CULTURAL, MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *STEMPHYLIUM VESICARIUM* CAUSING WHITE BLOTCH OF ONION

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DECEMBER, 2013

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REG. NO. 07-02192

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: DECEMBER, 2013

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This is to certify that the thesis entitled**,** "**CULTURAL, MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *STEMPHYLIUM VESICARIUM* CAUSING WHITE 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 result of a piece of bona fide research work carried out by **HOSNA ARA CHOWDHURY NISHA,** bearing Registration No**. 07-02192** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma in elsewhere.

I further certify that any help or sources of information as has been availed of during the course of this investigation have been duly acknowledged and the contents and style of the thesis have been approved and recommended for submission.

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ABSTRACT

An investigation comprising a set of experiments were conducted at the Molecular Plant Pathology Laboratory of the Department of Plant Pathology, Sher-e-Bangla Agricultural University and in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka during February'13 to July'14. A total of 24 isolates of *Stemphylium vesicarium* collected from different onion growing areas were characterized in terms of cultural, morphological and molecular aspects. Front colony colors were greenish brown to dirty white, deep grey to whitish, light grey to whitish, deep greenish white, light grey and dirty white to greenish. Reverse colony colors were brown, deep brown and light brown. Colony shapes were circular and irregular with umbonate, raised and flat type colony elevation. Colony texture were cottony, fluffy and velvety with entire, undulate and filiform type colony margin. Among the culture media, V-7 juice agar found to be the most suitable culture media for mycelial growth of *S. vesicarium.* The sporulation of the fungus was remarkably influenced by V-7 juice mixed with Potato Dextrose Agar, this media exhibited the highest sporulation (87.76-169.0/mm^2) of *S. vesicarium* in comparison to the other media. The minimum days (28.33 to 31.00 days) to conidia production was observed on V-7 juice agar medium. The length of conidia varied from 14.6 µm to 30.6 µm. Maximum length of conidia was 29.97 µm found in isolate DSSA and minimum 17.36 µm in isolate MSMM 02. The breadth of conidia ranged from 4.7 µm to 15.7 µm. Maximum breadth of conidia was 12.55 µm found in isolate DSSA and minimum 9.760 µm in isolate CCKH 02. The horizontal septation varied from 1-3. The longitudinal septation varied from 0-4. DNA extraction was successfully done by using CTAB method from 18 isolates out of 24 isolates for studying molecular variability. Testing of seven (7) decamer primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-13, OPB-04, OPB-18) showed no band of DNA of *S. vesicarium* isolates. Finally DNA sequencing was done by identifying the ITS regions from DSTR 01 isolate by using ITS primer (ITS1F and ITS4). SV-DSTR 01 showed no significant similarity with any gene through NCBI-BLAST program.

ACKNOWLEDGEMENTS

All praises to Almighty Allah, the great, gracious, merciful and supreme ruler of the universe who helped to complete the research work as well as to submit the thesis for the degree of Master of Science (M.S.) in Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh .

The author avails the opportunity of conveying her respect, deepest sense of gratitude and profound appreciation to her most reverend teacher and research supervisor, ***Dr. Md. Rafiqul Islam****, Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh for his scholastic guidance, keen inspiration, valuable suggestions, constructive criticisms, continuous inspiration and constant encouragement during the entire period of the research work and in the preparation of the manuscript.*

The author wishes to express her sincere appreciation and extreme gratitude to her co-supervisor, ***Dr. Mohammad Nurul Islam,*** *Professor, Department of Botany, University of Dhaka, Bangladesh for his precious instructions, guidence and cordial help to avail the laboratory facilities of the Department of Botany, University of Dhaka to complete the molecular characterization, the most technical part of the research.*

The author is indebted and thankful to Professor Dr. F. M. Aminuzzaman, Chairman, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh for his cordial inspiration and co-operation throughout the period of the research.

Cordial thanks and honors to Professor Mrs. Nasim Akhtar, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh for her valuable teaching, necessary suggestions and encouragement during the study period.

The author expresses heartfelt thanks and gratitude to her esteemed teachers, Professor Dr. Md. Salahuddin M. Chowdhury, Professor Nazneen Sultana, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka,

Bangladesh for their inspiration and co-operation throughout the period of the research work.

The author expresses her sincere appreciation to Associate Professor Khadija Akhter, Dr. Md. Belal Hossain, Dr. Fatema Begum, Dr. Nazmoon Nahar Tonu, and Assistant professor Abu Noman Faruk Ahmmed, Md. Tohidul Islam, Shukti Rani Chowdhury and Md. Ziaur Rahman Bhuiyan, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh for their valuable lesson and co-operation throughout the study period.

The author also extends special thanks to Mr. Sayed Mohammad Mohsin, Lecturer, Department of Plant Pathology, Sher-e- Bangla Agricultural University, Dhaka for his fellow feelings and cordial help in data analysis for the thesis work.

Thanks are extended to Senior Laboratory Assistant Pritilota, Laboratory Assistant Rehana Pervin and Khorshad Al-Alam and M.L.S.S., Nazrul Islam, Department of Plant Pathology, Sher-e- Bangla Agricultural University, Dhaka, Bangladesh for their help and co-operation throughout the period of the research work.

The author express her profound love and indebtedness to her grandparents, parents, brothers, friends and relatives for their blessings, inspirations and co–operation in all phases of this academic pursuit from beginning to the end.

The Author

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LIST OF SOME ABBREVIATE XORM AND THEIR ELABORATIONS

ABBREVIATE FORM	ELABORATION
%	Percentage
^{o}C	Degree Celsius
µl	Microliter
µm	Micrometer
AFLP	Amplified Fragment Length Polymorphism
AIS	Association for Information Systems
ANOVA	Analysis of variances
AP-PCR	Arbitrary Primed PCR
av.	Average
BARI	Bangladesh Agricultural Research Institute

BAU	Bangladesh Agricultural University
BBS	Bangladesh Bureau of Statistics
bp	Base Pair
$CaCO_3$	Calcium Carbonate
CARS	Centre for Advanced Research in Sciences
cm	Centimeter
CRD	Completely Randomized Design
CTAB	Cetyl Trimethyl Ammonium Bromide
CV	Coefficient of Variance
ddH_2O	De-ionized Distilled Water
DMRT	Dunkan's Multiple Range Test
dNTPs	Deoxinucleotide Tri-phosphate (s)
et al.	And Others
EDTA	Ethylene Di-amino Tetra Acetic Acid
EP tube	Eppendorf tube
FAO	Food and Agriculture Organization
g	Gram
ha	Hectare(s)
HCl	Hydro Chloric Acid
hr	Hour (s)
i.e.	That is
ISTA	International Seed Testing Association
ITS	Internal Transcribed Spacer
J.	Journal
LSD	Least Significant Difference
mm	Milimeter
ml	Mililitre

LIST OF SOME ABBREVIATE FORM AND THEIR ELABORATIONS (Cont'd)

ABBREVIATE FORM	ELABORATION
$MgCl_2$	Magnesium Chloride
NaOH	Sodium Hydro Oxide
NCBI	National Centre for Biotechnology Information
NUV	Near Ultra violet
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
psi	Per Square Inch
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length polymorphism
rpm	Rotation Per Minutes
SAU	Sher-e-Bangla Agricultural University
spp.	Species
TAE	Tris Acetate EDTA
TE	Tris EDTA
UP-PCR	Universally Primed PCR
V8	V8 juice agar
WP	Wettable Powder

CHAPTER 1

INTRODUCTION

Onion (*Allium cepa*) is one of the most important and familiar spices crop throughout the world. It is the member of the family Alliaceae. It is also used as popular vegetable in many countries of Asia and very common, favorite spice in Bangladesh. Onion has manifold uses as spices, vegetables, salad dressing etc. It is also used as condiments for flavoring a number of food and medicines (Vohra *et al.*1974; Hasan, 2007). It also relieves head sensation and insect bites due to its medicinal properties. Onion contains vitamin B and a trace of vitamin C and also trace of iron, calcium, and volatile oil known as allylpropyl- disulphide (Yawalkar, 1985). Out of 15 important vegetables and spice crops listed by FAO, onion stands second in terms of annual world production (Anonymous,1997). In Bangladesh onion covers 36% areas under spices and it ranks first in production (889000 MT) and second in area (125101 ha) (BBS, 2008).Among the onion producing countries China tops the list with 226.00 lakh ton onion production followed by India 163.09 lakh ton, U.S.A. 32.77 lakh ton and Iran 22.60 lakh ton, whereas the production of onion in Bangladesh is only 11.59 lakh ton (FAO, 2012) which is very lower compared to other onion growing countries in the world.

Onion grows in 22 districts of Bangladesh for cultivation of onion and its commercial cultivation is concentrated in the greater districts of Faridpur, Comilla, Jessore, Pabna, Rajshahi, Dinajpur, Mymensingh, Dhaka and Rangpur (BBS, 2010). The BARI released onion varieties namely BARI piyaz-1, BARI piyaz-2, BARI piyaz -3, and local varieties of Thakurgao, Faridpuri, Manikganj, Thaherpuri, and kalasnagar are commonly grown in Bangladesh. Few imported varieties like Indian big, Indian small also cultivated in Bangladesh. The national demand of onion is about 19.5 lac metric tons annually in the country but yearly onion production is 11.59 lac metric ton from 335000 acre of land (BBS, 2012) and national annual yield of onion is 8.54 ton/ha in the country (BBS, 2012). The onion production per unit area in Bangladesh is gradually decreasing due to disease problem (BBS, 2006). As a result, Bangladesh has to import a large quantity of onion bulb every year to fulfill the national demand at the cost of foreign currency. As per the literatures, onion crop is affected about 66 diseases including 38 fungal, 10 bacterial, 6 nemic, 3 viral, 1 mycoplasmal, 1 parasitic plant, and 7 miscellaneous disease and disorder. Among these diseases stemphylium blight commonly known as white blotch, caused by *Stemphylium vesicarium*, is an important disease throughout the world (Bose and Som 1986; Meah and

Khan, 1987 and Castesllanos-Linares *et al*. 1988). Onion production in Bangladesh is gradually decreasing due to white blotch/ stemphylium blight disease problem (BBS, 2001). *Stemphylium vesicarium*, the causal agent of white blotch of onion is considered as the pathogen that initiate the infection, which facilitates the subsequent infection of *Alternaria porri* causing purple blotch and hence presently the disease is named as Purple Blotch Complex of onion. In India the disease was first recorded by Rao and Pavgi in 1975. The disease has now become serious in Northern parts of the country. About 90% losses in seed yield were recorded in India (Anonymous, 1982). Severity of disease was also reported from other parts of the world, viz. Europe, Africa, North and West America (Ellis, 1971).

Stemphylium blight has become more widespread in Bangladesh in the onion growing region during recent years. Wet and warm conditions favors the disease spreading. Under tropical conditions, the disease is a limiting factor for yield of onion. Onion seed production is severely affected because the disease causes breaking of floral stalks (Munoz *et al*.1984). The first symptoms of disease appear on the leaves. The symptoms develop in the middle of leaf as small, yellow to orange streaks which soon develop into elongated, spindle shaped to ovate elongate diffusate spots surrounded by characteristics pinkish margin. These spots turn gray at the centre, later brown to dark olive brown with the development of conidiophores and conidia of the pathogen (Miller *et al.*, 1978). The infection is usually confined to the leaves and do not extend down to the scales of the bulb. Similar symptoms are developed on the inflorescence stalk of the onion. The symptoms develop only on the dorsal side of the leaf / stalk. The study of *Stemphylium* sp. is a problem particularly for its slow growth on artificial media. It grows mycelium in the culture media but does not sporulate before a month or more. Some researchers also reported that *Stemphylium* sp. do not sporulate well on ordinary synthetic media (Hashemi *et al*., 2004). For overcoming this problem many researchers have tried to find out the suitable culture media for the growth and sporulation of *Stemphylium* sp.. Many semi-synthetic media viz. Potato Dextrose Agar (PDA), Water Lentil Seed Agar (LSA), Ground Lentil Stem and Leaf Agar (SLA), Ground Lentil Stem, Leaf and Seed Agar (SLSA), Ground Wheat Straw Agar (GWSA) were used to culture *Stemphylium* sp. and wheat straw agar was reported to be suitable (Chowdhury *et al.,* 1996). But Hashemi *et al.,* (2004) while working with wheat straw agar for culturing *Stemphylium* sp. found little or no conidia production. Borges *et al.*, (1976) harvested 7×10^4 conidia ml^{-1} of *S. botryosum* in V8-juice agar while working a disease resistance study in alfalfa. Salter and Leath (1991) also used V8-juice agar and achieved 1×10^4 to 5×10^4 conidia ml^{-1}. In Bangladesh, V8-juice agar

medium is unavailable and somewhat costly (16,000 tk/500g media). Thus, for conducting research on *Stemphylium* sp. development of easily available and cost effective media is necessary. A lot of research work have been conducted on the management of white blotch of onion (Nizam, 2005; Khatun, 2007). Application of Rovral 50 WP (0.2 %), Dithane M- 45 (0.2 %) and some other options are suggested as as foliar spraying against the disease. But now-a-days the fungicides are not working properly against the disease. Reason might be the development of fungicide resistance of the causal pathogen 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 *Stemphylium vesicarium* in our country. As the onion growers are being discouraged for onion cultivation due to the severe losses of the disease, the problem need to give urgent attention.

Molecular characterization may help in understanding the genetic and/or functional differentiation i.e. genetic variability among individuals in a species. In recent years, random amplified polymorphic DNA (RAPD) and ribosomal DNA (rDNA) analyses have been extensively employed in phylogenetic and taxonomic studies of fungi (Achenback and Patrick, 1996; Achenback *et al*., 1997; Machado *et al*., 1997; Ouellet and Seifert, 1993; Williams *et al*., 1990). The internal transcribed spacer (ITS) region of the rDNA has been useful in distinguishing relationships at the species level (Achenback *et al*., 1997). As no previous molecular analyses of *Stemphylium vesicarium* from onion plants in Bangladesh is available, the present study was undertaken to assess the genetic diversity of the *Stemphylium vesicarium* isolates from onion plants utilizing RAPD and rDNA analyses.

Keeping these facts and views in mind, the study was undertaken to achieve the following objectives:

1) To collect, isolate, identify and preservation of causal organism of white blotch of onion collected from major growing areas of the country.

2) To identify a cheap and suitable culture medium for the growth and sporulation of *Stemphylium vesicarium.*

3) To determine the cultural, morphological and genetic variability of *Stemphylium vesicarium.*

CHAPTER 2

REVIEW OF LITERATURE

Stemphylium blight (white blotch), a common disease of onion in Bangladesh is considered as serious one as it has destructive effect in reducing the yield of onion. Now it is an acute problem in the country both for the researchers and the onion growers. Characterization of this pathogen on the basis of morphological and molecular parameters are being explored in

many countries of the world. Literatures in relation to Stemphylium blight of onion are presented in this chapter:

2.1. Stemphylium blight: cause, symptoms and epidemiology

Rao and Pavgi (1975) stated about *Stemphylium vesicarium*, a serious pathogen causing Stemphylium blight, which confined to leaves and florescence stalk.

Miller *et al.* (1978) reported on Stemphylium blight of onion in South Texas, a previously unrecognized disease of onion cause significant damage during 1976, both alone and with *Alternaria porri*. Lesions were nondelinated, light yellow to brown, water- soaked and form 1 cm in length to the entire leaf, compared with the purple lesions of *Alternaria porri*. Isolation from light yellow areas of lesion in variably yielded *Stemphylium vesicarium.* Density of spores (244/ cm^2) and lesions were greater on the southeast side of leaves facing the prevailing wind, where as fewer spores (14/ cm^2) were observed onthe opposite green side of the leaf. Inoculations with *Stemphylium vesicarium* on the short day onion cultivars, White Granex, Ben shamen and New Maxico Yellow Grano produced lesions identical to those in the field.

Wu (1979) surveyed on the seed-borne diseases of vegetables. Results of the survey on onion showed that *Alternaria porri* and *Stemphylium botryosum (Pleospora herbarum)* reduced germination of onion seeds.

Miller (1983) reported that measurements of infected leaves were taken weekly from bulb initiation to bulb maturity of onion. They observed that the leaf damage levels were significantly lower on younger than older leaves. Leaves emerging 9, 8, 7, 6 and 5 week before bulb maturity required 51/2, 5, 4 ½, 31/2, and 2 ½ weeks respectively to reach 50 % damage within 2 weeks.

Blancard *et al.* (1986) studied 19 isolates of *Stemphylium sp*. obtained from tomatoes of various countries, where 12 were *Stemphylium vesicarium,* 4 *Stemphylium solani* and 3 *Stemphylium flondanum.*

Bello *et al.* (1989) studied *Stemphylium loti* and 4 isolates of *Stemphylium* sp. obtained from necrotic leaf spots on plants in pasture fields. In inoculation test, symptoms produced by *Stemphylium loti* were similar to those found in the field, but those of the *Stemphylium sp.* were more severe than occurring naturally.

Shishkoff and Lorbeer (1989) isolated *Stemphylium vesicarium* from lesions on leaves of onion plants. In controlled inoculations the fungus caused lesions on leaves of all stages of onion plants, especially on older leaves. Rubbing leaves of green house grown onion plants with bleached, non absorbent cotton to damage the cuticle increased the number of lesions/leaf.

Dar *et al.* (1992) isolated *Stemphylium botryosum (Pleospora herbarum) from dark brown lesions on Phaseolus vulgaris leaves in several fields in the Kashmir valley during July 1990 and 1991. Pathogenicity was confirmed on this local cultivar in inoculation tests.*

Patil et al. (1992) observed leaf spot of onion in Nasik district of Maharashtra, India. Microscopic examination of the disease spots revealed conidia of Stemphylium sp.. The fungus was isolated and pathogenicity was confirmed, the fungus was identified as Stemphylium vesicarium in *Maharashtra state.*

Bassallote et al. (1993) surveyed 125 garlic fields in Southern Spain during 1989-91 and observed 31%, irrespective of cultivar, were affected by dark purple and white leaf spots indistinctly produced mainly on older leaves. Stemphylium vesicarium was isolated from these spots and *pathogenicity was confirmed.*

Boiteux et al. (1994) studied the causal agent of a foliar disease of garlic in the Brasilia area of Brazil and identified it as Stemphylium vesicarium. Disease symptoms were reproduced on garlic leaves at 8-10 days after inoculation in a greenhouse (temp. range 22-26^{o} *C).*

Hill (1995) and Basallotte-Ureba *et al.* (1999) stated that stemphylium leaf blight (SLB) of onion is characterized by elongated spindle-like lesions on leaves, initially small and white, which later become sunken with a purple colour surrounded by a whitish margin.

Gupta *et al*. (1996) stated Stemphylium blight (*Stemphylium vesicarium*) and purple blotch (*Alternaria porri*) are important diseases causing considerable damage to onion crops in India. Diseases are found severe during the rainy season especially when thrips are also associated with the crop.

Basallote et al. (1999) surveyed the major garlic production areas of Spain and identified a new leaf spot disease, characterized by white and purple lesions followed by extensive necrosis. Isolation and pathogenicity test with fungal isolates taken from these spots indicated

that *Stemphylium botryosum was the causal agent. Pseudothecia* of the teleomorph stage, *Pleospora sp.* were found on leaf debris from affected plants. Inoculation of garlic and onion plants with residues carrying mature *Pseudothecia, resulted in the development of white and purple leaf spots. Wetness periods longer than 24 hr were required for symptom development under controlled conditions. Isolates of Stemphylium botryosum from garlic, onion and asparagus caused disease in all 3 hosts.*

Lakra (1999) conducted an experiment at the Choudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India and found numerous purple spots/ blotches on older leaves and scapes when fortnightly dew fall was >1.0 mm, mean maximum relative humidity >75% and mean maximum temperature 20-30°C with>18 hr favorable temperature (10-30) duration. Exposure of leaf and/or scape to wetness for 8 hr was a pre-requisite for conidial germination with increasing disease intensity, every yield component was adversely affected; the most severe infection reduced the number of scapes/plant, the height of scape, the number of 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.

Suheri et al. (2000) studied the infection of onion by *Alternaria porri* and *Stemphylium vesicarium* under a range of controlled temperatures (4-25^{0}C) and leaf wetness periods (0-24h). Conidia of *Alternaria porri* and *Stemphylium vesicarium* germinated within 2 h when incubated at 4^{0}C. Terminal and intercalary appressoria were produced at similar frequencies at or above 10^{0}C. The maximum number of appressoria was produced after 24h at 25^{0}C. Penetration of leaves by both pathogens was via the epidermis and stomata, but the frequency of stomatal penetration exceeded that of epidermal penetration. There was a strong correlation (R2>90%) between appressorium formation and total penetration at all temperatures. Infection of onion leaves occurred after 16h of leaf wetness at 15^{0}C and 8h of leaf wetness at 10-25^{0}C and infection increased with increasing leaf wetness duration to 24h at all temperature. Interruption of a single or double leaf wetness period by a dry period of 4-24h had little effect on lesion numbers. Conidia of *Alternaria porri* and *Stemphylium vesicarium* separately or in mixtures caused similar number of lesions. *Alternaria porri* and *Stemphylium vesicarium,* both potentially important pathogens in winter grown *Allium* crops. Purple leaf blotch symptoms were considered to be a complex caused by both pathogens.

Everts et al. (2001) observed leaf spot symptoms on spinach (*Spmacia oleracea* cv. Seven R) at the University of Maryland. Leaf spot lesions were small (0.2-0.7 cm), circular, tan and papery. Naked visual signs of fungal infection *Stemphylium botryosum (Pleospora herbarum) was consistently reisolated from leaf spot lesions in plants tested in the laboratory and greenhouse trials. The pathogenicity test was repeated and recorded similar results.*

Mwakutuya et al. (2002) reported that the stemphylium blight, a defoliating fungal disease caused by *Stemphylium vesicarium* was detected regularly in Saskatcheewan lentil fields for many years, but was poorly understood. Up to 62% yield loss was reported from Bangladesh and eastern India. A research project was initiated to study the biology of the pathogen. Culture age and different light regimes did not effect on germination. High temperatures favoured the germination of *Stemphylium botryosum and the optimum temperature for conidial germination was between 25* ^{o} C and 30 ^{o} C.

