GENETICS OF RESISTANCE TO STEMPHYLIUM LEAF BLIGHT OF LENTIL (*LENS CULINARIS*) IN THE CROSS BARIMASUR-4 × CDC MILESTONE

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Abstract

Stemphylium blight of lentil caused by *Stemphylium botryosum* Wallr., is a serious problem in Bangladesh, northeast India and Nepal causing more than 60 % yield losses under epidemic conditions. The pathogen started to appear on lentil in Saskatchewan in recent years and is widely distributed throughout western Canada but it is not well understood. An investigation of inheritance of resistance to stemphylium blight was done in the lentil cross Barimasur-4 × CDC Milestone. In order to develop a reliable indoor screening technique for this inheritance study, a suitable isolate of *Stemphylium botryosum*, a suitable culture medium for inoculum production and an appropriate plant age for indoor inoculation were identified. The maximum differential of disease severity was observed when lentil genotypes were inoculated at 14 days after planting (DAP). At 14 DAP, lentil plants rapidly defoliated but were capable of regrowth which caused variability in scoring for disease reaction. Inoculation at 42 DAP, close to the flowering stage, was found to be better for consistently scoring disease reaction. V8P medium was most suitable for inducing conidia production. Based on ability to sporulate, the isolate SB-19 from Saskatchewan was identified as suitable for conducting genetic studies of resistance to stemphylium blight. It was compared to isolate SB-BAN from Bangladesh for aggressiveness on two lentil cultivars. The SB-BAN isolate was found to be more aggressive. A preliminary screening of local and exotic germplasm done with the two isolates revealed considerable variability for disease resistance. Resistance to *S. botryosum* appeared to be quantitatively inherited in the cross Barimasur-4 × CDC Milestone according to both field and indoor screenings. The results of this study also confirmed that Precoz, one of the parents of Barimasur-4, was resistant to *S. botryosum*.

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List of Abbreviations Used

ANOVA	Analysis of Variance
AUDPC	Area Under Disease Progress Curve
BARI	Bangladesh Agricultural Research Institute
CDC	Crop Development Centre
DAI	Days After Inoculation
DAP	Days After Planting
DSD1	Disease Scoring Date 1
DSD2	Disease Scoring Date 2
DSD3	Disease Scoring Date 3
DSD4	Disease Scoring Date 4
GWSA	Ground Wheat Straw Agar
ICARDA	International Centre for Agricultural Research in the Dry Areas
MAS	Marker Assisted Selection
PDA	Potato Dextrose Agar
PRC	Pulse Research Centre
QTL	Quantitative Trait Loci
RCBD	Randomized Complete Block Design
RIL	Recombinant Inbred Lines
SB-19	*Stemphylium botryosum* Isolate # 19
SB-BAN	*Stemphylium botryosum* Isolate from Bangladesh
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

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1. Introduction

Lentil (*Lens culinaris* Medik.) is one of the oldest cultivated crops and has been a major food source of many civilizations for more than 8000 years. It is an important pulse crop rich in protein and carbohydrate, and crop residues are used as animal feed. In the developing world it is often referred to as "poor man's meat" because of its high protein content and easy accessibility by the lower economic class. Like many other pulses, it is rich in cholesterol-lowering soluble fibre and high in folate, a valuable functional food in the human diet.

World lentil production in 2005-2006 was 4.17 million metric tonnes with a forecasted decline of 17 % in 2006-2007 (AAFC, 2006). Today, Canada is the largest lentil exporter in the world. In Canada, production of lentil has increased many folds since 1969 when commercial cultivation of lentil first started. The total lentil production of Saskatchewan in 2006 declined to 0.69 million tonnes compared to 1.26 million tonnes in 2005 and 0.95 million tonnes in 2004 (SAF, 2005a, 2007). The marketing of lentil is mainly based on seed coat and cotyledon colour (yellow versus red), with further subclasses based on seed diameter referred to as large, medium and small seed. Canada has been a major contributor to the global green lentil exports and has the potential to further increase area and production to increase its share in the red lentil market.

Lentil has a wide range of variability in its gene pool for various qualitative and quantitative traits, including resistance to biotic and abiotic stresses. In Canada, the main lentil diseases are ascochyta blight caused by *Ascochyta lentis,* Vassiljevski, anthracnose caused by *Colletotrichum truncatum*, Schwein., botrytis grey mold caused by *Botrytis cinerea,* Pers. and sclerotinia stem and pod rot caused by *Sclerotinia sclerotiorum,* Lib. Stemphylium blight caused by *Stemphylium botryosum,* Wallr. (Holzgang and Pearse, 2001) and powdery mildew caused by *Erysiphe* spp. (Banniza et al., 2004) have appeared in recent years and may be potential threats to future lentil production in Saskatchewan.

