CULTURAL, MORPHOLOGICAL, PATHOGENIC AND MOLECULAR CHARACTERIZATION OF *Alternaria porri* CAUSING PURPLE BLOTCH OF ONION

SAYED MOHAMMAD MOHSIN

DEPARTMENT OF PLANT PATHOLOGY
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
DHAKA-1207

DECEMBER, 2013

CULTURAL, MORPHOLOGICAL, PATHOGENIC AND MOLECULAR CHARACTERIZATION OF *Alternaria porri* CAUSING PURPLE BLOTCH OF ONION

BY

SAYED MOHAMMAD MOHSIN

Registration No. 07-02490

A Thesis
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
IN
PLANT PATHOLOGY

SEMESTER: JULY-DECEMBER, 2013

Approved by-

.....	.....
(Dr. Md. Rafiqul Islam)	**(Abu Noman Faruq Ahmmed)**
Professor	Assistant Professor
Department of Plant Pathology	Department of Plant Pathology
Sher-e-Bangla Agricultural University	Sher-e-Bangla Agricultural University
Supervisor	**Co-Supervisor**

.....
Prof. Dr. F. M. Aminuzzaman
Chairman
Examination Committee

Department of Plant Pathology
Sher-e-Bangla Agricultural University

Department of Plant Pathology
Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207

PABX: +88029144270-9
Fax: +88029112649
Web site: www.sau.edu.bd

CERTIFICATE

This is to certify that the thesis entitled, *"CULTURAL, MORPHOLOGICAL, PATHOGENIC AND MOLECULAR CHARACTERIZATION OF Alternaria porri CAUSING PURPLE BLOTCH OF ONION"* submitted to the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of *MASTER OF SCIENCE IN PLANT PATHOLOGY* embodies the results of a piece of bona fide research work carried out by **SAYED MOHAMMAD MOHSIN** bearing Registration No**. 07-02490** 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 been duly acknowledged.

Dated: 20 November, 2014
Place: Dhaka, Bangladesh

.....
(Dr. Md. Rafiqul Islam)
Professor
Department of Plant Pathology

Supervisor

ACKNOWLEDGEMENT

First of all I express my deep sense of gratitude to the almighty Allah-Rabbul Alamin, the most gracious who has enabled me to perform this research work and to submit the thesis successfully for the degree of Master of Science (M.S.) in Plant Pathology.

This is my greatest opportunity to convey my sincere respect to my supervisor Dr. Md. Rafiqul Islam, Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for his scholastic guidance, continuous encouragement and support during the total tenure of my research work. I am grateful to him for introducing me to the intricacies of science. He has given me the full liberty in my work, which helped me to grow the ability of working independently and widen the sphere of my thinking. He has helped me by giving suggestions for execution of my ideas in the way of my research whenever I needed them. I am really thankful to him for his competent guidance and uncompromising attitude during the thorough assessment of this manuscript.

It is my pleasure to express my deep respect to my Co-supervisor, Abu Noman Faruq Ahmmed, Assistant Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for his guidance, criticism, co-operatives to conduct the research work.

I express my indebtedness to Dr. F. M. Aminuzzaman, Professor and Chairman, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for his valuable advices, suggestions and continuous inspiration. I found him always helpful to provide the research facilities as a chairman of the department.

My deepest respect goes to Dr. M. Salahuddin M. Chowdhury, Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for his intellectual advices and academic instructions to conduct the research.

I am extremely grateful to Dr. Mohammad Nurul Islam, Professor, Dept. of Botany, University of Dhaka, for his genius guidance and providing the lab facilities to carry out the research work in the Plant Breeding and Biotechnology Laboratory.

I wishes to express my cordial respect to Professor Mrs. Nasim Akhtar, Professor Nazneen Sultana, Associate Professor Khadija Akhter, Dr. Nazmunnahar Tonu, Dr. Md. Belal Hossain, Dr. Fatema Begum, Assistant Professor Md. Tohidul Islam, Shukti Rani Chowdhury, Md. Ziaur Rahman Bhuiyan and Lecturer Hosna Ara Chowdhury Nisha,

Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for their teaching, academic advices, and encouragement during the study period.

Cordial thanks to Rita Sarah Borna, Lecturer, Department of Botany, University of Dhaka, for her friendly manner and co-operation to conduct the molecular part of the research.

I thankful to lab technician Rahana Akhter, Pritilata Sarder and Khorshed Al-Alam, laboratory staff of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for their cordial assistance during the research work.

I am grateful to Mst. Rahmat Ara and Mosammat Afruza Begum, the Ph.D. Students, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for their generous help and co-operation during the research work.

I wish to extend thanks to my friends Arif, Moudud, Refaie, Faria and Salma for their friendly co-operation and inspiration during the study period.

Finally, I recall indebtedness to my beloved parents and the family members for their boundless affection and well wishes during the whole study period.

The Author

CULTURAL, MORPHOLOGICAL, PATHOGENIC AND MOLECULAR CHARACTERIZATION OF *Alternaria porri* CAUSING PURPLE BLOTCH OF ONION

ABSTRACT

Purple blotch of onion (*Alternaria porri)* causes severe foliar damage to onion plants. Twenty seven (27) isolates of *Alternaria porri* were isolated from diseased samples collected from different onion growing regions of Bangladesh and studied their cultural, morphological, pathogenic and molecular characterization. Colony color varied between light to dark olivacious and grayish white. Shapes of colonies were irregular, regular with and without concentric ring. Margin of colonies were entire, irregular and wavy with effuse, fluffy and velvety texture. Isolates impregnated media with color ranging between grey to brown. Growth rate of isolates was between 2.43 to 3.95 mm/day with fast growth in isolate DSTR 02 and least in MMBH. Morphological variation in conidia production was between 7.720×10^3 to 47.02×10^3 per mm^2 with sporulation time 3.33 to 11.00 days. The conidial shape was straight to curve with light to deep brown color. Conidial septation ranged from 3.00 to 6.00 × 1.00 to 2.00 with size from 11.20 to 39.20 × 4.76 to 11.43 µm. Isolates shown variations in lesions size exhibited the pathogenic variation exist in the environment. In molecular study no band were found by using seven (7) RAPD primers in polymerase chain reaction (PCR) but band found by using ITS primer ITS1F and ITS4 from three isolates (DSSA, DSTR 02 and RBHR 02). Band producing isolates DSSA and RBHR 02 were explored for sequencing. The sequenced designated sample A.DSSA found 75% similarities with *Alternaria porri* genomic DNA (LN482533.1), *Alternaria alternata* partial sequence (KF269242.1), *Alternaria tenuissima* partial sequence (KM513592.1) and 74% similarities with *Alternaria mali* partial sequence (JF817299.1). Another sample A.RBHR 02 found 86% similarities with *Alternaria* sp. UFMGCB 4425 fungus complete genome (KJ404206.1) and 81% similarities with *Alternaria tenuissima* HLJ-KS-BH-TY-A (KF996744.1). The results demonstrated the existence of considerable variation in cultural, morphological, pathogenic and molecular characters of *Alternaria porri* isolates prevalent in Bangladesh environment.

CHAPTER	TITLE	PAGE NO.

CONTENTS

CONTENTS (cont'd)

CHAPTER	TITLE	PAGE NO.

CONTENTS (cont'd)

CHAPTER	TITLE	PAGE

CONTENTS (cont'd)

CONTENTS (cont'd)

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF SYMBOLS AND ABBREVIATIONS

% = Percentage

et al.	=	And others
spp.	=	Species
J.	=	Journal
No.	=	Number
viz.	=	Namely
df.	=	Degrees of freedom
&	=	And
etc.	=	Etcetera
^{0}C	=	Degree Celsius
@	=	At the rate of
cm	=	Centimeter
cfu	=	Colony forming unit
ppm	=	Parts per million
NaCl	=	Sodium chloride
Kg	=	Kilogram
g	=	Gram
ml	=	Milliliter
WP	=	Wettable Powder
hr	=	Hour (s)
cv.	=	Cultivar (s)
i.e.	=	That is
T	=	Treatment
ft	=	Feet (s)
pv.	=	Pathovar
var.	=	Variety
mm	=	Milimiter
µl	=	Microliter
µm	=	Micrometer

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

SAU	=	Sher-e-Bangla Agricultural University
BAU	=	Bangladesh Agricultural University
BARI	=	Bangladesh Agricultural Research Institute
BBS	=	Bangladesh Bureau of Statistics
FAO	=	Food and Agriculture Organization
AIS	=	Association for Information Systems
ISTA	=	International Seed Testing Association
USA	=	United State of America
UK	=	United Kingdom
DAE	=	Department of Agricultural Extension
PDA	=	Potato Dextrose Agar (media)
DAP	=	Days after Planting
NUV	=	Nearly Ultra Violet
CRD	=	Completely Randomized Design
ANOVA	=	Analysis of variances
LSD	=	Least Significant Difference
DMRT	=	Dunkan’s Multiple Range Test
CV%	=	Percentages of Co-efficient of Variance
RAPD	=	Randomly Amplified Polymorphic DNA
DNA	=	Deoxyribonucleic acid
RFLP	=	Restriction Fragment Length Polymorphism
AFLP	=	Amplified Fragment Length Polymorphism
PCR	=	Polymerase Chain Reaction
AP-PCR	=	Arbitrary Primed PCR
UP-PCR	=	Universally Primed PCR
rDNA	=	Ribosomal DNA
ITS	=	Internal Transcribed Spacer
CTAB	=	Cetyl Trimethylammonium Bromide
TAE	=	Tris Acetate EDTA

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

TE	=	Tris EDTA
EDTA	=	Ethylene Di-amino Tetra Acetic Acid
DNTP	=	Deoxinucleotide Tri-phosphate
NCBI	=	National Centre for Biotechnology Information
CARS	=	Centre for Advanced Research in Sciences

CHAPTER I

INTRODUCTION

Onion (*Allium cepa*) is an important spices crop commercially grown in many countries of the world. It is used as important and popular vegetables in Australia, Belgium, India, Japan, United Kingdom, USA and many other countries. Out of 15 important vegetables and spice crops listed by FAO, onion stands second in terms of annual world production (Anon. 1997). It ranks first in production among the spices crops cultivated in Bangladesh. It belongs to the family Alliaceae. It grows extensively during winter season in Bangladesh but at present it also grows in the summer season. Spices are important constituents of food items. A good number of spices crops are grown in Bangladesh. The major ones are Onion, Garlic, Zinger, Turmeric, Coriander, Chili, etc. Onion has manifold uses; as spice, vegetable, salad, dressing etc. It is also used as condiments for flavoring a number of foods and medicines (Vohora *et al*., 1974; Hassan *et al*., 2006). Recently, bunching onion (*Allium fistulatum*) is coming up as a popular vegetable too. It does not form bulbs but grows in clusters with long white stems (Benoit and Ceustermans, 1987). Global vegetable production of nearly 36 million tons onion per annum, next to tomatoes and cabbages bears importance (FAO, 2012). The

production of onion is nearly 11.59 lac metric tons from 335000 acres of land (BBS, 2012). Our requirement of onion per year is around 19.5 lac metric tons per year (BBS, 2012).

Among the onion producing countries of the world, Korea Republic tops the list with 65.25 t/ha onion production followed by USA (53.91 t/ha), Spain (52.06 t/ha), Japan (47.55 t/ha) (FAO, 2008). The production of onion in Bangladesh is 8.95 t/ha (AIS, 2011) which is very lower in comparison of other onion producing countries.

The major onion growing areas of the country are Faridpur, Comilla, Manikganj, Dinajpur, Jessore, Pabna, Rajshahi, Mymensingh, Jamalpur, Patuakhali, Kishorganj, Tangail, Borishal, Bandarban, Khagrachari, Sylhet, Bogra, Rangamati, Kustia, Dhaka, Chittagong, and Rangpur. The highest yield (208935 metric ton) was in Faridpur in 78695 acre of land (BBS, 2007).

The local varieties namely Faridpuri and Taherpuri are commonly grown in Bangladesh. The demand of bulb onion as well as the onion seeds is increasing every year in Bangladesh and the price of the true seed remains fairly high in each season.

Several constraints affect the onion production in our country. The use of low quality seeds, imbalanced fertilizers, improper irrigation and attack of various insect-pests and diseases are the reason to lower the production. In the world, onion is attacked by 66 diseases including 10 bacterial, 38 fungal, 6 nemic, 3 viral, 1 mycoplasmal, 1 parasitic plant and 7 miscellaneous diseases and disorders (Schwartz and Mohan, 2008; Schwartz, 2010).

Onions are attacked by ten diseases in Bangladesh caused by various pathogens (Ahmed and Hossain, 1985; Bose and Som, 1986). Most of the disease caused by the fungi and among the fungal diseases, the most important and damaging one purple blotch that is causes seed rot, germination failure, black mould, and white rot. Purple blotch of onion is noted as a major disease throughout the world including Bangladesh (Islam *et al.*, 2001; Ahmed and Hossain, 1985; Meah and

Khan, 1987; Bose and Som, 1986 and Castellanos-Linares *et al*., 1988). In India purple blotch of onion is considered as a major, devastating and widespread disease and causes serious yield reduction (Ahmed and Goyal, 1988). The disease is also a threat for seed production of onion (Gupta *et al*., 1986; Rahman *et al*. 1988 and Yazawa, 1993).

In Bangladesh the common diseases of onion are purple leaf blotch (*Alternaria porri*), stemphylium blight (*Stemphylium vesicarium)*, downey mildew (*Peronospora destructor*), basal/stem rot (*Fusarium* sp.; *Sclerotium* sp.; *Rhizoctonia* sp.), and damping off that may damage the crop and reduce the yield upto 100% (Brewster and James, 2008). Purple leaf blotch is caused by *Alternaria porri* and prevalent in all the onion growing areas. The fungus can cause a reduction in yield ranged from 30 to 50 % (Pascua *et al*. 1997). Bulb and seed yields of onions significantly reduce by purple blotch (Gupta and Pathak, 1988a). About 20 to 25% seed yield reduction has been recorded in India (Thind and Jhooty, 1982) and 41 to 44% in Bangladesh (Hossain *et al.*, 1993).

A lot of research works have been conducted on the management of purple blotch of onion (Ashrafuzzaman and Ahmed, 1976 and Rahman *et al.*, 1988 and Rahman, 1990). Rovral 50wp (0.2%), Dithane M-45 (0.2) and some other options are suggested as foliar spraying against the disease. But now a days the fungicides are not working properly against the disease. This might be due to the genetic variability or introduction of new races of the pathogen. But no initiative had yet been taken to determine the genetic variability of *Alternaria porri*, if any in the country. As the onion growers are being discouraged for onion cultivation due to the severe losses of the disease, the problem less need to give urgent attention.

One of the most important aspects of biology of an organism is the morphological and physiological characters of an individual within a species. 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. The variability is a well known phenomenon in genus

Alternaria and may be noticed as changes in spore shape and size, growth and sporulation, pathogenicity, etc. Diversity appears even in single spore isolates.

Understanding of pathogen population structures and mechanisms by which variation arises within a population is of paramount importance for devising a successful disease management strategy. This requires continuous monitoring of the development of pathogen variability for the breeding programme aimed at developing resistant genotypes to the given set of pathogenic races (Sartorato, 2002). Variation in pathogen populations can generally be detected with methods like morphological, cultural, pathogenic and molecular specificity. DNA markers have become a powerful tool to study taxonomy and molecular genetics of a variety of organisms. The random amplified polymorphic DNA (RAPD) allows quick assessment of genetic variability, and has been used to study inter- and intra-specifi variability amongst the isolates of several fungal species.

Keeping all these facts in mind the present study was undertaken to achieve the following objectives:

1. To collect the diseased specimen of onion from the major onion growing areas of Bangladesh.
2. To isolate, identify and characterize of *Alternaria porri* isolates.
3. To determinate the genetic variability of *Alternaria porri* isolates through molecular characterization (marker + sequencing).

CHAPTER II
REVIEW OF LITERATURE

Onion is one of the most important spice crops, which received much attention of the researchers throughout the world. Purple blotch of onion, incited by *Alternaria porri* is the most important disease of onion in Bangladesh and all over the onion growing region of the world. It causes serious yield reduction of the crop. The researshers in various parts of the world have worked on the purple blotch of onion caused by *Alternaria porri*. In Bangladesh very few works have been done in this respect. The available information in this conection over the world has been reviewed in this chapter.

2.1. Symptom of purple blotch of onion

Nuchnart Jonglaekha *et al.* (1982) observed that symptoms of purple blotch disease appearing on onion, shallat, multiphir onion, leek and garlic were similar except that the levels of susceptibility were different. They also observed that most of the conidia produce germ tubes and penetrate through wounds on leaves within 8 hrs. of inoculation. The conidia observed were club-shaped with transverse and longitudinal septa. This fungus produces spores when the temperature lies between 18-26° C.

Basallotte-Ureba *et al.*, (1999) reported that early symptoms appear on the older leaves as white flecks. Under suitable environmental conditions the white flecks expand and produce sunken purple lesions that are often elliptical with a yellow to pale-brown border.

2.2. Varietal resistance

Alves *et al.* (1983) studied the incidence of purple spot *(Alternaria porri* Ell. Cif.) on onion cultivars and hybrids in Manaus, Amazonia. Plants were divided into five classes on the basis of natural infection in the field. Incidence was 30-50% (class III) in most cases; only the hybrid Px76 having plants in class I (0-10%).

Gupta and Pathak (1988) studied 21 indigenous and exotic cultivars screened at 2 locations in India under artificial inoculations. All the exotic lines, except 2 from

the Sudan, were highly resistant to *Alternaria porri* while all the indigenous lines were susceptible. It is suggested that susceptible cultivars should be replaced by the resistant Pusa Red.

Bhonde *et al.* (1992) conducted a field trial during 1987-88 on 8 onion cultivars (Agrifound Light Red, Arka Niketan, L-102-1, Nasik Red and Pusa Red, Agrifound Dark Red, Arka Kalyan and Kharif Local). Agrifound Light Red had a good yield, and had the highest DM content and the lowest incidence and intensity of purple blotch in all cultivars.

Perez-Moreno *et al.* (1992) evaluated three commercial onion cultivars (Geminis, Blanca and Conjumatlan Morada), two hybrids (PVM-7 and PVM-3). Conjumatlan Blanca was the most susceptible cultivar, compared with the most tolerant hybrid PVM- 7. Highest economic yield was obtained with Geminis and the lowest with PVM- 3.

Sharma (1997) studied onion genotypes grown in Himachal Pradesh, India, for resistance to *Alternaria porri* during 1991-92. The lines IC48059, IC48179, IC39887, IC48025 and ALR found resistant and another 10 lines were moderately resistant.

Kibria (2010) reported that BARI piaz-3 gave lowest disease incidence and highest yield (12.67 t/ha) against purple blotch of onion (*Alternaria porri*) among nine onion cultivars viz. BARI piaz-1, BARI piaz-2, BARI piaz-3, Thakurgong local, Foridpur local, Manikgong local, Indian big, Indian small and Taherpuri. In case of disease reaction 8.00% observed in BARI piaz-3 and was graded as resistant.

Thirumalachar *et al.* (1953) reported about the existence of some varietal resistance and they stated that the fungus *Alternaria porri* (purple blotch) caused severe scorching of some onion varieties at the College of Agriculture Sabour; but the indigenous red variety had remained uninfected.

Shandhu *et al*. (1982) reported that none of 102 genotypes they screened was resistant to *Alternaria porri*. However, they could locate 12 genotypes which

showed moderate resistance reaction. The genotypes that had flat erect leaves showed moderately resistance reaction. Whereas all those with curved, drooping leaves were susceptible.

Gupta and Pathak, (1986) while working on sixty-days-old onion plants founded cv. Nasik Red was most susceptible cultivar to the purple blotch pathogen *(Alternaria porri)*. Plants inoculated at high RH (100%) for 120 hours resulted in maximum disease severity and shortest incubation period.

Ariosa-Terry and Herrera-Isla (1986) measured the damage of onion due to purple blotch caused by *A. porri*. The first symptoms appeared 50 days after sowing and disease intensity was the highest at 110 days. White onions were more affected than red onions.

2.3. Growth of *Alternaria porri in-vitro* and *in-vivo*

A study on the sporulation of *Alternaria porri* was conducted by Khare and Nema (1981). They observed maximum sporulation at 8-00 a.m. under field condition. A seasonal periodicity was also noted, indicating maximum sporulation immediately after rains. Under laboratory conditions maximum sporulation was at 22^0 C at 90% RH followed by 30^0 C. They also reported that temperature, humidity and nutrients seemed to play an important role for ensuring infection of *A. porri* on onion. Cent percent spore germination occurred *in vitro* within 4 hours at 22^0 C, while maximum germination was recorded within 6 hrs at 25^0 C on the host surface. Temperature lies between 22 to 25^0C is the best for development of onion leaf blotch disease.

Raju and Mehta (1982) demonstrated an experiment on certain nutritional aspects of *Alternaria porri* (Ellis) Ciferri on onion in *in vitro* and summarized that potato dextrose agar having pH 6.0 was best to culture the fungus. Temperature ranged from 22-25^0 C was found optimum for mycelial growth and sporulation of *Alternaria porri*.

Everts and Lacy (1990) examined formation of conidia by *Alternaria porri* under variable dew duration and controlled relative humidity (RH). Viable conidia

produced on lesions after 9 hrs of dew to 38 hrs and conidia formed during 16 hrs of dew duration caused typical lesions. Conidia were formed at all RHs tested (75-100%); numbers were very low at 75-85% RH but increased with increasing RH. Conidia formed on lesions on senescent leaves when incubated in dew chamber at 25^0 C and conidia formed repeatedly (up to eight cycles) on lesions to alternating low RH (35-50%) and high (100%) RH. The intensity and dynamics of *Alternaria porri* conidial germination were studied by Rodriguez *et al.* (1994) in different temperatures (5-40^0 C) and RH (76-100%). Conidia developed at 5-37.5^0 C, with an optimum temperature of 30^0 C. Germination started within 1 hr of incubation at 20-35^0 C and 50% of the conidia had germinated at 4 hrs of incubation.

Everts and lacy (1996) studied the factors influencing infection of onion leaves by *Alternaria porri* and subsequent lesion expansion. Conidia deposited on onion leaves formed single to several germ tubes and appressoria and often penetrated at more than one locus under conditions favorable. After 3 hrs in the dew chamber at 24^0 C following inoculation of onion leaves, 73% of conidia had germinated and 5% had formed appressoria. Infection hyphae were not observed until 6 h following inoculation, at which time 2% of conidia had formed infection hyphae and 0.5% of conidia had caused visible lesions. Length of dew period was significantly and positively correlated with lesion numbers but not with lesion size.

2.4. Epidemiology and its management

Srivastava *et al.* (1994) reported the high incidence (2.5 - 87.8%) of purple blotch (*Alternaria porri*) in both the kharif and rabi onions, when high humidity prevailed, during the 5 years of the survey (1988-93).

Miller (1983) reported that measurements of infected leaves were taken weekly from bulb initiation to bulb maturity. They observed that the leaf damage levels were significantly lowered on younger than older leaves. Leaves emerging 9, 8, 7, 6 and 5 week before bulbing maturity required $5^1/_2$, 5, $4^1/_2$, $3^1/_2$ and $2^1/_2$ weeks respectively to reach 50% damage.

Khare and Nema (1984) conducted an experiment to determine the effect of temperature and humidity of the development of symptoms of purple blotch of onion incited by *Alternaria porri* and noted that temperature between 22^0 C to 25^0 C and relative humidity 90% are the best for the development of leaf blotch symptom.

Gupta and Pathak (1988b) reported that bulb and seed yields and 1000 seed weight of Nashik Red onion were significantly reduced by *Alternaria porri* infection. Disease severity was computed in terms of the co-efficient of disease index (Codex). A linear relationship was found between yield and Codex.

Srivastava *et al*. (1996) conducted in vitro studies to determine the role of infected plant debris and soil in the perpetuation of disease and air borne spore of purple blotch (*Alternaria porri*) and Stemphylium blight (*S. vesicarium*) on onions in Haryana, India, in order to establish a forecasting system for effective control measures. The pathogens remained viable for 4 months on diseased plant debris, 3 months at soil in depths of 2.5, 5.0 and 7.5 cm and for 2 months at soil in depths of 10.0 and 15.0 cm. It was suggested that the inoculum load of *Alternaria porri* and *Stemphylium vesicarium* during ploughing of infected soil was higher during the winter.