Sharma *et al*. (2002) reported that onion seed production in punjab was reduced by 60-70% due to the severe downy mildew (*Peronospora destructor*) disease outbreak on seed stalks resulting in low seed recovery and poor seed health and vigor. They detected *Fusarium, Alternaria, Stemphylium* and *Aspergillus* spp.

Cova *et al.* (2003) reported that the leaf blight of onion as one of the most important diseases of the crop in Lara state. To determine the pathogens causing the disease and their relative importance, 22 leaf samples from 16 localities in four countries were analyzed through plant pathological standard techniques. Collected leaves showed visual symptoms of leaf blight. *Stemphylium botryosum* (*Pleospora herbarum*), *Alternaria alternata* and *Stemphylium vesicarium* were isolated from 93.7, 12.5 and 50.0% of the samples, respectively. *Alternaria alternata* was always associated with *Stemphylium botryosum.* Pathogenicity test with *Stemphylium botryosum* and *Alternaria alternata*, either individually or combined, reproduced disease symptoms similar to those observed in the field.

Hassan *et al.* (2007) reported that onion plants (*Allium cepa* cv. " Giza 6") in field condition exhibited symptoms of blight on the leaves and seed stalk . Initial symptoms on leaves consisted of tip necrosis followed by small white and/or large purple spots. The fungus consistently isolated from diseased tissue was identified as *Stemphylium vesicarium.*

Hussein *et al.* (2007) worked on Stemphylium blight disease of onion at the department of plant pathology in Assiut University of Egypt. They stated that blight symptoms of onion

started as small pale yellow lesion on onion leaves then enlarged and becoming ovate-elongate and dark brown to black when sporulation occurs.

Tesfaendrias (2012) stated that Stemphylium blight symptoms of onion start as small yellow to tan, water-soaked lesions that develop into elongated spots that turn dark olive brown to black when spores develop. Leaves may be completely blighted as the lesions coalesce. These foliar diseases of onions can prematurely defoliate the crop which can compromise bulb quality and make the crop more susceptible to secondary diseases that affect bulb quality.

2.2. Cultural characteristics of *Stemphylium vesicarium*

Mehta (2001) studied leaf blight disease of cotton (*Gossipium hyrsutum*) caused by *S. solani*. He collected the diseased specimen from three major cotton-growing states of Brazil. After isolation, he stated that *Stemphylium solani* is a very variable fungus. A range of different colony characteristics of *S. solani* were found among the cotton isolates grown on PDA medium. Colonies were slow growing on PDA, reaching a maximum of 66 mm in diameter 10 days after incubation and were dark brown to black. Mycelium was velvety, cottony or immersed. Some of the isolates produced a yellow pigment in the medium that turned deep red with age.

Byung - Soo *et al.* (2004) worked on gray leaf spot disease causing severe leaf spots in pepper and tomato plants in northern Gyeongbuk and Gangwon provinces of Korea. Two types of pathogen such as *Stemphylium solani* and *S. lycopersici* were found from each pepper and tomato plants. The pathogens were grown on PDA and V-8 juice agar media for characterization. Growth of *Stemphylium solani* and *S. lycopersici* on V8 juice agar media were Gray in color. Growth of *Stemphylium solani* on PDA was Gray in color but Gray to light brown with diffusion of brown pigment type growth was found in case of *S. lycopersici*. Both of the pathogens were cultured on V-8A (vegetable 8 juice agar); PCA (potato carrot agar) and PDA (potato dextrose agar) media for higher conidia production. Higher conidia production was found on V-8A medium (14.2×10^3- *S.solani,* 31.8×10^3 *S.lycopersici*) followed by PCA (2.5×10^3- *S. solani,* 3.0×10^3 - *S. lycopersici*) and PDA (0.8×10^3- *S. solani,* 3.1×10^3- *S. lycopersici*) media.

Koike *et al.* (2005) studied a new disease of spinach (*Spinacia oleracea*) crops in the Yuma region of Arizona a foliar disease that previously had not been diagnosed in this geographic area. The disease caused by fungus *Stemphylium botryosum* was identified based on the morphological characteristics of isolates onto V8 juice agar media. Mycelial growth of *Stemphylium botryosum* onto V8 juice agar media was found dark green-to-brown in color.

Kumar (2007) conducted an experiment at the University of Saskatchewan to identify a suitable culture medium for the sporulation of *Stemphylium botryosum*. V8 (V8 Juice Agar Medium), V8P (V8 Juice Potato Dextrose Agar Medium), V8P TD1 (V8P + 2% Tamarind Juice Medium), V8P TD2 (V8P + 4% Tamarind Juice Medium) respectively, were tested for their suitability for increasing conidia production by isolate SB-19. All the media used allowed the fungus to grow to sporulate profusely. The fungus produced the highest number of conidia ($84.7 \pm 6.0 \times 10^4$ conidia ml^{-1}) on V8P medium followed by V8 ($78.5 \pm 6.0 \times 10^4$ conidia ml^{-1}), V8PTD1 ($69.5 \pm 4.2 \times 10^4$ conidia ml^{-1}) and V8PTD2 ($41.5 \pm 6.1 \times 10^4$ conidia ml^{-1}).

Zheng *et al.* (2007) worked on leaf blight disease of garlic in Hubei province, China. The pathogen was identified as *Stemphylium solani* based on Ellis (1971). Isolations were made onto Potato Sugar Agar (PSA) giving white colonies. The centres turned grey, after 4 days on PSA, the agar became yellow-brown throughout the petridish. Colony of *Stemphylium solani* on potato sugar agar medium after 7 days of inoculation becoming grayish in color.

Hosen *et al.* (2009) characterized four isolates of *Stemphylium botryosum* namely MIH-1, MIH-2, MIH-3 and MIH-4 isolated from lentil in the terms of cultural traits such as colony type, color, shape and texture on culture media. Isolates MIH-1, MIH-2 and MIH-3 showed greenish brown and peripheral side white, only isolate MIH-4 showed different colony color which was dirty white. Remarkable variations were observed in colony shape. Isolate MIH-1, MIH-3 showed irregular, MIH-2 regular and MIH-4 roughly irregular shape of colony. They also varied in colony texture. Isolate MIH-1, MIH-3 showed velvety, MIH-2 effuse and MIH-4 fluffy type colony texture. The colonies of *Stemphylium botryosum* are velvety to cottony in texture with a grey, brown or brownish black in color. The maximum diameter of colony growth (54 mm) was observed on LDA followed by PDA (48 mm). The lowest diameter of colony growth (28 mm) was noted on WA. From their experiment, LDA medium was appeared to be the best for supporting the maximum mycelial radial growth of this fungal pathogen.

Hosen (2009) studied cultural and physiological parametes on PDA of *Botrytis cinerea* and *Stemphylium botryosum* causing gray mold in chickpea and stemphylium blight in lentil respectively. Marked variations were observed in colony characteristics i.e. colony color, texture and margin. Light ash colony color was observed in *B. cinerea* and greenish brown in *S. botryosum*. Distinguished comparison was noted on colony texture, velvet colony texture was found in *B. cinerea,* whereas effuse type was found in *S. botryosum* isolates. Entire and regular colony margin were found in *B. cinerea* and *S.botryosum*, respectively. *S. botryosum* colonies varied from velvety to cottony in texture with a gray brown or brownish black color, greenish brown to dirty white, regular to irregular shape.Variation exists in morphology and cultural characteristics of different isolates of *B. cinerea.* Five different nutrient media were also evaluated for their impact on the mycelial radial growth of *B. cinerea* and *S. botryosum*. The maximal colony diameter (80.16 mm) and spore (2.7×10^4) $ml^{-1\ 1}$ of *Botrytis cinerea* was found on Chickpea Dextrose Agar (CDA) and Lentil Dextrose Agar (LDA) respectively. The maximal colony diameter (57.00 mm) and spore (1.7×10^4) ml^{-} of *Stemphylium botryosum* was recorded in LDA.

Yong Wang *et al.* (2010) isolated *Stemphylium phaseolina* and *S. variabilis* from diseased leaves of *Phaseolus vulgaris* L. in Hebei Province, China, and from diseased leaves of *Allium sativum* L. in Angres, France. They reported colonies of *Stemphylium phaseolina* on PCA (Potato Carrot Agar) media were effuse, 26–33 mm diameter in 6 days, pale brown in color, cottony, whereas the colonies of *Stemphylium variabilis* on PCA media were smooth, 28–30 mm diameter in 5 days, pale brown, cottony in structure.

Yun-Fei *et al.* (2010) isolated three new species of *Stemphylium* (*Stemphylium luffae, S. lycii* and *S. cucumis*) from diseased leaves of *Luffa cylindrica, Lycium chinense* and *Cucumis melo* growing in the Sinkiang province of Northwest China. Colonies of *Stemphylium luffae* on PCA (Potato Carrot Agar) media spreading 32-36 mm diameter in 10 days, mycelia were pale brown in color and having cottony structure. Colonies of *Stemphylium lycii* on PCA media spreading 30 – 35 mm diameter in 10 days, mycelia were pale brown in color and having cottony structure. Colonies of *S. cucumis* on PCA media spreading 28 – 33 mm diameter in 10 days, mycelia were pale brown color and having cottony structure.

Mirjalili *et al.* (2011) isolated *Stemphylium sedicola* from the inner bark of *Taxus baccata* grown in Iran by the aseptic technique. For morphological study they cultured *Stemphylium sedicola* on PCA (Potato Carrot Agar) media. They found that, colonies of *Stemphylium*

sedicola on PCA were effuse, pale brown in color, and did not sporulate abundantly. The mycelium was septate and pale brown in color.

Arzanlou *et al.* (2012) reported that the colonies of *Stemphylium vesicarium* on PCA (Potato Carrot Agar) media were grey to brownish grey, formed concentric rings, were flat, attaining a diameter of 50 mm after 7 days with sparse aerial mycelium.

2.3. Morphological characteristics of *Stemphylium vesicarium*

Ellis (1971) conducted an experiment and described that the conidia of *Stemphylium vesicarium* were oblong to ovoid, densley verrucose with 1- 5 transverse and several longitudinal septa , 25 – 40 x 13-21 µm .

Simmons (1985) isolated *Stemphylium botryosum* from *Medicago sativa* and concluded that conidial dimensions of *Stemphylium botryosum* were from 33-35 x 24-26 µm, length/width ratio near 1.0-1.5, single conspicuous constriction at the median transverse septum was seen, having densely echinulate walls. Isolates of *Stemphylium alfalfa* were also separated from *Medicago* in Western Australia. Conidia were broadly ovoid, often conidia enlarge to a size range of about 32-35 x 16-19 µm (1/w near 2.0). Septa of most conidia thicken and darken in age.

Bayaa and Erskine (1998) stated that conidia of *Stemphylium botryosum* were olive brown, oblong or muriform in shape with three constricted transverse septa.

Camara *et al.* (2002) stated that the size of conidia varies from 13 × 8 to 78 × 24 µm, whereas the size of conidiophores varies from 25×2 to 285× 6 µm in different species of *Stemphylium.*

Byung - Soo *et al.* (2004) worked on gray leaf spot disease causing severe leaf spots in pepper and tomato plants in northern Gyeongbuk and Gangwon provinces of Korea. Two types of pathogen, *Stemphylium solani* and *S. lycopersici* were found from each pepper and tomato plants. Conidia of *Stemphylium solani* were oblong, with conspicuous median constriction, 3-4 transverse and 1-2 longitudinal septae, tan to light brown, round basal end, and asymmetrically tapered terminal end forming blunt pointed tip. Length and breadth ranges from 29.2-51.6 × 15.1-21.5 µm, average: 40.6 ± 5.1 × 18.0 ± 1.5 average l/w ratio:

2.25:1. Conidia of *S. lycopersici* were elongated elliptic, similar to bullets, light brown to orange brown, with 7-9 transverse septae and 2-3 longitudinal septae, 2-3 conspicuous transverse constrictions in outline, symmetrically pointed apical end, round basal end, and warty surface. Length and breadth ranges from 30.0-73.1×12.9-19.4 µm, average: 54.8 ± 8.3 × 16.4 ± 1.3 and average l/w ratio was 3.34.

Koike *et al.* (2005) studied a new foliar disease of spinach (*Spinacia oleracea*) crops in the Yuma region of Arizona disease that previously had not been diagnosed in this geographic area. The disease caused by fungus *Stemphylium botryosum* was identified based on the morphological characteristics of isolates onto V8 juice agar media. The conidia were brown, ellipsoidal to ovoid, verrucose, borne singly, and measured 17 to 28 × 13 to 19 µm.

Zheng *et al.* (2007) worked on Leaf blight disease of garlic in Hubei province, China. The pathogen was identified as *Stemphylium solani* based on Ellis (1971). Isolations were made onto potato sugar agar (PSA) medium. Conidia were pointed at the swollen apex of each conidiophore, clavate, with 1-3 transverse septa, 2-7 longitudinal or oblique septa, and 34-55 × 17-27 µm.

Lu *et al.* (2008) reported that the conidia of *Stemphylium solani* isolates isolated from *Allium sativum* and other crops were pointed at the swollen apex of each conidiophore, brown, oblong-ellipsoid or clavate, straight, smooth, with one to three dark coloured transverse septa and distinctly constricted at median septa, two to seven longitudinal or oblique septa, 29- 58 (45) × 14 - 28 (22) µm (mean) and mean length/width ratio was 2.03.

Hosen *et al.* (2009) conducted an experiment in the Plant Pathology laboratory of the Bangladesh Agricultural Research Institute (BARI) .They experimented four isolates of *Stemphylium botryosum* (MIH-1, MIH-2, MIH-3 and MIH-4) from lentil plants showing blight symptoms. The isolates were characterized in terms of morphological parameters. The length of conidia ranged from 10.00 to 25.00 µm. Mean length of conidia was maximum 16.04 µm in isolate MIH-2 and minimum 13.33 µm in isolate MIH-3. The breadth of conidia varied from 5.00 µm to 15.00 µm. The highest mean breadth 9.17 µm was observed in isolate MIH-2 and the lowest 6.46 µm in isolate MIH-3.

Yun-Fei *et al.* (2010) isolated three new species of *Stemphylium* (*Stemphylium luffae*, *S. lycii* and *S. cucumis*) from diseased leaves of *Luffa cylindrica*, *Lycium*

chinense and *Cucumis melo* growing in the Sinkiang province of Northwest China. Conidia of *Stemphylium luffae* found developing singly at the apex of each conidiophore, oblong, ovoid or broadly ellipsoidal, subacute or conical at the apex, and rounded or subtruncate at the base, with (1–) 3–5 transverse septa and 2–4 (–5) longitudinal or oblique septa, 32– 45×9 – 19 µm (av. 35.5 ± 3.1 × 14.5 ± 1.5 µm), L/W = 1.9 – 3.1 (av. 2.5 ± 0.2), usually a distinctly constricted at the median transverse septum, medium brown to dark brown in color. Conidia of *S. lycii* developed singly, ovoid, broadly ellipsoidal or oblong ellipsoidal, rounded at the apex, rounded or subtruncate at the base, with 1 – 3 transverse septa and 0 – 2 (–3) longitudinal or oblique septa, always a distinctly constricted at the median transverse septum, 23 – 31 × 13 – 19 µm (av. 26.5 ± 2.1 × 15.5 ± 1.8 µm), L/W = 1 .4 – 2.3 (av. 1.8 ± 0.2), medium brown to brown in color. Conidia of *S. cucumis* developed singly at the apex of each conidiophore, rectangular form, oblong, ovoid, rounded or subtruncate at the apex, rounded or subtruncate at the base, with 1– 3(– 4) transverse septa and 1 distinctly constricted, (1–)2 –4 longitudinal or oblique septa, 20 – 32 × 10 – 18 µm (av. 27.5 ± 3.1 × 13.1 ± 1.9 µm), L/W = 1.8 –2.8 (av. 2.2 ± 0.2), medium brown to brown in color.

Yong *et al.* (2010) isolated *Stemphylium phaseolina* and *S. variabilis* from diseased leaves of *Phaseolus vulgaris* L. in Hebei Province, China, and from diseased leaves of *Allium sativum* L. in Angres, France, respectively. They reported that the conidia of *Stemphylium phaseolina* developed singly, broadly obclavate or broadly ellipsoid, subtruncate at the apex, rounded at the base, with 2–4 distinct transverse and 1–3 longitudinal septa, 26–42 x 13–22 (av. 35 ± 3.6 x 19 ± 2.4) µm, L/W = 1.7–2.2, indistinctly constricted at the transverse septa, medium to dark brown, conspicuously punctate. And the conidia of *Stemphylium variabilis* were variable in shape, subspherical, oblong or broadly ellipsoid, with 1–3 transverse and 2–6 longitudinal septa, 15– 25 x 13–17 (av. 20.5 ± 3.8 x 14.8 ± 1.3) µm L/W = 1.2 – 1.6, one indistinctly constricted at the central transverse septum, medium brown, densely verrucose. Conidia often generate short secondary conidiophores, short and broad, 1–2 septate, 12– 18 µm long, smooth.

Hosen (2010) studied morphological comparison between *Botrytis cinerea* and *Stemphylium botryosum* on PDA media causing gray mold in chickpea and stemphylium blight in lentil. The conidial length of *B. cinerea* range from 7.75 to 15.35 µm and *S. botryosum* from 12.35 to 23.45 µm was observed. The breadth of conidia varied from 5.25 to 11.00 µm in *B. cinerea*

and 9.35 to 15.00 µm in *S. botryosum*. It appears that the conidial dimension (16.27 × 9.07 µm) of *S. botryosum* was comparatively larger than *B. cinerea* (11.74 × 8.12 µm).

Mirjalili *et al.* (2011) isolated *Stemphylium sedicola* from the inner bark of *Taxus baccata* grown in Iran by the aseptic technique. They cultured *Stemphylium sedicola* on PCA (Potato Carrot Agar) and reported that conidia of *Stemphylium sedicola* developed singly and almost entirely through a narrow pore at the apex of each conidiophore, medium brown in color, oblong to oblong-ellipsoid, subtruncate at the apex, rounded or subtruncate at the base, straight or slightly curved, with 1– 3 transverse septa, and usually distinctly constricted in the middle, 0 – 3 longitudinal or oblique septa, 15 – 30 x 12 – 18 µm (av. 21.84 x 14.06 l µm), L/W ratio was 1.4 - 2.16 (av. 2.0) dark, and thin-walled.

Arzanlou *et al.* (2012) reported that the immature conidia of *Stemphylium vesicarium* on PCA (Potato Carrot Agar) media were ellipsoid, rounded at the ends, mature conidia were 20 – 24 (-30)×12 – 15 µm, with length/width ratios approaching 1.5 – 2.3, solitary, acrogenous, oblong to broadly ellipsoid, sub-truncate basally, rounded to sub-truncate apically, golden-brown to olive-brown, with 1–3 transverse and 1–4 longitudinal or oblique septa, often constricted at one or more of the septa .

2.4. Molecular characterization

The identification of *Stemphylium* species is based principally on morphological characteristics of conidium and conidiophore, many of these characters often overlap among species, making species determinations difficult. In addition conidial characteristics of some species vary on different substrates and at different temperatures (Leach and Aragaki 1970). Shenoy *et al.* (2007) recognized that the morphology-based identification of a fungal species is not always easy and in many instances incorrect. DNA sequence data now are being used commonly to test morphological concept and other taxonomic hypotheses (Hunter *et al.* 2006). The ITS DNA sequence is a widely accepted DNA marker for identifying fungi (Nguyen and Seifert 2008).

Genetic diversity is commonly measured by genetic distance of genetic similarity both of which imply that there are either differences or similarities at genetic level (Weir, 1990). Availability of a large number of polymorphic markers enables precise classification of the

cultivars. Several molecular markers viz. RFLP, RAPD, SSRs, ISSRs and SNPS are presently available to assess the variability and diversity at molecular level (Joshi *et al*., 2000).

Mehta (2001) performed molecular analysis to assess the genetic diversity among the *S. solani* isolates from cotton, and to verify their relationship with representative *S. solani* isolates from tomato. Random amplified polymorphic DNA (RAPD) markers and internal transcribed spacers of ribosomal DNA (rDNA) were used to compare 33 monosporic isolates of *S. solani* (28 from cotton and 5 from tomato). An isolate of *Alternaria macrospora* from cotton was also used for comparison. RAPD analysis showed the presence of polymorphism between the genera and the species.

Yun-Fei *et al.* (2010) isolated three new species of *Stemphylium* from diseased leaves of *Luffa cylindrica, Lycium chinense* and *Cucumis melo* growing in the Sinkiang province of Northwest China. *Stemphylium luffae, S. lycii* and *S. cucumis* were described by morphological and molecular phylogenetic analyses. The principal morphological characteristics of these three species are congruent with those of *Stemphylium*. They were distinguished from morphologically similar *Stemphylium* species based on characteristics of their conidia and conidiophores. In order to establish the molecular phylogenetic status of these species, DNA loci including the internal transcribed spacer (ITS) of the nuclear rDNA region and the glyceraldehyde-3-phosphate dehydrogenase (gpd) genes were amplified and sequenced. Phylogenetic analyses using Maximum-Parsimony (MP) and Neighbor-Joining (NJ) were performed on the combined DNA sequences of ITS and gpd gene regions. Both morphological observations and molecular analyses support S. *luffae, S. lycii* and *S. cucumis* as three new taxa.

Wang (2010) described two new species of *Stemphylium* (anamorph *Pleospora*) on the basis of morphological characters and molecular phylogenetic analyses. *Stemphylium phaseolina* and *S. variabilis* were isolated respectively from diseased leaves of *Phaseolus vulgaris* L. in Hebei Province, China, and from diseased leaves of *Allium sativum* L. in Angres, France. The two species exhibit characteristic *Stemphylium* morphology but were distinct from similar species based on the morphology and development of conidia. The internal transcribed spacer (ITS) nuclear rDNA region and glyceraldehyde-3-phosphate dehydrogenase (gpd) genes were sequenced. The results of phylogenetic analyses of the combined DNA sequences of these two gene regions supported *S. phaseolina* and *S. variabilis* as two distinct phylogenetic species.

Sunflower leaf spot is one of the most common as well as important foliar diseases of sunflower in northern Iran. Arzanlou (2012) revealed *Stemphylium vesicarium* to be one of the causal agents of sunflower leaf spot based on morphological and molecular characteristics. The identitfication of the species was confirmed using sequence data from the ITS rDNA region and glyceraldehyde-3-phosphate dehydrogenase (gpd) gene sequences. This is the first report on the incidence of *S. vesicarium* on *H. annuus* anywhere in the world.

