disease of mustard caused by *Alternaria sp.* pathogen which is very virulent by nature. It causes high yield loss and quality deterioration in mustard, a major oilseed crop of Bangladesh.

Disease samples were collected from the central farm of Sher-e-Bangla Agricultural University and pathogen isolation followed by morphological, cultural and colonial study was done in the Molecular biology and plant virology laboratory under the department of Plant pathology, SAU. The causal agent responsible for the grey blight disease of mustard was identified as *Alternaria sp.* The mycelia of the isolate was hyaline, branched and septate. The spore of the isolate was muriform, beaked with longitudinal and transverse septation and dark colored. To study the growth and sporulation 11 different media were selected and the radial mycelial growth of the pathogen was recorded at 3, 6 and 9 DAI. After 9 days of inoculation the highest radial mycelial growth was observed in Cornmeal Agar Media (33mm) though the mycelial mass amount was very poor. The second highest radial mycelial growth was observed in Dehydrated Potato Dextrose Agar media (28mm) where the mycelial growth was very high and the mass amount of mycelia was very rich. According to the study, the best selected media for the radial mycelial growth of *Alternaria sp.* was Dehydrated Potato Dextrose Agar (D-PDA) media.

According to the study, in case of sporulation spore count was done by hemacytometer. The highest number of spore was found on Mustard Potato Glucose Agar (MPGA) media (9.33 a) which showed similarities to the Cabbage Potato Glucose Agar (CPGA) media (9.33 a). The spore concentration was found highest on Mustard Potato Glucose Agar (MPGA) media (2.2×10^7 spore/L) which showed similarities to the Cabbage Potato Glucose Agar (CPGA) media (2.1×10^7 spore/L). So to study sporulation the best selected media was Mustard Potato Glucose Agar (MPGA) and Cabbage Potato Glucose Agar (CPGA) media.

Based on septation, the highest longitudinal septation was observed on Mustard Potato Sucrose Agar (MPSA) media (6.33 a) which showed similarities to the Potato Sucrose Agar (PSA) media (5.67 ab), Potato Glucose Agar (PGA) media (5.33 ab) and Mustard Potato Glucose Agar (MPGA) media (5.67 ab). The highest transverse septation was observed on Mustard Potato Glucose Agar (MPGA) media (2.67 a) which showed similarities to the Cabbage Potato Sucrose Agar (CPSA) media (2.33 a).

The pathogenicity of *Alternaria* isolate was determined by pathogenicity test where it was found that the pathogen was highly virulent by nature and responsible for causing Grey blight disease of mustard. Molecular characterization of the pathogen was the highest level of authentication in this study. The molecular study was performed in the molecular laboratory and searches for similarity of nucleotide sequences were performed using the BLASTn on the NCBI website (https://www.ncbi.nlm.nih.gov) for matching with existing nucleotide sequences in the NCBI genbank data base to identify the fungal strain of *Alternaria sp.*

Considering the findings of the present study the following conclusions were drawn:

1. Grey blight disease of mustard caused by *Alternaria sp.* was highly depended on the environmental factors and geographical location favorable for the disease development.
2. For the *In vitro* study of growth and sporulation among the 11 different selected media the best result in case of radial mycelial growth was shown by Dehydrated Potato Dextrose Agar (D-PDA) media and in case of sporulation the best result was shown by Mustard Potato Glucose Agar (MPGA) media and Cabbage Potato Glucose Agar (CPGA) media.
3. Based on the molecular characterization the isolate was identified as *Alternaria sp.* and matches were found with the existing nucleotide sequences in the NCBI genbank data base to identify the fungal strain of *Alternaria sp.*

In this present study it can be concluded that more researches should be conducted to study the morphological, cultural, colonial and molecular characteristics of *Alternaria sp.* to control the grey blight disease of mustard successfully.

Chapter VI

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Chapter VII

Appendix

Appendix-I: Particulars of the Agro-ecological Zone of the Experimental site

Agro-ecological region: Madhupur Tract (AEZ-28).

Land Type: Medium high land.

General soil type: Non-Calcareous Dark gray floodplain soil

Soil series: Tejgaon

Topography: Up land

Location: SAU Farm, Dhaka.

Field level: Above flood level.

Drainage: Fairly good.

Firmness (consistency): Compact to friable when dry.

Appendix-II: ANOVA Table of the Laboratory Experiment

01: Radial mycelial growth (mm) at 3 days

Source of variance	Degree of freedom	Sum of squares	Mean of squares	F value	Probability
Replication	2	3.818	1.9091	5.74	0.0005
Factor	10	178.545	17.8545		
Error	20	62.182	3.1091		
Total	32	244.545			
Coefficient of Variance: 20.20 %					

02: Radial mycelial growth (mm) at 6 days

Source of variance	Degree of freedom	Sum of squares	Mean of squares	F value	Probability
Replication	2	13.879	6.9394	4.82	0.0014
Factor	10	520.970	52.0970		
Error	20	216.121	10.8061		
Total	32	750.970			
Coefficient of Variance: 20.94 %					

03: Radial mycelial growth (mm) at 9 days

Source of variance	Degree of freedom	Sum of squares	Mean of squares	F value	Probability
Replication	2	26.42	13.2121	4.36	0.0025
Factor	10	996.18	99.6182		
Error	20	456.91	22.8455		
Total	32	1479.52			
Coefficient of Variance: 21.61 %					

04: Number of conidia

Source of variance	Degree of freedom	Sum of squares	Mean of squares	F value	Probability
Replication	2	1.273	0.6364	46.06	0.0000
Factor	10	139.576	13.9576		
Error	20	6.061	0.3030		
Total	32	146.909			
Coefficient of Variance: 8.90%					

05. Longitudinal Septation of the conidia

Source of variance	Degree of freedom	Sum of squares	Mean of squares	F value	Probability
Replication	2	0.9697	0.48485	9.96	0.0000
Factor	10	48.3030	4.83030		
Error	20	9.6970	0.48485		
Total	32	58.9697			
Coefficient of Variance: 16.18%					

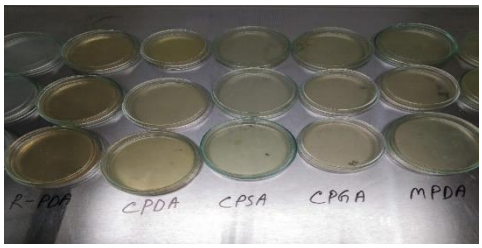
06. Transverse Septation of the conidia

Source of variance	Degree of freedom	Sum of squares	Mean of squares	F value	Probability
Replication	2	1.6970	0.84848	6.45	0.0002
Factor	10	9.5758	0.95758		
Error	20	2.9697	0.14848		
Total	32	14.2424			
Coefficient of Variance: 25.95%					

Appendix-II: Pictorial view of sample field

Appendix-III: Pictorial view of leaves used for media preparation

Appendix-IV: Pictorial view of lab experiment



Appendix-V: Pictorial view of Molecular experiment