Lakra (1999) found numerous purple spots / blotchs on older leaves and scapes when fortnightly dew fall was >1.0 mm, mean maximum relative humidity > 75% and mean maximum temperature 20-30^0 C with > 18 hr favourable temperature (10-30) duration. Exposure of leaf and/or scape to wetness for 8 hr was a prerequisite for conidial germination with increasing disease intensity, every tield component was adversely affected; the most severe infection reduced the number of scapes/plant, the height of scape, the number of umblets/umbel, the number of seeds/umblet, 1000-grain weight, number of seeds/plant and the seed yield/plant by 28.7, 74.5, 89.9, 41.7, 35.7, 95.7 and 97.3% respectively compared with healthy plants.

2.5. Characterization of *Alternaria porri*

2.5.1. Cultural and morphological characterization

Fanceli and Kimati (1991) conducted an experiment in Brazil to determine the influence of culture media and light on the sporulation of *Alternaria dauci*. They noted that Czapek's and host leaf extract medium yielded better sporulation of the fungus compared to other tested media.

Nuchart-Joglaekha *et al*. (1982) observed that most of the conidia produced germ tubes and penetrated wounds on leaves within 8 hours after inoculation. The conidia were club shaped with cross and longitudinal septa. This fungus produces spores when the temperature lies between 18 -26^0 C.

Ramegowda and Naik (2008) reported that the hyphal width of 9 *Alternaria macrospora* isolates varied from 2.87 to 6.95 µm.

Tetarwal *et al.* (2008) found conidiophores length and breadth among six isolates of *Alternaria alternata* varying from 18.90 to 27.40 and 4.23 to 5.75 µm, respectively.

Rotem (1966) found a wide variability in the spore dimensions of 42 isolates of *Alternaria solani*.

Simmons (1995, 1999a, b) reported that conidia produced by fungi in the *A. alternata* species group are 20-50 µm long, while conidia produced by the *A. porri* species group are generally more than 100 µm long.

Humperson-Jones and Phelps (1989) reported that conidiophores of majority of the species of *Alternaria* produce asexual spores (conidia) measuring between 160-200 µm long. Under in vitro conditions, sporulation occurs at a temperature range of 8-24 ºC, where mature spores occur after 14-24 h. Optimum temperatures are between 16 and 24 ºC where sporulation time ranges from 12 to 14 h. Moisture in the presence of rain, dew or high humidity are essential for infection and a minimum of 9-18 h are required for majority of the species.

2.5.2. Pathogenic characterization

Thrall *et al.* (2005) reported significant variations in the lesion size produced by *Alternaria brassicicola* isolates on wound inoculated *Cakile maritime* plants.

Kumar (2004) reported variation in lesion size and lesion number in *Alternaria triticina* isolates.

Quayyum *et al.* (2005) reported that he did not find any significant variation in the lesions produced by the isolates of *Alternaria panax* on detached leaflets of ginseng.

Shahzad (2003) reported the prevalence of *Alternaria* leaf blotch in all the districts of Kashmir with variable disease incidence and intensity. He also observed variation in susceptibility amongst different apple cultivars and attributed it to many factors including pathogenic variability.

2.5.3. Molecular characterization

Based on variation in the base sequence of nucleic acids, different methods have been developed and different approaches have been pursued to characterize remotely related fungi, as well as distinguishing between closely related strains. A range of methods include guanine-cytosine (GC) content of deoxyribonucleic acid (DNA), DNA-DNA hybridization, the analysis of restriction fragment length polymorphism (RFLP), the polymerase chain reaction (PCR), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and DNA sequencing.

Polymerase chain reaction has been considered as an important technique in molecular biology for many years. It was devised and named by Mullis and colleagues at Centus Corporation (Mullis and Faloona, 1987). The principle had been described in detail by Khorana and colleagues (Panet and Khorana, 1974) over a decade earlier. The use of PCR was limited until heat stable deoxyribonucleic acid (DNA) polymerase (Chien *et al*., 1976) became widely available. The PCR is a major development in the analysis of DNA and ribonucleic acid (RNA) because it has both simplified existing technology and enables the rapid development of new techniques that would not otherwise have been possible.

The diagnosis of disease by visual observation is extremely different and often inaccurate, particularly at the early stages of lesion development when appropriate disease control measures must be implemented. The internal transcribed spacer regions of ribosomal DNA have been used successfully to generate specific primers capable of differentiating many closely related fungal species (Mazzola *et al.*, 1996; Prasad *et al.*, 1996).

The potential use of PCR in detection and differentiation of plant pathogenic fungi has been realized in 1990s (Schesser *et al.*, 1991). The application of the PCR technique has radically changed the ability to isolate and analyze gene sequences.

Polymerase chain reaction can make a copy of one or a few target DNAs and amplify large amounts of DNA in a few hours by the use of a thermo-stable DNA polymerase (Saiki *et al.*, 1988). One of the first uses of PCR in fungi is to amplify ribosomal DNA sequences for determination of phylogenetic relationship (White *et al.*, 1990). PCR is used in many other areas of mycology including detection and identification of fungi (Foster *et al.* 1993; Johanson, 1994). Specifically, it has been used to analyze the genetic variation among isolates of *Pyrenophora* and *Fusarium* spp. (Blakeore *et al.*, 1994).

The PCR has recently been exploited to resolve genetic differences of clones among related organisms using small samples in a relatively short time (Saiki *et al*., 1985; Erlich 1989). Electrophoresis through agarose gels is the standard method for the separation, identification and purification of DNA and RNA fragments ranging in size from a few hundred to 20 Kb. It is simple, rapid perform and capable of resolving DNA fragments that cannot be separated adequately by other procedures such as density gradient centrifugation. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of intercalating fluorescent ethidium bromide dye under ultraviolet light. If necessarily, these bands of DNA can be recovered from the gel and used for a variety of cloning purpose (Chawla, 2002).

Sequencing of DNA is often conducted on internal transcribed spacer (ITS) of ribosomal DNA (rDNA) regions which exhibit a high degree of sequence divergence within the region, but which are flanked by conserved genes allowing for easy PCR amplification using primer sites within this conserved region. As the region is small enough, it can be sequenced for completion on a single gel run. Alignment of ITS sequence data of various fungi has proved to be invaluable for the study of specific and intra-specific variation (Mills, 1994).

For standard applications a primer concentration between 0.1-1µM is recommended (Rolfs *et al.*, 1992), and rarely the primers are completely used up during the reaction. Nevertheless, the primers have to complete with the accumulating product in finding their target sequence, which could become limiting for the reaction at late cycles.

Molecular methods involving the use of PCR have been recently described to resolve genetic variation between strains. One strategy is the use of defined PCR amplified fragments as substrate for RFLP or sequence analysis. Ribosomal DNA regions have often been chosen for taxonomic and polygenetic studies because sequence data are available and because they contain both variable conserved regions, allowing discrimination at the genus, species or intra-specific level. In fungi as well as other organisms, the non-coding regions of ribosomal DNA (rDNA) have been used as variable regions (Edel *et al*., 1995). The ITS of the rDNA can display variation within the genera and are used in the differentiation of species (Carbone and Kohn, 1993).

Polymerase chain reaction can be used to measure or analyse genetic variation by the so-called PCR fingerprinting techniques, random amplified polymorphic DNA (RAPD) (Fujimori and Okuda, 1994), arbitrary primed PCR (AP-PCR) (Williams *et al*., 1990; Micheli and Bova, 1997) and universally primed PCR (UP-PCR) (Bulat *et al*., 1994). Common for these techniques is that a single primer can be used under conditions that favor generation of numerous amplification products. This can be resolved by gel electrophoresis to obtain the fingerprints. One of the

advantages of the UP-PCR technique is the ability to distinguish among very similar strains of an organism because of the reliable generation of abundant (60-80) bands, including weak bands (Lubeck, 1997). The UP-PCR technique has been used for analysis of the genetic relatedness of *Trichoderma* and *Gliocladium* (Bulat *et al.*, 1998). This technique has also been used to identify fungal species in the genus *Clonostachys rosea* (Bulat *et al.*, 2000), *Trichoderma* (Cumagun *et al.*, 2000; Lubeck *et al.*, 2000), *Botrytis aclada* (Nielsen *et al.*, 2001), anastomosis groups of *Rhizoctonia solani* (Lubeck and Poulsen, 2001; Ali, 2002; Ali *et al.*, 2004) and *Pyricularia grisea* (Mian *et al.*, 2002 and 2003).

The reproducibility of the UP-PCR method has been demonstrated in *Trichoderma harzianum* (Lubeck *et al.*, 1999). Identical profiles of the same DNA has been obtained using different thermocyclers, different buffer compositions, and different polymerase and performing parallel experiments in different laboratories. This is in contrast to the well known difficulties with reproducibility of RAPD fingerprinting (Bulat *et al.*, 1994; Karp *et al.*, 1997; McDonald, 1997).

Porebski *et al*. (1997), Barnwell *et al*. (1998) and Csaikl *et al*. (1998) reported the most widely used method for avoiding co-precipitation of polysaccharides and DNA to keep the polysaccharides in solution at the DNA precipitation step, usually by using cationic hexadecyl trimethylammonium bromide (CTAB) as a selective precipitant of nucleic acids.

Barnwell *et al*. (1998) reported the presence of the polysaccharides prevents complete reddish solution of the DNA and access of enzymes to the DNA by inhibiting processes such as cutting with restriction enzymes, PCR or *in vitro* labeling.

DNA based molecular markers that are selectively neutral and randomly distributed in a genome are important tools in studies on genetic diversity of pathogen populations (Dini-Andreote *et al*., 2009).

RAPD analysis is extremely robust and can separate individuals having intra- and inter specific variability. It gives more comprehensive information regarding

genetic variability in pathogen populations as it is based on the entire genome of an organism (Achenback *et al.*, 1997).

Kumar *et al.* (2008) selected four random primers to study the genetic diversity within isolates of *Alternaria solani* because of their reproducible results of polymorphism between individuals.

Guo *et al.* (2004) reported that high genetic variation within *Alternaria alternata* isolates originating from *Pinus tabulaeformis* and *Alternaria alternate* appears to have the potential for relatively quick evolution which may lead to significant diversification.

Pusz (2009) reported that low genetic variability among the isolates of *Alternaria alternata* originating from *Amaranthus*; however, he stated that the low genetic variation may be as a result of high adaptive ability of fungus and due to the close proximity between collection sites and host plants.

Van der Waals *et al.* (2004) showed high genetic diversity among *Alternaria solani* isolates from South Africa and found no clustering of isolates according to geographical origin.

Kumar *et al.* (2008) also found no effect of origin of isolates; rather two isolates of *Alternaria solani* from two different locations were closer to each other.

Kumar *et al.* (2008) reported that groupings based on RAPD data could not be correlated with the ones based on cultural, morphology and pathogenicity.

Peever *et al.* (2002) also did not find any correlation between RAPD clustering of *Alternaria* isolates and pathogenicity.

Stackman *et al.* (1981) pointed out biological forms as threat in case of stem rust of wheat, similarly biological forms of *Alternaria mali* may pose a serious problem to breeding for resistance to *Alternaria* leaf blotch of apple.

CHAPTER III
MATERIALS AND METHODS

The experiment was conducted to determine the cultural, morphological, pathogenic and molecular variability of twenty seven isolates of *Alternaria porri* collected from major onion growing areas of Bangladesh.

3.1. Experimental period

The experiment was conducted during the period of February 2013 to July 2014.

3.2. Laboratory experiment

The experiment was conducted at the Molecular Plant Pathology Laboratory of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, and in the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka.

3.3. Collection of leaf diseased samples

Onion leaves having typical leaf spot symptoms (plate 1) were collected from 9 districts namely Dhaka, Mymensingh, Rajshahi, Gazipur, Comilla, Jamalpur, Manikgonj, Jessore, and Faridpur (Appendix I). The diseased leaves were cut from the plants grown in the field and put into a brown paper envelope. Then the brown paper envelopes of each collection were taken to the laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for isolation.

3.4. Isolation and identification of the pathogen

The pathogen was isolated by tissue planting method (Hasan, 2008). The surface of the working clean bench was sterilized with ethanol (70%). Then the infected onion samples were taken into the clean bench and cut into small pieces (0.5-1.0cm) the cut pieces were sterilized in $HgCl_2$ solution (1:1000) for 1 and half minutes and then taken out with the help of sterile forceps and put on sterile distilled water to wash the samples and washing was repeated 3 times.

After washing, the cut pieces were placed on sterilized blotter paper (Whatman No. 1) in petriplates and also placed onto the PDA plates, incubated at 25^0C under near ultraviolet light following ISTA rules (ISTA, 1996). Five to seven days after

incubation the fungal culture were studied under stereoscope (Model: Motic, SMZ-168) and compound microscope (Model: Omano, OMTM-85) for identification of the desired pathogen.

3.5. Preparation of culture medium and culture plates

Potato Dextrose Agar (PDA) was used for these experiments. The dehydrated PDA was hydrated in distilled water @ $39gL^{-1}$ in a conical flask (250ml) and autoclaved at 121^0C under 15 psi for 30 minutes. After autoclaved the media was kept few minutes for cool and added 25-30 drops of lactic acid then poured into sterile petriplates.

3.6. Purification and preservation of *Alternaria porri*

After identification of *Alternaria porri* it was purified for further study. The axenic culture of the isolates from the PDA were transferred to PDA slants using hyphal tip culture method and preserved in refrigerator at 4 ± 0.5^0C for further use.

3.7. Designation of collected isolates

The collected isolates were designated following Aminuzzaman *et al.* (2010) based on its locations and sources (Table 1). For example an isolate designated by JMSA 01 represents that this isolate was collected from district- Jessore (J), upazilla- Monirumpur (M), union- Shempur (S), village- Aminpur (A) and 01 denotes collection number.

Plate 1 Collected leaf sample showing typical symptom of purple blotch of onion

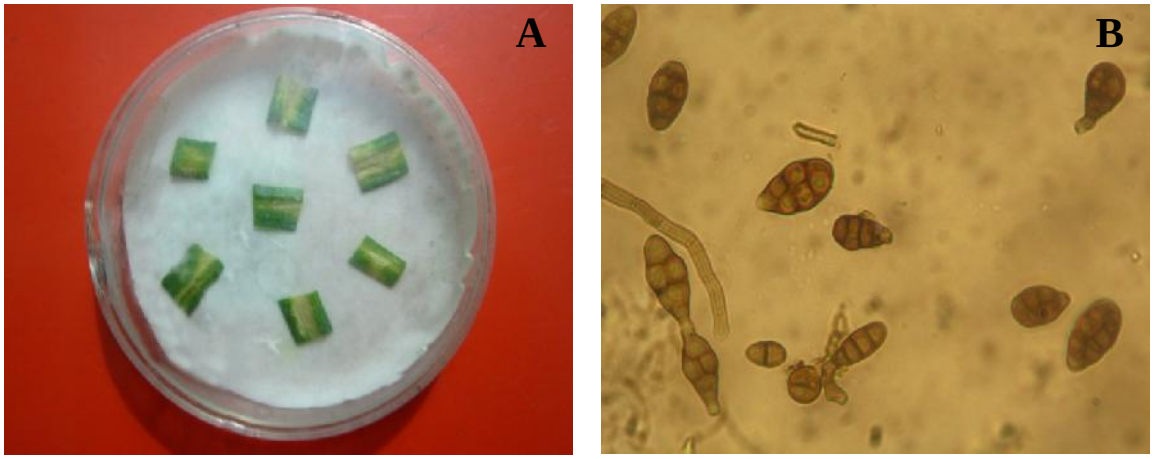


Plate 2 Isolation and identification of *Alternaria porri*, (A) Isolaion by blotter method (B) *Alternaria porri* conidia under compound microscope (400x)

Table 1 Sample collected area of purple blotch of onion caused by *Alternaria porri*

Sl.	Isolates	Sample collected region

No		District/ Region	Upazila/ Thana	Union/ Organization	Village/ Place
1	DSSA	Dhaka (SAU, Savar)	Sher-e-Bangla Nagar	SAU	Agronomy Field
2	DSTR 01		Savar	Tetuljhora	Rajfulbaria
3	DSTR 02				
4	MMBH	Mymensingh (BAU, Trishal)	Mymensingh Sadar	BAU	Horticulture Field
5	MTBB 01		Trishal	Balipara	Balipara
6	MTBB 02				
7	RBHR 01	Rajshahi (Taherpur)	Bagmara	Hamirkutssa	Ramrama
8	RBHR 02				
9	RBHR 03				
10	GJBS	Gazipur (BARI, Gazipur Sadar)	Joydebpur	BARI	Spices Field
11	GGBB 01		Gazipur Sadar	Baria	Bandan
12	GGBB 02				
13	CCKH 01	Comilla (Chandina)	Chandina	Keronkhali	Harong
14	CCKH 02				
15	CCKH 03				
16	JJLL 01	Jamalpur (Nandina)	Jamalpur Sadar	Lakhirchor	Lakhirchor
17	JJLL 02				
18	JJLL 03				
19	MSMM 01	Manikganj (Shibalaya)	Shibalaya	Mahadebpur	Mahadebpur
20	MSMM 02				
21	MSMM 03				
22	JMSA 01	Jessore (Monirumpur)	Monirumpur	Shempur	Aminpur
23	JMSA 02				
24	JMSA 03				
25	FFKU 01	Faridpur (Faridpur Sadar)	Faridpur Sadar	Kanaipur	Ulukanda
26	FFKU 02				
27	FFKU 03				

SAU = Sher-e- Bangla Agricultural University

BAU = Bangladesh Agricultural University

BARI = Bangladesh Agricultural Research Institute

3.8. Cultural variability of *Alternaria porri*

Mycelial discs (5 mm) of 7 days old culture of *Alternaria porri* isolates were transferred to the centre of PDA plates and cultural characteristics were noted on the potato dextrose agar (PDA) after four days of incubation at 24±1°C. Three replications were maintained for each isolate in a completely randomized design. The colony diameter was recorded on 4^{th}, 6^{th}, 8^{th}, 10^{th}, 12^{th}, and 14^{th} days after inoculation. Growth per day was calculated by the formula:

$$\text{Growth per day} = \frac{\text{Growth observed on a day} - \text{Growth on previous observation}}{2}$$

The other cultural properties on colony color, shape, margin, texture and substrate color was recorded.

3.9. Morphological variability of *Alternaria porri*

Fifteen (15) days old cultures of all the isolates were studied for morphological variations. In terms of conidia color, shape, size, septation, time of sporulation and number of conidia production were observed on PDA medium.

Length and breadth of conidia was measured using digital microscope (Model: Motic, BA-210) and motic software. Twenty recording per replication were made for the purpose. The conidia produced per unit surface area were estimated using haemacytometer, digital microscope and the formula of Chauhan and Pandey (1995) as follows:

$$\text{Conidia produced per unit surface area} = \frac{\text{Number of conidia/ml} \times \text{Volume of water of suspension}}{\text{Total surface area of suspension}}$$

3.10. Pathogenic variability of *Alternaria porri*

3.10.1 Collection of onion bulbs

For testing the virulence levels of *Alternaria porri* isolates, a local onion cultivar, Taherpuri collected from Savar Bazar, Dhaka was used in this experiment. Taherpuri is a popular local variety, which is commonly cultivated in Savar, Manikgonj, Faridpur and other onion growing areas of the country for its attractive size and shape, demand and higher market price.

3.10.2. Preparation of pot

Air dried sandy loam soil and cowdung were mixed thoroughly at the ratio of 4:1 and filled in earthen pots (20 cm diameter). No chemical fertilizers were used in the pot soil.

3.10.3. Experimental design

The earthen pots were arranged in net house following CRD maintaining three replications.

3.10.4. Sowing of bulbs

Three bulbs per pot were sown at 4-5 cm depth in the soil. Plants were watered as per requirement.

3.10.5. Preparation of inoculum

The conidia suspension of *Alternaria porri* was prepared with sterilized water using 10 days old PDA culture incubated at 22-24^{0}C under NUV light (12/12). The suspension was sieved through a double layered of cheese cloth to remove mycelia fragments and conidiophores. One drop of tween-20 (polyoxyethylene 20 sorbitan monolaurate) was added to the suspension to maintain uniform dispersion of conidia in suspension. The concentration of conidial suspension was adjusted to 21×10^5 per milliliter.

3.10.6. Inoculation and incubation

The plants were inoculated by 27 isolates of *Alternaria porri* with 21×10^5 spore/ml at 30 days after planting. At first the onion leaves were injured by sterile

toothpick. Then the injured surfaces were inoculated with a drop of inoculums suspension by a micropipette. The inoculated plants were covered with polyethylene sheet to maintain high relative humidity (% RH) and also to prevent natural contamination with other fungal conidia or spores. The plants were kept in a net house at 25±2^{0}C and humid condition was maintained by gently spraying sterilized distilled water.

3.10.7. Collection of data on leaf infection

After 5 days of inoculation on onion leaves the size of lesions was recorded on 5th, 7th, 9th, 11th, 13th, 15th, and 17th days after inoculation. Size of lesions increased per day was calculated by the formula:

$$\text{Leaf infection per day} = \frac{\text{Leaf infection observed on a day} - \text{Leaf infection on previous observation}}{2}$$

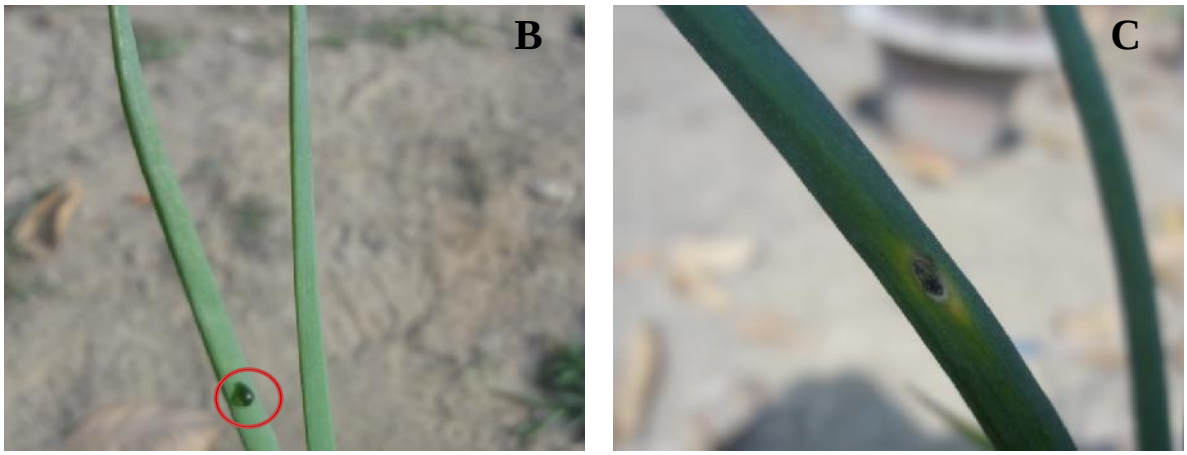


Plate 3 Pathogenicity test of *Alternaria porri*, (A) Preparation of conidial suspension (B) Inoculation of onion leaf (C) Showing symptom on onion leaf

3.11. Molecular variability of *Alternaria porri*

3.11.1. Collection of mycelium for genomic DNA isolation

To extract genomic DNA, mycelium of *Alternaria porri* was used of 15 days old pure culture of twenty seven (27) isolates.

3.11.2. Preparation of stock and working solutions

For conducting the isolation procedures, the following stock solutions and working solutions were prepared.

3.11.2.1. 1M Tris HCl pH 8.0 (100 ml)

12.14 g of Trizma Base (MW=121.14) was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0 by adding about 5 ml of concentrated HCl. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4 ºC.

3.11.2.2. 0.5M EDTA pH 8.0 (100 ml)

18.61 g of EDTA (EDTA. $2H_2O$, MW = 372.24) was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Added approximately 2 g of NaOH pellets to adjust the final pH to 8.0. The final volume of the solution was adjusted to 100 ml by adding sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4 ºC.

3.11.2.3. β-Mercaptoethanol

β-Mercaptoethanol was obtained as a 14.4M solution and stored in a dark bottle at room temperature.

3.11.2.4. Ribonuclease A

10 mg RNAase A was dissolved in 1 ml of deionized distilled water and store in -20 °C.

3.11.2.5. Tris-HCl saturated phenol

It was prepared by following procedure:

- **i)** The crystal phenol was melted in a water bath at 65 ºC for 30 minutes.
- **ii)** Then 100 ml of melted phenol was mixed with same volume of Tris-HCl (pH 8.0) with a magnetic stirrer for 10 minutes and was left in rest for 5 minutes.
- **iii)** At this stage, two distinct phases were visible, colorless upper phase and colored lower phase.
- **iv)** The upper phase was removed with the help of a dropper.