CHAPTER 3

MATERIALS AND METHODS

The experiment was conducted to determine the cultural, morphological and molecular characterization of twenty four isolates of *Stemphylium vesicarium* collected from major onion growing areas of Bangladesh.

3.1. Experimental site

The experiment was laid out during February'13 to July'14 at the Molecular Plant Pathology Laboratory of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh and in the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Bangladesh.

3.2. Collection of diseased samples

Leaves bearing typical symptoms of white blotch (Figure1 and Figure 2) of onion were collected from eight different districts namely Dhaka, Mymensingh, Rajshahi, Gazipur, Comilla, Jamalpur, Manikgonj and Faridpur. The diseased leaves were collected from the diseased plants grown in the field and then put into a brown paper envelope again covered with polythene. The brown paper envelopes of each lot were taken to the Molecular Plant Pathology laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

Figure 1. White blotch disease of onion caused by *Stemphylium vesicarium*

Figure 2. Diseased leaves showing white blotch symptom

3.3. Isolation, identification, purification and preservation of the isolates of *Stemphylium vesicarium* collected from diseased samples of different onion growing areas of Bangladesh

Stemphylium vesicarium was isolated by tissue planting **methods** from diseased samples. The surface of the working place was sterilized with ethanol (70%). Then the samples were cut into small pieces (0.5-1.0cm). The cut pieces were surface-sterilized by dipping in 0.1% mercuric chloride solution for 1 min and rinsed in sterile distilled water (SDW) for three times. After washing, cut pieces were placed on three layered sterilized moist blotter paper (Whatman No. 1) (Plate 1). The plates were incubated at 25^0C under NUV light. After 7-10 days of incubation, the fungal cultures were observed under stereoscopic binocular

microscope. The presence of *Stemphylium vesicarium* was confirmed by preparing temporary slides and examined under the compound microscope (Figure 3). After identification, the conidia of *Stemphylium vesicarium* were carefully picked up and transferred on to fresh PDA plate and incubated at 25^0 C for 7 days. The pure culture (Plate 2) of *Stemphylium vesicarium* was preserved in refrigerator at 4^0C for further use.

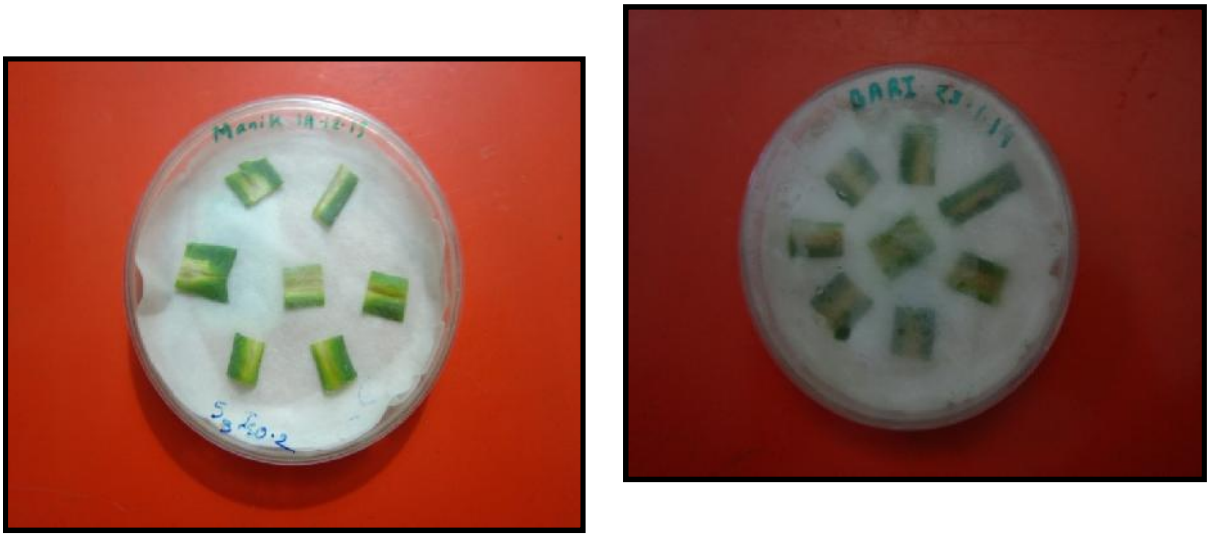


Plate 1. Isolation of *Stemphylium vesicarium* by tissue planting methods

Figure 3. Conidia of *Stemphylium vesicarium* seen under compound microscope (40X)

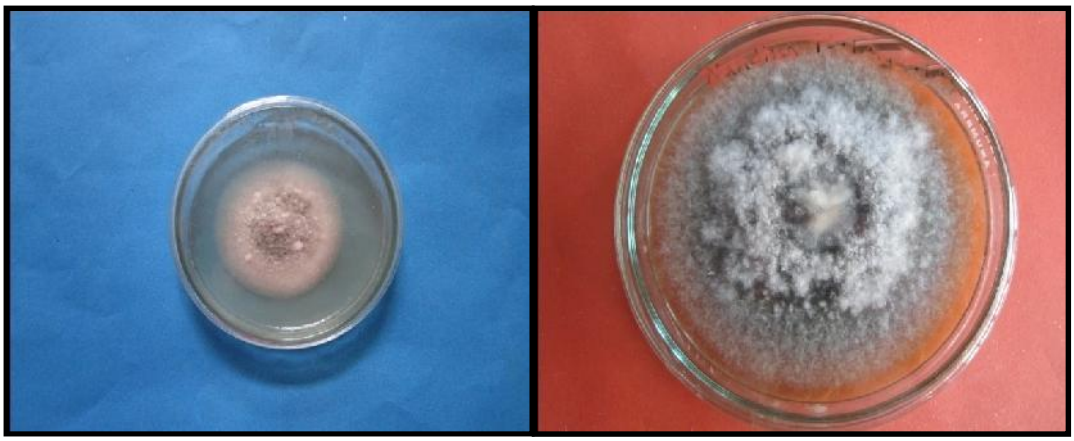


Plate 2. Pure culture of *Stemphylium vesicarium*

Table1. Locations of collection of white blotch affected onion sample

Sl. No	Isolates	Locations of collection			
		District/ Region	Upazila/ Thana	Union/ Organization	Village/ Place
1	DSSA	Dhaka (SAU and Savar)	Sher-Bangla Nagar	SAU	Agronomy Field
2	DSTR 01		Savar	Tetuljhora	Rajfulbaria
3	DSTR 02				
4	MMBH	Mymensingh (BAU and 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 and 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	FFKU 01	Faridpur (Faridpur Sadar)	Faridpur Sadar	Kanaipur	Ulukanda
23	FFKU 02				
24	FFKU 03				

SAU = Sher-e- Bangla Agricultural University

BAU = Bangladesh Agricultural University

BARI = Bangladesh Agricultural Research Institute

3.4. Designation of isolates

The isolates were designated based on Aminuzzaman *et al.* (2004). For example an isolate designated by CCKH 01 represents that this isolate was collected from district Comilla (C), upazilla Chandina (C), union Keronkhali (K), village Harong (H) and 01 denotes the collection number (Table 1).

3.5. Selection of culture media for *Stemphylium vesicarium*

The selection of suitable culture media was carried out at central laboratory of the department of Plant Pathology, SAU, during January'14 to June'14. The effect of different culture media on the growth of *Stemphylium vesicarium* were studied. Different cultural media (Table 2) were assayed for this experiment.

3.5.1. Preparation of PDA: Potato Dextrose Agar (PDA) was used in this experiment (Table 2). At first potato extract was taken in a conical flask (500ml) by boiling 100 g potato slice in 500 ml water. Then 8.5 g agar and 10 g dextrose was dissolved in potato extract. The PDA medium was autoclaved at 121^0C under 15 psi for 30 minutes. After autoclaving the medium was kept few minutes for cooling and added 50-60 drops of lactic acid. About 15-20 ml medium was poured into each sterile petridish for use.

3.5.2. Preparation of V-7 juice agar: Extract of 7 vegetables was made by boiling seven vegetables (Table 2) in water. Then the extract was taken in a conical flask (500ml) and adjusted the volume up to 500 ml by adding distilled water. Then 8.5 g agar and 1 g $CaCO_3$ was dissolved in the extract. The pH of the extract was adjusted to 5.0 - 5.5 by adding HCl acid and autoclaved at 121^0C under 15 psi for 30 minutes. After autoclaving the medium was kept few minutes for cooling. Then 15-20 ml medium was poured into each sterile petridish for use.

3.5.3. Preparation of combined PDA+V-7 juice agar: Extract of potato (100ml) PDA was added with 400 ml V-7 juice agar medium in a conical flask (500ml). After that 6.8 g agar, 8 g dextrose and 1 g $CaCO_3$ was dissolved in the medium (Table 2). The pH was adjusted to 5.0 - 5.5 by adding HCl acid and autoclaved at 121^0C under 15 psi for 30 minutes. After autoclaving the medium was kept few minutes for cooling. Then 15-20 ml medium was poured into each sterile petridish for use.

Table 2. Composition of different culture media for 1 litre

Name of media	Composition	
	Ingredient	Amount
PDA (Potato Dextrose Agar)	Potato slice Dextrose Agar Distilled water	200 g 20 g 17 g 1000 ml
V-7 juice agar (Vegetables juice agar)	Tomato Carrot Celery Spinach Beet Lettuce Onion Agar $CaCO_3$ Distilled water	174 ml 8 ml 4 ml 4 ml 4 ml 4 ml 2 ml 15 g 2 g 800 ml
Combined PDA + V-7 juice agar	Components of PDA Components of V-7 juice agar media	200 ml 800 ml

3.6. Cultural variability study of *Stemphylium vesicarium* isolates

Mycelial discs (5 mm) of seven days old culture of *Stemphylium vesicarium* isolates were transferred to the centre of the culture media. The culture plates were incubated at 25^0c in incubator. Radial mycelial growth was noted on the different media [PDA, V-7 juice agar and combined PDA+V-7 juice agar] after 3 days of incubation at 25^0c. Three replications were maintained for each isolate in a completely randomized design.

The radial mycelial growth was recorded on 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 11^{th}, 13^{th} and 15^{th} days after inoculation. Growth per day was calculated by the formula:

$$\text{Growth rate/day} = \frac{\text{Growth on a day} - \text{Growth on previous day}}{2}$$

Characters of 24 isolates of *Stemphylium vesicarium* in the terms of colony color, shape, elevation, margin, texture and substrate color were recorded on PDA. Conidia production and sporulation time were measured and recorded on different media [PDA, V-7 juice agar and

combined PDA+V-7 juice agar].The culture of 30, 40 and 90 days old culture were considered for cultural variability.

The conidia produced per unit surface area were measured using haemacytometer and digital microscope. The measurement was done by using the formula of Chauhan and Pandey (1995) as follows:

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

3.7. Morphological variability study of *Stemphylium vesicarium* isolates

Characters of 24 isolates of *Stemphylium vesicarium* in the terms of shape, color, size and septation of conidia on PDA were measured and recorded. Ninety days old cultures were considered for morphological variability.

3.8. Molecular variability study of *Stemphylium vesicarium*

3.8.1. Collection of mycelium for genomic DNA isolation

Genomic DNA of mycelia of 24 isolates of *Stemphylium vesicarium* were collected from 20 days old culture.

3.8.2. Preparation of stock and working solutions used for genomic DNA isolation

For conducting the genomic DNA isolation procedure, the following stock solutions and working solutions were prepared.

3.8.2.1. 1M Tris HCl pH 8.0 (100 ml)

12.14 gm 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.

3.8.2.2. 0.5M EDTA pH 8.0 (100 ml)

18.61 gm of EDTA (EDTA. $2H_2O$, MW = 372.24) was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 gm of NaOH pellets was added 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.

3.8.2.3. β-Mercaptoethanol

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

3.8.2.4. Ribonuclease A

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

3.8.2.5. Tris-HCl saturated phenol

It was prepared in 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 used during preparation of phenol.

3.8.2.6. Chloroform: Isoamyl alcohol (24:1) (50 ml)

48 ml 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.

3.8.2.7. 70% Ethanol (100 ml)

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

3.8.2.8. $T_{10}E_1$ (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° C.

3.8.2.9. 7.5M ammonium acetate pH 5.2 (100 ml)

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

3.8.2.10. Preparation of extraction buffer

To prepare extraction buffer the following components and concentrations were used. **Table 3: 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 as the 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 5.0 with HCl and was made up to 100 ml by adding sterile de-ionized distilled water

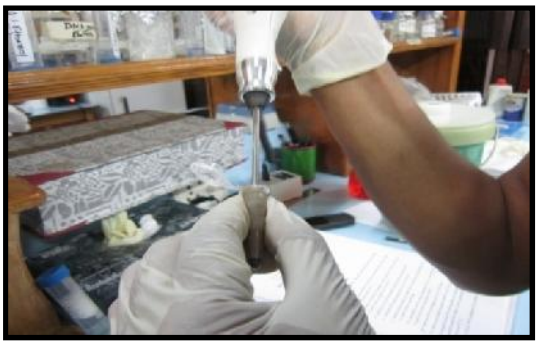
Freshly prepared extraction buffer solution was used. β-mercaptoethanol was added to the solution prior to use. The whole task was performed in the fume hood.

3.8.3. Protocol used for genomic DNA isolation

DNA was isolated using the modified CTAB method (Figure 4). The method is described below:

1. 1.5 g freshly harvested fungal mycelium was taken in effendorf tubes and placed them in liquid nitrogen. The fungal mycelium was grinded to get fine powder with homogenizer machine.
2. 300 µl extraction buffers were added in each effendorf tube.
3. The tubes were transferred to 65º C preheated water bath for 30 minutes and tubes were agitated 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 the steps 5 and 6 until there was a clear interphase.
7. For precipitation, 2/3 volume isopropanol (550µl) was added, pre cooled at -20^0C, 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 the supernatant and washed the pellet in 70% ethanol.
10. Carefully removed the washing–buffer, air dried the pellete and resuspended the pellet in 100 µl TE supplemented with RNAse A (final concentration 10 mg/ml) and incubated for 30 min at 37^0C.
11. 100 µl 7.5M NH_4-Acetate and 750 µl absolute ethanol 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) or in ethanol.
14. Allowed DNA to dissolve overnight at 4^0C.

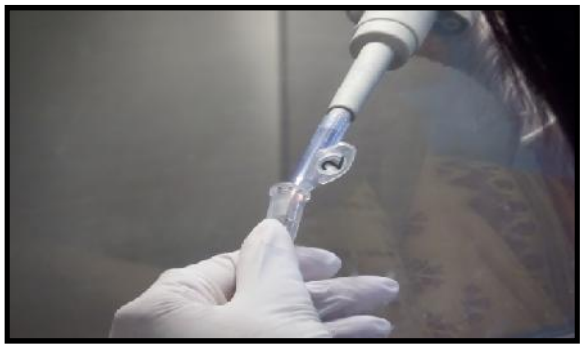
(A)



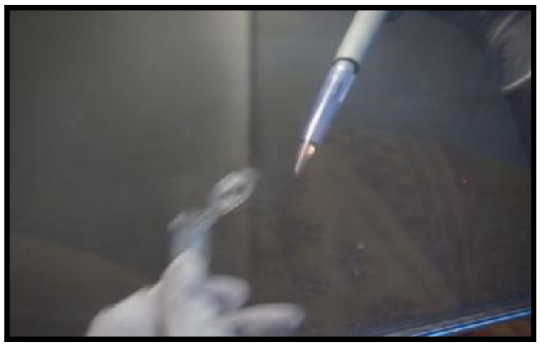
(B)

(C)

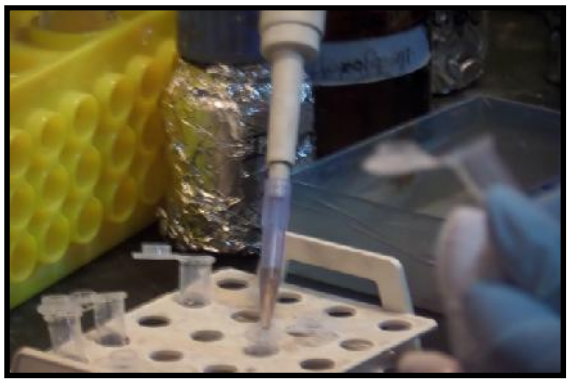
(D)



(E)



(F)



(G)

(H)

Figure 4. Photographs of steps done for genomic DNA extraction (A) Collection of *Stemphylium* mycelium in EP tubes (B) Crushing of mycelium by homogenizer (c) Keeping of crushed mycelium in water bath (D) Centrifugation of crushed mycelium (E and F) Separation of

supernatant portion from EP tube after centrifugation (G) Transfer of supernatant portion in another EP tube (H) DNA under EP tube in pellet form

3.8.4. Estimation of quality and quantity of isolated DNA samples

Before PCR amplification it is important to know the quality and quantity of genomic DNA because different DNA extraction methods produced DNA of widely different purity. It is necessary to optimize the amount of DNA to achieve reproducibility and strong signal in PCR assay. Excessive genomic DNA may result smears lack of clearly defined Bands in the gel. On the other hand, too little DNA will give non-reproducible band patterns (Williams *et al*. 1990). Measurement of isolated DNA concentration can be done by comparing DNA with the standard DNA on agarose gel electrophoresis or by estimating the absorbance of DNA by spectrophotometer at 260 nm. Agarose gel electrophoresis method was carried out in this investigation.

3.8.5. Preparation of stock solutions used for Gel electrophoresis

For conducting the gel electrophoresis, the following stock solutions and other solutions were prepared.

3.8.5.1. 50 ò TAE buffer (P^H 8.3) (1 liter)

242 gm 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.

3.8.5.2. 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 6× 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 6× DNA loading dye was added to DNA samples to achieve a final dye concentration of 1X.

B. 6X loading dye manufactured by Thermo Scientific.

3.8.5.3. 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.

3.8.6. 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 gm 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 1×TAE buffer.

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

3.8.7. 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.

3.8.8. RAPD analysis using PCR (Polymerase Chain Reaction)

To perform the RAPD analysis, a single oligonucleotide of arbitrary DNA sequence was 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).

3.8.8.1. 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.

3.8.8.2. Primer test

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

Table 4. 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

3.8.8.3. 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 nanomole 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.

3.8.8.4. 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.

3.8.8.5. 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.

3.8.8.6. Preparation of PCR reaction mixture

The following components were used to prepare PCR reaction mixture:

Table 5. Components of PCR reaction mixture

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.

3.8.8.7. 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 cycles	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.

3.8.8.8. 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 1×TAE 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.

3.8.9. Identification of ITS region and confirm through sequencing

3.8.9.1. DNA isolation

DNA was isolated from *Stemphylium vesicarium* isolates following CTAB method.

3.8.9.2. Primer

Sl. No.	Primer Type	Primer Name	Primer Sequence 5′-3′

1	Forward primer	ITS1F	CTTGGTCATTTAGAGGAAGTAA
2	Reverse primer	ITS4	TCCTCCGCTTATTGATATGC

3.8.9.3. Preparation of PCR reaction mixture

The following components were used to prepare PCR reaction mixture:

Table 6. Components of PCR reaction mixture

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.

3.8.9.4. 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^{o}C	for	5 minutes
30 cycles	Denaturation	at	94^{o}C	for	1 minutes
	Annealing	at	49^{o}C	for	1 minutes
	Extension	at	72^{o}C	for	3 minutes
	Final extension	at	72^{o}C	for	10 minutes

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

3.8.9.5. 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.

3.8.9.6. Purification of PCR products

Alcohol precipitation:

i. Added equal volume of chloroform and isoamyl alcohol (24:1) then centrifuged at 13000 rpm for 5 minutes.
ii. Taken the upper aqua's phase and added equal volume of chloroform then centrifuged at13000 rpm for 5 minutes.
iii. Taken the upper aqua's phase and added 1/10th 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.

3.8.9.7. Sequencing of PCR positive product

PCR amplified products were sequenced through automated sequencer in Centre for Advanced Research in Sciences, University of Dhaka, Dhaka-1000. .

3.9. Data analysis

The data of cultural and morphological variation were analyzed by using Completely Randomized Design (CRD) with the help of the statistical software, MSTAT-C computer package program. The data were subjected to an analysis of variance and LSD (Least Significant Difference) were used to separate means and compared with DMRT (Dunkan's Multiple Range Test) where F values indicated significantly differences at 5% level of probability. For molecular variability sequence was analyzed using database searches with *Stemphylium* sp. Sequences were carried out by NCBI-BLAST program (http://blast.ncbi.nlm.nih.gov).

CHAPTER 4

RESULTS

4.1. Cultural variability of *Stemphylium vesicarium*

4.1.1. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on PDA

Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* significantly varied on PDA (Table 7 and Plate 3). After 3 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (2.800 cm) was observed in CCKH 01, which was statistically similar to GGBB 02 (2.480 cm) followed by JJLL 03 (2.450 cm), DSTR 02 (2.400 cm), JJLL 02 (2.397 cm), DSSA (2.383 cm), MMBH (2.367 cm), FFKU 03 (2.353 cm), RBHR 02 (2.350 cm), FFKU 01 (2.327 cm), GGBB 01 (2.323 cm) and RBHR 03 (2.323 cm). The minimum radial mycelial growth (2.050 cm) was recorded in DSTR 01. which was statistically similar to CCKH 03 (2.123 cm) preceded by RBHR 01 (2.157 cm**),** RBHR 01 (2.173 cm**),** MTBB 02 (2.183 cm**),** GJBS (2.210 cm), MSMM 01 (2.217 cm), FFKU 02 (2.227 cm), JJLL 01 (2.233 cm), MSMM 02 (2.240 cm), MSMM 03 (2.260 cm) and RBHR 03 (2.300 cm).

After 5 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (3.190 cm) was measured in FFKU 01, which was statistically similar GGBB 02, JJLL 03 and FFKU 03 (3.177 cm) followed by CCKH 01 and MSMM 03 (3.147 cm), JJLL 01 (3.133 cmm), MSMM 02 (3.130 cm), DSTR 02 and MMBH (3.123 cm), JJLL 02 (3.117 cm) and DSSA (3.097 cm). The minimum radial mycelial growth (2.850 cm) was recorded in DSTR 01. which was statistically similar to CCKH 03 (2.887 cm) preceded by GJBS (2.917 cm), GGBB 01 (2.933 cm), FFKU 02 (2.967 cm), MSMM 01 (2.987 cm), RBHR 01 (2.993 cm), RBHR 01 (3.010 cm), MTBB 02 (3.043 cm), MTBB 01, MTBB 01 and RBHR 03 (3.050 cm).