Stemphylium species are pathogenic to plants, humans and animals and are distributed throughout the world. In plants, *Stemphylium* spp. have a wide range of hosts including leguminous and non-leguminous crops. *Stemphylium* spp. are also pathogenic on many horticultural cash crops and cause losses up to 100% yield loss in cotton in Brazil (Mehta, 1998). The fungus survives on many different hosts in the eastern and midwestern USA and in Canada.

Stemphylium blight of lentil is a foliar fungal disease and attacks the crop in the early pod setting stage. The infected leaves are shed, leaving only the terminal leaves on the stems, thereby severely affecting the assimilation of photosynthates, decreasing crop yield and reducing the quality of seed. The disease is of particular importance to the lentil crop in Bangladesh, northeast India and Nepal. It is widely distributed in Saskatchewan, where it is considered to be minor but not well understood. Stemphylium blight may become a more serious problem in the future and there is little or no understanding of the host resistance against the disease and potential management practices for its control (Pearse, 2005). Under severe conditions it causes yield losses of up to 62% (Bakr, 1991). With the breeding efforts of the International Center for Agricultural Research in Dry Areas (ICARDA), the stemphylium blight resistant lentil cultivar 'Barimasur-4' was released in Bangladesh in 1995 (Erskine and Sarker, 1997). Barimasur-4 was significantly higher yielding than Uthfala, a susceptible local check.

In order to develop effective disease management practices, it is necessary to understand the epidemiology of the fungus, the racial structure of the population, the interactions with different hosts and the factors affecting disease development. Investigation of differences among isolates for their virulence on lentil is essential to assess their potential to cause economic losses. A detailed understanding of spatial diversity and population structure of the pathogen is important for producing source material for resistance breeding. Selection of a virulent *S. botryosum* isolate for use in indoor screening for resistance breeding in lentil, is part of this study.

There are many challenges when a pathogen has not been studied in a host. The environment is a dynamic factor with multiple interactions of temperature, light, humidity etc. that greatly affect the development of disease. It is also not possible to

study and compare isolates from different geographical regions without a standard technique. One of the problems with *S. botryosum* is that it does not sporulate well on ordinary synthetic media (Hashemi et al., 2004). Sporulation ability of *S. botryosum* isolates from lentil was tested on wheat straw substrate (Chowdhury et al., 1996), but this medium was not suitable for mass scale culture of conidia. The large-scale conidia production system of *S. botryosum* isolates of lentil has not been optimized to allow large pathogenicity studies.

No published reports exist of appropriate methods for inoculation of lentil plants with *S. botryosum* in controlled environments. To begin the process of understanding the host-pathogen system, it is necessary to determine the optimal stage of lentil plant development to allow the maximum differential in genetic resistance studies.

Genetic resistance is a cost effective and ecosystem friendly approach to disease management. Therefore, exploring sources of resistance from the available cultivated gene pool is often a first step before exploring secondary or tertiary gene pool. Only a few reports of resistance to stemphylium blight in lentil are available and these are limited to screening of cultivated germplasm from several parts of the world, including the Crop Development Centre (CDC) at the University of Saskatchewan (Beare, 2002). Resistance to *S. vesicarium* in onion was reported to be under dominant gene control (Pathak et al., 2001), but there is no information available on the mode of inheritance of stemphylium blight resistance in lentil. Information about inheritance of resistance and possibly finding linkages with other morphological and molecular markers may lead to development of reliable and cost effective breeding tools.

Keeping the above-mentioned problems in view, the following were the main objectives of this study:

1. To compare *S. botryosum* isolates and identify a suitable culture medium to be used for producing inoculum for indoor screening for stemphylium blight resistance.
2. To develop a robust method for stemphylium blight screening in lentil.

3. To characterize genotypes and parents of available recombinant inbred lines (RILs) for their reaction to stemphylium blight.

4. To determine the inheritance pattern for resistance from the RILs developed from the cross Barimasur-4 × CDC Milestone.