This step was performed for six times which took about 3.5 hours to obtain pH 7.75. After saturation, the phenol became the half of the initial volume. As phenol is very much corrosive and highly toxic, protective measures (Apron, Gloves, and Musk) were adopted.

3.11.2.6. Chloroform: isoamyl alcohol (24:1) (50 ml)

Forty eight milliliters of Chloroform and 2 ml of Isoamyl alcohol were added and mixed properly using vortex mixture. Mixing was done under fume hood for safety. The solution was then stored at 4 °C and was shaken before every use. Chloroform: Isoamyl alcohol mixture is caustic and produces fumes. So, was used only in a fume hood wearing gloves and eye protection.

3.11.2.7. 70% ethanol (100 ml)

Thirty milliliters double distilled water was added in 70 ml absolute ethanol to prepare 100 ml 70% ethanol.

3.11.2.8. Wash buffer

Absolute ethanol 76% was mixed with 10 mM ammonium acetate to use as wash buffer.

3.11.2.9. $T_{10}E_1$ (Tris-HCl EDTA) buffer pH 8.0 (100 ml)

1 ml of 1M Tris-HCl was added with 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4 $^{\circ}$C.

3.11.2.10. 7.5M ammonium acetate pH 5.2 (100 ml)

Ammonium acetate 57.81 g mixed with 70 ml of ddH_2O and adjusted the final volume to 100 ml with ddH_2O and pH was adjusted at 5.2. Then the solution was sterilized by autoclaving.

3.11.2.11. Preparation of extraction buffer

To prepare extraction buffer the following components and concentrations were used. For the economic use of chemicals, different volume of solution may be prepared as shown in tabular form (Table 2).

Table 2 Preparation of extraction buffer

Chemical Names	Molecular Weight	Stock Con.	Reference Con./ Working Con.	Working Volume	
				100 ml	1000 ml
CTAB			2%	2 gm	20 gm
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (P^H 8)	372.24	0.5 M	20 mM	4 ml	40 ml
Tris-HCl (P^H 8)	121.1	1 M	100 mM	10 ml	100 ml
β-Mercaptoethanol		14.4 M	100 mM	700 µl	7ml

Steps of extraction buffer preparation (100 ml)

Extraction buffer was prepared by following steps (100 ml):

i. 10 ml of 1 M Tris HCl (pH 8.0) was taken in a 250 ml conical flask.
ii. 28 ml of 5 M NaCl was added to it.
iii. 4 ml of 0.5 M EDTA (pH 8.0) was added next.
iv. The solution was then autoclaved.
v. After autoclaving 1 g PVP and 2 g CTAB was added and was stirred in the magnetic stirrer.
vi. 800 µl β-mercaptoethanol was added prior to use and was mixed by glass rod.
vii. The pH of the solution was adjusted at pH 5 with HCl and was made up to 100 ml by adding sterile de-ionized distilled water

Freshly prepared extraction buffer solution was used, β-mercaptoethanol was added to the solution prior to use. The whole task was performed in the fume hood.

3.11.3. Protocol used for genomic DNA isolation

DNA was isolated using the modified CTAB method. The method is described below:

1. 1.5 g freshly harvested fungal mycelium taken in effendorf tubes and placed them in liquid nitrogen. The fungal mycelium was grinded to fine powder with homogenizer machine.
2. 300 µl extraction buffers were added in each effendorf tubes.
3. The contents transferred to 65º C preheated water bath for 30 minutes and tubes were inverted every 5-10 min to allow mixing. The samples were taken from the water bath and cooled down to room temperature.
4. Added 800 µl Chloroform: Isoamyl alcohol (24:1) and mixed gently to avoid shearing of genomic DNA.
5. The samples were centrifuged at 13,000 rpm for 10 min at room temperature to remove non-soluble debris.
6. The supernatants were transferred to fresh tubes and repeated steps 5 and 6 in order to obtain a clear sample.

7. For precipitation, added 2/3 volume isopropanol (550µl), pre cooled at -20^{0}C, mixed gently and monitored precipitation of DNA.
8. The samples were centrifuged at 13,000 rpm for 10 min at room temperature and DNA pellet formed.
9. Discarded supernatant and washed the pellet in 100 µl Wash Buffer.
10. Carefully removed the washing buffer and resuspended the pellet in 100 µl TE supplemented with RNAase A (final concentration 10 mg/ml) and incubated for 30 min at 37^{0}C.
11. Added 100 µl 7.5M NH_4-Acetate and 750 µl absolute ethanol and mixed gently.
12. The samples were centrifuged at 13,000 rpm for 10 min at room temperature.
13. Discarded supernatant completely, dried the pellet and resuspended in 100 µl reinst water (TE Buffer).
14. Allowed DNA to dissolve overnight at 4^{0}C.

3.11.4. Estimation of quality and quantity of isolated DNA samples

Measurement of isolated DNA concentration was done by agarose gel electrophoresis method in this investigation.

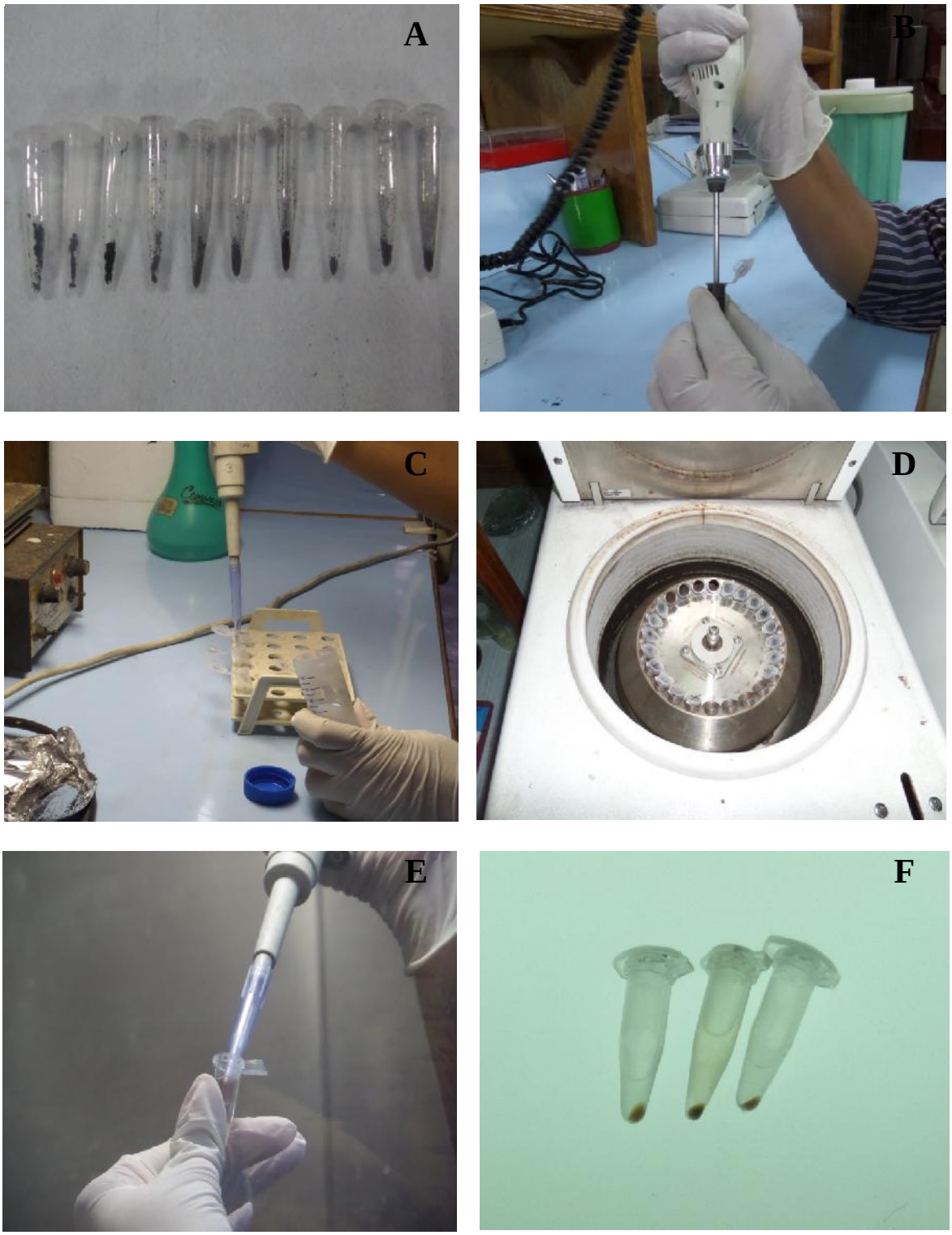


Plate 4 Extraction of genomic DNA by CTAB method, (A) Collected mycelium (B) Mycelium grinded by homogenizer (C) Added CI mix (D) Centrifuged (E)

Collected upper phase (F) Formed DNA pellet

3.11.5. Preparation of stock solutions used for gel electrophoresis

For conducting the gel electrophoresis, the following solutions were prepared.

3.11.5.1. 50 ò TAE buffer (P^H 8.3) (1 liter)

242 g Trizma Base (MW=121.14) was dissolved into 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (P^H 8.0) was added in it. They were mixed well. The P^H of the solution was adjusted by mixing concentrated HCl at pH 8.3. The final volume of the solution was adjusted to 1000 ml.

3.11.5.2. 6ò Loading dye

A. This is required to load samples in gel electrophoresis for further visualization.

Preparation of Stock Solutions

i. 10 ml of a 2% bromophenol blue stock solution.

ii. 10 ml of a 2% xylene cyanol stock solution.

iii. 50% glycerol solution.

The stock solutions were diluted to prepare 10 ml of the final 6× loading dye with the following component concentrations:

i. 30% glycerol

ii. 0.3% bromophenol blue

iii. 0.3% xylene cyanol

The 6x loading dye solution can be stored indefinitely in the refrigerator. The bromophenol blue, xylene cyanol and glycerol stock solutions can be stored indefinitely at room temperature.

The 6x DNA loading dye is added to DNA samples to achieve a final dye concentration of 1x.

B. 6x loading dye manufactured by Thermo Scientific.

3.11.5.3. Ethidium bromide solution

For 1ml solution, ethidium bromide 10 mg was added to 1 ml of sterile de-ionized distilled water. It was then mixed by hand shaking. The solution was then transferred to a dark bottle and stored at room temperature. Stock solution of 10 mg/ml can be purchased directly from companies.

3.11.6. Agarose gel electrophoresis

i. The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described by Sharp *et al.* 1973.

ii. 1.0 g of agarose was heated to melt into 100 ml of TAE buffer; ethidium bromide was added as 0.5 µg/ml final concentration and poured into gel-tray fixed with appropriate combs.

iii. After the gel was solidified it was placed into gel-running kit containing 1x TAE buffer.

iv. Digested plant DNA solutions were loaded with 6x gel loading dye and electrophoresis was continued until DNA fragments were separated well.

3.11.7. Documentation of the DNA sample

i. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed in Gel Documentation System (CSL-MDOCUV254/365 1D, Cleaver Scientific LTD, USA) for checking the DNA Bands.

ii. The DNA was observed as Band and photographed using Gel Documentation system.

3.11.8. RAPD analysis using polymerase chain reaction (PCR)

To perform the RAPD analysis, a single oligonucleotide of arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermo stable DNA polymerase, dNTPs, H_2O and suitable buffer, and then subjected to thermal cycling conditions typical to the polymerase chain reaction (PCR).

3.11.8.1. Preparation of working solution (25 ng/µl) of DNA samples for PCR reaction

Original stock solution concentration of each DNA sample was adjusted to a unique concentration (25 ng/µl) using the following formula:

$S_1 \times V_1 = S_2 \times V_2$

$V_1 = S_2 \times V_2 / S_1$

Where,

S_1= stock DNA concentration (ng/µl)

V_1= required volume (µl)

S_2= working DNA concentration (ng/µl)

V_2= working volume of DNA solution (µl)

Original stock DNA (2 µl) was taken in a 2 ml eppendorf tube and required amount of TE buffer calculated from the above formula was added to it.

3.11.8.2. Primer test

Primarily, seven (7) decamer primers were tested for RAPD amplification. The details of the primers are given in Table 3.

Table 3 Nucleotide sequences of primers used in RAPD

Primer Name	Sequence (5′ to 3′)
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	ATGCAGCCAC
OPA-04	AATCGGGCTG
OPA-13	CAGCACCCAC
OPB-04	GGACTGGAGT
OPB-18	CCACAGCAGT

3.11.8.3. Preparation of primers

The supplied primers were diluted to 100 µM following the deduction given below:

It is known that,

$$n = cv$$

Where, n = amount of supplied primer in mole.

c = molarity

v = volume

Stock solution was prepared at a concentration of 100 µM.

Therefore, here c = 100 µM. The supplied primer was 53.4 nano mole in each vial. So, n =53.4 nanomole; and v = Required volume of TE buffer to add in the supplied vial to make 100 µM main stock.

Therefore, 534 µl of TE buffer was added to the vial to make 100 µM main stocks. Using the above deduction method, all primers pairs were diluted to 100 µM main stocks. All primers were diluted to 50 times i.e., 2 µM to make working solution for use.

3.11.8.4. Preparation of purified Taq DNA polymerase

0.0174g of PMSF (0.5mM) was first dissolved in minimal volume of isopropanol (~l ml). Then 1.2I g of Tris (50mM), 0.745g of KCl (50mM), 0.0074g of EDTA (0.1mM) and 0.03084 g of DTT (1.0mM)were added and mixed thoroughly with de-ionized water after adjusting the pH to 7.9, the final volume was made 75ml with ddH2O and filter sterilized through 0.2/µm Millipore. Meanwhile, 50% glycerol was prepared from commercially available glycerol (98%) and autoclaved. 125ml of the 50% glycerol was added to 75ml of the mixture of storage buffer and stored at 4°C.

3.11.8.5. Preparation of dNTPs mixture

100µl each of dATP, dGTP, dCTP, dTTP (their concentrations being l0mM each) were mixed in a fresh autoclaved eppendorf tube and the final volume was made l000µl by adding 600µl of TE solutions (l0mM Tris-HCI, 0.lmM EDTA, pH 8.0) and dispensed as aliquots in tubes and stored at -20°C. The final concentration of each of the nucleotide in the above mixture was 1.0 mM.

3.11.8.6. Preparation of PCR reaction mixture

The following components were used to prepare PCR reaction mixture (Table 4).

Table 4 Components of PCR reaction mixture for RAPD

Sl. No.	Components	Amount per sample
1	Template DNA 50 ng/µl	3.00µl
2	Taq Buffer A 10X	2.50µl
3	dNTPs	2.50µl
4	Primer	1.00µl
5	Taq DNA Polymerase	0.05µl
6	H_2O	15.95µl
	Total =	25.00µl

During the experiment, PCR buffer, dNTPs, Primers and DNA sample solution were thawed from frozen stocks, mixed well by vortexing and kept on ice.

3.11.8.7. PCR amplification for RAPD

PCR amplification was done in an oil-free thermal cycler (Biometra, UNO II). The optimum amplification cycle was as follows:

	Initial denaturation	at	95^{o}C	for	5 minutes
30 cycle	Denaturation	at	94^{o}C	for	1 minutes
	Annealing	at	30^{o}C	for	1 minutes
	Extension	at	72^{o}C	for	3 minutes
	Final extension	at	72^{o}C	for	5 minutes

After completion of cycling programme, the reactions were held at 4^{o}C.

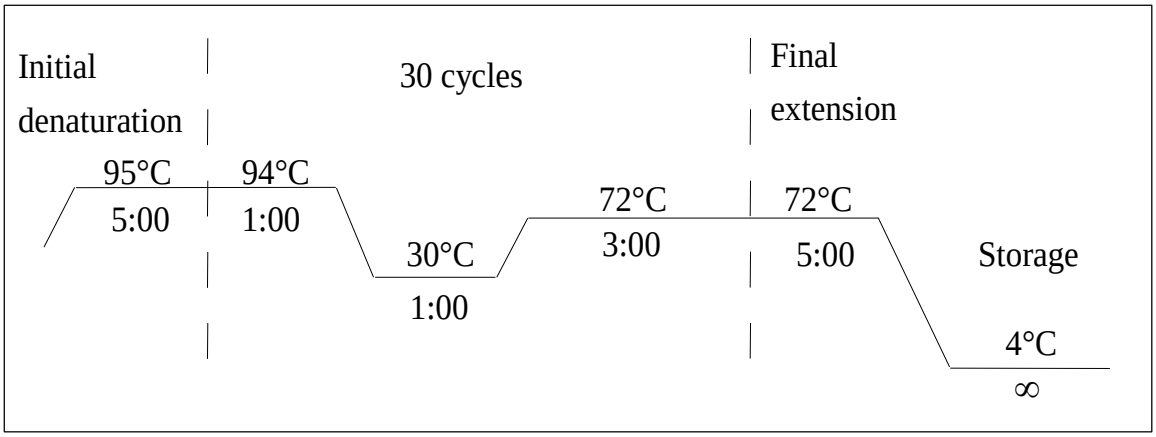


Figure 1 Scheme illustrating the temperature profile for the RAPD

3.11.8.8. Electrophoresis of the amplified products and documentation

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide and 100 ml 1xTAE buffer. Agarose gel electrophoresis was conducted in 1× TAE buffer at 50 Volts and 100 mA for 1.5 hours.

One molecular weight marker 1kb DNA ladder was electrophoresed alongside the RAPD reactions. DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system.

3.11.9. Identification of ITS region and confirm through sequencing

3.11.9.1. DNA isolation

DNA was isolated from *Alternaria porri* isolate following CTAB method.

3.11.9.2. Primer

Sl. No.	Primer Type	Primer Name	Primer Sequence 5'-3'
1	Forward primer	ITS1F	CTTGGTCATTTAGAGGAAGTAA
2	Reverse primer	ITS4	TCCTCCGCTTATTGATATGC

3.11.9.3. Preparation of PCR reaction mixture

The following components were used to prepare PCR reaction mixture (Table 5).

Table 5 Components of PCR reaction mixture for ITS region

Sl. No.	Components	Amount per sample
1	Template DNA 50 ng/µl	3.00µl
2	Taq Buffer A 10X	2.50µl
3	$MgCl_2$	2.50µl
4	dNTPs	2.00µl
5	Forward Primer	0.1µl
6	Reverse Primer	0.1µl
7	Taq DNA Polymerase	0.2µl
8	H_2O	14.60µl
	Total =	25.00µl

During the experiment, PCR buffer, dNTPs, Primers and DNA sample solution were thawed from frozen stocks, mixed well by vortexing and kept on ice.

3.11.9.4. PCR amplification

PCR amplification was done in an oil-free thermal cycler (Biometra, UNO II). The optimum amplification cycle was as follows:

	Initial denaturation	at	95°C	for	5 minutes
30 cycle	Denaturation	at	94°C	for	1 minutes
	Annealing	at	49°C	for	1 minutes
	Extension	at	72°C	for	3 minutes
	Final extension	at	72°C	for	10 minutes

After completion of cycling programme, the reactions were held at 4°C.

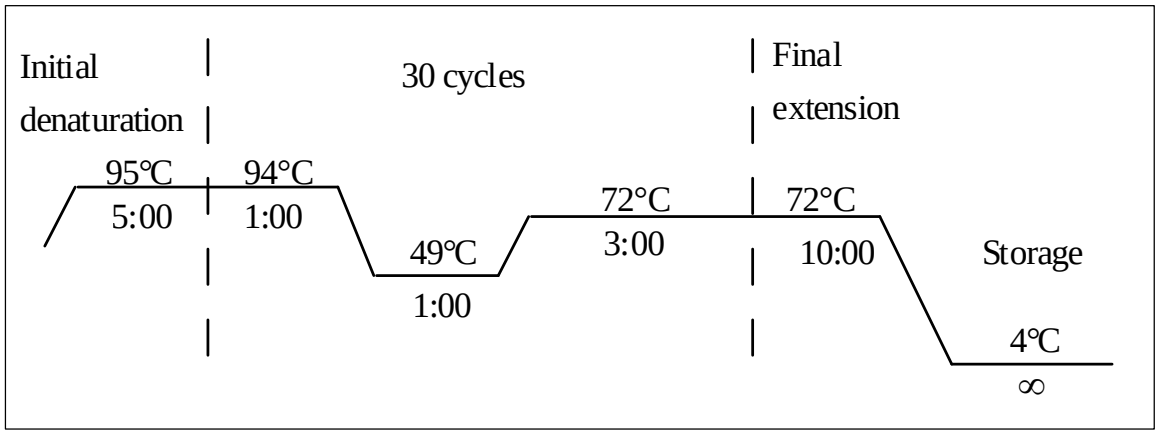


Figure 2 Scheme illustrating the temperature profile for the ITS

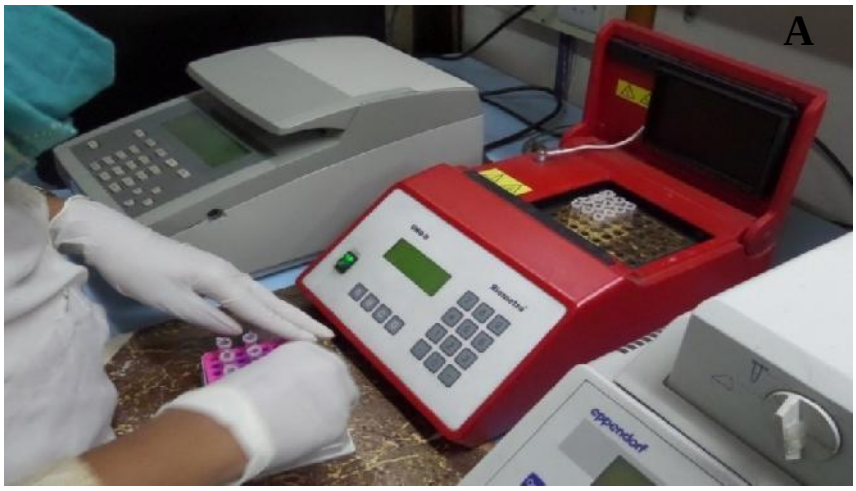
3.11.9.5. Gel electrophoresis

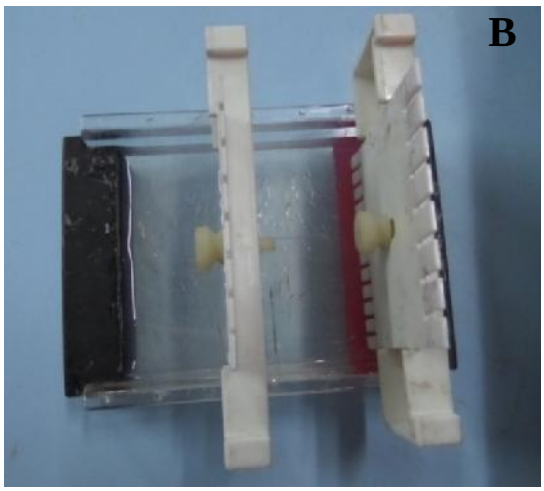
The PCR amplified DNA was run on 0.8% agarose gel and stained with ethidium bromide (0.5µl/ml). DNA amplification was seen as clear Band at expected size viewed by gel documentation system.

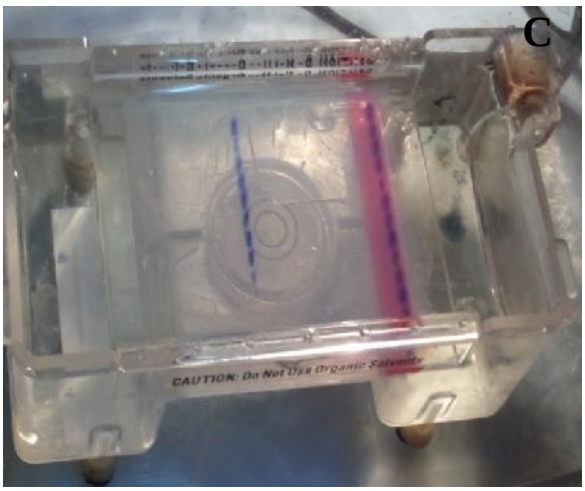
3.11.9.6. Purification of PCR products

Alcohol precipitation

i. Added equal volume of chloroform and Isoamyl alcohol (24:1) then centrifuged at13000 rpm for 5 minutes.
ii. Taken the upper aquase phase and added equal volume of chloroform then centrifuged at13000 rpm for 5 minutes.
iii. Taken the upper aquase phase and added $1/10^{th}$ volume of 3M Sodium Acetate and double volume of ice cold absolute alcohol then kept in -20^{0}C for 30 minutes.
iv. Centrifuged at13000 rpm for 20 minutes.
v. Washed the pellet with 70% alcohol (2 times)
vi. Air dried and the pellet dissolved in TE.