After 7 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (4.207 cm) was observed in FFKU 01, which was statistically similar to RBHR 01 (4.197 cm) followed by CCKH 01 (4.193 cm), MMBH (4.183 cm), MSMM 02 (4.163 cm) GGBB 02 (4.110 cm), JJLL 02 (4.093 cm), MSMM 03 (4.050 cm), JJLL 03 (4.037 cm), DSSA and JJLL 01 (4.027 cm). The minimum radial mycelial growth (3.630 cm) was recorded in GJBS. which was statistically similar to DSTR 01 (3.637 cm) preceded by FFKU 02 (3.667 cm), CCKH 03 (3.683 cm), RBHR 01 (3.693 cm), GGBB 01 (3.757 cm), MSMM 01 (3.787 cm),

RBHR 03 (3.800 cm), MTBB 02 (3.853 cm), RBHR 02 (3.863 cm), MTBB 01 (3.907 cm), FFKU 03 (3.970 cm) and DSTR 02 (3.973 cm).

After 9 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (5.317 cm) was counted in CCKH 01, which was statistically similar to GGBB 02 (5.233 cm) followed by RBHR 01 (5.107 cm), MSMM 03 (5.097 cm), MSMM 02 (5.050 cm), MMBH (5.943 cm), MTBB 02 (5.937 cm), JJLL 02 (5.930 cm), FFKU 01 (5.923 cm), DSTR 02 (5.900 cm) and MTBB 01 (5.880 cm). The minimum radial mycelial growth (4.463 cm) was recorded in GJBS. which was statistically similar to DSTR 01 (4.483 cm) preceded by RBHR 03 (4.527 cm), RBHR 01 (4.540 cm), CCKH 03 and FFKU 02 (4.580 cm), GGBB 01 (4.650 cm), FFKU 03 (4.697 cm), MSMM 01 (4.713 cm), JJLL 03 (4.757 cm), RBHR 02 (4.793 cm) and DSSA (4.840 cm).

After 11 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (6.207 cm) was observed in DSTR 02, which was statistically similar to CCKH 01 (6.160 cm) followed by FFKU 01 (6.147 cm), GGBB 02 (6.027 cm), MSMM 03 (6.983 cm), RBHR 01 (6.980 cm), JJLL 01 (6.880 cm), JJLL 03 (6.853 cm), DSSA (6.840 cm), RBHR 02 (6.823 cm) and MTBB 01 (6.810 cm). The minimum radial mycelial growth (5.430 cm) was recorded in GGBB 01. which was statistically similar to GJBS (5.493cm) preceded by DSTR 01 (5.567 cm), FFKU 02 (5.580 cm), RBHR 03(5.587 cm), RBHR 01 (5.653 cm), MTBB 02 and MSMM 01 (5.660 cm), CCKH 03 (5.700 cm), JJLL 02 (5.720 cm), MMBH (5.750 cm), FFKU 03 (5.777 cm) and MSMM 02 (5.780 cm).

After 13 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (7.130 cm) was measured in DSTR 02, which was statistically similar to MTBB 01 (7.113 cm) followed by RBHR 01(6.997 cm), CCKH 01 (6.977 cm), GGBB 02 (6.963 cm), FFKU 01 (6.943 cm), JJLL 01 (6.933 cm), MSMM 03 (6.930 cm), JJLL 02 (6.920 cm), RBHR 02 and MSMM 02 (6.900 cm) and JJLL 03 (6.877 cm). The minimum radial mycelial growth (6.420 cm) was recorded in GJBS. which was statistically similar to GGBB 01 (5.523cm) preceded by RBHR 03 (5.557cm), MMBH (6.610 cm), DSTR 01 (6.733 cm), CCKH 03(6.750 cm), MSMM 01 and FFKU 02 (6.753 cm), DSSA (6.787 cm), MTBB 02 (6.817 cm), RBHR 01 (6.823 cm) and FFKU 03 (6.850 cm).

After 15 days of inoculation, no significant difference was found in the terms of radial mycelial growth of *Stemphylium vesicarium*. However, the maximum radial mycelial growth of *Stemphylium vesicarium* (8.140 cm) was observed in DSTR 02, which was statistically

similar to JJLL 03 (8.060 cm) followed by MTBB 01 (7.960 cm), FFKU 02 (7.957cm), RBHR 01 (7.953cm), DSSA and GGBB 02 (7.907cm), MSMM 02 (7.900 cm), CCKH 01 (7.883 cm), MSMM 03 (7.850 cm), JJLL 01 (7.843 cm) and DSTR 01 (7.817 cm). The minimum radial mycelial growth (7.500 cm) was recorded in GJBS which was statistically similar to GGBB 01 (7.513 cm) preceded by MMBH (7.547 cm), CCKH 03 (7.607 cm), RBHR 03 (7.627 cm), MTBB 02 and JJLL 02 (7.740 cm), MSMM 01 (7.750 cm), RBHR 01 and RBHR 02 (7.767 cm), FFKU 03 (7.783 cm) and FFKU 01 (7.813 cm).

Table 7. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on PDA

Sl. No.	Isolates	Radial mycelial growth (cm)[1]							Mean radial mycelial growth/ Day (cm)
		3rd DAI	5th DAI	7th DAI	9th DAI	11th DAI	13th DAI	15th DAI	

1	DSSA	2.383 ab	3.097 a-c	4.027 a-e	4.860 b-i	5.840 a-f	6.787 ab	7.907 a-c	0.460 a
2	DSTR 01	**2.050 b**	**2.850 c**	3.637 f	4.483 hi	5.567 d-f	6.733 ab	7.817 a-c	0.480 a
3	DSTR 02	2.400 ab	3.123 a-c	3.973 a-f	4.900 b-g	**6.207 a**	**7.130 a**	**8.140 a**	0.480 a
4	MMBH	2.367 b	3.123 abc	4.183 a	4.943 a-f	5.750 b-f	6.610 ab	7.547 c	0.433 a
5	MTBB 01	2.293 b	3.050 abc	3.907 a-f	4.880 b-h	5.810 a-f	7.113 a	7.960 a-c	0.476 a
6	MTBB 02	2.183 b	3.043 a-c	3.853 a-f	4.937 a-f	5.660 c-f	6.817 ab	7.740 a-c	0.463 a
7	RBHR 01	2.157 b	3.010 a-c	4.197 a	5.107 a-c	5.980 a-d	6.997 ab	7.767 a-c	0.466 a
8	RBHR 02	2.350 b	3.050 a-c	3.863 a-f	4.793 c-i	5.823 a-f	6.900 ab	7.767 a-c	0.453 a
9	RBHR 03	2.300 b	3.050 abc	3.800 b-f	4.527 g-i	5.587 d-f	6.557 ab	7.627 bc	0.443 a
10	GJBS	2.210 b	2.917 abc	**3.630 f**	**4.463 i**	5.493 ef	**6.420 b**	**7.500 c**	0.440 a
11	GGBB 01	2.323 b	2.933 abc	3.757 c-f	4.650 e-i	**5.430 f**	6.523 ab	7.513 c	0.430 a
12	GGBB 02	2.480 ab	3.177 ab	4.110 a-c	5.233 ab	6.027 a-c	6.963 ab	7.907 a-c	0.453 a
13	CCKH 01	**2.800 a**	3.147 ab	4.193 a	**5.317 a**	6.160 ab	6.977 ab	7.883 a-c	0.423 a
14	CCKH 02	2.173 b	2.993 a-c	3.693 d-f	4.540 f-i	5.653 c-f	6.823 ab	7.953 a-c	0.483 a
15	CCKH 03	2.123 b	2.887 bc	3.683 d-f	4.580 f-i	5.700 c-f	6.750 ab	7.607 bc	0.460 a
16	JJLL 01	2.233 b	3.133 abc	4.027 a-e	4.990 a-e	5.880 a-e	6.933 ab	7.843 a-c	0.466 a
17	JJLL 02	2.397 ab	3.117 a-c	4.093 a-c	4.930 a-g	5.720 c-f	6.920 ab	7.740 a-c	0.446 a
18	JJLL 03	2.450 ab	3.177 ab	4.037 a-d	4.757 c-i	5.853 a-e	6.877 ab	8.060 ab	0.470 a
19	MSMM 01	2.217 b	2.987 a-c	3.787 c-f	4.713 c-i	5.660 c-f	6.753 ab	7.750 a-c	0.460 a
20	MSMM 02	2.240 b	3.130 a-c	4.163 ab	5.050 a-e	5.780 b-f	6.900 ab	7.900 a-c	0.470 a

Table 7. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on PDA (Cont'd)

Sl. No.	**Isolates**	**Radial mycelial growth (cm)[1]**							**Mean radial mycelial**
		3rd DAI	**5th DAI**	**7th DAI**	**9th DAI**	**11th DAI**	**13th DAI**	**15th**	

								DAI	growth/Day (cm)
21	MSMM 03	2.260 b	3.147 ab	4.050 a-d	5.097 a-d	5.983 a-d	6.930 ab	7.850 a-c	0.463 a
22	FFKU 01	2.327 b	**3.190 a**	**4.207 a**	4.923 a-g	6.147 ab	6.943 ab	7.813 a-c	0.456 a
23	FFKU 02	2.227 b	2.967 a-c	3.667 ef	4.580 f-i	5.580 d-f	6.753 ab	7.957 a-c	0.476 a
24	FFKU 03	2.353 b	3.177 ab	3.970 a-f	4.697 d-i	5.777 b-f	6.850 ab	7.783 a-c	0.453 a
	LSD (0.05)	0.370	0.237	0.3071	0.340	0.348	0.532	0.408	0.0519
	CV (%)	9.77%	4.74%	4.73%	4.28%	3.66%	4.75%	3.20%	6.40%

[1]Means of three replications for each isolate

Number with similar letter do not differ significantly at 5% level according to Dunkan's Multiple Range Test (DMRT)

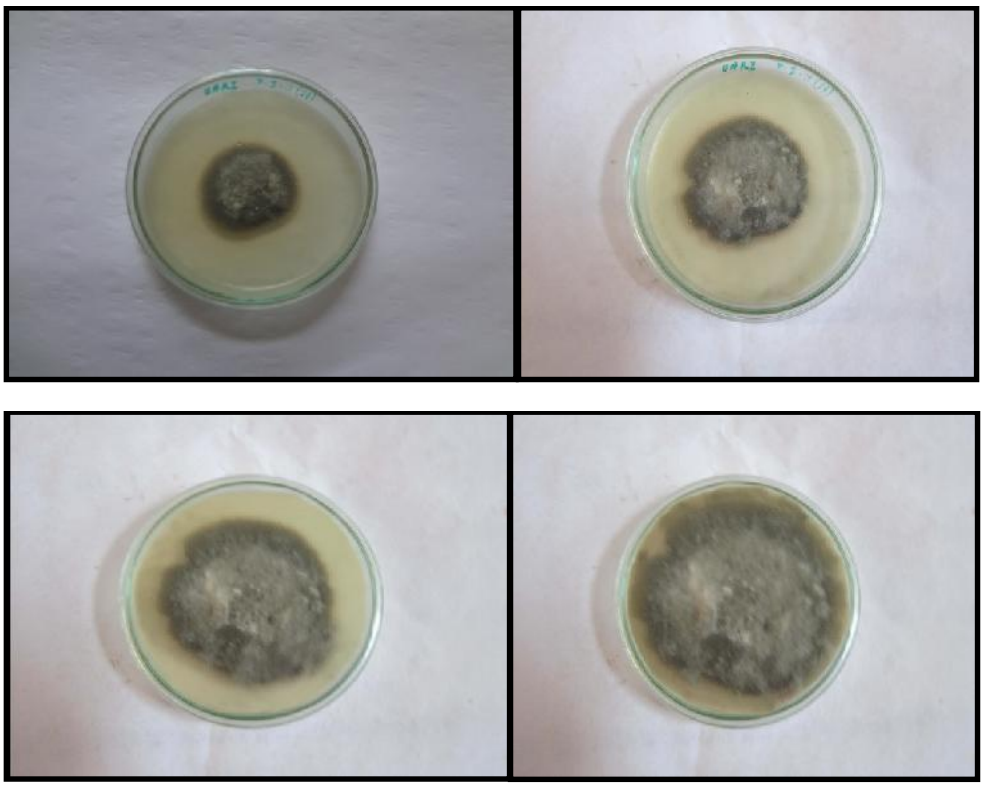


Plate 3. Radial mycelial growth pattern of *Stemphylium vesicarium* on PDA

4.1.2. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on V-7 juice agar

Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* varied on V-7 juice agar medium (Table 8 and Plate 4). After 3 days of inoculation the maximum radial mycelial growth (0.730 cm) was observed in GGBB 01, which was statistically similar to MTBB 02, MSMM 01 and FFKU 01 (0.716 cm) followed by FFKU 03 (0.700 cm), MTBB 01 (0.693 cm), DSSA and CCKH 02 (0.683 cm), JJLL 03 (0.676 cm), DSTR 02 and CCKH 02 (0.666 cm). The minimum radial mycelial growth (0.500 cm) was recorded in JJLL 02 which was statistically similar to GJBS and JJLL 01 (0.560 cm) preceded by RBHR 01 (0.566 cm), RBHR 02 and MSMM 03 (0.580 cm), RBHR 03, CCKH 01 and CCKH 03 (0.583 cm), MSMM 02 (0.633 cm), and MMBH (0.640 cm), DSTR 01 (0.643 cm) and FFKU 02 (0.650 cm).

After 5 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (1.847 cm) was observed in MTBB 02, which was statistically similar to GJBS (1.743 cm) followed by FFKU 01 (1.727 cm), GGBB 02 (1.717 cm), MSMM 01 (1.713 cm), GGBB 01 (1.710 cm), MTBB 01 and MSMM 03 (1.680 cm), DSTR 02 and MMBH (1.663 cm), CCKH 03 (1.653 cm), DSSA (1.647) and JJLL 03 (1.633 cm). The minimum radial mycelial growth (1.360 cm) was recorded in RBHR 02 which was statistically similar to JJLL 01 (1.427 cm) preceded by RBHR 01 (1.450 cm), RBHR 03 (1.480 cm), FFKU 02 (1.490 cm), JJLL 02 (1.507 cm), DSTR 01 (1.533 cm), MSMM 02 (1.550 cm) and CCKH 02 (1.567 cm).

After 7 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (3.170 cm) was counted in GGBB 02, which was statistically similar to MTBB 02 (3.127 cm) followed by GJBS (3.017 cm), MMBH and JJLL 03 (2.990 cm), MSMM 01 (2.950 cm), MTBB 01 (2.913 cm), FFKU 01 (2.897 cm), DSTR 02 (2.883 cm), MSMM 02 (2.867 cm), GGBB 01 (2.850 cm), MSMM 03 (2.847 cm), CCKH 01 (2.840 cm).The minimum radial mycelial growth (2.603 cm) was recorded in FFKU 02. which was statistically similar to RBHR 02 (2.673 cm) preceded by CCKH 02 (2.680 cm), CCKH 03 (2.727 cm), FFKU 03 (2.733 cm), DSSA and DSTR 01 (2.747 cm), JJLL 02 (2.763 cm), RBHR 03 (2.767 cm), RBHR 01 and JJLL 01 03 (2.817 cm).

After 9 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (4.380 cm) was measured in MMBH, which was statistically similar to JJLL 03 (4.377 cm) followed by MTBB 02 (4.367 cm), GGBB 02 (4.333 cm), MSMM 03 (4.313 cm), MTBB 01

and RBHR 03 (4.250 cm), MSMM 01 and FFKU 01 (4.223 cm), DSTR 02 (4.213 cm), GJBS (4.197 cm), CCKH 01 (4.183 cm). On the other hand the minimum radial mycelial growth (3.847 cm) was recorded in CCKH 02. which was statistically similar to RBHR 02 (3.877 cm) preceded by DSSA (3.950 cm), CCKH 03 and FFKU 02 (3.957 cm), FFKU 03 (3.993 cm), JJLL 02 (4.033 cm), JJLL 01 (4.050 cm), DSTR 01 (4.063 cm), GGBB 01 (4.110 cm), RBHR 01 and MSMM 02 (4.133 cm).

After 11 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (5.777 cm) was observed in MMBH, which was statistically similar to RBHR 03 (5.733 cm) followed by MTBB 01 (5.690 cm), JJLL 03 (5.667 cm), MTBB 02 (5.647 cm), GGBB 02 (5.627 cm), FFKU 01 (5.590 cm), MSMM 03 (5.557 cm), DSTR 02 (5.537 cm), MSMM 02 (5.533 cm), CCKH 01 (5.510 cm) and MSMM 01 (5.497 cm). The minimum radial mycelial growth (5.093 cm) was recorded in RBHR 02 which was statistically similar to CCKH 02 (5.130 cm) preceded by FFKU 02 (5.187 cm), CCKH 03 (5.190 cm), DSSA (5.237 cm), FFKU 03 (5.267 cm), JJLL 02 (5.330 cm), DSTR 01 (5.333 cm), GGBB 01 (5.347 cm), GJBS (5.410 cm), RBHR 01 (5.457 cm) and JJLL 01(5.460 cm).

After 13 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (7.100 cm) was observed in RBHR 03, which was statistically similar to MTBB 01 (7.020 cm) followed by 01 MMBH (7.000 cm), RBHR 01 (6.983 cm), JJLL 01 (6.920 cm), FFKU 01 (6.910 cm), MSMM 03 (6.900 cm), CCKH 01 (6.883 cm), JJLL 03 (6.870 cm), DSTR 01 (6.857 cm), DSTR 02 (6.850 cm) and MSMM 02 (6.843 cm). The minimum radial mycelial growth (6.513 cm) was recorded in RBHR 02 which was statistically similar to CCKH 03 (6.550 cm) preceded by CCKH 02 (6.603 cm), FFKU 03 (6.627 cm), DSSA (6.677 cm), GGBB 01 (6.703 cm), MSMM 01 (6.717 cm), MTBB 02 (6.733 cm), FFKU 02 (6.743 cm), GJBS (6.813 cm) and GGBB 02 (6.827 cm).

After 15 days of inoculation no significant difference was found in the terms of radial mycelial growth of *Stemphylium vesicarium*. However, the highest radial mycelial growth of *Stemphylium vesicarium* (8.353 cm) was observed in RBHR 01, which was statistically similar to MMBH (8.307 cm) followed by MTBB 01 and RBHR 03 (8.267 cm), MSMM 03 (8.263 cm), CCKH 01 (8.257 cm), JJLL 03 (8.250 cm), MSMM 02 (8.220 cm), JJLL 01 (8.210 cm), GJBS (8.200 cm), DSTR 01 (8.197 cm) and FFKU 01 (8.190 cm). The minimum radial mycelial growth (8.003 cm) was recorded in CCKH 03 isolate which was statistically similar to FFKU 03 (8.033 cm) preceded by CCKH 02 (8.060 cm), RBHR 02

(8.070 cm), DSSA and JJLL 02 (8.083 cm), GGBB 01 and MTBB 02 (8.150 cm), GGBB 02 and FFKU 02 (8.163 cm), MSMM 01 (8.167 cm) and DSTR 02 (8.180 cm).