2. Review of Literature

2.1 Origin, Taxonomy and Distribution of Lentil

Lentil (*Lens culinaris* Medik.) is a short, slender, bushy annual plant of the family *Leguminosae* with a fairly erect growth habit (Webb and Hawtin, 1981) and originally from Turkey (Ladizinsky, 1979). The Latin name of the species, *Lens culinaris,* was first published by Medikus in 1787 (Anonymous, 1981). *Lens culinaris* is believed to originate from *Lens orientalis* (Ladizinsky, 1979). The genus *Lens* Miller comprises five annual species (*L. culinaris, L. odemensis, L. nigricans, L. ervoides* and *L. lamottei*). *Lens culinaris* is the only cultivated species and is comprised of the sub-species *L. culinaris* subsp. *culinaris, L. culinaris* subsp. *orientalis.* However, a recent study on the genus proposed that *L. odemensis* and *L. tomentosus* are also sub-species of *L. culinaris*, on the basis of crossability and similarity of isozymes and RAPD markers (Ferguson et al., 2000).

Lentil originated from the Mediterranean region and is well adapted to the cool seasons there. It can be grown in other parts of world with a similar climate (Anonymous, 1981). The crop spread to other regions of the world such as central Asia, where it is grown in temperate summers and to south Asia, where it is grown in winter during the dry season after the monsoon rise in the northern part of the sub-continent.

Current world production averages about three million metric tonnes each year (SAF, 2005). Lentil is mostly consumed as dhal or soup, mainly in India, Bangladesh, Nepal, Pakistan, Sri Lanka, Turkey and Egypt. India was the largest producer of lentil, but Canada surpassed their production in 2005 (FAO, 2005). India is the largest consumer of lentil while Canada is now the largest exporter in the world (FAO, 2005). The cultivated area of lentil has increased more than ten-fold in the last 20 years in Saskatchewan (Anonymous, 2003). Saskatchewan grows more than 95% of the Canadian lentil crop, valued at about $ 150 million at the farm gate (SPG, 2005). Canadian domestic consumption has increased significantly and has reached 175,000

metric tonnes in 2004 (SPG, 2005). Australia, Turkey, Syria, Nepal, the USA, Bangladesh and Morocco are other major producers of lentil (FAO, 2005).

2.2 Diseases of Lentil

Lentil is infected by a wide range of pathogens throughout its geographic distribution. It is mainly affected by alternaria blight (*Alternaria* spp.), ascochyta blight (*Ascochyta lentis*), anthracnose (*Colletotrichum truncatum*), botrytis stem and pod rot (*Botrytis cinerea*), rust (*Uromyces fabae*), sclerotinia stem and pod rot (*Sclerotinia sclerotiorum*), stemphylium blight (*Stemphylium botryosum*), wilts (*Pythium* spp., *Rhizoctonia* spp., *Fusarium oxysporum*), and downy mildew (*Peronospora lentis*) (Khare, 1981). Ascochyta blight, anthracnose, botrytis grey mould and sclerotinia stem and pod rot are major problems in Canada (Chongo et al., 2002). Stemphylium blight has started to appear in fields in Saskatchewan in recent years (Holzgang and Pearse, 2001). Recently, powdery mildew has also been reported on lentil in Saskatchewan (Banniza et al., 2004).

2.3 Distribution and Losses Caused by *Stemphylium* spp. in Lentil and Other Hosts

The distribution of, and economic loss in lentil due to, *S. botryosum* are geographically limited. *Stemphylium botryosum* of lentil was first identified in 1987 in Bangladesh (Bakr and Zahid, 1987). Occurrence of the disease in lentil has also been reported in north-east India (Sinha and Singh, 1991). Disease intensity as high as 83% was observed on an unsprayed local susceptible lentil cultivar in Bihar state of India, causing more than 90% yield loss (Sinha and Singh, 1993). As the cultivated area of lentil increases in western Canada, stemphylium blight may become a potential threat to lentil production, particularly if higher levels of resistance are achieved for other major foliar diseases like ascochyta blight and anthracnose (Hashemi *et al.*, 2005a).

Many species of *Stemphylium* are pathogenic on plants, humans and other animals. In humans these may cause allergic reactions and lung infection (Wu et al., 2004). *Stemphylium* spp. are pathogenic on many crops throughout the world and cause

varying degrees of losses on different crops (Polfliet, 2002). On plants, the symptoms of stemphylium blights are usually reported as leaf spots and leaf blights.

Stemphylium blight of onion (*Allium cepa)* was reported in India by Gupta and Srivastava (1988). *Stemphylium vesicarium* was isolated from diseased plants and pathogenicity was confirmed in garlic (*Allium sativum)* in 1989-91 in South Africa (Aveling and Naude, 1992). An outbreak of stemphylium leaf spot caused by *S. vesicarium* in garlic was reported in 1992 in Spain (Basallote et al., 1993). Cho and Hun (1998) reported *S. vesicarium* on garlic and other *Allium* spp. in Korea. Suheri and Price (2000a; 2000b) reported stemphylium leaf blight of garlic caused by *S. vesicarium* in Australia. Asparagus (*Asparagus officinalis)* is also infected by *S. vesicarium* and the disease was described as purple spot in the USA (Lacy, 1982; Johnson, 1987).