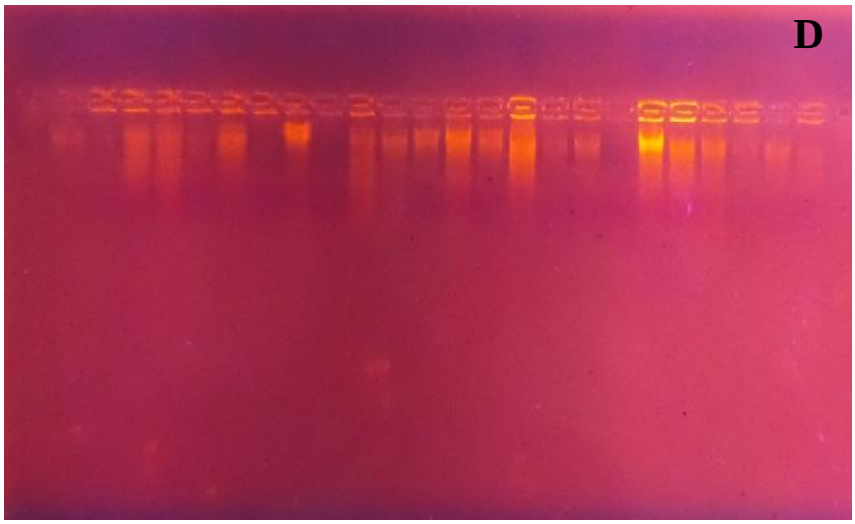


Plate 5 Polymerase chain reaction, gel electrophoresis and gel documentation, (A) PCR (B) Gel prepared (C) DNA loaded (D) Gel documented

3.11.9.7. Sequencing of PCR positive product

PCR amplified products were sequenced through automated sequencer in Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka - 1000. The sequences obtained through sequencing were analysed through BLAST search in order to confirm that it was from *Alternaria porri* genome.

3.12. Data analysis

The data were statistically analyzed (Gomez, 1984) for cultural, morphological and pathogenic variability and the treatment means were compared by Dunkan's Multiple Range Test (DMRT). The package used for analysis was MSTAT-C version-88, developed by Michigan State University, Agricultural University of Norway. For molecular variability sequences were analyzed using the software MEGA version 6.0. Database searches with *Alternaria* sp. sequences were carried out by NCBI-BLAST program (http://blast.ncbi.nlm.nih.gov).

CHAPTER IV

RESULTS

4.1. Cultural variation of *Alternaria porri*

4.1.1. Radial mycelial growth of 27 isolates of *Alternaria porri*

Radial mycelial growth of 27 isolates of *Alternaria porri* varied significantly on PDA media (Table 6). After 4 days of inoculation, maximum increase (13.67 mm) of colony diameter was recorded in CCKH 01, which was statistically similar to the isolates CCKH 02 (13.00 mm), MSMM 01(12.50 mm), FFKU 01 (12.33 mm), MSMM 02 (12.17 mm), FFKU 03 (12.17 mm), JMSA 01 (12.00 mm) and CCKH 03 (11.67 mm) followed by MMBH (11.33 mm). The minimum increment (9.00 mm) of colony diameter was recorded in GGBB 01 which was statistically similar to the isolates GJBS (9.167 mm), RBHR 01 (9.333 mm), DSTR 02 and RBHR 02 (9.50 mm), DSTR 01 (9.667 mm), RBHR 03 (9.833 mm), MTBB 01, MTBB 02 and GGBB 02 (10.17 mm), JJLL 02, JJLL 03 and MSMM 03 (10.50 mm) and JMSA 03 (10.83 mm), JJLL 01, JMSA 02 and FFKU 02 (11.17 mm).

After 6 days of inoculation, the maximum increment (21.33 mm) of colony diameter was recorded in CCKH 01 and CCKH 02, those were statistically similar to the isolates JMSA 01 (20.83 mm), DSSA (20.33 mm), FFKU 01 (19.83 mm), MSMM 02 (19.33 mm) followed by CCKH 03 (19.00 mm), MSMM 01 (18.83 mm), DSTR 01 (18.67 mm), FFKU 03 (18.50 mm), JMSA 02 and FFKU 02 (18.00 mm). The minimum increment (15.00 mm) of colony diameter was recorded in GJBS which was statistically similar to the isolates RBHR 01 (15.17 mm), GGBB 01 (15.33 mm), RBHR 03 (16.67 mm) and GGBB 02 (16.67 mm), JJLL 01 (16.83 mm), DSTR 02 and JJLL 03 (17.17 mm) followed by JMSA 03 (17.33 mm), RBHR 02 and JJLL 02 (17.50 mm), MMBH and MSMM 03 (17.67 mm), MTBB 01and MTBB 02 (17.83 mm).

Table 6 Radial mycelial growth of 27 isolates of *Alternaria porri*

Sl. No.	Isolate	Radial mycelial growth (mm)					
		4th DAI	**6th DAI**	**8th DAI**	**10th DAI**	**12th DAI**	**14th DAI**
1	DSSA	11.00 b-h	20.33 a-c	27.33 c-e	33.67 c-f	40.83 c-e	46.83 a-c
2	DSTR 01	9.667 f-h	18.67 b-f	26.50 c-f	35.33 b-d	42.00 a-c	47.00 a-c
3	DSTR 02	9.50 f-h	17.17 e-i	26.00 c-g	35.33 b-d	**43.50 a**	**49.00 a**
4	MMBH	11.33 b-g	17.67 d-f	22.33 jk	**25.67 k**	**30.17 l**	**35.67 f**
5	MTBB 01	10.17 d-h	17.83 d-f	24.33 f-j	33.50 d-g	41.50 b-d	46.83 a-c
6	MTBB 02	10.17 d-h	17.83 d-f	24.83 f-i	33.50 d-g	40.17 c-g	46.50 b-d
7	RBHR 01	9.333 gh	15.17 hi	22.67 i-k	28.67 j	36.83 k	44.83 cd
8	RBHR 02	9.50 f-h	17.50 d-g	24.50 f-j	34.00 c-e	40.83 c-e	46.83 a-c
9	RBHR 03	9.833 e-h	16.67 f-i	24.00 g-k	31.67 f-h	38.67 g-k	44.50 d
10	GJBS	9.167 gh	**15.00 i**	**21.67 k**	28.83 ij	37.33 jk	41.67 e
11	GGBB 01	**9.000 h**	15.33 g-i	22.67 i-k	29.17 ij	37.00 jk	44.83 cd
12	GGBB 02	10.17 d-h	16.67 f-i	23.50 h-k	30.50 h-j	37.83 i-k	45.50 b-d
13	CCKH 01	**13.67 a**	**21.33 a**	29.67 b	37.17 ab	43.50 a	46.83 a-c
14	CCKH 02	13.00 ab	**21.33 a**	28.17 bc	35.50 b-d	41.50 b-d	47.33 ab
15	CCKH 03	11.67 a-f	19.00 b-f	27.83 b-d	35.50 b-d	41.50 b-d	46.83 a-c
16	JJLL 01	11.17 b-h	16.83 f-i	24.50 f-j	32.50 e-h	40.83 c-e	46.50 b-d
17	JJLL 02	10.50 c-h	17.50 e-g	24.50 f-j	32.33 e-h	38.83 f-j	45.17 b-d
18	JJLL 03	10.50 c-h	17.17 e-i	23.83 g-k	32.17 e-h	39.83 d-h	46.00 b-d
19	MSMM 01	12.50 a-c	18.83 b-f	26.00 c-g	33.83 c-e	40.67 c-f	45.83 b-d
20	MSMM 02	12.17 a-d	19.33 a-e	28.00 bc	35.67 bc	41.50 b-d	46.50 b-d
21	MSMM 03	10.50 c-h	17.67 d-f	25.83 c-h	34.17 c-e	40.17 c-g	45.50 b-d
22	JMSA 01	12.00 a-e	20.83 ab	**33.17 a**	**37.67 a**	43.00 ab	46.83 a-c
23	JMSA 02	11.17 b-h	18.00 d-f	24.17 f-j	31.50 gh	38.67 g-k	45.17 b-d
24	JMSA 03	10.83 b-h	17.33 e-h	24.00 g-k	30.67 hi	38.17 h-k	44.83 cd
25	FFKU 01	12.33 a-d	19.83 a-d	27.50 b-d	35.50 b-d	42.83 ab	46.33 b-d
26	FFKU 02	11.17 b-h	18.00 d-f	25.00 e-i	32.33 e-h	39.33 e-i	45.67 b-d
27	FFKU 03	12.17 a-d	18.50 c-f	25.50 d-h	32.33 e-h	39.33 e-i	45.17 b-d
	LSD (0.05)	1.855	1.947	2.046	1.718	1.660	1.890
	CV (%)	10.40%	6.59%	4.90%	3.19%	2.54%	2.53%

After 8 days of inoculation, the maximum increment (33.17 mm) of colony diameter was recorded in JMSA 01 which was statistically different from all other isolates. This was followed by CCKH 01 (29.67 mm), CCKH 02 (28.17 mm), MSMM 02 (28.00 mm), CCKH 03 (27.83 mm), FFKU 01 (27.50 mm), DSSA (27.33 mm), DSTR 01 (26.50 mm), DSTR 02 and MSMM 01 (26.00 mm), MSMM 03 (25.83 mm), FFKU 02 and FFKU 03 (25.00 mm). The minimum increment (21.67 mm) of colony diameter was recorded in GJBS which was statistically similar to the isolates MMBH (22.33 mm), RBHR 01 and GGBB 01 (22.67 mm), GGBB 02 (23.50 mm), JJLL 03 (23.83 mm), RBHR 03 and JMSA 03 (24.00 mm) followed by JMSA 02 (24.17 mm), MTBB 01 (24.33 mm), RBHR 02, JJLL 01 and JJLL 02 (24.50 mm) and MTBB 02 (24.83 mm).

After 10 days of inoculation, the maximum increment (37.67 mm) of colony diameter was recorded in JMSA 01 which was statistically similar to the isolate CCKH 01 (37.17 mm) followed by MSMM 02 (35.67 mm), CCKH 02, CCKH 03 and FFKU 01 (35.50 mm), DSTR 01 and DSTR 02 (35.33 mm), MSMM 03 (34.17 mm), RBHR 02 (34.00 mm), MSMM 01 (33.83 mm), DSSA (33.67 mm), MTBB 01 and MTBB 02 (33.50 mm). The minimum increment (25.67 mm) of colony diameter was recorded in MMBH which was statistically different from all other isolates followed by RBHR 01 (28.67 mm), GJBS (28.83 mm), GGBB 01 (29.17 mm), GGBB 02 (30.50 mm), JMSA 03 (30.67 mm), JMSA 02 (31.50 mm), RBHR 03 (31.67 mm), JJLL 03 (32.17 mm), JJLL 02, FFKU 02 and FFKU 03 (32.33 mm) and JJLL 01 (32.50 mm).

After 12 days of inoculation, the maximum increment (43.50 mm) of colony diameter was recorded in DSTR 02 and CCKH 01, those were statistically similar to the isolates JMSA 01 (43.00 mm), FFKU 01 (42.83 mm) and DSTR 01 (42.00 mm) followed by MTBB 01, CCKH 02, CCKH 02 and MSMM 02 (41.50 mm), DSSA, RBHR 02 and JJLL 01 (40.83 mm), MSMM 01 (40.67 mm), MTBB 02 and MSMM 03 (40.17 mm). The minimum increment (30.17 mm) of colony diameter was recorded in MMBH which was statistically different from all other

isolates followed by RBHR 01 (36.83 mm), GGBB 01 (37.00 mm), GJBS (37.33 mm), GGBB 02 (37.83 mm), JMSA 03 (38.17 mm), RBHR 03 and JMSA 02 (38.67 mm), JJLL 02 (38.83 mm), FFKU 02 and FFKU 03 (39.33 mm) and JJLL 03 (39.83 mm).

After 14 days of inoculation, the maximum increment (49.00 mm) of colony diameter was recorded in DSTR 02 which was statistically similar to the isolates CCKH 02 (47.33 mm), DSTR 01 (47.00 mm), DSSA, MTBB 01, RBHR 02, CCKH 01, CCKH 03 and JMSA 01 (46.83 mm) followed by MTBB 02, JJLL 01 and MSMM 02 (46.50 mm), FFKU 01 (46.33 mm) and JJLL 03 (46.00 mm). The minimum increment (35.67 mm) of colony diameter was recorded in MMBH which was statistically different from all other isolates followed by GJBS (41.67 mm), RBHR 03 (44.50 mm), RBHR 01, GGBB 01 and JMSA 03 (44.83 mm), JJLL 02, JMSA 02 and FFKU 03 (45.17 mm), GGBB 02 and MSMM 03 (45.50 mm), FFKU 02 (45.67 mm) and MSMM 01 (45.83 mm).

The maximum increment rate of radial mycelial growth per day (3.950 mm) was recorded in DSTR 02 which was statistically similar to the isolates DSTR 01 and RBHR 02 (3.733 mm) followed by MTBB 01 (3.667 mm), MTBB 02 (3.633 mm), DSSA and GGBB 01 (3.583 mm), RBHR 01 and JJLL 03 (3.550 mm), GGBB 02 and JJLL 01 (3.533 mm), CCKH 03 (3.517 mm) and MSMM 03 (3.500 mm).The rate minimum increment of radial mycelial growth per day (2.433 mm) was recorded in MMBH which was statistically differed from all other treatments followed by GJBS (3.250 mm), FFKU 03 (3.300 mm), CCKH 01 (3.317 mm), MSMM 01 (3.333 mm), JMSA 02, JMSA 03 and FFKU 01 (3.400 mm), CCKH 02 and MSMM 02 (3.433 mm), FFKU 02 (3.450 mm), RBHR 03 and JJLL 02 (3.467 mm) and JMSA 01 (3.483 mm) (Figure 3).

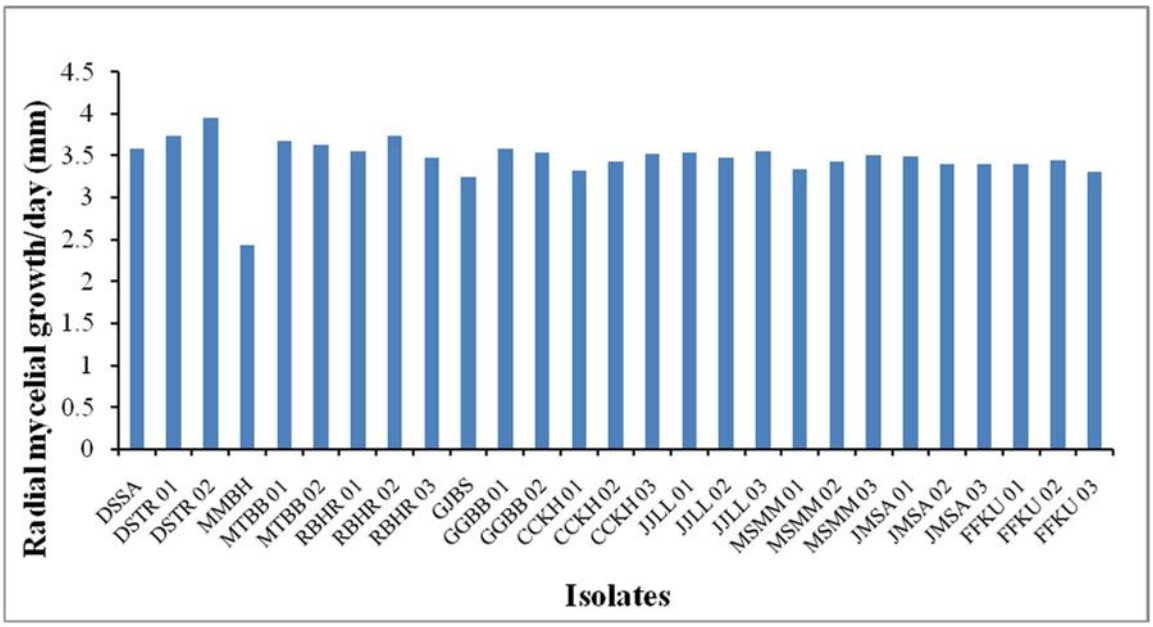


Figure 3 Radial mycelia growth of 27 isolates of *Altermaria porri* per day

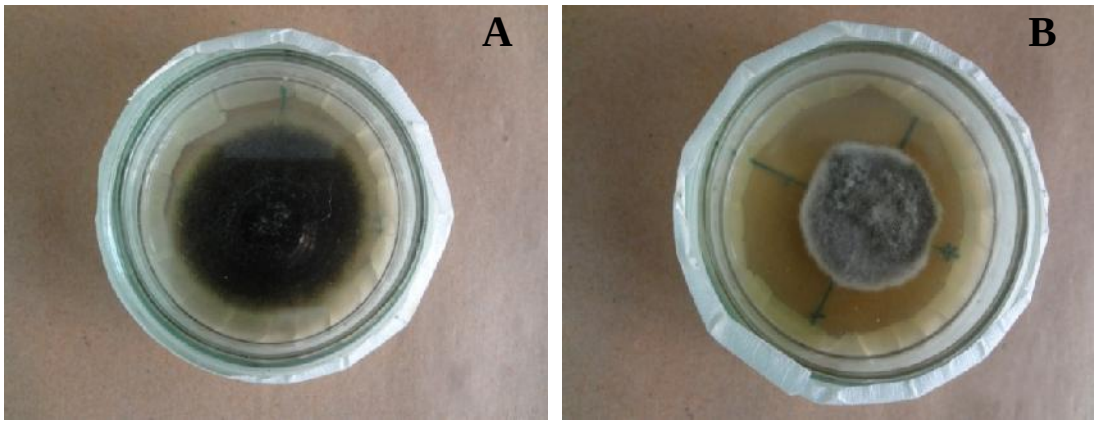


Plate 6 Radial mycelial growth 10 days after inoculation, (A) Isolate DSTR 02 (B) Isolate MMBH

4.1.2. Colony characterization of 27 *Alternaria porri* isolates

All the isolates exhibited variation in colony characteristics in respect of color, shape, margin and texture and substrate color. Colony colors were dark olivaceous green, grayish white, light olivaceous green and olivaceous green. Colony shapes were irregular, regular with concentric ring and regular without concentric ring. Colony margins were entire, irregular and wavy. Colony texture were effuse, fluffy and velvet. Substrate colors were grey, deep brown and light brown (Table 7).

Dark olivaceous green colony color was found in isolates DSSA, RBHR 01, GJBS, GGBB 01, GGBB 02, JJLL 01 and JMSA 01; grayish white in isolate MMBH; light olivaceous green in isolates DSTR 01, DSTR 02, RBHR 02, RBHR 03, CCKH 01, CCKH 03, JJLL 02, JMSA 02, JMSA 03, FFKU 01, FFKU 02 and FFKU 03; and olivaceous green in MTBB 01, MTBB 02, CCKH 02, JJLL 03, MSMM 01, MSMM 02 and MSMM 03 (Table 7 and Plate 7).

Marked variability was found in colony shape. Irregular colony shape was found in isolates MMBH, RBHR 01, GJBS, CCKH 01 and JJLL 02; regular with concentric ring in isolates DSSA, DSTR 01, DSTR 02, RBHR 02, RBHR 03, GGBB 01, GGBB 02, CCKH 03, JJLL 01, JJLL 03, MSMM 02, MSMM 03, JMSA 01, JMSA 02, JMSA 03 and FFKU 02 and regular without concentric ring in isolates MTBB 01, MTBB 02, CCKH 02, MSMM 01, FFKU 01 and FFKU 03 (Table 7 and Plate 8).

Entire colony margin was found in isolates DSSA, DSTR 02, MTBB 02, RBHR 02, RBHR 03, GJBS, GGBB 02, CCKH 01, CCKH 02, CCKH 03, JJLL 01, JJLL 02, JJLL 03, MSMM 02, MSMM 03, JMSA 01, JMSA 03, FFKU 01 and FFKU 02; irregular in isolates MTBB 01, RBHR 01, GGBB 01, JMSA 02 and FFKU 03; and wavy in isolates DSTR 01, MMBH and MSMM 01 (Table 7 and Plate 9).

Table 7 Colony characterization of 27 *Alternaria porri* isolates on PDA

Isolates	Characteristics features
	Colony color
DSSA, RBHR 01, GJBS, GGBB 01, GGBB 02, JJLL 01, JMSA 01.	Dark olivaceous green
MMBH.	Grayish white
DSTR 01, DSTR 02, RBHR 02, RBHR 03, CCKH 01, CCKH 03, JJLL 02, JMSA 02, JMSA 03, FFKU 01, FFKU 02, FFKU 03.	Light olivaceous green
MTBB 01, MTBB 02, CCKH 02, JJLL 03, MSMM 01, MSMM 02, MSMM 03.	Olivaceous green
	Colony shape
MMBH, RBHR 01, GJBS, CCKH 01, JJLL 02.	Irregular
DSSA, DSTR 01, DSTR 02, RBHR 02, RBHR 03, GGBB 01, GGBB 02, CCKH 03, JJLL 01, JJLL 03, MSMM 02, MSMM 03, JMSA 01, JMSA 02, JMSA 03, FFKU 02.	Regular with concentric ring
MTBB 01, MTBB 02, CCKH 02, MSMM 01, FFKU 01, FFKU 03.	Regular without concentric ring
	Colony margin
DSSA, DSTR 02, MTBB 02, RBHR 02, RBHR 03, GJBS, GGBB 02, CCKH 01, CCKH 02, CCKH 03, JJLL 01, JJLL 02, JJLL 03, MSMM 02, MSMM 03, JMSA 01, JMSA 03, FFKU 01, FFKU 02.	Entire
MTBB 01, RBHR 01, GGBB 01, JMSA 02, FFKU 03.	Irregular
DSTR 01, MMBH, MSMM 01.	Wavy

Table 7 Contd.

Isolates	Characteristics features
	Colony texture

DSSA, MTBB 01, MTBB 02, RBHR 01, GJBS, GGBB 01, GGBB 02, CCKH 01, CCKH 02, CCKH 03, JJLL 03, JMSA 01, JMSA 02, JMSA 03, FFKU 01, FFKU 02, FFKU 03.	Effuse
MMBH.	Fluffy
DSTR 01, DSTR 02, RBHR 02, RBHR 03, JJLL 01, JJLL 02, MSMM 01, MSMM 02, MSMM 03.	Velvet
	Substrate color
MMBH, GJBS.	Grey
DSSA, MTBB 01, MTBB 02, RBHR 01, RBHR 02, GGBB 01, GGBB 02, CCKH 01, CCKH 02, JJLL 03, JMSA 01, JMSA 02, JMSA 03, FFKU 01, FFKU 03.	Deep brown
DSTR 01, DSTR 02, RBHR 03, CCKH 03, JJLL 01, JJLL 02, MSMM 01, MSMM 02, MSMM 03, FFKU 02.	Light brown

Distinct differences of the 27 isolates were obtained in terms of colony texture. Effuse colony texture was found in isolates DSSA, MTBB 01, MTBB 02, RBHR 01, GJBS, GGBB 01, GGBB 02, CCKH 01, CCKH 02, CCKH 03, JJLL 03, JMSA 01, JMSA 02, JMSA 03, FFKU 01, FFKU 02 and FFKU 03; fluffy in isolate MMBH and velvet in isolates DSTR 01, DSTR 02, RBHR 02, RBHR 03, JJLL 01, JJLL 02, MSMM 01, MSMM 02 and MSMM 03 (Table 7 and Plate 10).