Table 8. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on V-7 juice agar

Sl. No.	Isolates	Radial mycelial growth (cm)[1]							Mean radial mycelial growth/Day (cm)
		3rd DAI	5th DAI	7th DAI	9th DAI	11th DAI	13th DAI	15th DAI	
1	DSSA	0.683 a	1.647 a-c	2.747 a-c	3.950 a	5.237 a-c	6.677 a	8.083 a	**0.616 a**

2	DSTR 01	0.643 a	1.533 a-c	2.747 a-c	4.063 a	5.333 a-c	6.857 a	8.197 a	0.630 a
3	DSTR 02	0.666 a	1.663 a-c	2.883 a-c	4.213 a	5.537 a-c	6.850 a	8.180 a	0.626 a
4	MMBH	0.640 a	1.660 a-c	2.990 a-c	**4.380 a**	**5.777 a**	7.000 a	8.307 a	0.640 a
5	MTBB 01	0.693 a	1.680 a-c	2.913 a-c	4.250 a	5.690 a-c	7.020 a	8.267 a	0.633 a
6	MTBB 02	0.716 a	**1.847 a**	3.127 ab	4.367 a	5.647 a-c	6.733 a	8.150 a	0.620 a
7	RBHR 01	0.566 a	1.450 bc	2.817 a-c	4.133 a	5.457 a-c	6.983 a	**8.353 a**	**0.650 a**
8	RBHR 02	0.580 a	**1.360 c**	2.673 c	3.877 a	**5.093 c**	**6.513 a**	8.070 a	0.626 a
9	RBHR 03	0.583 a	1.480 bc	2.767 a-c	4.250 a	5.733 ab	**7.100 a**	8.267 a	0.640 a
10	GJBS	0.560 a	1.743 ab	3.017 a-c	4.197 a	5.410 a-c	6.813 a	8.200 a	0.640 a
11	GGBB 01	**0.730 a**	1.710 ab	2.850 a-c	4.110 a	5.347 a-c	6.703 a	8.150 a	0.620 a
12	GGBB 02	0.666 a	1.717 ab	**3.170 a**	4.333 a	5.627 a-c	6.827 a	8.163 a	0.626 a
13	CCKH 01	0.583 a	1.630 a-c	2.840 a-c	4.183 a	5.510 a-c	6.883 a	8.257 a	0.640 a
14	CCKH 02	0.683 a	1.567 a-c	2.680 c	**3.847 a**	5.130 bc	6.603 a	8.060 a	**0.616 a**
15	CCKH 03	0.583 a	1.653 a-c	2.727 bc	3.957 a	5.190 a-c	6.550 a	**8.003 a**	**0.616 a**
16	JJLL 01	0.560 a	1.427 bc	2.817 a-c	4.050 a	5.460 a-c	6.920 a	8.210 a	0.636 a
17	JJLL 02	**0.500 a**	1.507 a-c	2.763 a-c	4.033 a	5.330 a-c	6.767 a	8.083 a	0.630 a
18	JJLL 03	0.676 a	1.633 a-c	2.990 a-c	4.377 a	5.667 a-c	6.870 a	8.250 a	0.633 a
19	MSMM 01	0.716 a	1.713 ab	2.950 a-c	4.223 a	5.497 a-c	6.717 a	8.167 a	0.620 a
20	MSMM 02	0.633 a	1.550 a-c	2.867 a-c	4.133 a	5.533 a-c	6.843 a	8.220 a	0.633 a
21	MSMM 03	0.580 a	1.680 a-c	2.847 a-c	4.313 a	5.557 a-c	6.900 a	8.263 a	0.640 a
22	FFKU 01	0.716 a	1.727 ab	2.897 a-c	4.223 a	5.590 a-c	6.910 a	8.190 a	0.623 a
23	FFKU 02	0.650 a	1.490 bc	**2.603 c**	3.957 a	5.187 a-c	6.743 a	8.163 a	0.626 a

Table 8. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on V-7 juice agar (Cont'd)

Sl. No.	**Isolates**	**Radial mycelial growth (cm)[1]**							**Mean radial mycelial growth/D**
		3rd	**5th DAI**	**7th DAI**	**9th DAI**	**11th DAI**	**13th DAI**	**15th DAI**	

		DAI							**ay (cm)**
24	FFKU 03	0.700 a	1.627 a-c	2.733 bc	3.993 a	5.267 a-c	6.627 a	8.033 a	0.610 a
	LSD (0.05)	0.194	0.284	0.352	0.475	0.521	0.495	0.344	0.0519
	CV (%)	18.87%	10.74 %	7.51%	6.98%	5.82%	4.44%	2.55%	2.72%

[1]Means of three replications for each isolate

Number with similar letter do not differ significantly at 5% level according to Dunkan's Multiple Range Test (DMRT)

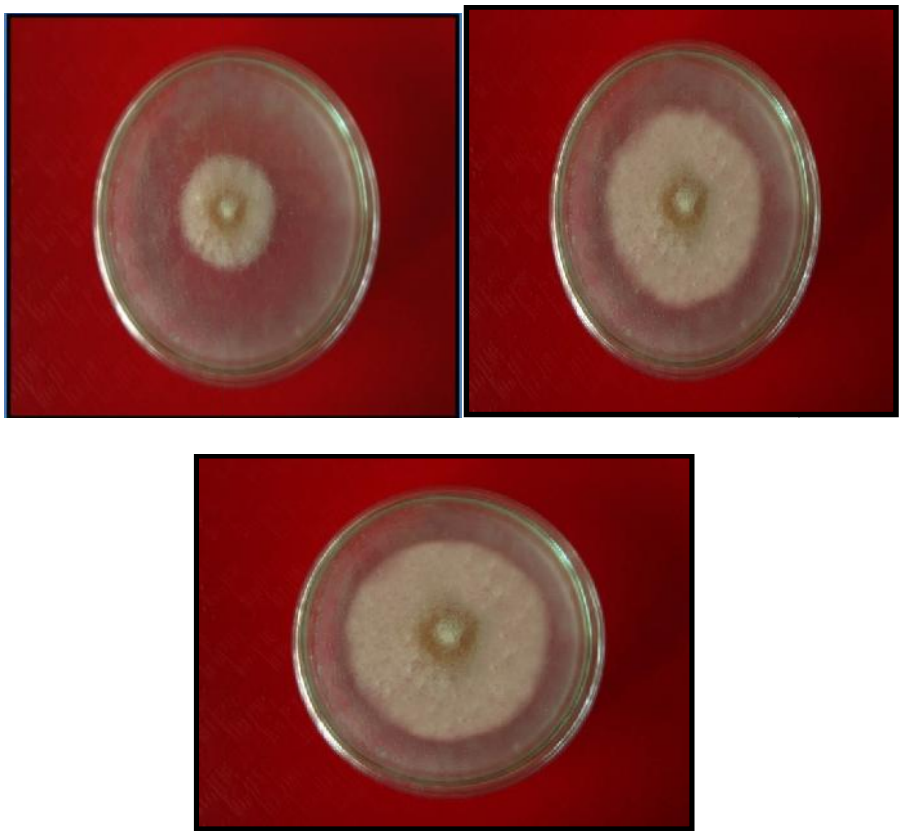


Plate 4. Radial mycelial growth pattern of *Stemphylium vesicarium* on V-7 juice agar

4.1.3. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on combined PDA+ V-7 juice agar

Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* significantly varied on combined PDA+V-7 juice agar medium (Table 9). After 3 days of inoculation maximum

radial growth (3.20 cm) was observed in DSSA, which was statistically similar to DSTR 02 (3.187 cm), JJLL 02 (3.140 cm) followed by FFKU 01 (3.133 cm), GGBB 02 (3.080 cm), RBHR 02 (3.067 cm), JJLL 01 and MSMM 02 (3.050 cm) RBHR 01 (3.037 cm) , DSTR 01 (3.023 cm), MSMM 03 (3.017 cm) and FFKU 03 (3.013 cm). The minimum radial mycelial growth (2.813 cm) was recorded in MSMM 01 which was statistically similar to FFKU 02 (2.867 cm) preceded by MTBB 02 (2.880 cm), GJBS (2.893 cm), CCKH 01 (2.930 cm), CCKH 02 and RBHR 03 (2.933 cm), MTBB 01(2.943 cm), MMBH (2.950 cm), CCKH 03 (2.957 cm), RBHR 03 (2.933 cm) and JJLL 03 (3.00 cm).

After 5 days of inoculation maximum radial growth of *Stemphylium vesicarium* (4.203 cm) was observed in MSMM 02, which was statistically similar to DSSA (4.093 cm) followed by JJLL 02 (4.083 cm), DSTR 02 (4.063 cm), FFKU 01 (4.037 cm), GGBB 02 (3.997 cm), FFKU 02 (3.987 cm) , JJLL 01 (3.980 cm) , MSMM 03 (3.917 cm) DSTR 01(3.903 cm), CCKH 01 (3.897 cm), MTBB 01 and RBHR 03 (3.873 cm) , CCKH 02 (3.857 cm). The lowest radial mycelial growth (3.657 cm) was recorded in GJBS which was statistically similar to MSMM 01 (3.673 cm) preceded by CCKH 03 (3.703 cm), RBHR 02 (3.710 cm) , FFKU 03 (3.727 cm) , MTBB 02 (3.760 cm), RBHR 02 and MMBH (3.783 cm) , RBHR 01 (3.817 cm) , GGBB 01 and JJLL 03 (3.863 cm).

After 7 days of inoculation maximum radial mycelial growth of *Stemphylium vesicarium* (5.087 cm) was recorded in RBHR 01, which was statistically similar to MSMM 03 (5.000 cm) followed by CCKH 01 (4.993 cm), MSMM 02 (4.983 cm), MMBH (4.960 cm), FFKU 01 (4.920 cm), JJLL 03 (4.887 cm), DSTR 02 (4.883 cm) , GGBB 02 (4.880 cm) JJLL 02 (4.877 cm), MTBB 01 (4.870 cm), JJLL 01 (4.863 cm), FFKU 02 (4.850 cm) and MTBB 02 (4.827 cm). The minimum radial mycelial growth (4.480 cm) was recorded in RBHR 03 which was statistically similar to GJBS (4.500 cm) preceded by FFKU 03 (4.560 cm), GGBB 01 (4.663 cm), DSTR 01(4.737 cm), CCKH 03 (4.740 cm), MSMM 01 (4.743 cm), CCKH 02 (4.770 cm), RBHR 02 (4.783 cm) and DSSA (4.803 cm).

After 9 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (6.033 cm) was counted in RBHR 01, which was statistically similar to MSMM 03 (6.017 cm) followed by CCKH 01 (5.970 cm), MSMM 02 (5.967 cm), DSSA (5.947 cm) JJLL 01 (5.913 cm), DSTR 02 and GGBB 02 (5.910 cm), JJLL 02 (5.897 cm) , MTBB 02 (5.883 cm), FFKU 02 (5.853 cm) and RBHR 02 (5.847 cm). The minimum radial mycelial growth (5.293 cm) was recorded in RBHR 03 which was statistically similar to GGBB 01 (5.453 cm)

preceded by GJBS (5.470 cm) FFKU 03 (5.547 cm), MSMM 01 (5.613 cm), CCKH 03 (5.647 cm), CCKH 02 (5.690 cm), CCKH 03 (4.740 cm), MSMM 01 (4.743 cm), CCKH 02 (4.770 cm), RBHR 02 (4.783 cm) and DSSA (4.803 cm).

After 11 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (7.143 cm) was measured in MTBB 01, which was statistically similar to MTBB 02 (7.100 cm) followed by JJLL 02 (7.060 cm), RBHR 01 (7.057 cm) DSTR 01 (7.050 cm), DSSA (7.043 cm), JJLL 01 (6.997 cm) , DSTR 02 (6.990 cm), MSMM 03 (6.920 mm) , CCKH 01 (6.913 mm), GGBB 02 (6.903 mm), RBHR 02 (6.893 cm) and JJLL 03 (6.877 cm). The minimum radial mycelial growth (6.393 cm) was recorded in RBHR 03 which was statistically similar to FFKU 03 (6.427 cm) preceded by MSMM 01 (6.463 cm), GGBB 01 (6.477 cm), GJBS (6.517 cm), MMBH (6.677 cm), CCKH 03 (6.750 cm), FFKU 01 (6.763 cm), CCKH 02 (6.770 cm), FFKU 02 (6.823 cm), MSMM 02 (6.853 cm).

After 13 days of inoculation the maximum radial mycelial growth of *Stemphylium vesicarium* (8.150 cm) was observed in DSTR 01, which was statistically similar to DSTR 02 (8.017 cm) followed by DSSA (7.990 cm), JJLL 03 (7.970 cm) RBHR 01 (7.950 cm) , MTBB 01 (7.933 cm), CCKH 02 (7.917 cm), MSMM 03 (7.890 cm), JJLL 01(7.830 cm), FFKU 02 and MTBB 02 (7.820 cm), and GGBB 02 (7.817 cm). The minimum radial mycelial growth (7.470 cm) was recorded in GGBB 01 which was statistically similar to RBHR 03 (7.477 cm) preceded by FFKU 03 (7.503 cm), GJBS (7.523 cm), MMBH (7.643cm), CCKH 03 (7.703 cm), RBHR 02 (7.737 cm), JJLL 02 (7.773 cm), CCKH 01 (7.793 cm), MSMM 01 (7.710 cm), FFKU 01 (7.800 cm) and MSMM 02 (7.810 cm).

After 15 days of inoculation, no significant difference was found in the terms of radial mycelial growth of *Stemphylium vesicarium*. However, the highest radial mycelial growth of *Stemphylium vesicarium* (8.880 cm) was observed in JJLL 01, which was statistically similar to CCKH 02 and MSMM 02 (8.870 cm) followed by FFKU 01 (8.867 cm), CCKH 01 and JJLL 03 (8.863 cm), RBHR 03 and GGBB 02 (8.860 cm) , DSSA, MTBB 02, MSMM 03 and FFKU 02 (8.857 cm), MMBH and GJBS (8.853 cm), JJLL 02 (8.850 cm), DSTR 01, MTBB 01 and RBHR 01 (8.840 cm), GGBB 01 (8.837 cm), DSTR 02, CCKH 03 and MSMM 01 (8.833 cm), RBHR 02 (8.830 cm) and FFKU 03 (8.807 cm).

Table 9. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on combined PDA+V-7 juice agar

Sl. No.	Isolates	Radial mycelial growth (cm)[1]							Mean radial mycelial growth/Day (cm)[1]
		3rd DAI	5th DAI	7th DAI	9th DAI	11th DAI	13th DAI	15th DAI	
1	DSSA	**3.200 a**	4.093 ab	4.803 a-e	5.947 ab	7.043 a-c	7.990 b	8.857 ab	0.473 a
2	DSTR 01	3.023 a-c	3.903 b-h	4.737 b-f	5.747 b-e	**7.050 a-c**	8.150 a	8.840 ab	0.483 a
3	DSTR 02	3.187 ab	4.063 a-c	4.883 a-d	5.910 a-c	6.990 a-d	8.017 ab	8.833 ab	**0.470 a**
4	MMBH	2.950 a-c	3.783 d-h	4.960 a-c	5.763 b-e	6.677 e-g	7.643 hi	8.853 ab	0.493 a

5	MTBB 01	2.943 a-c	3.873 b-h	4.870 a-d	5.900 a-c	**7.143 a**	7.933 b-e	8.840 ab	0.490 a
6	MTBB 02	2.880 a-c	3.760 e-h	4.827 a-d	5.883 abc	7.100 ab	7.820 c-g	8.857 ab	0.500 a
7	RBHR 01	3.037 a-c	3.817 c-h	**5.087 a**	**6.033 a**	7.057 a-c	7.950 b-d	8.840 ab	0.486 a
8	RBHR 02	3.067 a-c	3.710 gh	4.783 a-f	5.847 a-d	6.893 a-e	7.737 f-h	8.830 ab	0.480 a
9	RBHR 03	2.933 a-c	3.873 b-h	**4.480 f**	**5.293 g**	**6.393 h**	7.477 j	8.860 ab	0.496 a
10	GJBS	2.893 a-c	**3.657 h**	4.500 ef	5.470 fg	6.517 f-h	7.523 ij	8.853 ab	0.496 a
11	GGBB 01	3.013 a-c	3.863 b-h	4.663 c-f	5.453 fg	6.477 gh	**7.470 j**	8.837 ab	0.486 a
12	GGBB 02	3.080 a-c	3.997 a-e	4.880 a-d	5.910 a-c	6.903 a-e	7.817 c-g	8.860 ab	0.480 a
13	CCKH 01	2.930 a-c	3.897 b-h	4.993 ab	5.970 ab	6.913 a-e	7.793 d-h	8.863 ab	0.496 a
14	CCKH 02	2.933 a-c	3.857 b-h	4.770 a-f	5.690 c-e	6.770 de	7.917 b-e	8.870 ab	0.496 a
15	CCKH 03	2.957 a-c	3.703 gh	4.740 b-f	5.647 d-f	6.750 d-f	7.703 gh	8.833 ab	0.490 a
16	JJLL 01	3.050 a-c	3.980 a-f	4.863 a-d	5.913 a-c	6.997 a-d	7.830 c-g	**8.880 a**	0.486 a
17	JJLL 02	3.140 ab	4.083 ab	4.877 a-d	5.897 a-c	7.060 a-c	7.773 e-h	8.850 ab	0.476 a
18	JJLL 03	3.000 a-c	3.863 b-h	4.887 a-c	5.760 b-e	6.877 a-e	7.970 bc	8.863 ab	0.490 a
19	MSMM 01	**2.813 c**	3.673 gh	4.743 b-f	5.613 ef	6.463 gh	7.710 gh	8.833 ab	**0.503 a**
20	MSMM 02	3.050 a-c	**4.203 a**	4.983 a-c	5.967 ab	6.853 b-e	7.810 c-g	8.870 ab	0.486 a
21	MSMM 03	3.017 a-c	3.917 b-g	5.000 ab	6.017 a	6.920 a-e	7.890 b-f	8.857 ab	0.486 a
22	FFKU 01	3.133 a-c	4.037 a-d	4.920 a-c	5.717 c-e	6.763 de	7.800 d-h	8.867 ab	0.480 a

Table 9. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on combined PDA+V-7 juice agar (Cont'd)

Sl.	Isolates	Radial mycelial growth (cm)[1]	Mean

No.		3rd DAI	5th DAI	7th DAI	9th DAI	11th DAI	13th DAI	15th DAI	radial mycelial growth/Day (cm)[1]
23	FFKU 02	2.867 bc	3.987 a-e	4.850 a-d	5.853 a-d	6.823 c-e	7.827 c-g	8.857 ab	0.500 a
24	FFKU 03	3.013 a-c	3.727 f-h	4.560 d-f	5.547 ef	6.427 gh	7.503 ij	**8.807 b**	0.483 a
	LSD (0.05)	0.264	0.214	0.269	0.187	0.226	0.137	0.0519	0.0519
	CV (%)	5.36%	3.37%	3.39%	1.99%	2.02%	1.08%	0.32%	2.84%

[1]Means of three replications for each isolate

Number with similar letter do not differ significantly at 5% level according to Dunkan's Multiple Range Test (DMRT)

4.1.4. Radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on different [PDA, V-7 juice agar and combined PDA+ V-7 juice agar] culture media:

The effect of different culture media viz. PDA, V-7 juice agar and combined PDA+ V-7 juice agar on radial mycelial growth per day of *Stemphylium vesicarium* is presented in Table 10. The fungus grew well on V-7 juice agar medium. The maximum (0.610-0.650 cm/day) radial mycelial growth of *Stemphylium vesicarium* was found on V-7 juice agar medium. The minimum (0.423-0.483 cm/day) radial mycelial growth was recorded on PDA preceded by combined PDA+ V-7 juice agar medium (0.470-0.503 cm/day).

Table10. Per day radial mycelial growth of 24 isolates of *Stemphylium vesicarium* on different culture media

Sl. No.	Isolates	Radial mycelial growth/day (cm) on PDA[1]	Radial mycelial growth/day (cm) on V-7 juice agar[1]	Mean radial mycelial growth/day (cm) on combined PDA +V-7 juice agar medium[1]
1	DSSA	0.460 a	0.616	0.473
2	DSTR 01	0.480 a	0.630	0.483
3	DSTR 02	0.480 a	0.626	**0.470**
4	MMBH	0.433 a	0.640	0.493
5	MTBB 01	0.476 a	0.633	0.490
6	MTBB 02	0.463 a	0.620	0.500
7	RBHR 01	0.466 a	**0.650**	0.486
8	RBHR 02	0.453 a	0.626	0.480
9	RBHR 03	0.443 a	0.640	0.496
10	GJBS	0.440 a	0.640	0.496
11	GGBB 01	0.430 a	0.620	0.486
12	GGBB 02	0.453 a	0.626	0.480
13	CCKH 01	0.423 a	0.640	0.496
14	CCKH 02	0.483 a	0.616	0.496
15	CCKH 03	0.460 a	0.616	0.490
16	JJLL 01	0.466 a	0.636	0.486
17	JJLL 02	0.446 a	0.630	0.476
18	JJLL 03	0.470 a	0.633	0.490
19	MSMM 01	0.460 a	0.620	**0.503**
20	MSMM 02	0.470 a	0.633	0.486
21	MSMM 03	0.463 a	0.640	0.486
22	FFKU 01	0.456 a	0.623	0.480
23	FFKU 02	0.476 a	0.626	0.500
24	FFKU 03	0.453 a	**0.610**	0.483
	LSD (0.05)	0.0519	0.0519	0.0519

	CV (%)	6.40%	2.72%	2.84%

[1]Means of three replications for each isolate

Number with similar letter do not differ significantly at 5% level according to Dunkan's Multiple Range Test (DMRT)

4.1.5. Colony characters of 24 isolates of *Stemphylium vesicarium* on PDA

The isolates of *Stemphylium vesicarium* exhibited variations in colony characters like color, shape, elevation, margin, texture and substrate color (Table11 and plate 5, 6, 7 and 8). Colony colors were greenish brown to dirty white, deep grey to whitish, light grey to whitish, deep greenish white, light grey and dirty white to greenish. Colony shapes were circular and irregular. Colony elevation were umbonate, raised and flat. Colony margins were entire, undulate, filiform. Colony texture were cottony, fluffy and velvety. Substrate colors were brown, deep brown and light brown.

Remarkable variation was found in colony color of 24 isolates of *Stemphylium vesicarium*. Greenish brown to dirty white colony color was found in DSSA, DSTR 02, RBHR 03, GGBB 01, GGBB 02, CCKH 01; Deep grey to whitish colony color was found in MTBB 01, MTBB 02, RBHR 02, GJBS, CCKH 02, CCKH 03, JJLL 01, JJLL 02 , JJLL 03, MSMM 01, MSMM 02, MSMM 03, FFKU 01, FFKU 03; Deep greenish white color was found in DSTR 01; Light grey colony color was recorded in MMBH and dirty white to greenish colony color was found in RBHR 01 (Table 11 and plate 4).

Circular colony shape was found in DSTR 02, MMBH, MTBB 02, RBHR 01, RBHR 02, GJBS, GGBB 01, GGBB 02, CCKH 01, CCKH 02, JJLL 01, JJLL 03, MSMM 03, FFKU 01, FFKU 02; Irregular colony shape was found in DSSA, DSTR 01, MTBB 01, RBHR 03, CCKH 03, JJLL 02, MSMM 01, MSMM 02, FFKU 03 (Table 11 and plate 6).