Stemphylium solani has been reported primarily on cotton (*Gossypium hirsutum*) and tomato (*Lycopersicon esculentum*). Epidemics caused by *S. solani* on cotton were reported from Parana state of Brazil during 1994 and 1995, and caused up to 100% yield losses in a local susceptible cotton cultivar in India (Mehta, 1998). Grey leaf spot on tomato plants (*S. lycopersici*) was first observed in Korea in 1994 (Min et al., 1995).

Legumes are reportedly infected by *S. sarciniforme* and *S. botryosum.* Stemphylium leaf spot, caused by *S. sarciniforme* occurs on red clover at high severity levels in the northeastern USA and Canada during most seasons (Berg and Leath, 1996). *Stemphylium botryosum* has been reported on alfalfa (*Medicago sativa*) (Cowling and Gilchrist, 1982; Rokaibah, 1996). *Stemphylium botryosum* infection is not specific to legumes since it has also been reported to cause a leaf spot on spinach (*Spinacia oleracea* L.) in the USA (Koike et al., 2001; Everts and Armentrout, 2001), and a stem spot and needle blight on asparagus, first observed in Greece (Elana, 1996). In 1996, leaf spot of Drummond phlox (*Phlox drummondii*) caused by *S. botryosum* was observed in Japan (Takeuchi and Horie, 1997).

Four new species of *Stemphylium* were recently described in China as *S. gossypii., S. lactuci, S. momordi* and *S. alli-cepae* affecting cotton, lettuce (*Lactuca sativa*), bitter gourd (*Momordica charantia*) and onion (Zhang et al., 2003).

2.4 Biology of *Stemphylium* spp.

Since almost no published work is available for *Stemphylium botryosum* in lentil, the epidemiology of *Stemphylium* spp. in other hosts has been reviewed to characterize the pathogen.

2.4.1 Taxonomy and Nomenclature

Stemphylium is the anamorph of genus Pleospora, which belongs to the kingdom Fungi, phylum Ascomycota, class Ascomycetes, order Pleosporales, family Pleosporaceae (Anonymous, 2004), but the pathogen is commonly referred to as *Stemphylium*, its asexual stage or anamorph (Bayaa and Erskine, 1998). More than 20 species are known (Anonymous, 2005) and four more were recently described (Zhang et al., 2003).

Molecular based discrimination has been used in the taxonomy of *Stemphylium*. Results of a phylogenetic study of the relationship among the species and isolates of the genus *Stemphylium* supported a monophyletic origin (Camara et al., 2002). Chaisrisook et al., 1995 cited by Camara et al., 2002 noted at least five genetically distinct species of *Stemphylium*, those can cause leaf spot of alfalfa. Results from another phylogenetic study revealed that *Embellisia* and *Nimbya* species clustered within a large monophyletic *Alternaria-Nimbya-Embellisia-Ulocladium* clade with *Stemphylium* as the sister taxon (Pryor and Bigelow, 2003).

Morphological and developmental characters such as size and shape of the conidia, conidiophores and ascospores and the size and time of maturation of pseudothecia were useful for diagnosing species variation (Camara et al., 2002). However, other morphological characters such as septum development and small variations in conidial wall ornamentation were not reliable.

The phylogenetic relationships of 43 isolates representing 16 species of *Stemphylium* were inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene sequence data (Marcos et al., 2002). The results generally agreed with current morphological species concepts. Species that were primarily pathogenic to

alfalfa were resolved into two separate groups. *Stemphylium botryosum* and two isolates with morphological characters similar to *S. globuliferum* had identical sequences at both loci. These two loci in *S. vesicarium*, *S. alfalfae* and *S. herbarum* were nearly identical but differed from *S. botryosum*. *Stemphylium lycopersici* and *S. xanthosomatis* had identical ITS sequences and a single nucleotide difference in the *gpd* region.

The mating system in *Stemphylium* spp. was recently studied by Inderbitzin et al. (2005). MAT loci, which regulate sexual reproduction in *Stemphylium* spp. were demonstrated to have a single gene, either MAT1-1 or MAT1-2 in some species, whereas others contained a unique fusion of the MAT1-1 and MAT1-2. Species with fused MAT regions were able to self.