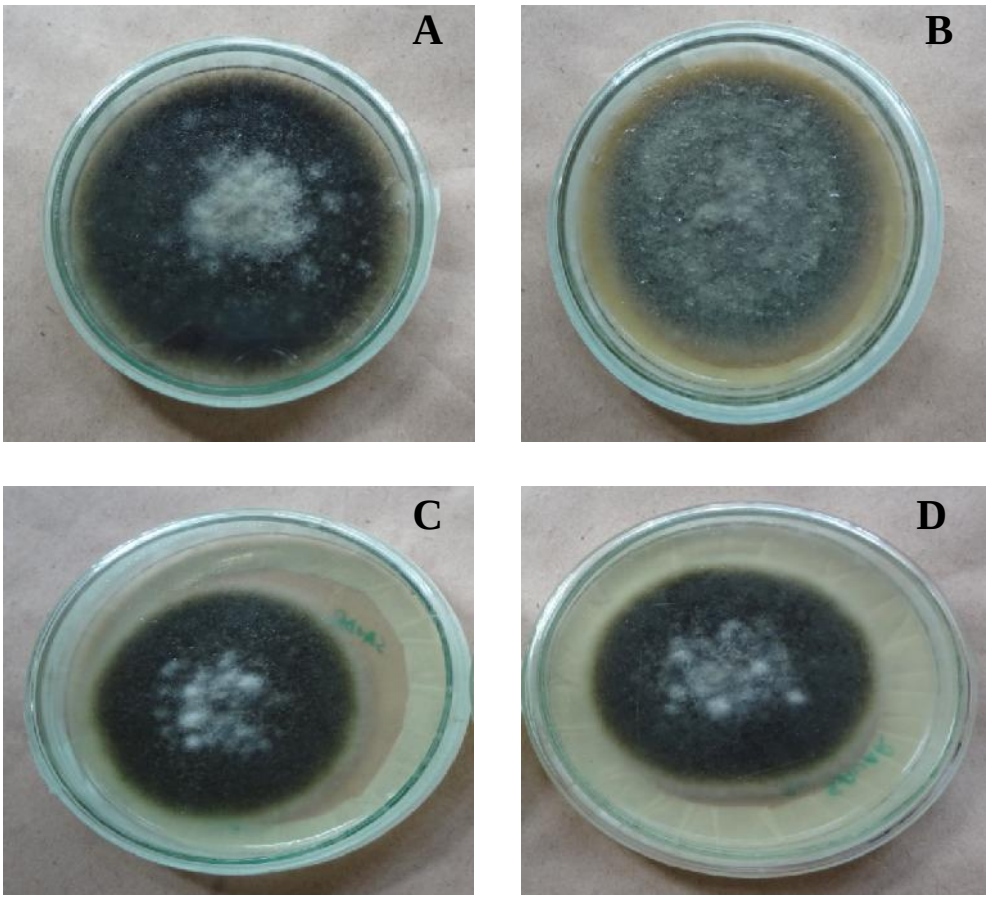


Plate 7 Colony color of *Alternaria porri* isolates, (A) Dark olivaceous green, (B) Grayish white, (C) Light olivaceous green and (D) Olivaceous green

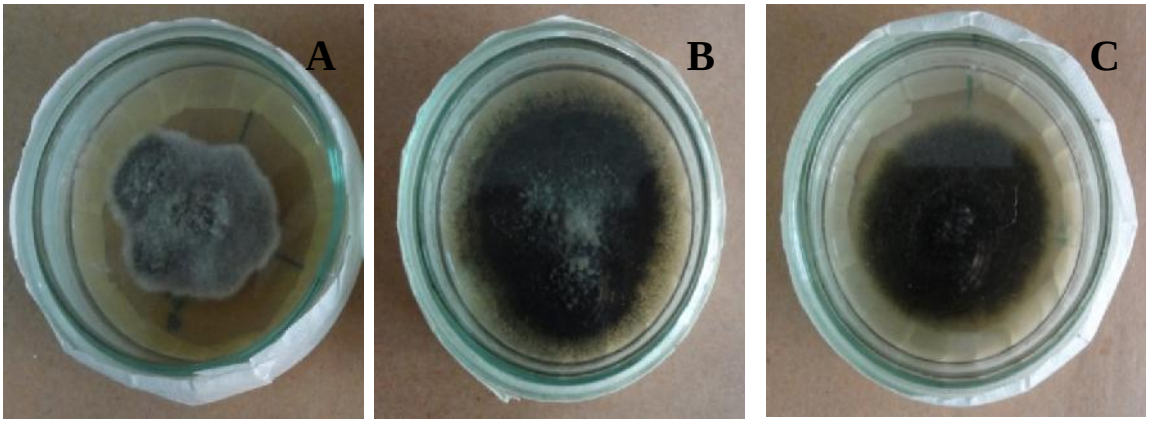


Plate 8 Colony shape of *Alternaria porri* isolates, (A) Irregular, (B) Regular with concentric ring and (C) Regular without concentring ring

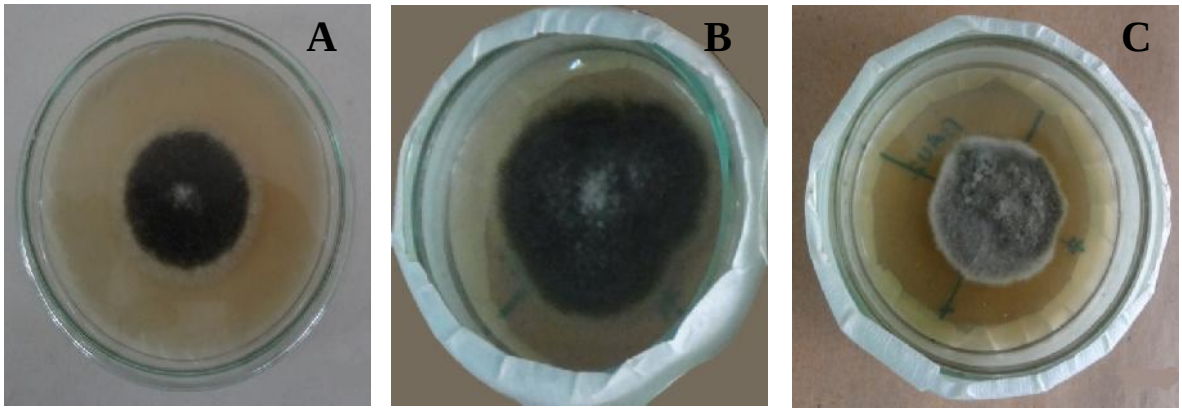


Plate 9 Colony margin of *Alternaria porri* isolates, (A) Entire, (B) Irregular and (C) Wavy

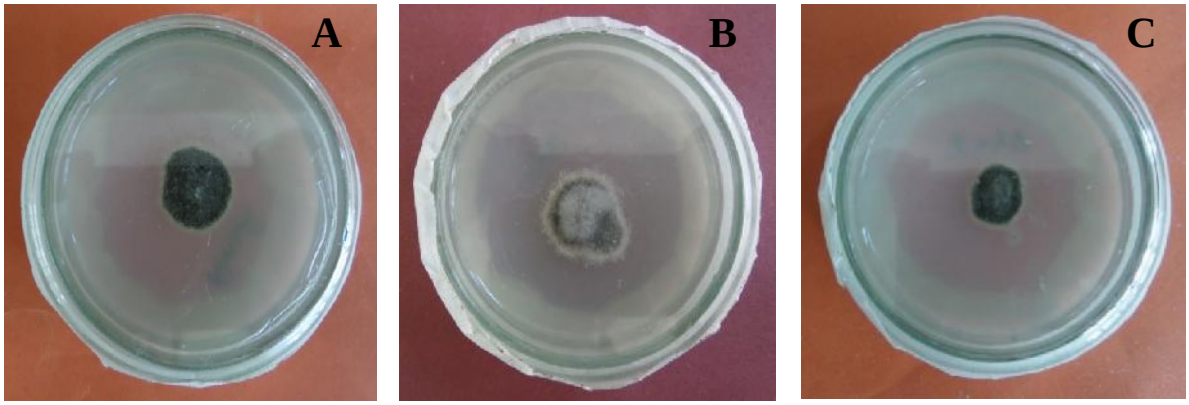


Plate 10 Colony texture of *Alternaria porri* isolates, (A) Effuse, (B) Fluffy and (C) Velvet

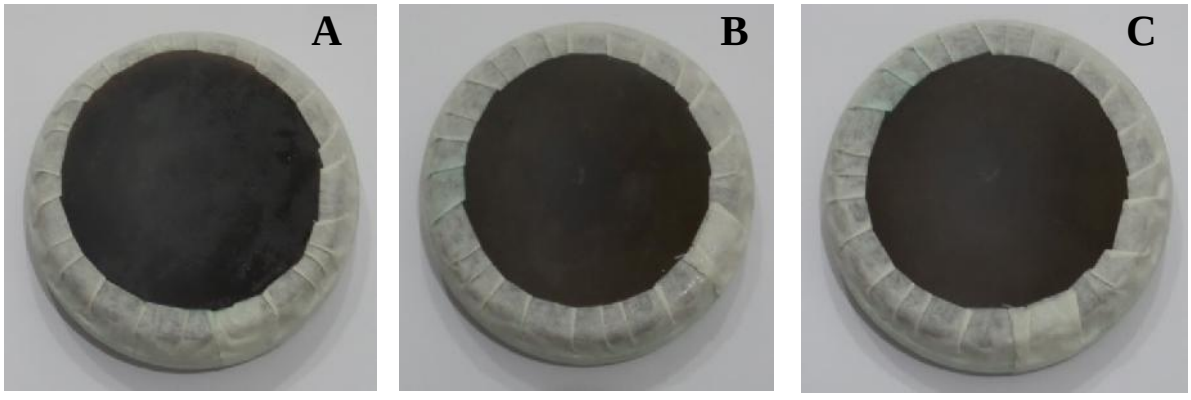


Plate 11 Substrate color of *Alternaria porri* isolates, (A) Grey, (B) Deep brown and (C) Light brown

Grey substrate color was found in isolates MMBH and GJBS; deep brown in isolates DSSA, MTBB 01, MTBB 02, RBHR 01, RBHR 02, GGBB 01, GGBB 02, CCKH 01, CCKH 02, JJLL 03, JMSA 01, JMSA 02, JMSA 03, FFKU 01 and FFKU 03; and light brown in isolates DSTR 01, DSTR 02, RBHR 03, CCKH 03, JJLL 01, JJLL 02, MSMM 01, MSMM 02, MSMM 03 and FFKU 02 (Table 7 and Plate 11).

4.2. Morphological variation of *Alternaria porri*

4.2.1. Conidia production, sporulation, shape and conidia color of 27 isolates of *Alternaria porri*

Marked variation was found in conidia production of 27 different isolates of *Alternaria porri* (Table 8). The highest production of conidia (47.02×10^3/ mm^2) was recorded in DSSA which was statistically different from all other isolates. This was followed by GGBB 01 (40.65×10^3/ mm^2), DSTR 01 (40.40×10^3/ mm^2), DSTR 02 (37.60×10^3/ mm^2), GJBS (36.92×10^3/ mm^2), MTBB 01 (34.20×10^3/ mm^2), GGBB 02 (33.86×10^3/ mm^2), JJLL 01 (33.61×10^3/ mm^2), MTBB 02 (32.85×10^3/ mm^2), CCKH 01 (31.06×10^3/ mm^2), JJLL 02 (30.55×10^3/ mm^2), JJLL 03 (29.11×10^3/ mm^2), RBHR 01 (27.92×10^3/ mm^2) and FFKU 03 (26.22×10^3/ mm^2). On the other hand the lowest production of conidia (7.720×10^3/ mm^2) was recorded in MMBH which was statistically different from all other isolates followed by RBHR 03 (20.62×10^3/ mm^2), RBHR 02 (22.83×10^3/ mm^2), MSMM 02 (23.00×10^3/ mm^2), MSMM 03 (23.26×10^3/ mm^2), JMSA 02 (23.42×10^3/ mm^2), MSMM 01 (23.93×10^3/ mm^2), FFKU 01 (24.44×10^3/ mm^2), FFKU 02 (24.61×10^3/ mm^2), JMSA 01 (25.03×10^3/ mm^2), CCKH 03 (25.46×10^3/ mm^2), CCKH 02 (25.55×10^3/ mm^2) and JMSA 03 (25.89×10^3/ mm^2).

Table 8 Conidia production, sporulation, shape and color of conidia of 27 isolates of *Alternaria porri*

Sl. No	Isolate	Number of conidia/mm^2 ($\times 10^3$)	Sporulation time (days)	Shape	Color
1	DSSA	**47.02 a**	4.000 b	Straight/Curved	Brown
2	DSTR 01	40.40 b	3.667 b	Straight	Brown
3	DSTR 02	37.60 bc	3.667 b	Straight	Brown
4	MMBH	**7.720 j**	**11.00 a**	Straight	Deep Brown
5	MTBB 01	34.20 c-e	**3.333 b**	Straight	Brown
6	MTBB 02	32.85 c-e	3.667 b	Straight	Brown
7	RBHR 01	27.92 e-h	4.333 b	Straight	Brown
8	RBHR 02	22.83 hi	3.667 b	Straight	Brown
9	RBHR 03	20.62 i	3.667 b	Straight	Brown
10	GJBS	36.92 b-d	4.333 b	Straight	Light Brown
11	GGBB 01	40.65 b	4.000 b	Straight	Brown
12	GGBB 02	33.86 c-e	3.667 b	Straight	Brown
13	CCKH 01	31.06 d-f	4.000 b	Straight/Curved	Brown
14	CCKH 02	25.55 f-i	4.000 b	Straight	Brown
15	CCKH 03	25.46 f-i	3.667 b	Straight	Brown
16	JJLL 01	33.61 c-e	4.000 b	Straight	Brown
17	JJLL 02	30.55 e-g	3.667 b	Straight	Brown
18	JJLL 03	29.11 e-h	3.667 b	Straight	Brown
19	MSMM 01	23.93 hi	4.000 b	Straight	Brown
20	MSMM 02	23.00 hi	**3.333 b**	Straight/Curved	Deep Brown
21	MSMM 03	23.26 hi	4.000 b	Straight	Brown
22	JMSA 01	25.03 f-i	3.667 b	Straight	Brown
23	JMSA 02	23.42 hi	3.667 b	Straight	Light Brown
24	JMSA 03	25.89 f-i	4.000 b	Straight	Brown
25	FFKU 01	24.44 g-i	4.000 b	Straight/Curved	Brown
26	FFKU 02	24.61f-i	4.000 b	Straight	Brown
27	FFKU 03	26.22f-i	4.000 b	Straight	Brown
	LSD (0.05)	5.507	1.374		
	CV (%)	11.68%	20.47%		

The highest sporulation time (11.00 day) was recorded in MMBH which was statistically different from all other isolates. On the other hand the lowest sporulation time (3.33 day) was recorded in MTBB 01 and MSMM 02 those were statistically similar to all other isolates without MMBH (Table 8).

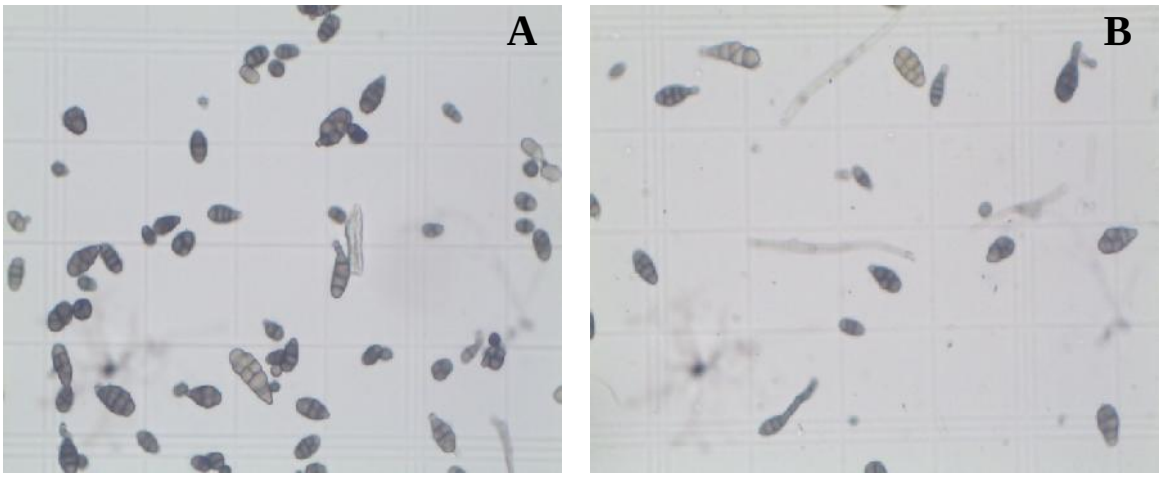


Plate 12 Counting of *Alternaria porri* conidia by using haemacytometer and digital microscope, (A) Isolate DSSA (B) Isolate MMBH

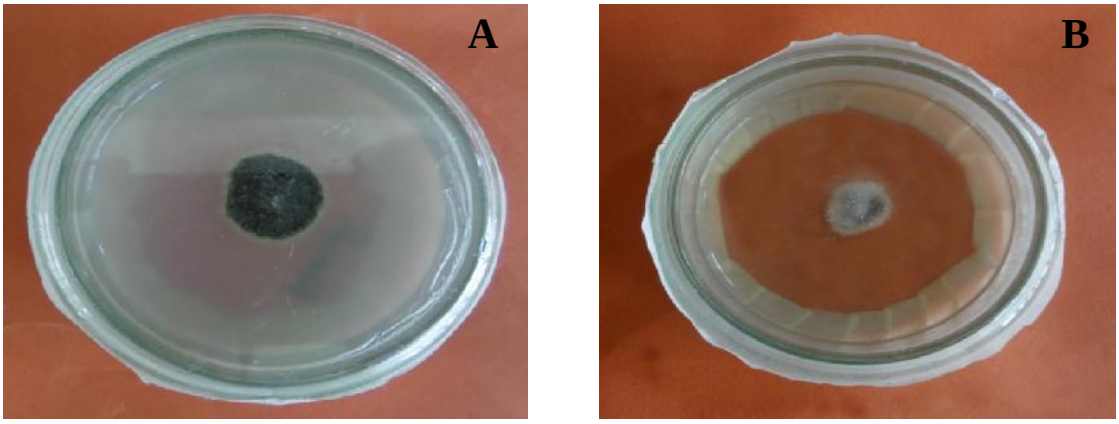


Plate 13 Sporulation 5 days after inoculation, (A) Isolate MTBB 01 (B) Isolate MMBH

Slight variation was observed in shape of conidia of 27 isolates of *Alternaria porri* (Table 8). All of the isolates contained straight shape conidia among them some isolates DSSA, CCKH 01, MSMM 02 and FFKU 01 contained both straight and curved shape conidia.

Slight variation was observed in color of conidia of 27 isolates of *Alternaria porri* (Table 8). Most of the isolates contained brown color conidia. Some isolates

MMBH and MSMM 02 contained deep brown and GJBS and JMSA 02 contained light brown conidia.

4.2.2. Septation of conidia of 27 isolates of *Alternaria porri*

Slight variations were observed in horizontal septation of conidia of 27 different isolates of *Alternaria porri* (Table 9). The horizontal septation of conidia varied from 3.00 to 6.00, whereas the highest mean horizontal septation (3.567) was recorded in isolate DSSA and the lowest (3.200) in isolate GGBB 01. But no significant variation found.

Significant variations were observed in longitudinal septation of conidia of 27 different isolates of *Alternaria porri* (Table 9). The longitudinal septation of conidia varied from 1.00 to 2.00. The highest mean longitudinal septation (1.400) was recorded in isolates DSSA and DSTR 02 whereas the lowest (1.133) in isolates MTBB 02, JJLL 03 and MSMM 03.

4.2.3. Size of Conidia of 27 Isolates of *Alternaria porri*

Significant variations were observed in length of conidia of 27 different isolates of *Alternaria porri* (Table 10). The length of conidia varied from 11.20 to 39.20µm, whereas the highest mean length (28.31µm) was recorded in isolate MMBH and the lowest (19.38 µm) in isolate RBHR 01.

Significant variations were observed in breadth of conidia of 27 different isolates of *Alternaria porri* (Table 10). The breadth of conidia varied from 4.76 to 11.43µm. The highest mean breadth (8.147µm) was recorded in isolate MSMM 01 whereas the lowest (6.740µm) in isolate RBHR 03.

Table 9 Septation of conidia of 27 isolates of *Alternaria porri*

Sl. No	Isolates	Septation of conidia		Range of variation	
		Horizontal	Longitudinal	Horizontal	Longitudinal
1	DSSA	**3.567**	**1.400 a**	3-5	1-2
2	DSTR 01	3.467	1.300 ab	3-4	1-2
3	DSTR 02	3.267	**1.400 a**	3-4	1-2
4	MMBH	3.367	1.267 ab	3-6	1-2
5	MTBB 01	3.267	1.233 ab	3-4	1-2
6	MTBB 02	3.267	**1.133 b**	3-4	1-2
7	RBHR 01	3.433	1.233 ab	3-5	1-2
8	RBHR 02	3.300	1.300 ab	3-4	1-2
9	RBHR 03	3.433	1.267 ab	3-5	1-2
10	GJBS	3.267	1.233 ab	3-4	1-2
11	GGBB 01	**3.200**	1.300 ab	3-4	1-2
12	GGBB 02	3.300	1.200 ab	3-4	1-2
13	CCKH 01	3.267	1.233 ab	3-4	1-2
14	CCKH 02	3.267	1.200 ab	3-5	1-2
15	CCKH 03	3.267	1.200 ab	3-4	1-2
16	JJLL 01	3.400	1.200 ab	3-6	1-2
17	JJLL 02	3.233	1.233 ab	3-4	1-2
18	JJLL 03	3.267	**1.133 b**	3-4	1-2
19	MSMM 01	3.233	1.267 ab	3-5	1-2
20	MSMM 02	3.367	1.167 b	3-4	1-2
21	MSMM 03	3.333	**1.133 b**	3-5	1-2
22	JMSA 01	3.433	1.200 ab	3-6	1-2
23	JMSA 02	3.233	1.167 b	3-4	1-2
24	JMSA 03	3.333	1.200 ab	3-4	1-2
25	FFKU 01	3.267	1.200 ab	3-5	1-2
26	FFKU 02	3.300	1.167 b	3-5	1-2
27	FFKU 03	3.300	1.300 ab	3-4	1-2
	LSD (0.05)	0.3191(NS)	0.1717		
	CV (%)	5.85%	8.51%		

Table 10 Size of conidia of 27 isolates of *Alternaria porri*

Sl. No	Isolates	Size of conidia		Range of variation	
		Length (µm)	Breadth (µm)	Length (µm)	Breadth (µm)
1	DSSA	19.91 ij	7.290 b-d	11.30 - 33.80	5.76 - 9.73
2	DSTR 01	23.20 d-g	7.713 a-c	17.60 - 33.60	6.46 - 10.46
3	DSTR 02	20.45 h-j	7.537 a-c	12.70 - 31.56	4.76 - 9.76
4	MMBH	**28.31 a**	7.337 a-d	19.40 - 39.00	6.23 - 9.30
5	MTBB 01	19.73 j	7.573 a-c	11.20 - 30.10	6.03 - 9.6
6	MTBB 02	20.27 h-j	7.637 a-c	13.60 - 29.40	5.96 - 8.40
7	RBHR 01	**19.38 j**	7.180 cd	12.70 - 30.67	6.70 - 9.56
8	RBHR 02	20.90 h-j	7.460 a-d	11.60 - 28.59	6.17 - 8.67
9	RBHR 03	19.82 j	**6.740 d**	13.20 - 30.63	5.90 - 8.30
10	GJBS	19.49 j	7.417 a-d	16.10 - 23.90	6.36 - 8.83
11	GGBB 01	19.87 ij	7.273 cd	14.56 - 24.58	6.30 - 8.40
12	GGBB 02	20.13 h-j	7.513 a-d	14.67 - 29.45	6.13 - 8.16
13	CCKH 01	24.55 cd	7.677 a-c	16.80 - 31.50	6.56 - 10.50
14	CCKH 02	22.15 e-h	7.683 a-c	15.79 - 28.30	6.83 - 8.80
15	CCKH 03	21.40 f-j	7.450 a-d	16.40 - 30.80	6.10 - 8.76
16	JJLL 01	27.23 ab	8.130 a	20.30 - 35.40	6.93 - 10.60
17	JJLL 02	23.46 d-f	7.707 a-c	20.80 - 35.90	6.93 - 9.80
18	JJLL 03	22.05 e-i	7.690 a-c	20.40 - 34.50	6.20 - 9.40
19	MSMM 01	26.24 bc	**8.147 a**	15.60 - 37.10	6.33 - 9.93
20	MSMM 02	23.09 d-g	7.910 a-c	14.80 - 39.20	6.43 - 9.06
21	MSMM 03	21.18 g-j	7.800 a-c	15.30 - 33.70	6.46 - 8.76
22	JMSA 01	25.74 bc	8.093 ab	21.10 - 34.30	6.26 - 11.43
23	JMSA 02	23.59 de	7.847 a-c	20.20 - 33.10	5.96 - 10.26
24	JMSA 03	23.54 d-f	7.673 a-c	18.30 - 34.10	5.93 - 10.86
25	FFKU 01	22.12 e-h	7.547 a-c	15.20 - 30.40	5.96 - 8.76
26	FFKU 02	21.27 g-j	7.173 cd	14.10 - 29.20	5.76 - 8.53
27	FFKU 03	20.43 h-j	7.287 b-d	14.20 - 37.30	6.93 - 8.53
	LSD (0.05)	1.849	0.6670		
	CV (%)	5.09%	5.38%		

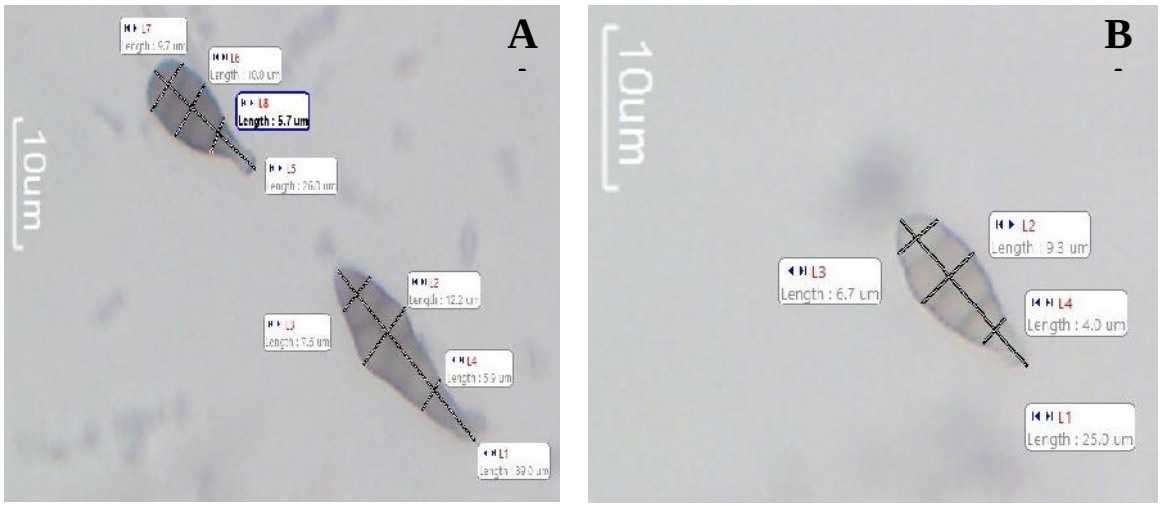


Plate 14 Measurement of *Alternaria porri* conidia, (A) Isolate MMBH (B) Isolate RBHR 01

4.3. Pathogenic variation of 27 isolates of *Alternaria porri*

The leaf infection of onion by 27 different *Alternaria porri* isolates varied significantly in the pot experiment (Table 11). After 5 days of inoculation the leaf infection varied from 2.333 to 4.000 mm, whereas the maximum infection (4.000 mm) of onion leaf was recorded in isolate MMBH and the minimum infection (2.333 mm) in isolates DSTR 02, MTBB 02, GGBB 02, CCKH 02, JJLL 01, JMSA 02 and FFKU 02.