Table 11. Colony characteristics of 24 isolates of *Stemphylium vesicarium* on PDA

Isolate	**Front colony color**
DSSA, DSTR 02, RBHR 03, GGBB 01, GGBB 02 , CCKH 01	Greenish brown to dirty white
MTBB 01, MTBB 02, RBHR 02, GJBS, CCKH 02, CCKH 03, JJLL 01, JJLL 02, JJLL 03, MSMM 01, MSMM 02, MSMM 03, FFKU 01 , FFKU 03	Deep grey to whitish
FFKU 02	Light grey to whitish
DSTR 01	Deep greenish white
MMBH	Light grey
RBHR 01	Dirty white to greenish
	Colony Shape
DSTR 02, MMBH, MTBB 02, RBHR 01, RBHR 02, GJBS, GGBB 01, GGBB 02, CCKH 01, CCKH 02, JJLL 01, JJLL 03, MSMM 03, FFKU 01, FFKU 02	Circular
DSSA, DSTR 01, MTBB 01, RBHR 03, CCKH 03, JJLL 02, MSMM 01, MSMM 02, FFKU 03	Irregular
	Colony Elevation
DSTR 01, GJBS, GGBB 01, CCKH 02, CCKH 03, JJLL 02, JJLL 03, FFKU 01, FFKU 02, FFKU 03, MSMM 01, MSMM 02, MSMM 03 (13)	Umbonate
DSSA, DSTR 02, MMBH, MTBB 01, MTBB 02, RBHR	Raised

02, RBHR 03, GGBB 02, CCKH 01, JJLL 01	
RBHR 01	Flat
	Colony Margin
DSTR 01, DSTR 02, MMBH, MTBB 02, RBHR 01, GJBS, GGBB 01, GGBB 02, CCKH 01, MSMM 01, FFKU 02	Entire
DSSA, RBHR 03, CCKH 03, JJLL 01, MSMM 02	Undulate
MTBB 01, RBHR 02, CCKH 02, JJLL 02, JJLL 03, MSMM 03, FFKU 01, FFKU 03	Filiform
	Colony Texture
DSSA, DSTR 01, DSTR 02, MMBH, RBHR 03, GGBB 01, GGBB 02 , FFKU 02	Cottony
MTBB 01, MTBB 02, RBHR 02, GJBS, CCKH 02, CCKH 03, JJLL 01, JJLL 02, JJLL 03, MSMM 01 , MSMM 02, MSMM 03, FFKU 01, FFKU 03	Fluffy
RBHR 01, CCKH 01	Velvety

Table 11. Colony characteristics of 24 isolates of *Stemphylium vesicarium* on PDA (Cont,d)

Isolates	**Reverse colony color**
DSSA, DSTR 01, DSTR 02, MTBB 01, MTBB 02, MSMM 03, FFKU 01, FFKU 03	Deep Brown
RBHR 01,RBHR 02, GJBS, CCKH 02, CCKH 03, JJLL 01, JJLL 02, JJLL 03, MSMM 01 , MSMM 02	Brown
RBHR 03, GGBB 01, GGBB 02 , CCKH 01, MMBH, FFKU 02	Light brown

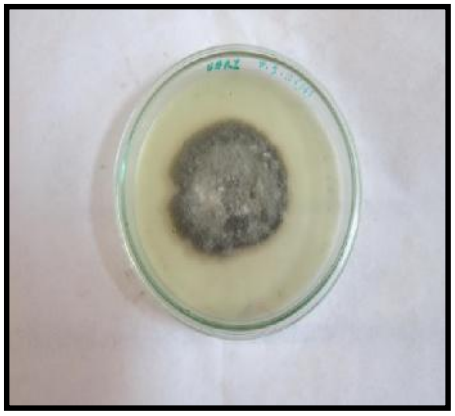
Umbonate type colony elevation was found in DSTR 01, GJBS, GGBB 01, CCKH 02, CCKH 03, JJLL 02, JJLL 03, FFKU 01, FFKU 02, FFKU 03, MSMM 01, MSMM 02, MSMM 03; In DSSA, DSTR 02, MMBH, MTBB 01, MTBB 02, RBHR 02, RBHR 03, GGBB 02, CCKH 01, JJLL 01 raised colony elevation was found. RBHR 01 showed flat colony elevation (Table 11 and plate 7).

Entire colony margin was found in DSTR 01, DSTR 02, MMBH, MTBB 02, RBHR 01, GJBS, GGBB 01, GGBB 02, CCKH 01, MSMM 01, FFKU 02. Undulate colony margin was found in DSSA, RBHR 03, CCKH 03, JJLL 01, MSMM 02. MTBB 01, RBHR 02, CCKH 02, JJLL 02, JJLL 03, MSMM 03, FFKU 01, FFKU 03 showed filiform colony margin (Table 11 and plate 8).

Cottony colony texture was found in DSSA, DSTR 01, DSTR 02, MMBH, RBHR 03, GGBB 01, GGBB 02, FFKU 02; MTBB 01, MTBB 02, RBHR 02, GJBS, CCKH 02, CCKH 03, JJLL 01, JJLL 02, JJLL 03, MSMM 01, MSMM 02, MSMM 03, FFKU 01, FFKU 03 showed fluffy colony texture; RBHR 01 and CCKH 01 showed velvety colony texture (Table 11 and plate 9).

Deep brown reverse colony color was found in DSSA, DSTR 01, DSTR 02, MTBB 01, MTBB 02, MSMM 03, FFKU 01, FFKU 03; RBHR 01, RBHR 02, GJBS, CCKH 02, CCKH 03, JJLL 01, JJLL 02, JJLL 03, MSMM 01, MSMM 02 showed brown reverse colony color with light brown reverse colony color in RBHR 03, GGBB 01, GGBB 02, CCKH 01, MMBH, FFKU 02 (Table 11 and plate 10).

(A)



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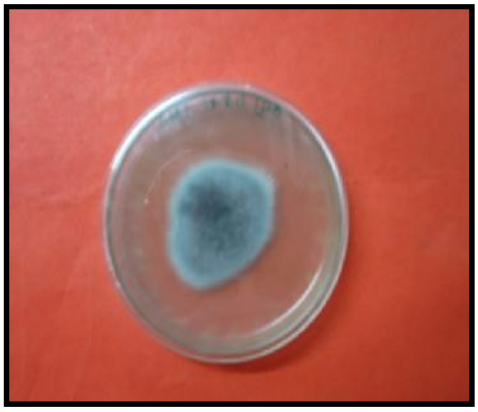
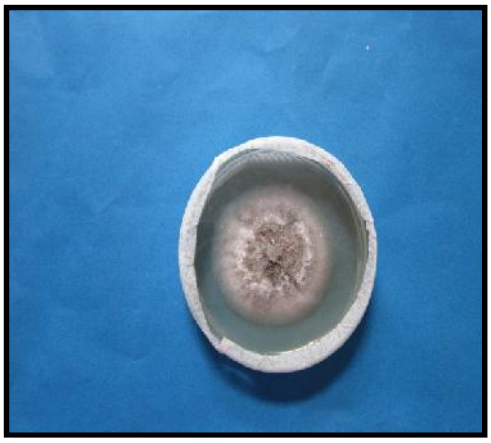


(C)

(D)



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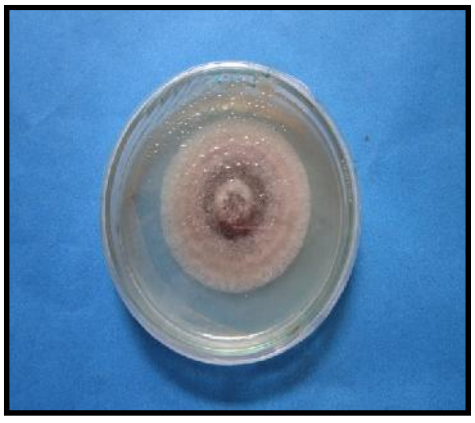
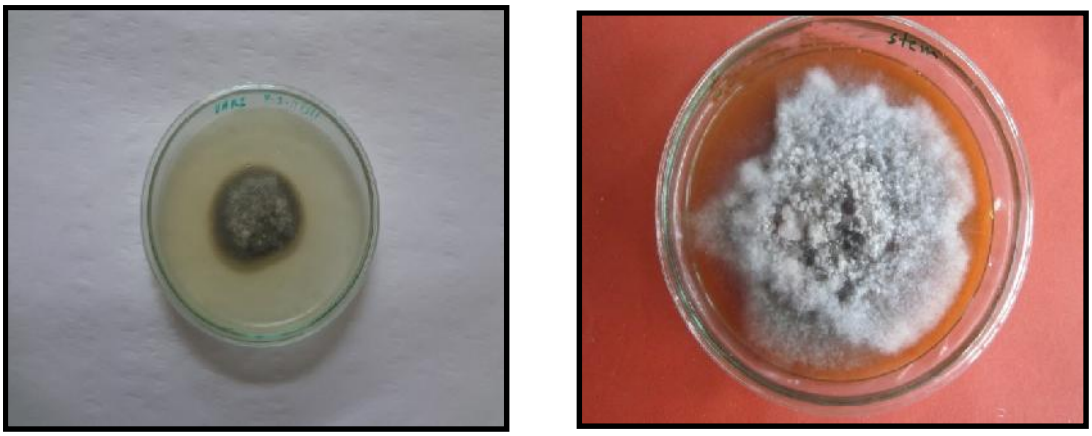


(F)

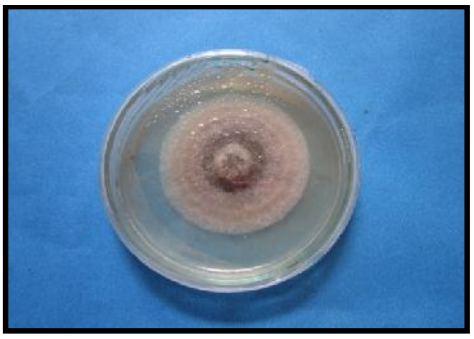
Plate 5. Colony color of *Stempylium vesicarium* isolates: (A) Greenish brown to dirty white (B) Deep grey to whitish (C) Light grey to whitish (D) Deep greenish white (E) Light grey (F) Dirty white to greenish



(A) **(B)**

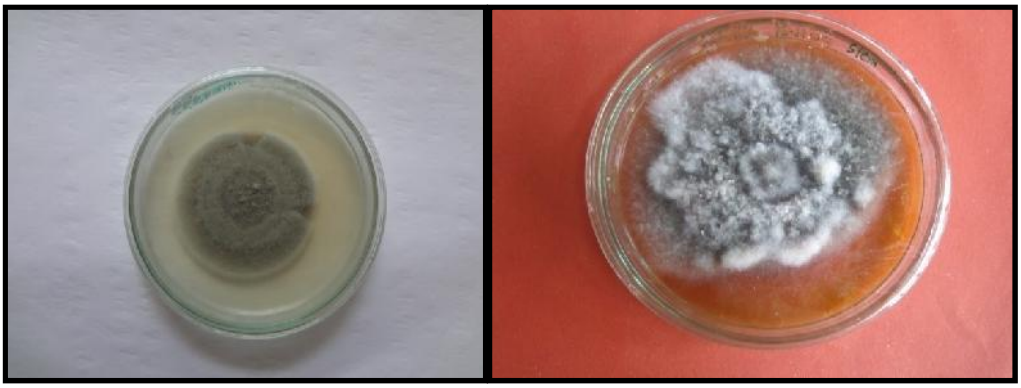
Plate 6. Colony shape of *Stempylium vesicarium* isolates: (A) Circular B) Irregular

(A) **(B)**



(C)

Plate 7. Colony elevation of *Stempylium vesicarium* isolates: (A) Umbonate (B) Raised (C) Flat

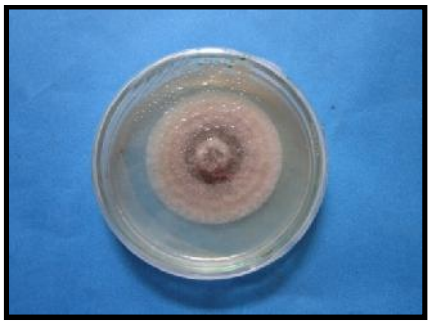


(A) **(B)**

(C)

Plate 8. Colony margin of *Stempylium vesicarium* isolates: (A) Entire (B) Undulate (C) Filiform

(A) **(B)**



(c)

Plate 9. Colony Texture of *Stempylium vesicarium* isolates: (A) Cottony (B) Fluffy (C) Velvety

Plate 10. Reverse colony color of *Stempylium vesicarium* isolates: (A) Deep brown (B) Brown (C) Light brown

4.1.6. Conidia production of *Stemphylium vesicarium*

4.1.6.1. Conidia production of *Stemphylium vesicarium* on PDA

Marked variation was found in conidia production of 24 isolates of *Stemphylium vesicarium* (Table 12 and Figure 9) on PDA. The highest conidia production (36.67/mm^2) was recorded in MSMM 01 followed by MSMM 02 (32.73/ mm^2), DSTR 02 (30.78/mm^2), RBHR 03 (29.89/mm^2), GJBS (29.26/mm^2) , RBHR 02 and MSMM 03 (29.24/mm^2) , CCKH 03 and FFKU 01 (27.50/mm^2), DSSA (27.07/mm^2), GGBB 02 (26.63/mm^2), MTBB 01 (26.62/mm^2), RBHR 01 (25.77/ mm^2). The lowest conidia production (20.73/mm^2) and (20.95/mm^2) were recorded in MMBH and JJLL 02 preceded by FFKU 02 (21.78/mm^2), FFKU 03 (22.26/mm^2), MTBB 02 and CCKH 02 (23.13/mm^2), JJLL 01 (23.31/mm^2), JJLL 03 (23.56/mm^2), GGBB 01 (23.57/mm^2).

4.1.6.2. Conidia production of *Stemphylium vesicarium* on V-7 juice agar

Marked variation was found in conidia production of 24 different isolates of *Stemphylium vesicarium* (Table 12) on V-7 juice agar medium. The highest conidia production (92.12/mm^2) was found in MSMM 01, which was statistically similar to MSMM 02 (88.62/mm^2), JJLL 02 and FFKU 03 (88.19/mm^2), MSMM 03 (83.39/mm^2), CCKH 03 (82.5/mm^2) followed by JJLL 03 (77.28/mm^2), DSTR 01 and JJLL 01 (74.66/mm^2), CCKH 02 (71.60/mm^2), RBHR (70.73/mm^2) , MTBB 01 (69.42/mm^2), FFKU 01 (68.99/mm^2), RBHR 02 (68.54/mm^2). The lowest conidia production (55.01/mm^2) was recorded in MMBH, preceded by RBHR 01 (56.32/mm^2), GGBB 02 (57.20/mm^2), CCKH 01 (58.50/mm^2), DSTR 02 (58.94/mm^2), GJBS (60.68/mm^2), MTBB 02 (62.87/mm^2), GGBB 01 (63.74/mm^2), FFKU 02 (65.49/mm^2) and DSSA (66.80/mm^2).

4.1.6.3. Conidia production of *Stemphylium vesicarium* on combined PDA+V-7 juice agar

Marked variation was found in conidia production of 24 different isolates of *Stemphylium vesicarium* (Table 12) on combined PDA+V-7 juice agar medium. The maximum conidia production (169.0/mm^2) was recorded in JJLL 03 followed by RBHR 02 (162.4/mm^2), MSMM 01, MSMM 02 and FFKU 03 (161.1/mm^2), JJLL 01 (157.2/mm^2), FFKU 01 (154.1/mm^2), GGBB 02 (151.9/mm^2), MTBB01 (141.5/mm^2), MSMM 03 (140.6/mm^2), RBHR 01 (138.8/ mm^2), CCKH 02 (137.5/mm^2) and those were statistically different. The lowest conidia production (87.76/mm^2) was recorded in GJBS preceded by DSSA (89.06/mm^2), DSTR 01 and FFKU 02 (98.24/mm^2), CCKH 01 (106.1/mm^2), DSTR 02 (114.0/mm^2), MTBB 02 (119.2/mm^2), JJLL 02 (120.5/mm^2), RBHR 03 (124.4/mm^2), CCKH 03 (125.8/mm^2) and GGBB 01 (133.6/mm^2).

Table 12. Conidia production of 24 isolates of *Stemphylium vesicarium* on PDA, V-7 juice agar and combined PDA+V-7 juice agar media

Sl. No	Isolates	Number of conidia/mm^2 on PDA[1]	Number of conidia/mm^2 on V-7 juice agar[1]	Number of conidia/mm^2 on combined PDA+V-7 juice agar medium[1]
1	DSSA	27.07 b-h	66.80 c-g	89.06 i
2	DSTR 01	29.47 b-e	74.66 b-d	98.24 hi
3	DSTR 02	30.78 bc	58.94 f-h	114.0 fg
4	MMBH	**20.73 h**	**55.01 h**	124.4 d-f
5	MTBB 01	26.62 b-h	69.42 c-f	141.5 c
6	MTBB 02	23.13 e-h	62.87 e-h	119.2 ef
7	RBHR 01	25.77 c-h	56.32 gh	138.8 c
8	RBHR 02	29.24 b-e	68.54 c-f	162.4 ab
9	RBHR 03	29.89 b-d	70.73 c-e	124.4 d-f
10	GJBS	29.26 b-e	60.68 e-h	**87.76 i**
11	GGBB 01	23.57 d-h	63.74 d-h	133.6 cd
12	GGBB 02	26.63 b-h	57.20 gh	151.9 b
13	CCKH 01	28.35 b-f	58.50 f-h	106.1 gh
14	CCKH 02	23.13 e-h	71.60 c-e	137.5 c
15	CCKH 03	27.50 b-g	82.52 ab	125.8 de
16	JJLL 01	23.31 e-h	74.66 b-d	157.2 b
17	JJLL 02	20.95 h	88.19 a	120.5 ef
18	JJLL 03	23.56 d-h	77.28 bc	**169.0 a**
19	MSMM 01	**36.67 a**	**92.12 a**	161.1 ab
20	MSMM 02	32.73 ab	88.62 a	161.1 ab
21	MSMM 03	29.24 b-e	83.39 ab	140.6 c
22	FFKU 01	27.50 b-g	68.99 c-f	154.1 b
23	FFKU 02	21.78 gh	65.49 d-h	98.24 hi
24	FFKU 03	22.26 f-h	88.19 a	161.1 ab
	LSD (0.05)	5.439	9.708	9.802

	CV (%)	12.44%	8.33%	4.51%

[1]Means of five replications for each isolate

Number with similar letter do not differ significantly at 5% level according to Dunkan's Multiple Range Test (DMRT)

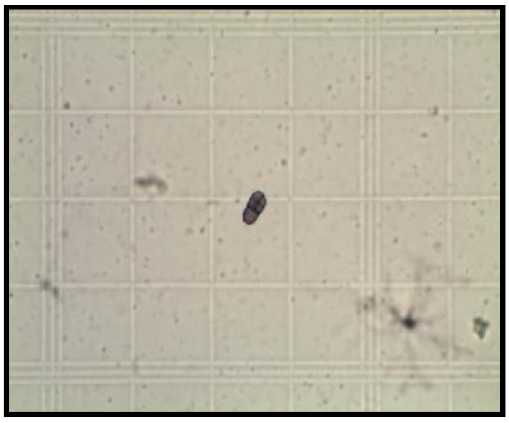


Figure 5: Conidia of *Stemphylium vesicarium* on PDA under compound microscope (10X)

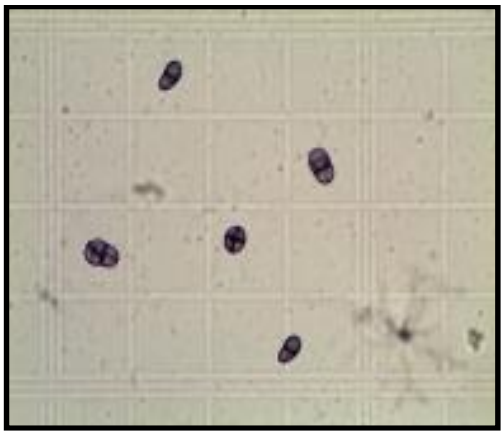


Figure 6: Conidia of *Stemphylium vesicarium* on V-7 juice agar under

compound microscope (10X)

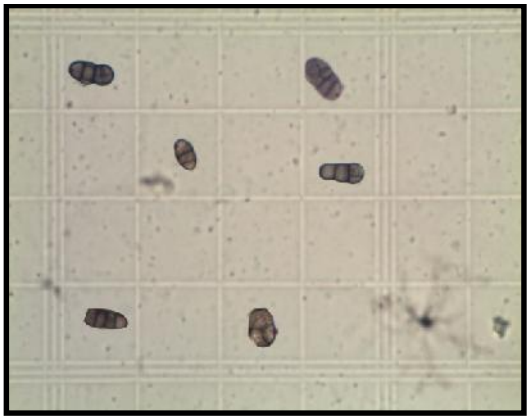


Figure 7. Conidia of *Stemphylium vesicarium* on combined PDA+V-7 juice agar under compound microscope (10X)

4.1.6.4. Comparison of conidia production of *Stemphylium vesicarium* on PDA, V-7 juice agar and combined PDA+V-7 juice agar media

The effect of different culture media [PDA, V-7 juice agar and combined (PDA+V-7 juice agar)] on conidia production of *Stemphylium vesicarium* is presented in Figure 15. The highest number (87.76 to 169.0/mm^2) of conidia was found on combined (PDA+V-7 juice agar) medium. The minimum number (20.73 to 36.67/mm^2) of conidia was found on PDA preceded by V-7 juice agar medium (55.01 to 92.12/mm^2).

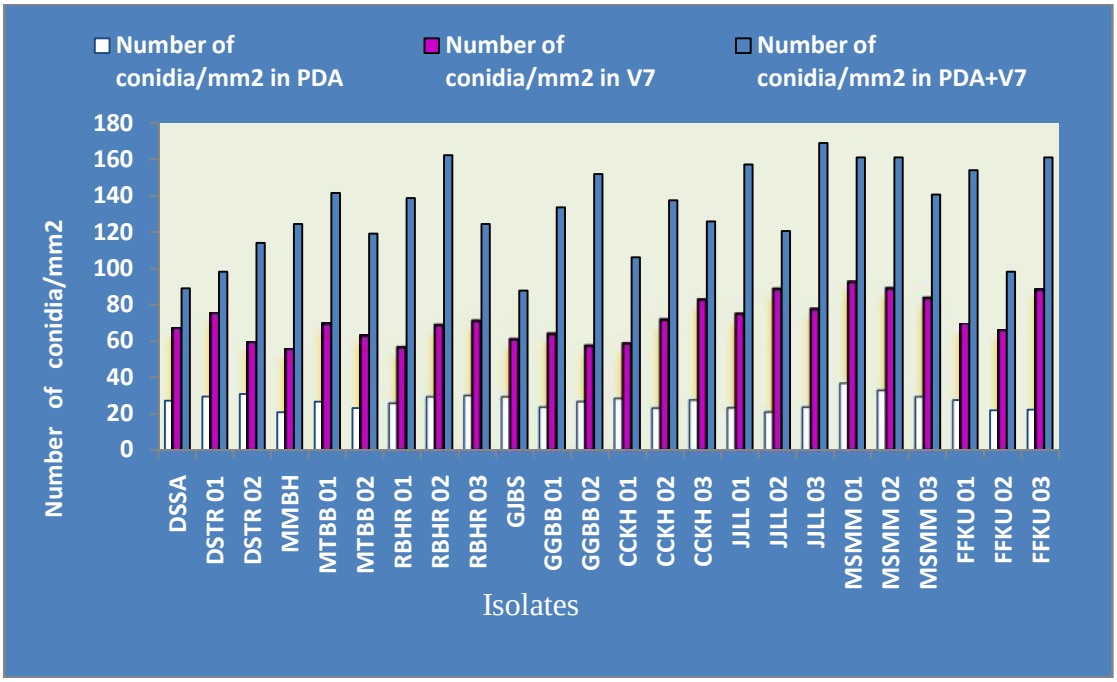


Figure 8: Conidia production of 24 isolates of *Stemphylium vesicarium* on different media [PDA, V-7 juice agar and combined PDA+V-7 juice agar]

4.1.7. Sporulation time of *Stemphylium vesicarium*

4.1.7.1. Sporulation time on PDA

The sporulation time of *Stemphylium vesicarium* on PDA is presented in Table 13. Significantly maximum sporulation time (90.67 days) of *Stemphylium vesicarium* on PDA was required in GGBB 02 and MSMM 02 which was statistically similar with DSTR 01, RBHR 01, JJLL 02, FFKU 01, FFKU 03. In case of MMBH, MTBB 01, GGBB 01, CCKH 03, JJLL 03 the required time for sporulation was 89.67 days. The minimum sporulation time (86.33 days) was recorded in GGBB 01 preceded by RBHR 02, MSMM 01 and FFKU 02

(87.67 days). For the rest of the isolates, the range of sporulation time was 88.00-89.33 days after inoculation.