2.4.2 Morphology and Microscopic Structure

The asexual stage of the lentil pathogen is *Stemphylium botryosum* while the sexual stage is known as *Pleospora herbarum* (Bayaa and Erskine, 1998). Conidiophores are short, aseptate, swollen at the apex, and may be single or in a group. Conidiophores proliferate further after a conidium is produced, producing new cells and new conidia.

Conidia are olive brown, oblong or muriform in shape with three constricted transverse septa (Bayaa and Erskine, 1998; Raid and Kucharek, 2005). 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* (Camara et al., 2002). Perithecia are globuse, membranous and black and sometimes have a slender neck (Bayaa and Erskine, 1998). Asci are oblong with outer and inner walls measuring 183-267× 27-37 µm with elongate to ovate and yellowish to brown ascospores measuring 32-48× 12-21 µm (Bayaa and Erskine, 1998).

2.4.3 Disease Symptoms

The symptoms of stemphylium blight in lentil appear as small brown to tan colored spots which later spread and cover the whole leaf leading to complete leaf shedding (Bakr, 1991). In alfalfa, *S. botryosum* causes leaf spot followed by chlorosis

and leaf defoliation (Lucas et al., 1973). In spinach, symptoms start with round to oval, 2-5 mm leaf spots which later coalesce and form a necrotic leaf tissue (Koike et al., 2001). Similar disease symptoms were caused by *S. vesicarium* on garlic leaves 8 to 10 days after inoculation in a greenhouse (Boiteux et al., 1994) and on onion and leek (*Allium ampeloprasum* L.) in field conditions with natural inoculum (Cho and Hun, 1998). Stemphylium blight usually affect different crops from the flowering stage onwards. Defoliation of lower branches is very common in many host species (Polfliet, 2002). Massive defoliation and stem bending was observed in leaf blight of lentil caused by *S. botryosum* (Bayaa and Erskine, 1998).

2.4.4 Infection Process

The pathogen survives on debris of plants. Pseudothecia of *Pleospora*, the teleomorph of *Stemphylium*, were observed on debris of overwintered garlic leaves affected by leaf spots caused by *S. botryosum* (Basallote et al., 1993).

Airborne conidia of *S. botryosum* land on host tissue and germinate when conditions are favorable. Generally penetration occurs through stomata but *Stemphylium* spp. also produce the toxin stemphol that may aid host infection (Solfrizzo et al., 1994). Penetration of leaves may occur directly through the epidermis, but the frequency of stomatal penetration by *S. vesicarium* exceeds that of epidermal penetration under favorable environment conditions (Suheri and Price, 2000a). Penetration through stomata is also affected by host resistance but is governed by environmental factors as reported for *S. botryosum* by Cowling and Gilchrist (1982). The hyphae of the fungus enter the cell and spread inside, deriving water and nutrients from the surrounding cells. Eventually these cells die causing tissue to turn brown and appear blighted. Small tan colored necrotic spots gradually enlarge in size and eventually cover the whole leaf.

2.4.5 Factors Affecting Disease Development

Infection of the host plant is a very complex process that is influenced by environmental interactions. The availability of moisture is critical during the time of conidial germination. Under moist conditions, disease incidence increases rapidly.

Excessive vegetative growth in combination with high humidity favours disease development.

Stemphylium vesicarium required wet conditions for growth on garlic (Aveling and Naude, 1992). Severe foliar damage with subsequent yield losses occurred when leaf wetness periods exceeded 24 continuous hours. Warm, humid conditions were conducive to the development of severe epidemics (Aveling and Naude, 1992). In *S. vesicarium* on garlic, a strong correlation was observed between the amount of rainfall and the aerial concentration of ascospores and conidia (Basallote et al., 1993). The role of relative humidity was critical in cases of low precipitation. Field observations during a survey indicated that outbreaks of garlic leaf spot were favoured by foggy and rainy weather in spring, followed by dry warm days (Basallote et al., 1993). Modeling of environmental conditions played a dynamic role in forecasting incidence of *S. vesicarium* in pear (Boshuizen et al., 2003). In lentil, one of the important factors determining the appearance, development and spread of *S. botryosum* was the number of cloudy and foggy days during the November-February cropping season in the Indian sub-continent (Sinha and Singh, 1991).