After 7 days of inoculation the leaf infection varied from 7.667 to 16.33 mm, whereas the maximum infection (16.33 mm) of onion leaf was recorded in isolate MMBH and the minimum infection (7.667 mm) in isolate DSTR 02 (Table 11).

After 9 days of inoculation the leaf infection varied from 14.00 to 35.33 mm, whereas the maximum infection (35.33 mm) of onion leaf was recorded in isolate MMBH and the minimum infection (14.00 mm) in isolate DSTR 02 (Table 11).

Table 11 Pathogenic variability of 27 isolates of *Alternaria porri*

Sl. No.	Isolate	Leaf infection (mm)						
		5th DAI	7th DAI	9th DAI	11th DAI	13th DAI	15th DAI	17th DAI
1	DSSA	2.667 ab	12.33 b	18.33 bc	26.67 b-e	37.00 bc	48.67 bc	57.00 b-d
2	DSTR 01	3.333 ab	8.667 bc	15.00 bc	21.67 f-i	26.00 ij	34.67 j-l	41.33 jk
3	DSTR 02	**2.333 b**	**7.667 c**	**14.00 c**	**18.67 i**	**24.33 j**	**29.67 l**	**35.67 k**
4	MMBH	**4.000 a**	**16.33 a**	**35.33 a**	**51.33 a**	**64.33 a**	**82.67 a**	**94.67 a**
5	MTBB 01	3.333 ab	9.333 bc	17.00 bc	23.67 c-h	30.00 e-i	37.67 g-k	49.00 e-i
6	MTBB 02	**2.333 b**	8.333 bc	16.67 bc	23.33 c-i	28.67 g-j	36.33 i-k	45.67 g-j
7	RBHR 01	2.667 ab	9.667 bc	16.33 bc	21.67 f-i	28.33 g-j	35.00 j-l	43.33 ij
8	RBHR 02	2.667 ab	8.667 bc	14.67 bc	22.00 e-i	29.00 f-j	38.00 f-k	47.00 f-j
9	RBHR 03	2.667 ab	8.333 bc	14.33 bc	20.00 hi	27.33 h-j	34.67 j-l	43.67 ij
10	GJBS	2.667 ab	9.333 bc	16.33 bc	25.00 b-g	33.00 c-g	41.67 d-i	49.33 e-i
11	GGBB 01	3.333 ab	9.000 bc	17.00 bc	27.00 b-d	36.00 b-d	47.33 b-d	58.00 bc
12	GGBB 02	**2.333 b**	8.667 bc	15.33 bc	22.67 d-i	30.67 d-i	39.00 e-k	47.33 f-j
13	CCKH 01	2.667 ab	11.00 bc	19.00 b	28.67 b	40.33 b	50.33 b	62.33 b
14	CCKH 02	**2.333 b**	9.667 bc	17.67 bc	26.67 b-e	35.33 b-e	44.00 b-g	51.67 c-h
15	CCKH 03	3.000 ab	9.000 bc	18.00 bc	27.67 bc	35.00 b-e	44.00 c-g	51.67 c-h
16	JJLL 01	**2.333 b**	8.333 bc	18.00 bc	26.33 b-f	34.33 c-f	42.33 c-i	50.00 d-i
17	JJLL 02	2.667 ab	9.000 bc	17.33 bc	25.00 b-g	33.00 c-g	41.00 d-j	48.67 e-i
18	JJLL 03	3.000 ab	9.000 bc	16.33 bc	24.33 b-h	32.67 c-h	40.33 e-j	48.67 e-i
19	MSMM 01	3.000 ab	9.333 bc	14.67 bc	21.33 ghi	26.67 ij	33.33 kl	41.00 jk
20	MSMM 02	3.000 ab	10.33 bc	17.33 bc	24.67 b-h	31.33 d-i	39.00 e-k	46.00 g-j
21	MSMM 03	2.667 ab	9.000 bc	15.33 bc	22.33 d-i	30.00 e-i	37.00 h-k	44.33 h-j
22	JMSA 01	3.000 ab	9.333 bc	18.33 bc	27.00 b-d	36.00 b-d	45.33 b-e	54.00 c-f
23	JMSA 02	**2.333 b**	8.667 bc	17.00 bc	26.00 b-g	35.00 b-e	44.33 b-f	52.67 c-g
24	JMSA 03	3.000 ab	8.667 bc	18.00 bc	26.33 b-f	35.33 b-e	43.00 c-h	51.67 c-h
25	FFKU 01	3.000 ab	10.00 bc	18.33 bc	27.00 b-d	35.00 b-e	43.00 c-h	49.67 e-i
26	FFKU 02	**2.333 b**	11.67 bc	18.67 bc	28.67 b	37.33 bc	47.33 b-d	55.00 c-e
27	FFKU 03	2.667 ab	9.000 bc	16.00 bc	23.67 c-h	32.67 c-h	41.67 d-i	49.00 e-i
	LSD (0.05)	1.234	3.354	3.956	4.051	4.673	5.499	6.211
	CV (%)	27.01%	21.41%	13.87%	9.69%	8.52%	7.95%	7.49%

After 11 days of inoculation the leaf infection varied from 18.67 to 51.33 mm, whereas the maximum infection (51.33 mm) of onion leaf was recorded in isolate MMBH and the minimum infection (18.67 mm) in isolate DSTR 02 (Table 11).

After 13 days of inoculation the leaf infection varied from 24.33 to 64.33 mm, whereas the maximum infection (64.33 mm) of onion leaf was recorded in isolate MMBH and the minimum infection (24.33 mm) in isolate DSTR 02 (Table 11).

After 15 days of inoculation the leaf infection varied from 29.67 to 82.67 mm, whereas the maximum infection (82.67 mm) of onion leaf was recorded in isolate MMBH and the minimum infection (29.67 mm) in isolate DSTR 02 (Table 11).

After 17 days of inoculation the leaf infection varied from 35.67 to 94.67 mm, whereas the maximum infection (94.67 mm) of onion leaf was recorded in isolate MMBH and the minimum infection (35.67 mm) in isolate DSTR 02 (Table 11).

The rate of leaf infection per day maximum increment (7.553 mm) was recorded in MMBH which was statistically different from all other isolates. On the other hand the rate of leaf infection per day minimum increment (2.777 mm) was recorded in DSTR 02 which was statistically similar with isolates DSTR 01and MSMM 01 (3.167 mm) (Figure 4).

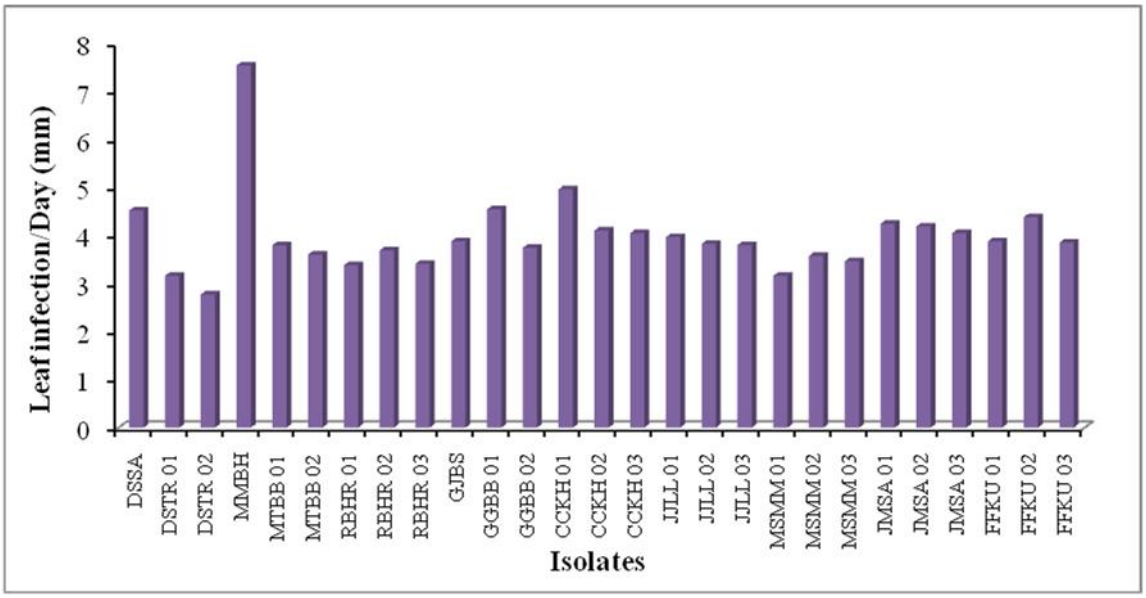


Figure 4 Infection of onion leaf per day by 27 isolates of *Alternaria porri*

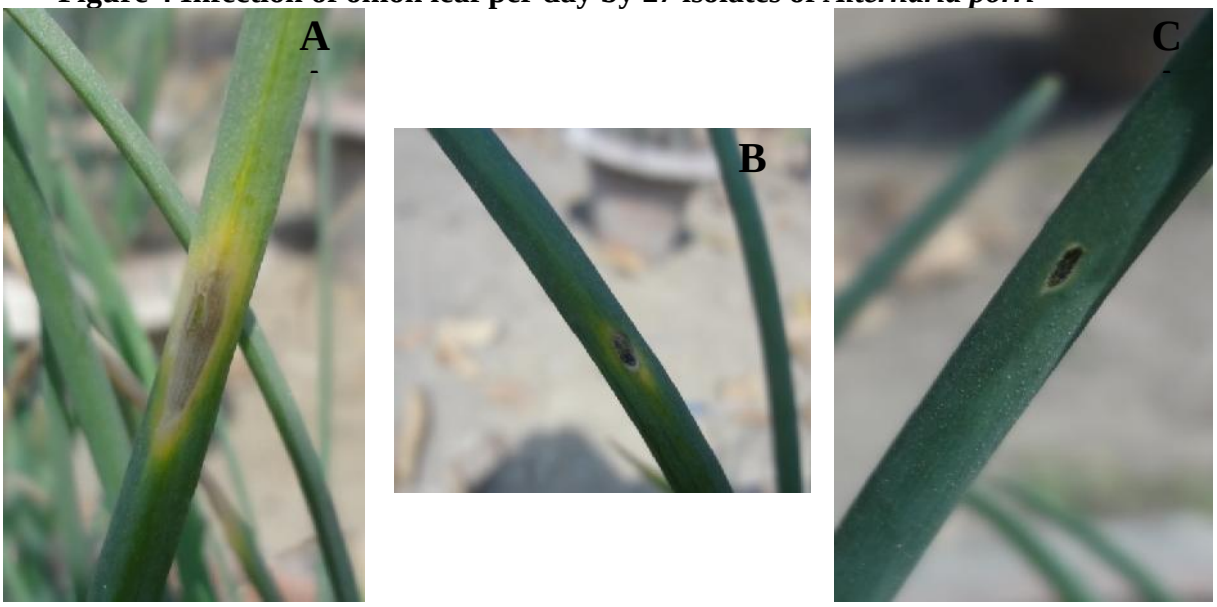


Plate 15 Disease developed 7 days after inoculation, (A) Isolate MMBH (B) Isolate DSTR 02 (C) Control

4.4. Molecular variation of *Alternaria porri*

4.4.1. Extraction of genomic DNA

Genomic DNA of 24 isolates was extracted by CTAB extraction method and this was confirmed by gel running of genomic DNA. The genomic DNA could not be extracted from the isolates DSTR 01, FFKU 01and FFKU 02 (Plate 16).

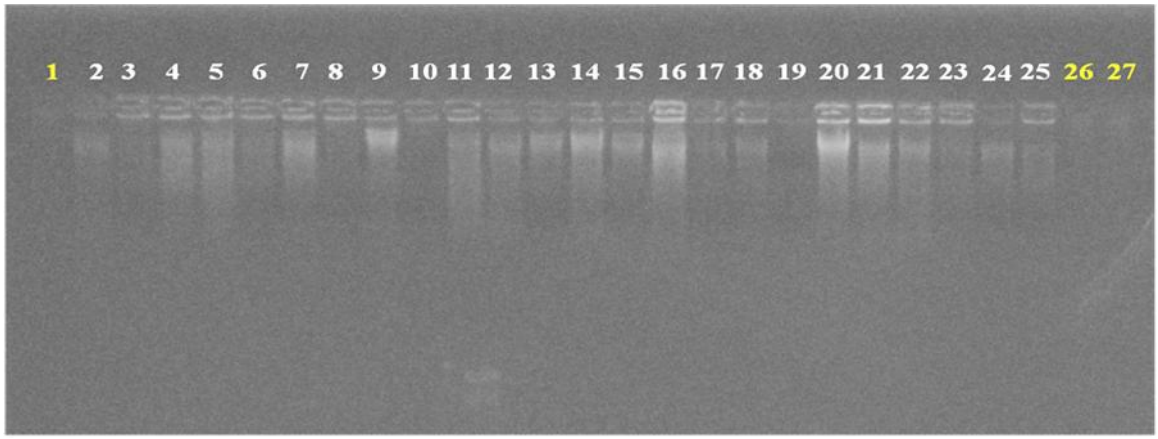


Plate 16 Gel electrophoresis of the genomic DNA of *Alternaria porri* isolates

4.4.2. RAPD analysis

To perform the RAPD analysis, seven (7) decamer primers were tested. These primers were selected for fingerprinting of twenty four (24) isolates of *Alternaria porri*. But there was no band found by using these primers (plate 17).

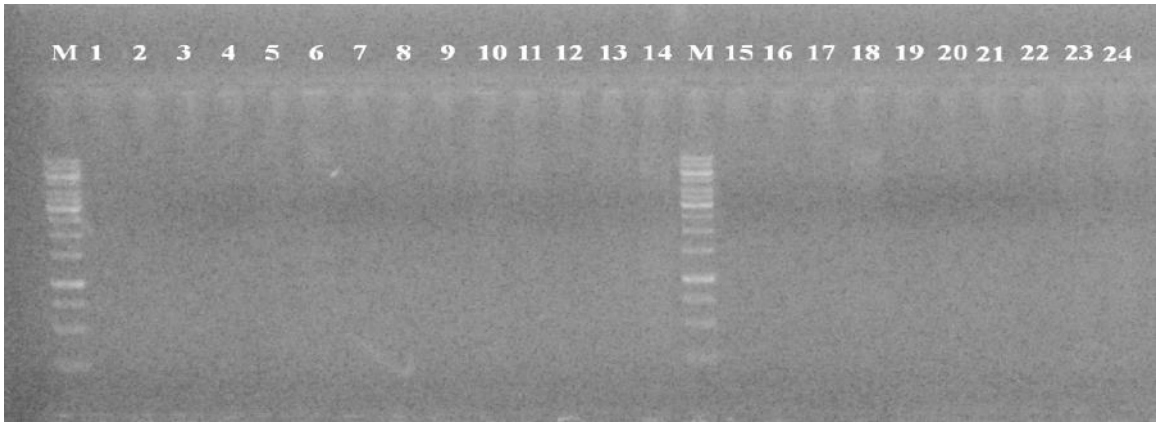


Plate 17 Gel electrophoresis of the PCR product performed by RAPD

4.4.3. ITS region identification

For ITS region identification two ITS primers ITS1F and ITS4 were used as forward primer and reverse primers respectively. By using these primer the ITS

regions were identified from three (3) isolates among twenty four (24) and those isolates were DSSA, DSTR 02 and RBHR 02 (Plate 18).

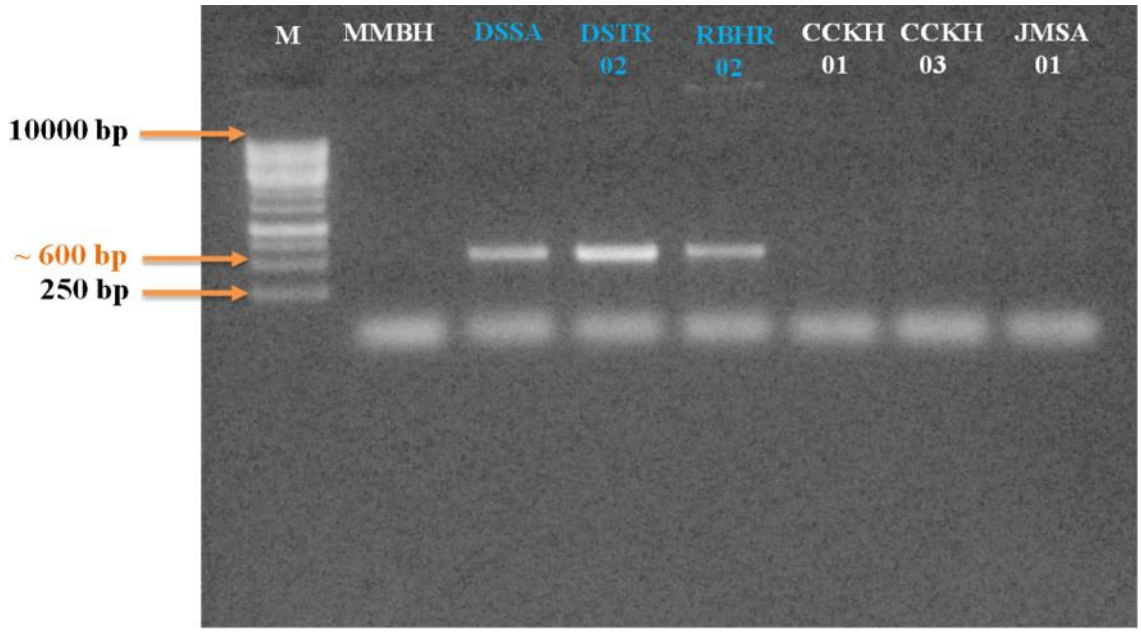


Plate 18 Gel electrophoresis of the PCR product performed by ITS1F and ITS4 primers

4.4.4. PCR products purification

Purification of PCR products was done by alcohol precipitation. Two (2) isolates DSSA and RBHR 02 were purified among three (3) and isolate DSTR 02 was missed during purification.

4.4.5. Sequencing of the PCR products

In order to confirm at the gene sequence level, PCR amplified bands were subjected to automated sequencing. For this purpose, a 600 bp band from two samples were used for sequencing. The sequenced samples were designated as A.DSSA and A.RBHR 02, respectively.

4.4.6. Analysis of the sequence results

Partial sequences of two samples were analyzed through NCBI-BLAST program database search system. Obtained results from the BLAST database showed that one sample A.DSSA have 75% nucleotide (nt) identities with *Alternaria porri*, *Alternaria alternata*, *Alternaria tenuissima* and 74% nucleotide (nt) identities with *Alternaria mali* fungus gene another sample A.RBHR 02 have 86% nucleotide (nt) identities with *Alternaria* sp. UFMGCB 4425 and 81% nucleotide (nt) identities with *Alternaria tenuissima* fungus gene reported from this geographic area as well as with others from worldwide.

4.4.6.1. A.DSSA [387nt]

```
....|....| ....|....| ....|....| ....|....| ....|....|
    5          15         25         35         45
TTAAAAAAAC TGGCTTCCCT CCCTGTAACC TGAGTCAAAG TTTGAAAAAA

....|....| ....|....| ....|....| ....|....| ....|....|
   55         65         75         85         95
AAGGCTTAAT GGATGCTATA CCTTCGTTGA AAAATAAGCC ATTTGGGCTG

....|....| ....|....| ....|....| ....|....| ....|....|
  105        115        125        135        145
CGTCTCCCTT CTCCCTATGC CGACTGCTGT TACCTTAAGG GGAGCCTCCA

....|....| ....|....| ....|....| ....|....| ....|....|
  155        165        175        185        195
CCAAAACTAC AACAAAACGC CCAAGCCAAG CAAATCTTGT GGGTAAAAAA

....|....| ....|....| ....|....| ....|....| ....|....|
  205        215        225        235        245
CACTCGAACA ACATCCCCTT TGGAACACTA AGTGTCAATC GCTTTCAACA

....|....| ....|....| ....|....| ....|....| ....|....|
  255        265        275        285        295
TTCTATGATG CAATGATTCT GCGATTTACA CAATTCATCG CATCTCCCTA

....|....| ....|....| ....|....| ....|....| ....|....|
  305        315        325        335        345
CGTTTTTACT CCATTCAGAA CAAGATTCCC TGTTGATGTT TTACATTATT

....|....| ....|....| ....|....| ....|..
  355        365        375        385
TTTACTCATC TGATCCATGC AAAAGTTTAT TTTCTCC
```

Table 12 Percent identities of nucleotide of A.DSSA isolate with selected *Alternaria* fungus reported worldwide

Accession	Description	Max score	Total score	Query coverage (%)	E-value	Identity
LN482533.1	*Alternaria porri* genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, strain TUHT137	167	167	80%	1e-37	75%
KF269242.1	*Alternaria alternata* isolate GZUBCEC2-12-2 18S ribosomal RNA gene, partial sequence;	170	170	80%	1e-38	75%

	internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence					
KM513592.1	*Alternaria tenuissima* strain JR9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	167	167	80%	1e-37	75%
JF817299.1	*Alternaria mali* strain CanR-77 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	170	170	86%	1e-38	74%

The NCBI-BLAST database output showed that this A.DSSA isolate shares more than 70% nt identities with previously reported *Alternaria*. In this case have 75% nt similarities with *Alternaria porri* genomic DNA (LN482533.1), *Alternaria alternata* partial sequence (KF269242.1), *Alternaria tenuissima*

partial sequence (KM513592.1) and 74% nt similarities with *Alternaria mali* partial sequence (JF817299.1) (Table 12).