4.1.7.2. Sporulation time on V-7 juice agar

The sporulation time of *Stemphylium vesicarium* on V-7 medium did not differ significantly (Table 13). The sporulation time ranged from 28.33 to 31.00 days on V-7 juice agar medium. The maximum sporulation time (31.00 days) was required in JJLL 03 and the minimum sporulation time (28.33 days) was required in JJLL 01, GJBS after inoculation.

4.1.7.3. Sporulation time on combined PDA +V-7 juice agar

The sporulation time of *Stemphylium vesicarium* on V-7 juice agar medium is presented in Table 13. The sporulation time ranged from 35.00 to 39.67 days on combined PDA+ V-7 juice agar medium. The maximum sporulation time (39.67 days) was required in CCKH 02 and the minimum sporulation time (35.00 days) was required in DSSA and RBHR 03, respectively. Most of the isolates were statistically similar in respect of sporulation time.

Table 13. Sporulation time of 24 isolates of *Stemphylium vesicarium* on PDA, V-7 juice agar and combined PDA+V-7 juice agar media

Sl. No	Isolates	Sporulation time (days) on PDA	Sporulation time (days) on V-7 juice agar	Sporulation time (days) on combined PDA+V-7 juice agar
1	DSSA	89.33 ab	30.33 a	**35.00 b**
2	DSTR 01	90.33 a	29.33 a	36.00 ab
3	DSTR 02	88.00 ab	30.00 a	36.67 ab
4	MMBH	89.67 ab	29.67 a	36.67 ab
5	MTBB 01	89.67 ab	29.67 a	36.67 ab

6	MTBB 02	89.33 ab	28.67 a	37.67 ab
7	RBHR 01	90.00 a	30.67 a	35.67 ab
8	RBHR 02	88.67 ab	29.00 a	35.67 ab
9	RBHR 03	89.00 ab	29.33 a	**35.00 b**
10	GJBS	**86.33 b**	28.67 a	37.00 ab
11	GGBB 01	89.67 ab	30.33 a	36.67 ab
12	GGBB 02	**90.67 a**	29.67 a	37.33 ab
13	CCKH 01	89.33 ab	30.00 a	38.67 ab
14	CCKH 02	88.33 ab	29.67 a	**39.67 a**
15	CCKH 03	89.67 ab	30.00 a	36.67 ab
16	JJLL 01	88.33 ab	28.33 a	37.67 ab
17	JJLL 02	90.00 a	30.00 a	36.67 ab
18	JJLL 03	89.67 ab	31.00 a	38.67 ab
19	MSMM 01	87.67 ab	30.33 a	37.33 ab
20	MSMM 02	**90.67 a**	29.33 a	39.33 a
21	MSMM 03	89.33 ab	29.00 a	36.33 ab
22	FFKU 01	90.00 a	29.67 a	37.67 ab
23	FFKU 02	87.67 ab	29.67 a	37.00 ab
24	FFKU 03	90.00 a	30.00 a	38.67 ab
	LSD (0.05)	2.843	2.330	3.357
	CV (%)	1.94%	4.78%	5.51%

[1]Means of three replications for each isolate

Number with similar letter do not differ significantly at 5% level according to Dunkan's Multiple Range Test (DMRT).

4.1.7.4. Comparison of sporulation time of 24 isolates of *Stemphylium vesicarium* on PDA, V-7 juice agar and combined PDA+V-7 juice agar media

The effect of different culture media [PDA, V-7 juice agar and combined (PDA+V-7 juice agar) on sporulation time of *Stemphylium vesicarium* is presented in figure 19. The earliest (28.33 to 31.00 days) conidia production was observed on V-7 juice agar medium in respect of all the isolates. On PDA medium comparatively more days (86.33 to 90.00 days) were required for conidia production.

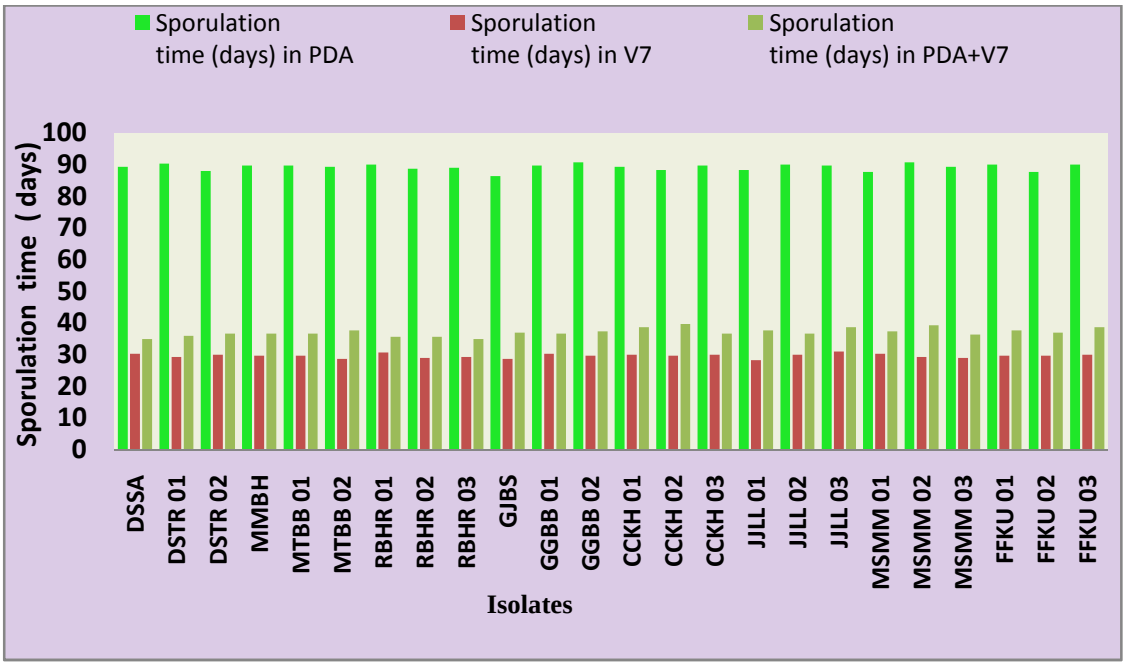


Figure 9. Sporulation time (days) of 24 isolates of *Stemphylium vesicarium* on different media

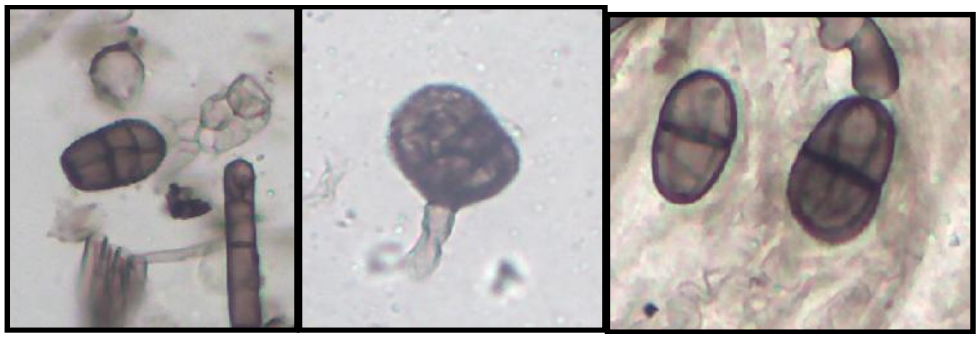
4.2. Morphological Variation of *Stempylium vesicarium*

4.2.1. Shape and conidia color of 24 isolates of *Stemphylium vesicarium* on PDA

Conidial shape of 24 isolates of *Stemphylium vesicarium* is presented in Table 14 and Figure 20. Distinct variations were observed in shape of conidia of 24 isolates of *Stemphylium vesicarium* on PDA. Ovoid shaped conidia was found in DSSA, MTBB 01, MTBB 02, RBHR 01, JJLL 01, JJLL 03, FFKU 02, FFKU 03; Oblong shaped conidia was found in DSTR 01, DSTR 02, RBHR 02, GGBB 01, GGBB 02, CCKH 01, CCKH 02, CCKH 03,

JJLL 02, MSMM 03, FFKU 01. The rest MMBH, RBHR 03, GJBS, MSMM 01, MSMM 02 possessed ovoid to oblong shaped conidia.

Conidial color of 24 isolates of *Stemphylium vesicarium* is presented in Table 14 and Figure 21. Remarkable variations was observed in conidial color of 24 isolates of *Stemphylium vesicarium* on PDA. The color of conidia of DSSA, MTBB 01, MTBB 02, GJBS, JJLL 01 and MSMM 01 was deep brown. The isolates DSTR 01, DSTR 02, MMBH, RBHR 03, GGBB 01, GGBB 02, CCKH 03, JJLL 03 and FFKU 01 possessed light brown colored conidia. The rest RBHR 01, RBHR 02, CCKH 01, CCKH 02, JJLL 02, MSMM 02, MSMM 03 and FFKU 03 exhibited brown colored conidia.



(A) **(B)** **(C)**

Figure 10. Conidial shape of *Stempylium vesicarium* (A) Ovoid (B) Oblong (C) Ovoid to oblong

(A) **(B)** **(C)**

Figure 11. Conidial color of *Stempylium vesicarium* (A) Deep brown (B) Light brown (C) Brown

Table 14. Shape and color of conidia of 24 isolates of *Stemphylium vesicarium* on PDA

Sl. No	Isolates	Shape	Color
1	DSSA	Ovoid	Deep Brown
2	DSTR 01	Oblong	Light Brown
3	DSTR 02	Oblong	Light Brown
4	MMBH	Ovoid to oblong	Light Brown
5	MTBB 01	Ovoid	Deep Brown
6	MTBB 02	Ovoid	Deep Brown
7	RBHR 01	Ovoid	Brown
8	RBHR 02	Oblong	Brown
9	RBHR 03	Ovoid to oblong	Light Brown
10	GJBS	Ovoid to oblong	Deep Brown
11	GGBB 01	Oblong	Light Brown
12	GGBB 02	Oblong	Light Brown
13	CCKH 01	Oblong	Brown
14	CCKH 02	Oblong	Brown
15	CCKH 03	Oblong	Light Brown
16	JJLL 01	Ovoid	Deep Brown
17	JJLL 02	Oblong	Brown
18	JJLL 03	Ovoid	Light Brown
19	MSMM 01	Ovoid to oblong	Deep Brown
20	MSMM 02	Ovoid to oblong	Brown
21	MSMM 03	Oblong	Brown
22	FFKU 01	Oblong	Light Brown
23	FFKU 02	Ovoid	Brown
24	FFKU 03	Ovoid	Deep Brown

4.2.2. Size of conidia of *Stempylium vesicarium* on PDA

Distinct variations were observed in length and breadth of conidia of 24 isolates of *Stempylium vesicarium* **(** Table 15 and figure 23).The length of conidia varied from 14.6 µm to 30.6 µm, whereas the maximum mean length (29.97 µm) was recorded in DSSA and minimum mean length (17.36 µm) in MSMM 02. Breadth of conidia ranged from 4.7 µm to 15.7 µm. The highest mean breadth (12.55 µm) was recorded in DSSA and the minimum mean breadth (9.760 µm) was in CCKH 02.

Table 15. Size of conidia of 24 isolates of *Stemphylium vesicarium* on PDA

Sl. no	Isolates	Conidial length[1] (µm)		Conidial breadth[1] (µm)	
		Range	Mean	Range	Mean
1	DSSA	29.5-30.6	**29.97 a**	4.7-14.5	**12.55 a**
2	DSTR 01	20.4-27.8	25.13 cd	7.7-15.1	9.800 ij
3	DSTR 02	23.5-26.2	24.43 e-g	6.0-15.3	11.89 b
4	MMBH	19-25.3	23.13 ij	6.9-14.5	11.10 d-f
5	MTBB 01	20.0-27.6	24.87 c-e	8.2-12.1	10.15 h-j
6	MTBB 02	23.7-25.2	24.60 d-f	9.6-14.7	11.36 cd
7	RBHR 01	19.8-27.9	23.86 gh	9.4-14.4	11.86 b
8	RBHR 02	22.4-23.1	22.67 j	8.1-13.8	10.81 fg
9	RBHR 03	21.2-24.9	22.60 j	9.3-13.7	10.89 e-g
10	GJBS	16.9-22.7	20.74 l	8.1-12.1	11.12 d-f
11	GGBB 01	17.6-25.7	21.27 l	9.0-14.6	10.98 d-f
12	GGBB 02	24.9-28.1	26.80 b	8.2-14.7	11.73 bc
13	CCKH 01	15-19.1	17.50 o	8.2-15.7	11.29 de
14	CCKH 02	20.2-27.7	25.42 c	6.7-16.2	**9.760 j**
15	CCKH 03	20.8-28.4	25.37 c	7.7-14.2	10.34 h
16	JJLL 01	19.9-26.4	24.02 fg	7.9-14.9	10.54 gh
17	JJLL 02	18.3-23.2	20.73 l	8.7-13.9	11.01 d-f
18	JJLL 03	16.3-21.4	19.47 m	7.3-13.3	10.95 d-g
19	MSMM 01	15.5-22.3	18.09 n	7.3-12.9	9.820 ij
20	MSMM 02	14.6-20.5	**17.36 o**	7.3-14.4	10.23 hi
21	MSMM 03	19-25.3	23.32 hi	9.2-12.8	10.13 h-j
22	FFKU 01	19.7-24.6	19.98 m	9.6-13.5	9.860 ij
23	FFKU 02	16.9-25.2	21.87 k	7.9-14.3	10.52 gh
24	FFKU 03	17.2-24.9	24.52 d-f	8.0-13.9	11.23 d-f

	LSD (0.05)		0.5639		0.3954
	CV (%)		1.50%		2.23%

[1]Means of ten replications for each isolate

Number with similar letter do not differ significantly at 5% level according to Dunkan's Multiple Range Test (DMRT)

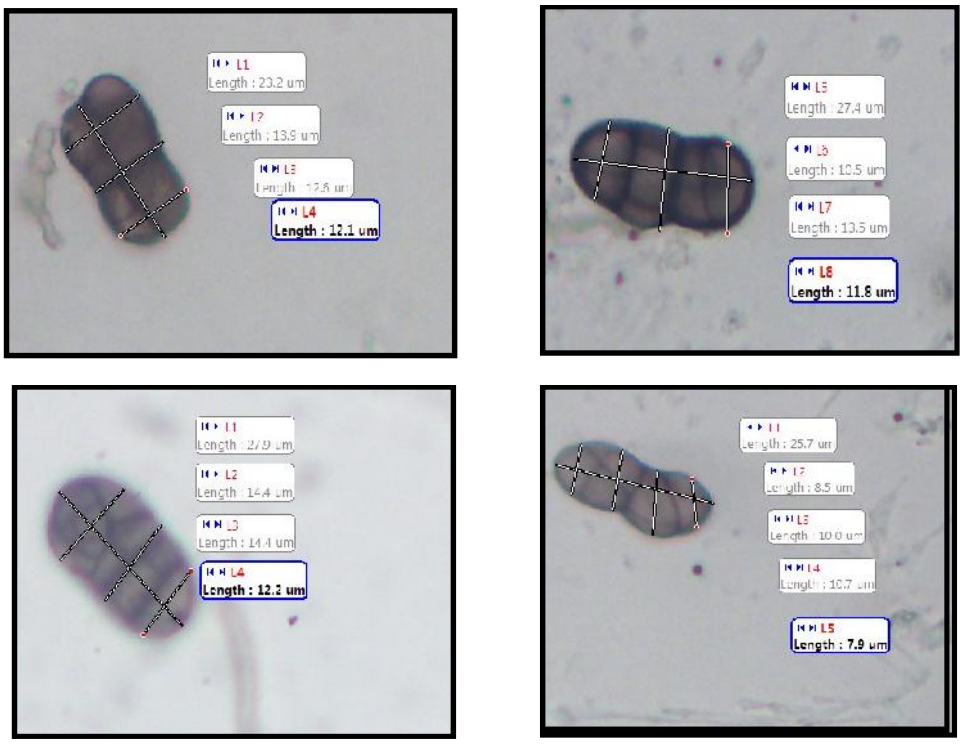


Figure 12. Conidia size of *Stemphylium vesicarium* observed under compound microscope (40X)

4.2.3. Septation of *Stemphylium vesicarium* conidia on PDA

The septation of *Stemphylium vesicarium* on PDA medium is presented in Table 16. Distinct variations were observed in horizontal and longitudinal septation of conidia of 24 isolates of *Stemphylium vesicarium*. The horizontal septation varied from 1-3, whereas the maximum mean number of horizontal septation was recorded in DSTR 01 (2.500) and minimum (1.600) was in MTBB 01, RBHR 01, GGBB 01 and MSMM 01. The longitudinal septation varied from 0-4, whereas the maximum mean number of longitudinal septation (2.500) was recorded in MMBH and minimum (1.400) in GGBB 02.

Table 16. Septation of conidia of 24 isolates of *Stemphylium vesicarium* on PDA

Sl. No	Isolates	Horizontal Septation[1]		Longitudinal Septation[1]	
		Range	Mean	Range	Mean
1	DSSA	1-3	2.100 cd	0-3	1.600 g-i
2	DSTR 01	1-3	**2.500 a**	1-4	2.267 a-c
3	DSTR 02	1-3	2.300 a-c	1-3	1.500 hi
4	MMBH	1-3	1.800 ef	0-4	**2.500 a**
5	MTBB 01	1-3	**1.600 f**	1-4	2.100 b-e
6	MTBB 02	1-3	1.800 ef	1-4	2.400 ab
7	RBHR 01	1-3	**1.600 f**	1-4	1.800 e-h
8	RBHR 02	1-3	2.000 de	0-4	2.400 ab
9	RBHR 03	1-3	1.800 ef	0-4	1.967 c-f
10	GJBS	1-3	1.800 ef	0-4	1.667 f-i
11	GGBB 01	1-3	**1.600 f**	0-4	1.667 f-i
12	GGBB 02	1-3	1.700 f	0-4	**1.400 i**
13	CCKH 01	1-3	2.000 de	0-4	1.933 c-g
14	CCKH 02	1-3	2.100 cd	0-3	1.633 f-i
15	CCKH 03	1-3	2.300 a-c	1-3	1.733 f-i
16	JJLL 01	1-3	2.400 ab	1-4	2.100 b-e
17	JJLL 02	1-3	2.133 cd	1-3	1.800 e-h
18	JJLL 03	1-3	2.200 b-d	0-4	1.500 hi

19	MSMM 01	1-3	**1.600 f**	0-3	1.800 e-h
20	MSMM 02	1-3	1.800 ef	1-4	2.133 b-e
21	MSMM 03	1-3	1.800 ef	1-4	1.600 g-i
22	FFKU 01	1-3	2.000 de	0-4	2.200 a-d
23	FFKU 02	1-3	2.300 a-c	1-4	1.900 d-g
24	FFKU 03	1-3	2.400 ab	1-3	1.700 f-i
	LSD (0.05)		0.2263		0.2937
	CV (%)		6.92%		9.41%

[1]Means of ten replications for each isolate

Number with similar letter do not differ significantly at 5% level according to Dunkan's Multiple Range Test (DMRT)

4.3. Molecular Characterization of *Stemphylium vesicarium*

4.3.1. Extraction of genomic DNA

To extract genomic DNA, mycelia of *Stemphylium vesicarium* were collected from twenty days old pure culture of twenty four (24) isolates. Eighteen (18) genomic DNA were found among twenty four (24) isolates by CTAB extraction method and this was confirmed by gel running of genomic DNA. The genomic DNA couldn't be extracted from the DSSA, DSTR 02, MTBB 01, RBHR 01, RBHR 03, JJLL 01 isolates. (Figure 25).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

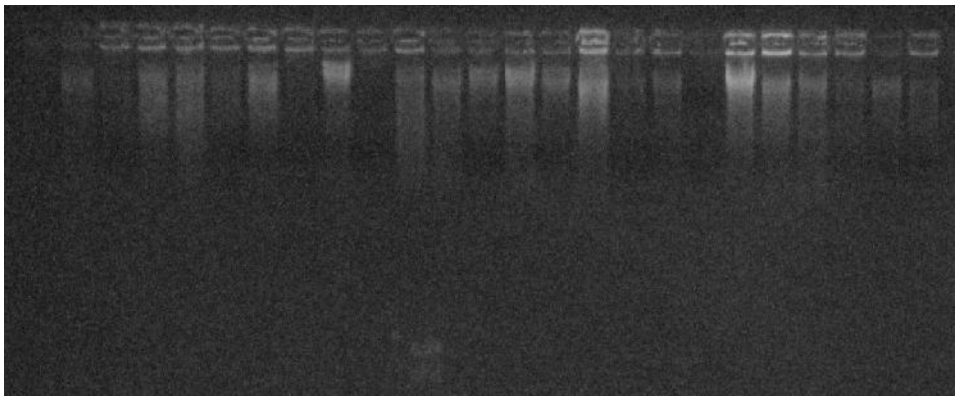


Figure 13: Gel

electrophoresis of the genomic DNA of *Stemphylium vesicarium* isolates

(In figure 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24 indicates DSTR 01,DSSA, DSTR 02, MMBH, MTBB 01, MTBB 02, RBHR 01, RBHR 02, RBHR 03, GJBS, GGBB 01, GGBB 02, CCKH 01, CCKH 02, CCKH 03, JJLL 01, JJLL 02, JJLL 03, MSMM 01, MSMM 02, MSMM 03, FFKU 01, FFKU 02, FFKU 03 isolates)

4.3.2. RAPD analysis

To perform the RAPD analysis, seven (7) decamer primers were tested. These primers were selected for fingerprinting of eighteen (18) isolates of *Stemphylium vesicarium*. But there was no band found by using these primers.