Temperature is another important factor in disease development. A study in onion showed that conidia of *S. vesicarium* germinated within 2 h when incubated at 4°C (Suheri and Price, 2000a). Terminal and intercalary appressoria were produced at higher frequency after 24 h at 25°C. In another study, using *S. vesicarium*, a positive relationship was reported between conidium concentration in the air and the number of hours with temperatures in the range of 12-21°C (Prados-Ligero et al., 2003)

High temperature favoured the germination of conidia of *S. botryosum* and under controlled conditions the optimum temperature for conidial germination was between 25°C and 30°C (Mwakutuya et al., 2004). In Bangladesh, *S. botryosum* initiated infection on lentil when the night temperature remained above 8°C with average day temperature above 22°C and the relative humidity in the plant canopy exceeded 95% (Bakr, 1991; Erskine and Sarker, 1997). In northeastern India, infection occurred when the average temperature reached around 18°C, relative humidity was above 50% and the daily mean sunshine was 7.7 hours or less due to cloudy and foggy weather (Sinha and Singh, 1993). In a recent study on the biology of *S. botryosum* in

lentil, the minimum latent period was 48 h and was observed at the ideal temperature of 25-30°C under controlled conditions. It increased with decreases in temperature and wetness period (Mwakutuya, 2006). The pathogen continued to develop despite interruptive dry periods. Infection level increased after 2 h of incubation at a temperature above 10°C and continued to increase with the expanding wetting period up to 48 h and temperature up to 30°C. Optimal conditions for infection were temperatures of more than 25°C combined with minimum 8 h of wetness.

2.4.6 Sources of Inoculum

Stemphylium botryosum is reported to spread by airborne conidia. It overwinters on seed and as mycelium on dead stems and leaves in many cropping systems. Limited information is available on whether the pathogen is seed-borne in nature on lentil (Bayaa and Erskine, 1998). In alfalfa, *S. botryosum* is spread by airborne and waterborne conidia (conidia and ascospores) and by sowing infected seed (Malvick, 1998).

Infection of *Stemphylium vesicarium* on pear increases every year as the inoculum accumulates in plant debris (Polfliet, 2002). This may be an indication that this genus may be influenced by changes in cropping practices like minimum tillage. Weedy infected asparagus seedlings during the harvest season of spinach in Washington State may act as a bridge to carry inoculum of *S. vesicarium* from one season to another. Ascospores and conidia of *Pleospora herbarum* on debris from the previous year's fern growth serve as primary inoculum for asparagus (Johnson, 1990).

2.4.7 Racial Structure

The racial structure of *Stemphylium* spp. is not clear in many hosts. The existence of variation in virulence and different races of *S. solani* and *S. vesicarium* were reported in tomato (Hernandez, 1985) and alfalfa (Irwin and Bray, 1991), respectively. In other studies with alfalfa, no race structure was described, but biotypes of *S. botryosum* were reported on the basis of adaptation to cool (18-20°C) or warm weather (23-27°C) (Cowling and Gilchrist, 1982). Differences in relative virulence of *S. botryosum* isolates were correlated with the frequency of stomatal penetration in

alfalfa. Isolates with high relative virulence produced a higher proportion of effective stomatal penetrations compared to isolates with low virulence (Cowling and Gilchrist, 1982).

2.5 Disease Rating for Stemphylium Blight

A descriptive scale from 1-5 was used for scoring leaf spot caused by *S. botryosum* in alfalfa (Salter and Leath, 1991). Koike et al. (1991) used a sign scale (- = no disease; + = small leaf spot <5mm; + + = medium leaf spot) to score leaf spot disease of spinach caused by *S. botryosum*. A 0-5 visual scale (0= no disease; 1= minute pinhead size spots, < 5% diseased leaf area; 2 = 5-25% diseased; 3= 26-50% diseased; 4= 51-75% diseased; 5= coalescing lesions with > 76% diseased area) was used to score cotton leaf spot disease caused by *S. solani*. A qualitative scale (HR, R, MR, S and HS) and a semi-quantitative scale of 1-5 (1= no symptoms; 2= < 5% infection; 3=6-25% infection; 4=26-50% infection and 5= >50% infection) was used to score stemphylium blight caused by *S. vesicarium* in onion and garlic (Anonymous, 1998).

Various methods have been described for disease assessment. Area under the disease progress curve (AUDPC) has proven to be a reliable index of disease progress over time, showing a positive correlation with single plant evaluation for ascochyta blight in lentil (Ahmed and Morrall, 1996). Differences in screening and disease assessment techniques also produce variable results. One example of an extreme difference in interpretation is with ascochyta blight (*Ascochyta rabiei*) reaction in chickpea (*Cicer arietinum*), where a dominant gene for resistance reported in one study was found to be recessive in another (Tekeoglu et al., 2000).