The phylogenetic analysis done by using MEGA 6.0 software revealed that the A.DSSA isolate form a cluster by separate clade. This analysis also revealed that this isolate is the major ancestor of all the strains (Figure 5).

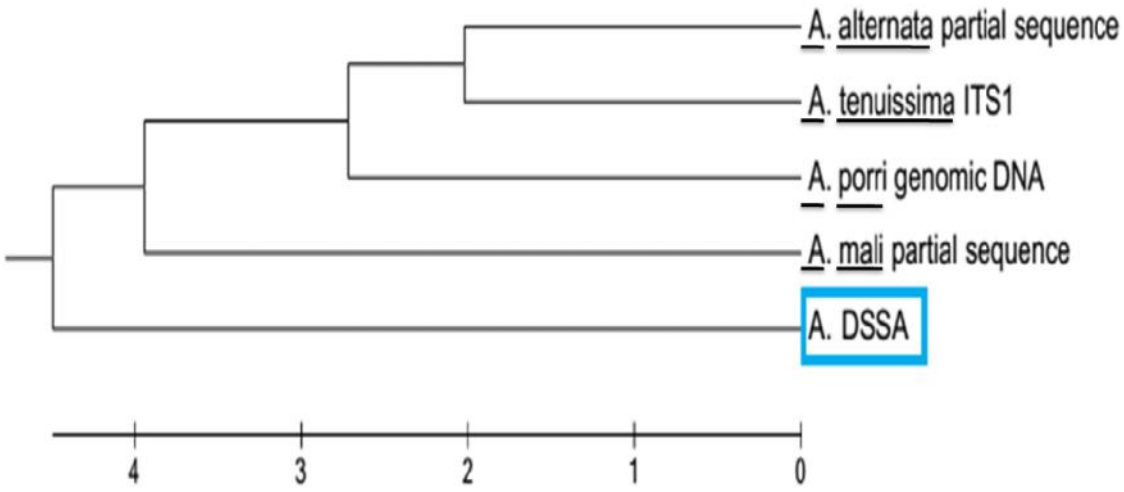


Figure 5 The phylogenetic tree for A.DSSA isolate showing ancestral relationship with other reported *Alternaia* isolates

4.4.6.2. A.RBHR 02 [263nt]

```
....|....| ....|....| ....|....| ....|....| ....|....|
    5          15         25         35         45
CAAATAGACC GGTCTACATA CGTGCACGAG TCAAGTGAAA AAAAGGCTTA

....|....| ....|....| ....|....| ....|....| ....|....|
    55         65         75         85         95
ATGGATGCTA GGACCTAGCG ATGGAAAGTG CGACTTGTGC TGCGCTCGAT

....|....| ....|....| ....|....| ....|....| ....|....|
   105        115        125        135        145
CTCTCCGCGG CTGCCGTTAC TTTGAGGGGA GCCTCCACCA AAGCTAGAGA

....|....| ....|....| ....|....| ....|....| ....|....|
   155        165        175        185        195
CAAGACGCCC TACACCAAGC AATTTTTGTC CCCTAAAATG ACTTTCTTAC

....|....| ....|....| ....|....| ....|....| ....|....|
   205        215        225        235        245
AGGTTTGCCC TTTGGAATTC CAGAGTTCTG ACTGTGAGTT AAACGATCTG

....|....| ...
   255
ACATTCCCTA ACT
```

The NCBI-BLAST database output showed that this A.RBHR 02 isolate shares more than 85% nt identities with previously reported *Alternaria* sp. UFMGCB 4425. In this case have 86% nt similarities with *Alternaria* sp. UFMGCB 4425 fungus complete genome (KJ404206.1) and 81% nt similarities with *Alternaria tenuissima* HLJ-KS-BH-TY-A (KF996744.1) (Table 13).

Table 13 Percent identities of nucleotide of A.RBHR 02 isolate with selected *Alternaria* fungus reported worldwide

Accession	Description	Max score	Total score	Query coverage (%)	E-value	Identity
KJ404206.1	*Alternaria* sp. UFMGCB 4425 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	69.4	69.4	24%	2e-08	86%
KF996744.1	*Alternaria tenuissima* isolate HLJ-KS-BH-TY-A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	147	147	72%	1e-31	81%

The phylogenetic analysis done by using MEGA 6.0 software revealed that the A.RBHR 02 isolate form a cluster by separate clade. This analysis also revealed that this isolate has close relationship with KF996744.1 (Figure 6).

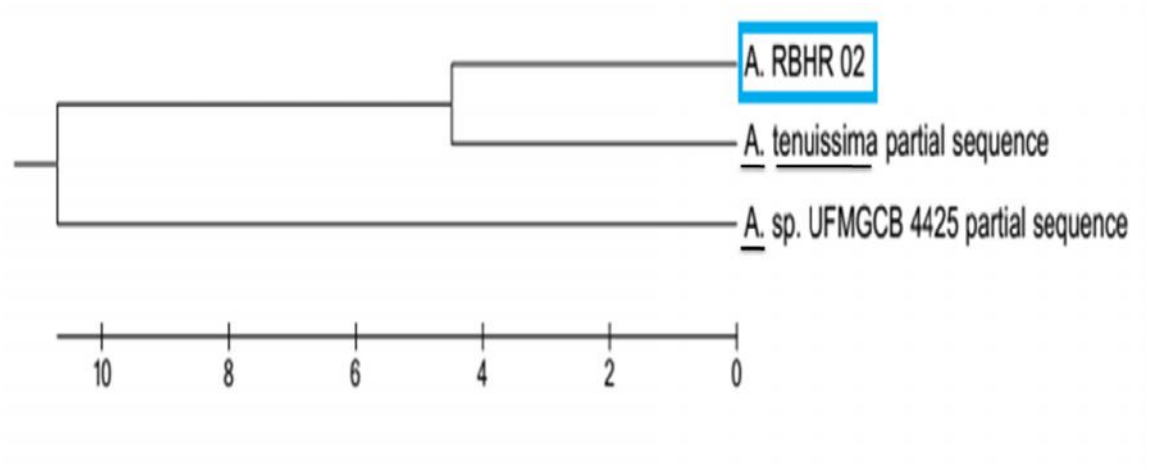


Figure 6 The phylogenetic tree for A.RBHR 02 isolate showing ancestral relationship with other reported *Alternaia* isolates

CHAPTER V

DISCUSSION

In the present research work, all the isolates showed variations in respect of their cultural, morphological, pathogenic and molecular characteristics.

Cultural variability

In respect of cultural characteristics, the isolates of *Alternaria porri* showed variation in growth rate, colony color, shape, margin and texture and substrate color.

The isolates of *Alternaria porri* depicted variation in growth rate (growth/day). Isolate DSTR 02 was exhibited the fastest growth among the isolates with mean growth rate of 3.950 mm/day while isolate MMBH exhibited the slowest growth with mean growth rate of 2.433 mm/day. Isolates in the present study showed periodic changes in their growth rates. All the isolates showed an increasing trend in growth rate from 4 days to 14 days. Pusz (2009) found colony diameter ranged from 4.8-6.8 cm while working with *Alternaria alternata*. Similar observations were also recorded by Thrall *et al.* (2005) and Rai and Kumari (2009).

All the isolates varied in colony color, shape, margin and texture and substrate color. Colony color varied from light to dark olivaceous green with grayish white. Mostly, the colony was irregular, regular with concentric ring and regular without concentric ring shape with entire, irregular and wavy margin. All of the isolates had effuse, fluffy and velvety mycelia growth with a color mostly grey to brown with some variations which were clearly visible from the underside of plates. The results are in agreement with Pusz (2009) who found that the colonies of *Alternaria alternata* isolated from *Amaranthus retroflexus* varied from light grey to dark grey. Similarly, Rai and Kumari (2009) observed loose, cottony, compact and dense colonies with light to dark black colour in *A. alternata* infecting Periwinkle. Hubballi *et al.* (2011) noted variation in the pigmentation of 15 *A. alternata* isolates producing black, brownish black, greenish black, brown and yellow pigmentation.

Morphological variability

In respect of morphological characteristics, the isolates of *Alternaria porri* showed variation in conidia production, sporulation, shape and color of conidia, septation of conidia and size of conidia. The conidia production of isolates varied from 7.720×10^3 to 47.02×10^3 per mm^2 with maximum in isolate DSSA and minimum in MMBH. Similarly, Daniel *et al*. (2008) observed *Alternaria alternata* isolates producing 2.8×10^5 to 17.2×10^5 conidia mL^{-1}.

In present study, the variation in sporulation time observed with the maximum sporulation time in isolate MMBH and minimum in MTBB 01 and MSMM 02. All the isolates produced light to deep brown color and straight or curved shape conidia. Similarly, Kaul and Saxena (1988) observed differences in color, shape and sporulation of the isolates of *Alternaria solani*.

Conidial septation both horizontal and longitudinal varied significantly among the isolates. Horizontal septa varied from 3 to 6 and longitudinal septa from 1 to 2. The highest mean number of horizontal septa was recorded in isolate DSSA (3.567) whereas the lowest in isolate GGBB 01(3.200). The highest mean number of longitudinal septa was recorded in isolate DSSA and DSTR 02 (1.400) whereas the lowest in isolates MTBB 02, JJLL 03 and MSMM 03 (1.133). In present study, the average conidial size varied from 11.20 -39.20 x 4.76 -11.43µm. Maximum (28.31µm) and minimum (19.38 µm) conidial length were noticed in isolate MMBH and RBHR 01, respectively, while maximum (8.147µm) and minimum (6.740µm) conidial breadth were recorded in isolates MSMM 01 and RBHR 03, respectively. Ramegowda and Naik (2008) reported that the hyphal width of 9 *A. macrospora* isolates varied from 2.87 to 6.95 µm. Tetarwal *et al.* (2008) found variation of conidiophore length and breadth among six isolates of *A. alternata* ranged from 18.90 to 27.40 and 4.23 to 5.75 µm, respectively. Rotem (1966) found a wide variability in the spore dimensions of 42 isolates of *A. solani*.

Pathogenic variability

A considerable pathogenic variability was observed in size of leaf infection among the isolates. Isolate MMBH was fastest among the isolates with mean

leaf infection rate of 7.553 mm/day while isolate DSTR 02 was slowest with mean leaf infection rate of 2.777 mm/day. Isolates in the present study depicted periodic changes in their leaf infection rates. All the isolates showed an increasing trend in leaf infection rate from 5 days to 17 days. The findings are in agreement with Thrall *et al.* (2005) who reported significant variations in the lesion size produced by *Alternaria brassicicola* isolates on wound inoculated *Cakile maritima* plants. Kumar (2004) also reported variation in lesion size and lesion number in *Alternaria triticina* isolates. However, present observations are contradictory to the findings of Quayyum *et al.* (2005) who did not find any significant variation in the lesions produced by the isolates of *Alternaria panax* on detached leaflets of ginseng.

Molecular variability

Seven (7) decamer primers were tested to perform the RAPD analysis. These primers were selected for fingerprinting of twenty four (24) isolates of *Alternaria porri*. But there was no band found by using these primers. The findings are agreement with Pusz (2009) who reported low genetic variability among the isolates of *Alternaria alternata* from *Amaranthus*. Kumer *et al.* (2008) also found no effect of origin of isolates; rather two isolates of *Alternaria solani* from two different locations were closer to each other. By using two ITS primer ITS1F and ITS4 the ITS regions were identified from three (3) isolates. The isolates were DSSA, DSTR 02 and RBHR 02. During purification isolate DSTR 02 was missed. Then a 600 bp band from two samples were used for sequencing. The sequenced samples were designated as A.DSSA and A.RBHR 02 respectively. The sequenced data of A.DSSA was analyzed through NCBI-BLAST database and the results showed that 75% nt similarities with *Alternaria porri* genomic DNA (LN482533.1), *Alternaria alternata* partial sequence (KF269242.1), *Alternaria tenuissima* partial sequence (KM513592.1) and 74% nt similarities with *Alternaria mali* partial sequence (JF817299.1). The NCBI-BLAST database output showed that 86% nt similarities with *Alternaria* sp. UFMGCB 4425 fungus complete genome (KJ404206.1) and 81% nt similarities with *Alternaria tenuissima* HLJ-KS-BH-

TY-A (KF996744.1). The results are in agreement with McKay *et al.*(1999) who reported that 99.6% level of homology in the ITS regions of *Alternaria alternata* and *Alternaria lini*. Similarly Kusaba and Tsuge (1995) also observed that, the close relationship between *Alternaria dauci* and *Alternaria bataticola,* as shown by the high level of homology between the two species. The phylogenetic analysis using MEGA 6.0 software revealed that the A.DSSA isolate form a cluster by separate clade. This analysis also revealed that this isolate is the major ancestor of all the strains. The A.RBHR 02 isolate form a cluster by separate clade. This analysis also revealed that this isolate has close relationship with KF996744.1.

CHAPTER VI

SUMMARY AND CONCLUSION

Onion (*Allium cepa*) is widely used as an essential spice and vegetable all over the world as well as in Bangladesh. The purple blotch of onion caused by *Alternaria porri* is the major constraint for lowering the onion yield in Bangladesh. The research was carried out to characterize *Alternaria porri* causing purple blotch of onion on the basis of cultural, morphological, pathogenic and molecular aspects. The experiment was laid out in the completely randomized design with three replications. Twenty seven isolates of *Alternaria porri* were collected from nine different onion growing regions. The observations were made on cultural, morphological, pathogenic and molecular characteristics of *Alternaria porri.* All the isolates showed variation in the terms of cultural, morphological and pathogenic characteristics.

Isolate DSTR 02 was exhibited the fastest growth among the isolates with mean growth rate of 3.950 mm/day while isolate MMBH exhibited the slowest growth with mean growth rate of 2.433 mm/day. All the isolates varied in colony color, shape, margin and texture and substrate color. Colony color varied from light to dark olivaceous green with grayish white. Mostly, the colony was irregular, regular with concentric ring and regular without concentric ring shape with entire, irregular and wavy margin. All of the isolates had effuse, fluffy and velvety mycelia growth with a color mostly grey to brown with some variations which were clearly visible from the underside of plates.

The conidia production of isolates varied from 7.720×10^3 to 47.02×10^3 per mm^2 with maximum in isolate DSSA and minimum in MMBH. Maximum sporulation time was recorded in isolate MMBH and minimum in MTBB 01 and MSMM 02. All the isolates produced light to deep brown color and straight or curved shape conidia. The highest mean number of horizontal septa was recorded in isolate DSSA (3.567) whereas the lowest in isolate GGBB 01(3.200). The highest mean number of longitudinal septa was recorded in

isolate DSSA and DSTR 02 (1.400) whereas the lowest in isolates MTBB 02, JJLL 03 and MSMM 03 (1.133). Maximum (28.31µm) and minimum (19.38 µm) conidial length were noticed in isolate MMBH and RBHR 01, respectively, while maximum (8.147µm) and minimum (6.740µm) conidial breadth were recorded in isolates MSMM 01 and RBHR 03, respectively.

Isolate MMBH was fastest among the isolates with mean leaf infection rate of 7.553 mm/day while isolate DSTR 02 was slowest with mean leaf infection rate of 2.777 mm/day.

Seven (7) decamer primers were tested to perform the RAPD analysis but no band was found by using these primers. Two ITS primers ITS1F and ITS4 were used to identify ITS regions from three (3) isolates. The isolates were DSSA, DSTR 02 and RBHR 02. During purification isolate DSTR 02 was missed. Then a 600 bp band from two samples were used for sequencing. The sequenced samples were designated as A.DSSA and A.RBHR 02 respectively. The sequenced data of A.DSSA was analyzed through NCBI-BLAST database and the results showed that 75% nt similarities with *Alternaria porri* genomic DNA (LN482533.1), *Alternaria alternata* partial sequence (KF269242.1), *Alternaria tenuissima* partial sequence (KM513592.1) and 74% nt similarities with *Alternaria mali* partial sequence (JF817299.1). The NCBI-BLAST database output showed that A.RBHR 02 86% nt similarities with *Alternaria* sp. UFMGCB 4425 fungus complete genome (KJ404206.1) and 81% nt similarities with *Alternaria tenuissima* HLJ-KS-BH-TY-A (KF996744.1).

At the end of the above results and discussion it can be concluded that-

- Cultural, morphological and pathogenic variability exists in purple blotch pathogen (*Alternaria porri*) prevailing in the onion growing regions of Bangladesh.
- Isolate MMBH exhibited the slowest growth rate (2.433 mm/day) and its colony color was grayish white which was different from all other isolates.

- The lowest conidia production ($7.720\times10^3/mm^2$) and highest sporulation time (11 days) was recorded in isolate MMBH which was different from other isolates.
- Isolate MMBH was fastest among the isolates with mean leaf infection rate (7.553 mm/day).
- The NCBI-BLAST database output showed that the isolate A.DSSA was 75% nt similarities with *Alternaria porri* genomic DNA (LN482533.1), *Alternaria alternata* partial sequence (KF269242.1), *Alternaria tenuissima* partial sequence (KM513592.1) and 74% nt similarities with *Alternaria mali* partial sequence (JF817299.1).
- The NCBI-BLAST database output showed the Isolate A.RBHR 02 was 86% nt similarities with *Alternaria* sp. UFMGCB 4425 fungus complete genome (KJ404206.1) and 81% nt similarities with *Alternaria tenuissima* HLJ-KS-BH-TY-A (KF996744.1).
- From all of the study the fungul isolates showed substantial identities with *Alternaria porri* and further detailed variation and diversity can be studied from the full genome sequence analysis. This could help us to reveal the true nature of this fungus from Bangladesh and proper sustainable management for purple blotch of onion disease can be implemented.

REFERENCES

Achenback, L.A., Patrick, J.A. and Gray, L.E. (1997). Genetic homogeneity among isolates of *Fusarium solani* that cause soybean sudden death syndrome. *Theor. Appl. Genet.* **95**:474-478.

Ahmed, H.U. and Hossain, M.M. (1985). Final report of project crop disease survey and establishment of a herbarium at BARI, Plant. Path. Divn., BARI, Joydebpur. PP.1670.

Ahmed, S.R. and Goyal, J.P. (1988). Control of purple blotch of onion with fungicides.. Department of Plant pathology, Agricultural Research Station, Banswara 327001, India, **20**(2): 185-186.

AIS (2011). Krishi Diary, DAE, Khamar Bari, Dhaka.

Ali, M.A. (2002). Biological variation and chemical control of *Rhizoctonia solani* causing rice sheath blight diseases in Bangladesh. A thesis submitted for the degree of Doctor of Philosophy of the University of London and for the Diploma of Imperial College. 202pp.

Ali, M.A., Kamal, M.M., Archer, S.A., Buddie, A. and Rutherford, M. (2004). Anastomosis and DNA fingerprinting the rice isolates of *Rhizoctonia solani* Kuhn using AFLP markers. *Bangladesh J. Plant Pathol.* **20** (1 &2): 1-8.

Alves, M.L.B., Paiva, W.O. and Assis, L.A.G. (1983). Incidence of purple spot (*Alternaria porri* Ell. Cif.) on onion (*Allium cepa* L.) cultivars and hybrids in Manaus, Amazonia. *Rev. Plant. Pathol.* **62**(10): 4564.

Aminuzzaman, F.M., Hossain, I. and Ahmed, F. (2010). Cultural variation and pathogenicity of *Bipolaris sorokiniana* on wheat in Bangladesh. *Int. J. Agric. Environ. & Biotech.* **3**:93-98.

Anonymous, (1997). On-farm trial on the integrated approach to control purple blotch of onion seed crop. Abstracts of Plant pathology Research, 1986-2005. Plant pathology Division, BARI, Joydebpur. p. 83.

Ariosa-Terry, M. and Herrera-Isla, L. (1986). Evaluation of damage caused by purple spot (*Alternaria porri*) in 2 onion varieties (*Allium cepa*) and in leek (*Allium porrum*). *Rev. Plant. Pathol.* **65**:4656.

Ashrafuzzaman, M.H. and Ahmed, M.U. (1976). Control of foliage disease of onion by spray fungicides. *Bangladesh J. Hort.* **4**(2): 25-30.

Barnwell, P., Balanchard, A.N., Bryant, J.A., Smirnoff, N. and Weir, A.F. (1998). Isolation of DNA from the highly mucilaginous succulent plant *Sedum telephium*. *Plant Mol. Biol. Report.* **16**: 133-138.

Basallotte-Ureba, M.J., Prados-Ligero, A.M., Melero-Vara, J.M., (1999). Aetiology of leaf spot of garlic and onion caused by *Stemphylium vesicariumin*. *Physiol. Plant Pathol.* **48**: 139-145.

BBS, (2007). Year Book of Agricultural Statistics of Bangladesh, 2006-07. Statistics Division, Ministry of Planning, Dhaka.

BBS, (2012). Year Book of Agricultural Statistics of Bangladesh, 2009-10. Statistics Division, Ministry of Planning, Dhaka.

Benoit, F. and Ceustermans, N. (1987). Japanese stem onion, Possibilities of the crop. Horticultural Abstr. 058-02097.

Bhonde, S. R., Srivastava, K. J. and Singh, K. N. (1992). Evaluation of varieties for late kharif (Rangda) crop of onion in Nasik area. Newsletter Associated Agricultural Development Foundation, Associated Agricultural Development Foundation, Nasik 422 001, India. 12: 1, 1-2.

Blakemore, E.J.A., Jaccoud Filho, D.S. and Reeves, J.C. (1994). PCR for the detection of *Pyrenophora* species, *Fusarium moniliformae, Stenocarpella maydis*, and the *Phomopsis/Diaporthe* complex. In: *Modern Assays For Plant Pathogenic Fungi Identication, Detection and Quantification.* (Eds. Schots, A., Dewey, F.M. and Olivier, R.P.). CAB Intrnational. Willingford, UK. 205-213pp.

Bose, T.K. and Som, G.M. (1986). Vegetable crops in India. Naya Prokash, Calcatta, India. Pp. 567-569.

Brewster and James (2008). Onion and other vegetable alliums. Crop Production Science in horti.

Bulat, S.A., Lubeck, M., Alekhina, I.A., Jensen, D.F. and Lubeck, P.S. (2000). Identification of the universally primed-PCR-derived sequence-characterized amplified region marker for an antagonistic strain of *Clonostachys rosea* and development of a strain-specific PCR detection assay. *Appl. Environ. Microbiol.* **66**: 4758-4763.

Bulat, S.A., Lubeck, M., Mironenko, N.V., Jensen, D.F. and Lubeck, P.S. (1998). UP-PCR analysis and ITSI ribotyping of strains of *Trichoderma* and *Glicladium. Mycol. Res.* **102**: 933-943.

Bulat, S.A., Mironenko, N.V., Lapteva, M.N. and Strelchenko, P.P. (1994). Polymerase chain reaction with universal primers (UP-PCR) and its application to plant genome analysis. In: *Conservation of Plant Genes II: Utilization of Ancient and Modern DNA.* (Eds. Adams RP, Miller JS and Goldenberg EM), Missouri Botanical Garden, St. Louis. 113-129pp.

Carbone, I. and Kohn, L.M. (1993). Ribosomal DNA sequence divergence within internal transcribed spacer I of the Sclerotiniaceae. *Mycologia.* **85**: 415-427.

Castellanos-Linares, J.J., Auchet-Jencens, F. and Garacia-Correosa, I. (1988). Effect of *Alternaria porri*. (Ell.) Cif. On onion seed production under experimental conditions in Cuba. *Rev. Plant. Pathol.* **67**: 2730.

Chauhan, S. and Pandey, B.N. (1995). Identification of *Bipolaris maydis* race T pathogenic to *Populus deltoids*. *Indian Phytopathol.* **48**(1):55-60.

Chawla, H.S. (2002). Basic Technology. In: *Intoduction to Plant Biotechnology. Second edition.* Oxford and IBH Publishing Co. Pvt. Ltd. New Delhi. 538pp.

Chien, A., Edgar, D.B. and Trela, J.M. (1976). Deoxyribonucleic acid polymerase from the extreme thermopile *Thermus aquaticus. J. Bacteriol.* **127**: 1550-1557.