4.3.3. ITS Region Identification

For ITS region identification two ITS primer ITS1F and ITS4 were used as forward primer and reverse primer respectively. By using these primer the ITS regions were identified from one (1) isolate among eighteen (18). The isolate was DSTR 01 (Figure 26).

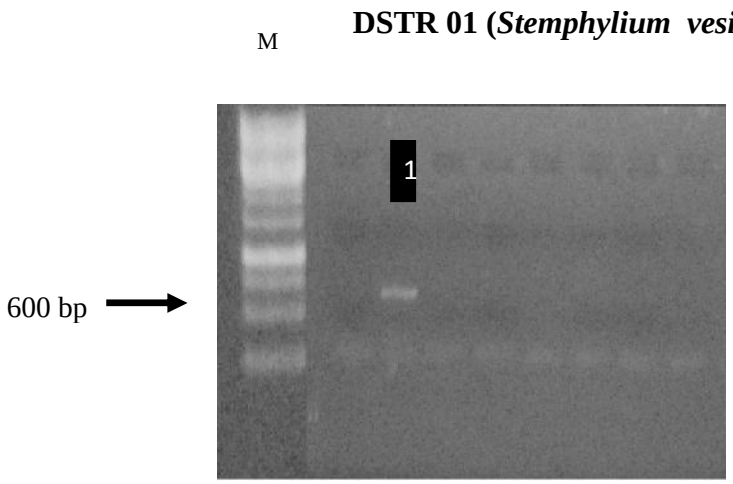


Figure 14: Gel electrophoresis of the PCR product performed by ITS1F and ITS4 primer and showing ~600 bp amplification

(In figure 26, 1 indicates DSTR 01isolate)

4.3.4. PCR Products Purification

Purification of PCR products was done by alcohol precipitation technique and the amplified ITS region of rDNA of DSTR 01 isolate was purified.

4.3.5. 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 of the sample identified in agarose gel electrophoresis was used for sequencing. The sequenced samples were designated as Sv-DSTR 01.

4.3.5.1. *Stemphylium vesicarium* (Sv)

```
 ....|....|  ....|....| ....|....| ....|....|  ....|....|
     5          15         25         35         45
CAATAGATTC TGCTCTCCCT ACCGGAACCT TGGGGTAAGA GTTCTAAAAA

 ....|....|  ....|....| ....|....| ....|....|  ....|....|
    55         65         75         85         95
AATGTGGTAA TGATGGGTTG GGCCTTAAAT CAAAAAAGAA AGGTGAAGAA

 ....|....|  ....|....| ....|....| ....|....|  ....|....|
   105        115        125        135        145
TTGTGATGAG TTCTTTTCCC AAAAAAGCCC GCTCCAATCA TTTGGGAGTT

 ....|....| ....|....|  ....|....| ....|....|  ....|....|
   155        165        175        185        195
TCTCCGAACA AAAATAACCA AATTCATCAA TCTTTACCTA AAATGAAATA

 ....|....| ....|....|  .
   205        215
CAAAAAATAC TATTTGAATC C
```

4.3.6. Analysis of the Sequence Results

The sequences of the Sv - DSTR 01 sample was analyzed through NCBI-BLAST program database search system. Obtained results from the BLAST database showed that the sample Sv - DSTR 01 showed no significant similarity with any gene.

CHAPTER 5

DISCUSSION

Onion (*Allium cepa*) is one of the most important and familiar spices crop throughout the world. It is the member of the family Alliaceae. It is also used as popular vegetable in many countries of Asia and very common, favorite spice in Bangladesh. The white blotch of onion is considered as the destructive disease of onion that seriously affects the bulb and seed production. *Stemphylium vesicarium,* the causal agent of white blotch of onion is considered as the pathogen that initiate the infection, which facilitates the subsequent infection of *Alternaria porri* causing purple blotch and hence presently the disease is named as purple blotch complex of onion.

Now-a-days the management options are not working properly against the disease. Reason might be the development of fungicide resistance of the causal pathogen 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 *Stemphylium vesicarium* in our country. As the onion growers are being discouraged for onion cultivation due to the severe losses of the disease. Molecular characterization may help in understanding the genetic and/or functional differentiation i.e. genetic variability among individual in a species. Thus, initiative was taken to study the variability of the isolates of *Stemphylium vesicarium*.

In the present research work, all 24 isolates (DSSA to FFKU 03) showed variations in respect of their cultural and morphological characteristics. In respect of cultural characteristics, the isolates of *Stemphylium vesicarium* showed variation in growth rate, colony color, shape, elevation, margin, textures, substrate color, conidia production and sporulation time. The remarkable effect of different culture media on radial mycelia growth was observed in *Stemphylium vesicarium*. The maximum (0.610-0.650 cm/day) radial mycelial growth of *Stemphylium vesicarium* was found on V-7 juice agar medium. The minimum (0.423-0.483 cm/day) radial mycelial growth was recorded on PDA medium preceded by combined (PDA+ V-7 juice agar) medium (0.470-0.503 cm/day). The present investigation showed that *Stemphylium vesicarium* grows well on V-7 juice agar medium. Arzanlou *et al.* (2012) reported that the colonies of *Stemphylium vesicarium* were attaining a diameter of 50 mm after 7 days of inoculation on PCA (Potato Carrot Agar) medium. Yun-Fei *et al.* (2010) stated that colonies of *Stemphylium luffae, Stemphylium lycii* and *S. cucumis* isolated from diseased leaves of *Luffa cylindrica, Lycium chinense* and *Cucumis* spreading 32-36 mm, 30–35 mm and 28–33 mm diameter in 10 days on PCA (Potato Carrot Agar) medium.

Remarkable variation was found in colony color of *Stemphylium vesicarium* on PDA medium. Most of the colony color was deep grey to whitish. Greenish brown to dirty white, light grey to whitish, deep greenish white, light grey and dirty white to greenish colony color was also found in the present study. The results are in agreement with Byung - Soo *et al.* (2004) who found that the colony color of *S. solani* isolated from pepper was gray in color and gray to light brown growth found in case of *S. lycopersici* isolated from tomato plants on PDA medium. Hosen *et al.* (2009) observed greenish brown and dirty white color colony of *S. botryosum* isolated from lentil plants. All the isolates of *Stemphylium vesicarium* colony had circular and irregular shaped with entire, undulate and filiform margin. The results are in agreement with Hosen (2009) who found that the colony margin of *S.botryosum* isolated from lentil plants was entire and regular.Umbonate, raised and flat colony elevation was found where most of the isolates colony had raised elevation. Arzanlou *et al.* (2012) found flat type colony elevation of *Stemphylium vesicarium* grown on PCA (Potato Carrot Agar) media. All the isolates colony had cottony, fluffy and velvety texture on PDA medium. Where most of the isolates showed fluffy texture. The results are in agreement with Mehta (2001) who found that the mycelium of *S. solani* isolated from cotton was velvety, cottony or immersed. Similarly, Hosen *et al.* (2009) observed velvety, effuse and fluffy type colony texture of

Stemphylium botryosum isolated from lentil plants. Substrate color was found deep brown, brown and light brown among the twenty four isolates of *Stemphylium vesicarium.*

The pathogen produces conidia on different culture media and conidia production was varied with media. The conidia production of all isolates of *Stemphylium vesicarium* on PDA medium varied from 20.73 to 36.67 per mm^2 with maximum in isolate MSMM 01 and minimum in MMBH. The conidia production of all isolates of *Stemphylium vesicarium* on V-7 medium varied from 55.01 to 92.12 per mm^2 where maximum was in isolate MSMM 01 and minimum in MMBH. On combined (PDA+ V-7 juice agar) medium, conidia production of all isolates of *Stemphylium vesicarium* varied from 87.76 to 169.0 per mm, ^{2} while maximum was in isolate JJLL 03 and minimum in GJBS. The present investigation clearly pointed that culture media showed profound effect on conidia production of *Stemphylium vesicarium.* The maximum conidia production occurred on combined (PDA+ V-7 juice agar) medium. Kumar (2007) identified a suitable culture medium for the sporulation of *Stemphylium botryosum* and found that the fungus produced the highest number of conidia ($84.7 \pm 6.0 \times 10^4$ conidia ml^{-1}) on V8P medium (V8 Juice Potato Dextrose Agar Medium). Byung - Soo *et al.* (2004) stated that the higher no. of conidia production was occurred on V-8 A medium (14.2×10^3 - *S. solani,* 31.8×10^3 *S. lycopersici*).

All the isolates showed variation in respect of sporulation time on different media. The sporulation time of *Stemphylium vesicarium* on PDA medium varied from 86.33 to 90.67 days with maximum sporulation time in isolate GGBB 02 and MSMM 02 respectively and minimum in GJBS isolate. On V-7 juice agar medium the sporulation time for all isolates varied from 28.33 to 31.0 days with maximum sporulation time in isolate JJLL 03 and minimum in JJLL 01. On combined PDA+ V-7 juice agar medium the sporulation time for all isolates varied from 35.0 to 39.67 days with maximum sporulation time in isolate CCKH 02 and minimum in DSSA and RBHR 03. The results of the present investigation showed that *Stemphylium vesicarium* produces conidia on V-7 juice agar medium earlier than PDA and combined (PDA+ V-7 juice agar) medium. All the isolates of *Stemphylium vesicarium* varied in terms of conidia shape, color, size and septation on PDA medium. Conidial shape was ovoid, oblong and ovoid to oblong. The present findings agreed with the report of Ellis (1971). Koike *et al.* (2005) who found oblong to ovoid shaped conidia of *Stemphylium sp.*. Simmons (1985) and Bayaa and Erskine (1998) reported that the conidia of *Stemphylium botryosum* were broadly ovoid. Oblong, ovoid or broadly ellipsoidal shaped conidia of *Stemphylium luffae, S. lycii* and *S. cucumis* was also found by Yun-Fei *et al.* (2010).

In the present research work, brown, deep brown and light brown colored conidia were found by all the isolates of *Stemphylium vesicarium* on PDA medium. The current findings are well supported by Byung-Soo *et al.* (2004) who worked on *Stemphylium solani* and *S. lycopersici* and observed tan to light brown colored conidia. Koike *et al.* (2005) and Lu *et al.* (2008) found brown colored conidia of *Stemphylium botryosum* and *Stemphylium solani*.

Significant variation was also observed in conidial size of *Stemphylium vesicarium* on PDA. The length and breadth of conidia ranged from 14.6 µm to 30.6 µm and 4.7 µm to 15.7 µm, whereas the highest mean length (29.97 µm) was recorded in DSSA minimum (17.36 µm) in MSMM 02 and the highest mean breadth 12.55 µm was observed in DSSA and the lowest 9.760 µm in CCKH 02. The present findings agreed with the report of Ellis (1971) and Arzanlou *et al.* (2012) who measured 25-40 x13-21 µm and 20–24 (-30)×12-15 µm conidial length and breadth of *Stemphylium vesicarium*. Simmons (1985), Koike *et al.* (2005), Hosen *et al.* (2009) and Hosen (2011) also observed 33-35 x 24-26 µm, 17 to 28 × 13 to 19 µm, 10 to 25×5 to 15 µm and 12.35 to 23.45 µm conidia of *Stemphylium botryosum*.

Conidia of different isolates of *Stemphylium vesicarium* was also varied in the terms of horizontal and longitudinal septation on PDA medium. The horizontal septation varied from 1-3, whereas the highest mean number of horizontal septation was recorded in isolate DSTR 01 (2.500) and minimum (1.600) in isolates MTBB 01, RBHR 01, GGBB 01 and MSMM 01. The longitudinal septation varied from 0-4, whereas the highest mean number of longitudinal septation (2.500) was recorded in isolate MMBH and minimum (1.400) in isolate GGBB 02. This finding collaborate with the reports of other workers (Arzanlou *et al.* 2012 and Ellis 1971) who found 1–3 transverse, 1–4 longitudinal or oblique septa and 1-5 transverse and several longitudinal septa per conidium of *Stemphylium vesicarium*. Lu *et al.* (2008) noted 1 to 3 dark coloured transverse septa and 2 to 7 longitudinal or oblique septa in *Stemphylium solani.* Yun-Fei *et al.* (2010) found (1–) 3–5 transverse septa and 2–4 (–5) longitudinal or oblique septa in *Stemphylium luffae*.

Genomic DNA was extracted from eighteen (18) isolates of *Stemphylium vesicarium* out of twenty four (24) isolates by using CTAB extraction method. This was confirmed by gel running of genomic DNA. The genomic DNA couldn't be extracted from the DSSA, DSTR 02, MTBB 01, RBHR 01, RBHR 03, JJLL 01 isolates. For fingerprinting of eighteen (18) isolates, seven (7) decamer primers were tested to perform the RAPD analysis. But there no

band was found by using these primers. The findings are in agreement with Mehta (2001) who reported that no variation was found by geographic region among the twenty eight (28) isolates of *Stemphylium solani* isolated from cotton.

By using two ITS primer (ITS1F and ITS4) the ITS regions were identified from one (1) isolate. The isolate was DSTR 01. A 600 bp band from the sample (DSTR 01) was used for DNA sequencing. The sequenced samples were designated as Sv-DSTR 01. The sequenced data of Sv-DSTR 01 was analyzed through NCBI-BLAST database and the NCBI-BLAST database showed no significant similarity with any gene.

CHAPTER 6

SUMMARY AND CONCLUSION

White blotch disease caused by *Stemphylium vesicarium* is the major constraint for lowering the onion yield in Bangladesh. The present research was carried out to characterize *Stemphylium vesicarium* causing white blotch of onion on the basis of cultural morphological and molecular aspects. The experiment was laid out in the completely randomized design with three replications. Twenty four isolates of *Stemphylium vesicarium* were collected from eight different onion growing districts. The observations were made on cultural, morphological and molecular characteristics of *Stemphylium vesicarium.* All the 24 isolates showed variation in the terms of cultural and morphological characteristics.

Among three different culture media, V-7 juice agar medium showed the best performance in the terms of radial mycelial growth. The lowest radial mycelial growth was observed on PDA. Results revealed that presence of seven different vegetables juice in culture medium favoured radial mycelial growth of *Stemphylium vesicarium.*

Colonies of twenty four isolates of *Stemphylium vesicarium* showed deep grey to whitish, greenish brown to dirty white, light grey to whitish, deep greenish white, light grey and dirty white to greenish color on PDA medium. Circular and irregular shaped colonies were found in all the isolates with entire, undulate and filiform margin on PDA medium. Umbonate, raised and flat type colony elevation were found among all the isolates with cottony, fluffy and velvety texture on PDA medium. Deep brown, brown and light brown substrate color was found among the twenty four isolates of *Stemphylium vesicarium.*

Conidia production varied with media used in this research work.The highest number of conidia production was recorded (87.76 to 169.0 per mm^2) on combined (PDA+ V-7 juice agar) medium irrespective of all the isolates of *Stemphylium vesicarium* with maximum in isolate JJLL 03 and minimum in GJBS. The lowest number of conidia production was recorded on PDA medium ranged from 20.73 to 36.67 per mm^2 with maximum in isolate MSMM 01 and minimum in MMBH. And the conidia production of all isolates of *Stemphylium vesicarium* on V-7 juice agar medium varied from 55.01 to 92.12 per mm^2 with maximum in isolate MSMM 01 and minimum in MMBH. The sporulation time of

Stemphylium vesicarium on PDA medium varied from 86.33 to 90.67 days with maximum sporulation time in isolate GGBB 02 and MSMM 02; minimum in GJBS. On V-7 juice agar medium the sporulation time for all isolates varied from 28.33 to 31.0 days with maximum sporulation time in isolate JJLL 03 and minimum in JJLL 01. On combined (PDA+ V-7 juice agar) medium the sporulation time for all isolates varied from 35.0 to 39.67 days with maximum sporulation time in isolate CCKH 02 and minimum in DSSA and RBHR 03. All the isolates produced ovoid, oblong and ovoid to oblong shape conidia which were deep brown, light brown and medium brown in color on PDA medium. The conidia size varied within a range of 14.6 µm to 30.6 µm in length and breadth from 4.7 µm to 15.7 µm. The highest mean length (29.97 µm) of conidia was recorded in DSSA isolate and minimum (17.36 µm) in isolate MSMM 02. The highest mean breadth (12.55 µm) of conidia was observed in isolate DSSA and the lowest (9.760 µm) in CCKH 02 isolate. The horizontal septation of conidia varied from 1-3, whereas the highest mean number of horizontal septation was recorded in isolate DSTR 01 (2.500) and minimum (1.600) in isolates MTBB 01, RBHR 01, GGBB 01 and MSMM 01. The longitudinal septation of conidia varied from 0-4, whereas the highest mean number of longitudinal septation (2.500) was recorded in isolate MMBH and minimum (1.400) in isolate GGBB 02.

Genomic DNA was extracted from eighteen (18) isolates out of twenty four (24) isolates of *Stemphylium vesicarium* by using CTAB extraction method. For fingerprinting of eighteen (18) isolates, seven (7) decamer primers were tested to perform the RAPD analysis. But no band was found by using these primers. ITS regions were identified from one (1) isolate (DSTR 01) by using two ITS primer (ITS1F and ITS4). For DNA sequencing, a 600 bp band from the sample (DSTR 01) was used. The sequenced samples were designated as Sv-DSTR 01. The sequenced data of Sv- DSTR 01 was analyzed through NCBI-BLAST database and the NCBI-BLAST database showed no significant similarity with any gene.

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

- Variability exists in white blotch pathogen (*Stemphylium vesicarium*) prevailing in the onion growing areas of Bangladesh.

- V-7 juice agar was appeared to be the best medium for supporting the mycelial growth of this fungal pathogen.
- Combined (PDA+ V-7 juice agar) medium was appeared to be the best for the sporulation of *Stemphylium vesicarium*.
- Less time (28.33 to 31.0 days) was seemed to be required for sporulation of *Stemphylium vesicarium* on V-7 juice agar.
- More research should be done on molecular characterization by collecting white blotch disease samples of onion plants from all over the country to find out the genetic variability in *Stemphylium vesicarium*.

CHAPTER 7

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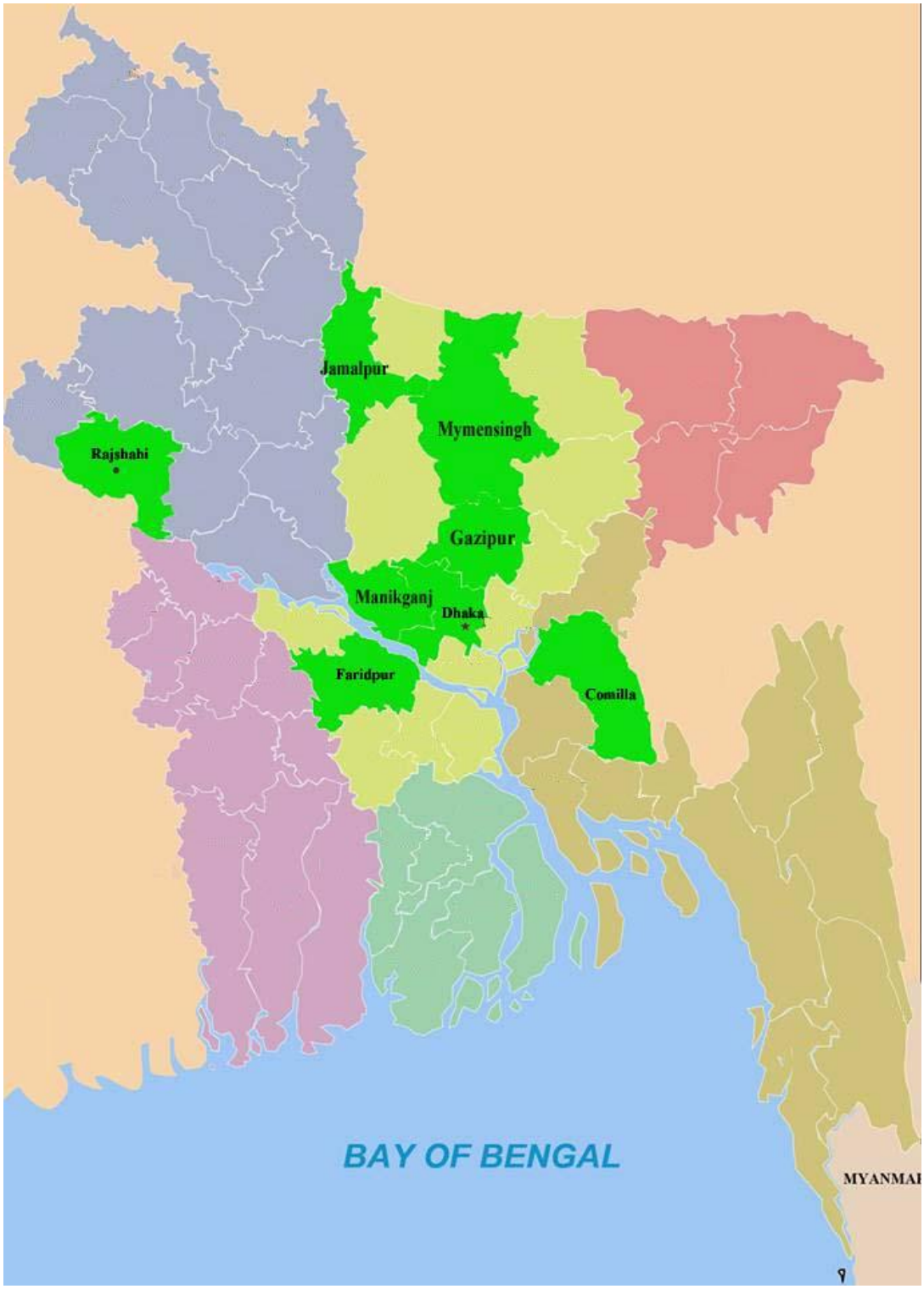
APPENDIX



Appendix I. Geographical origin of 24 isolates of *Stempylium vesicarium* collected from Bangladesh