2.6 Control Measures for *Stemphylium* spp.

Fungicide treatments applied as sprays after disease appearance effectively controlled stemphylium blight caused by *S. vesicarium* in onion (Gupta and Srivastava, 1988). During 1982-85, four fungicides (copper oxychloride, mancozeb, carbendazim and thiram) applied as a spray, one month after transplanting, prevented disease

development. The cost-benefit ratio revealed that a preventive spray of 25% mancozeb gave the highest net financial return.

Similarly, the fungicide Pristine 38WG (propiconazole) and Tanos 50DF (25 % famoxadon) were effective in limiting the development of purple spot caused by *S. vesicarium* in asparagus. Pristine 38WG alternated with Bravo WeatherStik (chlorothalonil) was the only treatment to significantly reduce stemphylium leaf blight when compared to the untreated control (Hausbeck and Bousds, 2005). Incorporation of debris into the soil in late autumn and in late winter provided effective cultural control for stemphylium blight in asparagus cultivation (Johnson, 1990).

Tebuconazole, procymidone and fosetyl sprays applied prior to artificial inoculation significantly reduced leaf spot in garlic caused by *S. vesicarium* (Basallote et al., 1998). A significant increase in garlic yield was observed in experiments conducted under environmental conditions conducive to disease development (Basallote et al., 1998). Few research reports are available covering control methods for stemphylium blight in legumes. In lentil, stemphylium leaf blight caused by *S. botryosum* was controlled most effectively by a foliar spray of Rovral 80WP (iprodione) at 0.2% (Bakr and Ahmed, 1992). Treated plots yielded 35% more than the controls.

2.7 Genetic Studies of Disease Resistance for *Stemphylium* spp.

Host resistance is a cost-effective method for controlling disease and it requires little alteration in existing cultural practices. Transfer of available resistance in a cultivated background is especially important for farmers lacking resources to control the disease. Little is known about genetic defense mechanisms against *Stemphylium* spp. in crop plants.

Significant cultivar differences were observed for lesion size and severity scores for stemphylium leaf spot caused by *S. sarciniformae* in red clover (Berg and Leath, 1996).

A thick plant canopy also plays an important role by providing a microclimate conducive to disease development. French asparagus cultivars produce relatively short,

compact fern growth, thereby providing an ideal plant canopy for disease development (Broadhurst, 1996).

Regarding the possible mechanism of resistance, the role of a novel *PR-5* gene coding for a secreted protein (AoPRT-L), which is also responsive to salicylic acid, was confirmed in monocots against *S. vesicarium* (Darby et al., 2000). Resistance to *S. vesicarium* has been reported to be under single dominant gene control in onion (Pathak et al., 2001).

A resistance gene (*Sm*) originating from the wild tomato (*Lycopersicon pimpinellifolium*), was reported and used to improve resistance in tomato to *S. solani* for subtropical and tropical disease conditions (Behare et al., 1991; Laterrot, 1998).

A narrow-leafed lupin (*Lupinus angustifolius*) germplasm accession SNLL87 was reported to have resistance to *S. solani* (Miller et al., 1988). The narrow-leafed lupin cultivar Wandoo is known to have resistance against *S. vesicarium*, but appears to carry a gene different from $gl_{1,}$ discovered in a spontaneous mutant of a bitter commercial cultivar of Australian origin. The second gene of resistance gl_2 was identified in a wild Portuguese accession PI 168530 in the cultivar Chittick (Gladstones, 1986).

The inheritance of resistance to *S. solani* was investigated in three different crosses in cotton. Genetic analysis showed significant additive genetic variation for resistance with a F_3 family ratio of 1:2:1 for a single non-dominant gene segregation in two crosses and for the third one, a segregation pattern including two genes and epistasis (9:6:1) was reported (Mehta and Arias, 2001).

Differences in relative host resistance in alfalfa were expressed through hypersensitive response to effective stomatal penetration for *S. botryosum* (Cowling and Gilchrist, 1982). Resistance to *S. botryosum* in lettuce proved to be controlled by two genes, *Sm1* and *sm2*, which are dominant and recessive, respectively (Netzer et al., 1985).

Studies on defense structural factors such as epidermal hairs, thickness of epidermis and cortical layers revealed considerable variation for resistance to *S. botryosum* in lentil (Chowdhury et al., 1997). These phenotypic variations could be used in developing resistant cultivars. The genetic basis of resistance to stemphylium

blight of lentil has not been reported. Most of the reports are limited to screening of germplasm. The lentil cultivar Barimasur-4, released in Bangladesh, was reported to be resistant to *S. botrysum* (Erskine and Sarker, 1997). The lentil cultivar Precoz, also reported as ILL 4605, developed in Argentina (Riva, 1975), had also been reported to have resistance to *S. botryosum* (Erskine and Manners, 1996).