Csaikl, U.M., Bastian, H., Brettschneider, R., Gauch, S., Meir, A., Schauerte, M., Scholz, F., Sperisen, C., Vornam, B. and Ziegenhagen, B. (1998).

Comparative analysis of different DNA extraction protocols: A fast, universal maxi-preparetion of high quality plant DNA for genetic evaluation and phylogenetic studies. *Plant Mol. Biol. Report.* **16**: 69-86.

Cumagun, C.J.R., Hockehull, J. and Lubeck, M. (2000). Characterization of *Trichoderma* isolates from Philippines rice fields by UP-PCR and rDNA-ITS1 analysis: Identification of UP-PCR markers. *J. Phytopathol.* **148**: 109-115.

Daniel, D.C.C., Eduardo, A., Tereza, R.S.B., Renato, B.C. and Eloisa, A.G.I.L. (2008). Comparison of methodologies for conidia production by *Alternaria alternate* from citrus. *Braz. J. Microbiol*. **39**:792-798.

Dini-Andreote, F., Pietrobon, V.C., Andreote, F.D., Romao, A.S., Sposito, M.B. and Araujo, W.L. (2009). Genetic variability of Brazilian isolates of *Alternaria alternata* detected by AFLP and RAPD techniques. *Braz. J. Microbiol.* **40**:670-677.

Edel, V., Steinberg, C., Avelange, I., Laguerre, G. and Alabouvette, C. (1995). Comparison of three molecular methods for the characterization of *Fusarium oxysporum* strains. *Phytopathology*. **85**(5): 579-585.

Erlich, H.A. (1989). PCR Technology: Principle and Application from DNA Amplification. New York: Stockton Press.

Everts, K.L. and Lacy, M.L. (1990). The influence of dew duration, relative humidity and leaf senescence on conditional formation and infection of onion by *Alternaria porri*. *Phytopathology.* **80**(11): 1203-1207.

Everts, K.L. and Lacy, M.L. (1996). Factors influencing infection of onion leaves by *Alternaria porri* and subsequent lesion expansion. *Plant. Dis.* **80**(3): 276-280.

Fancelli, M.I. and Kimati, H. (1991). Influence of culture media and fluorescent light on the sporulation of *Alternaria daci. Rev. Plant. Pathol.* **70** (10): 806.

FAO (2008). Production Year Book (SAARC Agricultural Information Centre).

FAO (2012). Production Year Book for 1990. Food and Agriculture Organization, Rome.

Foster, L.M., Kozak, K.R., Loftus, M.G., Stevens, J.J. and Ross, I. (1993).The polymerase chain reaction and its application to filamentous fungi. *Mycol. Res.* **97**: 769-781.

Fujimori, F. and Okuda, T. (1994). Application of the random amplified polymorphic DNA using the polymerase chain reaction for efficient elimination of duplicate strains in microbial screening. *J. Antibiot*. **47**: 173-182.

Gomez, K.A. (1984). Statistical Procedure for Agricultural Research. John Wiley and Sons, New York.

Guo, L.D., Xu, L., Zheng, W.H., Hyde, K.D. (2004). Genetic variation of *Alternaria alternata*, an endophytic fungus isolated from *Pinus tabulaeformis* as determined by random amplified microsatellites (RAMS). *Fungal Divers.* **16**:53-65.

Gupta, R.B.L. and Pathak, V.N. (1986). Effect of host of inoculums density and duration of high relative humidity on development of purple blotch of onion. *Phytophylactia* **18**(3) 151-152.

Gupta, R.B.L. and Pathak, V.N. (1988a). Yield losses in onions due to purple blotch disease caused by *Alternaria porri*. *Phytophylactica* **20** (1): 21-23.

Gupta, R.B.L. and Pathak, V.N. (1988b). Reaction of onion cultivars to purple blotch (*Alternaria porri*). *Int. J. Trop. Pl. Dis.* **6**: 129-131.

Gupta, R.P., Srivastava, V.K. and Panday, U.B. (1986). Control of purple blotch disease of onion seed crop. *Indian Phytopathol*. **39** (2): 303-304.

Hasan, M.H.A., Allam, A.D.A., Abo-Elyousr, K.A.M. and Hussein, M.A.M. (2006). New Disease Report. Plant Pathology Department, Faculty of Agriculture, Assiut University, 71526 Assuit, Egypt.

Hasan, A. (2008). Control of purple blotch complex of onion through fertilizer and fungicide application. MS thesis. Department of Plant Pathology, SAU. Dhaka.

Hossain, M., Chowdhury, M.N. and Khan, A.L. (1993). Effect of fungicides on the production of healthy onion seeds. Abs. Fifth Botanical Conf., Bangladesh Phytopath. Soc., pp: 7.

Hubballi, M., Sornakili, A., Nakkeeran, S., Anand, T. and Raguchander, T. (2011).Virulence of *Alternaria alternata* infecting noni associated with production of cell wall degrading enzymes. *J. Plant Protect. Res*.**51**:87-92.

Humpherson-Jones, F.M. and Phelps, K. (1989). Climatic factors influencing spore production in *Alternaria brassicae* and *Alternaria brassicicola*. *Ann. Appl. Biol.* **114**: 449-458.

Islam, M.R., Akter, N., Chowdhury, S.M., Ali, M. and Ahamed, K.U. (2001). Evaluation of fungicides against *Alternaria porri* causing purple blotch of onion. *J. Agric. Sci. Tech.* **2**(1): 27-30.

ISTA (1996). International Rules for Seed Testing. *Seed Science and Technology*. **24**: 20-22.

Johanson, A. (1994). PCR for detection of the fungi that cause sigatoka leaf spots of banana and plantain. In: *Modern Assays for Plant Pathologenic Fungi: Indentification, Detection and Quantification.* (Eds. Schots, A. F., Dewey, M. And Oliver, R. P.). CAB International. Wallingford, UK. 215-222pp.

Karp, A., Edwards, K.J., Bruford, M., Funk, S., Vosman, B., Morgante, M., Seberg, O., Kremer, A., Boursot, P., Arctander, P., Tautz, D. and Hewitt, G.M. (1997). Molecular technologies for biodiversity evaluation: Opportunities and challenges. *Nat. Biotechnol.* **15**: 625-628.

Kaul, A.K. and Saxsena, H.K. (1988). Physiological specialization in *Alternaria solani* causing early blight of tomato. *Indian J. Mycol. Plant Pathol.* **18**: 128-132.

Khare, U.K. and Nema, K.G. (1981). Studies on purple blotch of onion sporulation on host and dispersal of conidia. *Indian Phytopathol*. **34**(2): 214-218.

Khare, U.K. and Nema, K.G. (1984). An experiment to determine the effect of temperature and humidity of the development of symptoms of purple blotch of onion incited by *Alternaria porri*. *Indian Phytopathol*. **36**(2): 234-235.

Kibria, G.M. (2010). Screening of different onion varieties against *Alternaria porri* causing purple blotch disease. Plant Path. Dept. Sher-e Bangla Agril. Univ.pp.1-49.

Kumar, A. (2004). Cultural and physiological variability among theisolates of *Alternaria triticina* from Bihar causing leaf blight of leaf. *Plant Dis. Res.* **19**:10-15.

Kumar, V., Haldar, S., Pandey, K.K., Singh, R.P., Singh, A.K., Singh, P.C. (2008). Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of *Alternaria solani* in India. *World J. Microbiol. Biotechnol.* **24**:1003-1009.

Kusaba, M. and Tsuge, T. (1995). Phylogeny of *Alternaria* fungi known to produce host-specificc toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. *Curr. Gen.* **28**:491-498.

Larka, B.S. (1999). Development of purple blotch incited by and its losses in seed crop of onion. *Indian J. of Agrial. Sci*. **69**:(2) 144-146.

Lubeck, M. (1997). Marker Gene and PCR Based Approaches as Tools for Monitoring Fungi in Soil-with Emphasison UP-PCR and Fungi from The Genera *Trichoderma* and *Gliocladium*. Ph.D. Thesis. Department of Plant Biology, Plant Pathology Section, The Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Lubeck, M., Alekhina, I.A., Lubeck, P.S., Jensen, D.F. and Bulat, S.A. (1999). Delineation of *Trichoderma harzianum* into two different genotypic groups by a highly robust fingerprinting method, UP-PCR and UP-PCR product cross hybridization. *Microbiol. Res.* **103**: 289-298.

Lubeck, M. and Poulsen, H. (2001). UP-PCR cross blot hybridization as a tool for identification of anastomosis groups in the *Rhizoctonia solani* complex. *FEMS Microbiol. Lett.***201**: 83-89.

Lubeck, M., Poulsen, S.K., Lubeck, P.S., Jensen, D.F. and Thrane, U. (2000). Identification of *Trichoderma* strains from building materials by ITSI ribotyping, UP-PCR fingerprinting and UP-PCR cross hybridization. *FEMS Microbiol. Lett.* **185**: 129-134.

Mazzola, M., Wong, O.T. and Cook, R.J. (1996). Virulence of *Rhizoctonia oryzae* and *R. Solani* AG-8 on wheat and detection of *R. Oryzae* in plant tissue by PCR. *Phytopathology.* **86**(4): 354-360.

McDonald, B.A. (1997). The population genetics of fungi: tools and techniques. *Phytopathology*. **87**: 448-453.

McKay, G.J., Brown, A.E., Bjourson, A.J. and Mercer, P.C. (1999). Molecular characterisation of *Alternaria linicola* and its detection in linseed. *Eur. J.Plant Pathol.***105**: 157-166.

Meah, B. and Khan, A.A. (1987). Checklist of vegetables and fruit diseases in Bangladesh. Department of Plant Pathology, BAU. Mymensingh. p.22.

Mian, M.S., Stevans, C. and Mia, M.A.T. (2002). Methods for DNA fingerprinting to differetiate strains of *Pyricularia grisea*. *Bangladesh J. Plant Pathol.* **18**(1 & 2): 19-26.

Mian, M.S., Stevans, C. and Mia, M.A.T. (2003). Diversity of the rice blast pathogen *Pyricularia grisea* from Bangladsh analysed by DNA fingerprinting. *Bangladesh J. Plant Pathol.* **19**(1 & 2): 81-85.

Micheli, M.R. and Bova, R. (1997). Fingerprinting Methods Based on Arbitrarily Primed PCR. Springer-Verlag, Berlin, Heidelberg, New York.

Miller, M.E. (1983). Relationship between onion leaf age and susceptibility to *Alternaria porri*. *Plant dis.* **67**(3): 283-286. Texas Agric. Expt. Sta. Weslaco, USA.

Mills, P.R. (1994). DNA based methods for identification and characterization. In: *The identification and characterization of pest organisms.* (Ed. Hawksworth, D. L.), CAB International, U.K.

Mullis, K.B. and Faloona, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. *Meth. Enzymol.* **155**: 335-350.

Nielsen, K., Justesen, A.F., Jensen, D.F. and Yohalem, D.S. (2001). University primed polymerase chain reaction alleeles and internal transcribed spacer restriction fragment length polymorphisms distinguish two subgroups in *Botrytis aclada* distinct from *B. byssoidea. Phytopathology*. **91**: 527-533.

Nuchart-Jonglaekha, Witcha-Saatsut, Sombat, S. (1982). Studies on purple blotch of onion, garlic and fungicide tests for control. Chiang Mai University. Chiang Mai (Thailand). Dept. of Plant Pathology, Chiang Mai (Thailand).

Panet, A. and Khorana, H. G. (1974). *J.. Biol. Chem.* **249**: 5213-5221.

Pascua, M.S.C. Kik Patena, L.S. and Badar, A.J. (1997). Breeding for purple blotch resistant in garlic and shallot. *Philippine J. Crops Sci.* **5**:72-75.

Perez Moreno, Luis, Chavez-Hernadez, luis-Felipe. (1992). Genotype and fungicide evaluation for control of purple spot *(Alternaria porrf)* and downy mildew peronospora destructor (Berk) caps of onion *(Allium cepa* L) in Irapauto, GTO, Universidad de GTO. Universidad de Guanjuato, (Mexico), Esculea de Agronomia Y Zooteenia, Apdo, Postal 311, irapauto, GTO, 36500. Ravista-Mexicana-de-Fitopattologia (Mexico). (1992). V. 10(l).P.29-34.

Peever, T.L., Ibanez, A., Akimitsu, K., Timmer, L.W. (2002). Worldwide phylogeography of the citrus brown spot pathogen, *Alternaria alternata*. *Phytopathology*. **92**:794-802.

Porebski, S., Bailey, L.G. and Baum, B.R. (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Report.* **15**: 8-15.

Prasad, S.S., Sharada, K., Brown, A.F. and Mills, P.R. (1996). PCR-based detection of *Colletotrichum acculatum* of strawberry. *Plant Pathol*. **45**: 650-655.

Pusz, W. (2009). Morpho-physiological and molecular analyses of *Alternaria alternata* isolated from seeds of *Amaranthus*. *Phytopathology*. **54**:5-14.

Quayyum, H.A., Dobinson, K.F., Traquair, J.A. (2005). Conidial morphology, virulence, molecular characterization and host-parasite interactions of selected *Alternaria panax* isolates on American ginseng. Can. *J. Bot.* **83**:1133-1143.

Rahman, M.L., Ahmed, H.U. and Mian, I.H. (1988). Efficacy of fungicides in controlling purple leaf blotch of onion. *Bangladesh J. Plant Pathol.* **4**(1&2): 71-76.

Rahman, M.L. (1990). Efficacy of Fungicides in controlling purple leaf blotch (*Alternaria porri*) of onion (*Allium cepa*). M.S. Thesis in Plant Pathology. SAU.

Rai, P.K., Kumari, L. (2009). Variability in *Alternaria alternata* infecting periwinkle (*Catharanthus roseus*). *Progr. Agric*. **9**:269-272.

Raju, K.S. and Metha, B.K. (1982). Certain nutritional aspects of *Alternaria porri* of onion. *India J. Mycol*. **12**(1): 96-98.

Ramegowda, G., Naik, M.K. (2008). Morphological, cultural, and physiological diversity in isolates of *Alternaria* spp. infecting Bt-cotton. *J. Mycol. Plant Pathol.* **38**:267-271.

Rodriguez, F., Herrera, I. and Vinagera, E. (1994). Influence of the temperature and relative humidity on the germination of *Alternaria porri* conidia, causal agent of purple blotch of onion. *Rev. Plant. Pathol.* **73**:2941.

Rolfs, A., Schuller, I., Finchk, U. and Weber-Rolfs, I. (1992). PCR: Clinical Diagnostics and Research. Spinger-Verlag. Berlin.

Rotem, J. (1966). Variability in *Alternaria porri* f.sp. *solani*. *Israel J. Bot.* **15**:47-57.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Aenheim, N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis of diagnosis of sickle cell anemia. *Science* **230**: 1350-1354.

Sartorato, A. (2002). Identification of *Phaeoisariopsis griseola* pathotypes from five states in Brazil. *Fitopathol. Brasil*. **27**:78-81.

Schesser, K., Lunder, A. and Henson, J.M. (1991). Use Polymerase Chain Reaction to detect the take-all fungus, *Gaeumanomyces graminis* in infected wheat plants. *Appl. Environ. Microbiol.* **57**: 553-556.

Schwartz, H.F. and Mohan, S.K. (2008). Compendian of onion and garlic disease, 2nd edition. American Phytopathological Society Press.

Schwartz, H.F. (2010). Soil borne diseases of onion. Colardo State University Extension Service.

Shahzad, A. (2003). Studies on *Alternaria* leaf blotch of apple in Kashmir. Ph.D. Thesis. Post Graduate Faculty, SKUAST-Kashmir, Shalimar, J and K, India.

Shandhu, K.S., Gill, S.S. and Hari Singh (1982). Effect of cultural practices in purple blotch disease in onion seed crop. *J. Res*. (1982) **19**(2): 118-120. Punjab Agric. Univ. Ludhiana, India.

Sharma, I.M. (1997). Screening of onion varieties/lines against purple blotch caused by *Alternaria porri* under field conditions. *Plant Disease Research*, Regional Horticultural Research Station, Bajaura (Distt. Kullu), Himachal Pradesh 175125, India. 12: 1, 60-61.

Sharp, P.A., Sugden, B. and Sambrook, J. (1973). Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. *Biogeochemistry* **12**:3055-3063.

Simmons, E.G. (1995). *Alternaria themes* and variations (112–144). *Mycotaxon.* **55**:55–163.

Simmons, E.G. (1999a). *Alternaria themes* and variations (226–235): classification of citrus pathogens. *Mycotaxon.* **70**:263–323.

Simmons, E.G. (1999b). *Alternaria themes* and variations (236–243): host-specific toxin producers. *Mycotaxon.* **70**:325–369.

Srivastava, P.K., Bhardwaj, B.S. and Gupta, R.P. (1994). Status of field diseases and insect pests of onion in India. News Let. Natl. Hort. Res. Dev. Found. 14(2): 11-14.

Srivastava, P.K., Tiwar, B.K. and Srivastava, K.J. (1996). Studies on integrated diseases management of onion. National Horti. Res. Development Foundation, Nashik, India. 19:4, 7-9. (cab abstract 2000/08-2002/07).

Stackman, L.C., Parker, J.H., Dioemeisel, F.J. (1981). Can biologic forms of stem rust on wheat change rapidly enough to interfere with breeding for rust resistance? *J. Agric. Res.* **14**:111-123.

Tetarwal, M.L., Rai, P.K., Shekhawat, K.S. (2008). Morphological and pathogenic variability of *Alternaria alternata* infecting senna (*Cassia angustifolia*). *J. Mycol. Plant Pathol.* **38**:375-377.

Thind, T.S. and Jhooty, J.S. (1982). Association of thrips with purple blotch infection on onion plants caused by *Alternaria porri. Indian Phytopathol.* **35**: 696-698.

Thirumalachar, M.J. and Mishra (1953). Some diseases of economic plants in Bihar, India. I and II. FAO, Pl. Prot. Bull. 1(10): 145-146; 2(1): 11-12 (R.A.M. 33; 338).

Thrall, P.H., Barrett, L.G., Burdon, J.J., Alexander, H.M. (2005). Variation in pathogen aggressiveness within a metapopulation of the *Cakile maritime-Alternaria brassicicola* host-pathogen association. *Plant Pathol.* **54**:265-274.

Van der, W.J.E., Korsten, L., Slippers, B. (2004). Genetic diversity among *Alternaria solani* isolates from potatoes in South Africa. *Plant Dis.* **88**:959-964.

Vohora, S.B., Rizman, M. and Khan, J.A. (1974). Medicinal Uses of Common Indian Vegetables. *Plant Medica*. **23**(4): 381-393.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531-6535.

White, T.J., Bruns, T.D., Lee, S. and Taylor, J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic. In: *PCR Protocols: A Guide to Methods and Applications.* (Eds. Innis M, Gelfand DH, Sninsky JJ and White TJ), Academic Press, San Diego. 315-322pp.

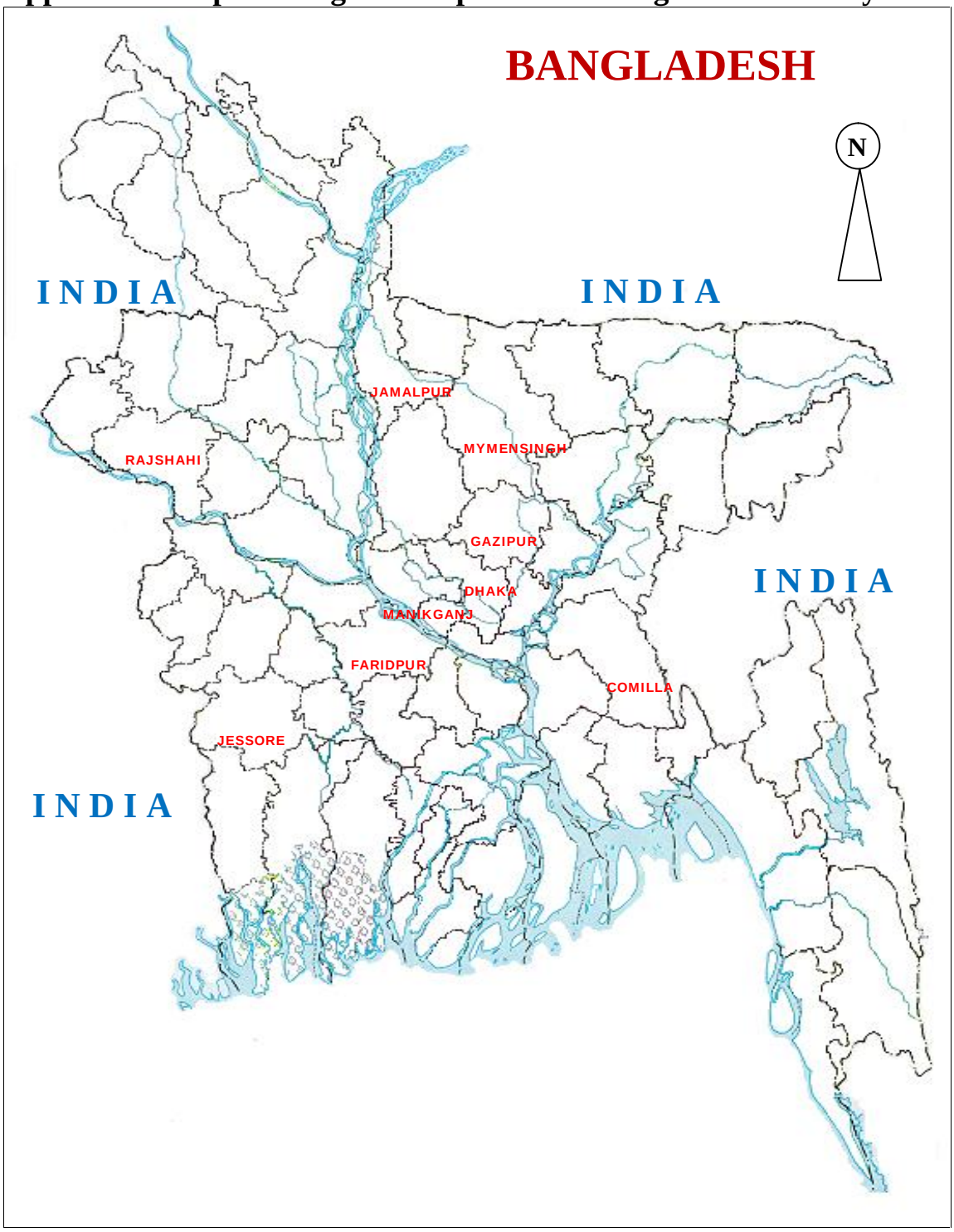
Yazawa, S. (1993). Onion seed production in Srilanka. *Rev. Plant. Pathol.* **72**(7): 526.

Website consultation

http://blast.ncbi.nlm.nih.gov

APPENDICES

Appendix I. Map showing the sample collected region under study

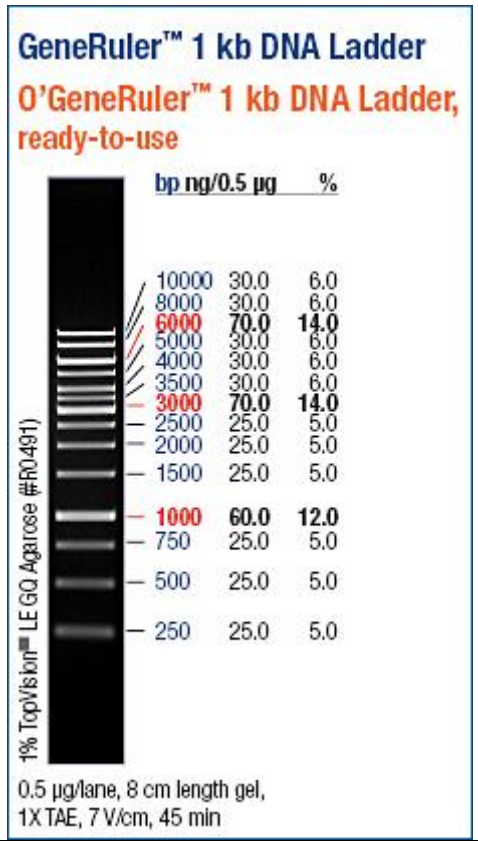


Appendix II. 1kb DNA ladder

Appendix III. Preparation of media

The culture media used in this thesis work are given below:

Potato Dextrose Agar (PDA)



Sl. No.	Components	Amount per sample
1	Peeled potato	200 g
2	Dextrose	20 g
3	Agar	17 g
4	Distilled water	1000 ml