3. Material and Methods

3.1 Pathological Study

3.1.1 Identification of a Suitable Isolate of *Stemphylium botryosum* to be Used in Indoor Screening for Stemphylium Blight Resistance

Three isolates of *Stemphylium botryosum*, (SB-16, SB-17 and SB-19) from Saskatchewan were selected for testing to find a suitable isolate for use in indoor screening using conidial suspensions. They were selected based on their superior ability to sporulate, evident from a preliminary study by Hashemi et al. (2004).

A fourth isolate, SB-BAN was collected from a farmer's field near Ishurdi, Bangladesh during a visit to the stemphylium blight screening nursery in January 2005. This nursery is managed by the Pulses Research Centre of the Bangladesh Agricultural Research Institute (BARI) and was used in this study as a reliable screening site for resistance to stemphylium blight in lentil.

All four isolates were tested for sporulation ability in an experiment in a RCBD with sub-sampling and six replications, where each plate was considered an experimental unit. Plugs of each isolate, obtained from single conidium cultures, were grown on V8 medium in 90×15-mm sterile Petri dishes. An optimal temperature of 27°C for mycelial growth had been determined in preliminary experiments. Cultures were placed in an incubator for 15 days with an alternating period of two days dark and one-day light following 7 days of continuous fluorescent light. After incubation for two weeks, the plates were washed using 5 ml sterile distilled water and the conidia were dislodged using a soft brush. Using a hemacytometer, the number of conidia was estimated from two sub-samples taken from two different pipettings from the conidial suspension in each plate.

3.1.2 Identification of a Suitable Culture Medium

Seven different media: 25% Potato Dextrose Agar (PDA), Water Lentil Seed Agar (LSA), 25% Ground Lentil Stem and Leaf Agar (SLA), 25% Ground Lentil Stem, Leaf and Seed Agar (SLSA), 25% Ground Wheat Straw Agar (GWSA), V8 Juice Agar (V8), V8 Juice with PDA (V8P) had been tested for sporulation ability with 23 *Stemphylium* isolates collected from Saskatchewan in preliminary experiments (Hashemi et al., 2004). It became apparent that *S. botryosum* does not sporulate readily in common culture media, which creates challenges for artificial inoculation. Therefore, finding an appropriate medium to achieve required conidia concentrations for inoculation was important.

The isolate SB-19 was chosen from the preliminary experiments and further tested on four V8-based media. Tamarind pulp is a natural source of vitamins (thiamin, niacin and ascorbic acid), minerals (potassium, calcium, iron and phosphorous) and tartaric acid (Mathur, 2004). Tartaric acid has been successfully used in slight adjustment of pH in wine making (Collings, 2002). Hypothesizing that a slight increase in acidity of the media by tartaric acid might facilitate release of conidia from mycelia, V8, V8P, V8PTD1 (V8P + 2% tamarind juice) and V8PTD2 (V8P + 4% tamarind juice) media were tested in a RCBD with 6 replications.

3.1.2.1 Preparation of V8, V8P, V8PTD1 and V8PTD2 Media

V8 juice agar was prepared using 112.5 ml V8 Original Blend Vegetable Cocktail (Campbell Co., Canada), 11.3 g of Difco^{TM} Agar, Granulated (Becton Dickinson and Co., Sparks, MD, USA), 1.13 g $CaCO_3$ (EMD Chemicals Inc., Darmstadt, Germany) and 637.5 ml distilled water. To prepare V8P medium, 112.5 ml V8 juice, 7.5 g of Difco^{TM} Agar, Granulated, 7.5 g of Difco^{TM} Potato Dextrose Agar (Becton Dickinson and Co., Sparks, MD, USA), 2.25 g $CaCO_3$ and 637.5 ml distilled water were combined. After shaking well, the media were sterilized in an isothermal autoclave for 25 min on liquid cycle. Media were later cooled in a water bath (54ºC) before pouring into 90×15mm sterile Petri dishes.

The V8-based media were modified as V8PTD1 and V8PTD2 by adding 10 ml of 2% and 4% (w/v) tamarind juice, respectively made from tamarind concentrate

(Aeroplane brand manufactured by ADF Foods Ltd. Gujarat, India) available at a local supermarket. Other ingredients in the tamarind media were the same as in the V8P medium. The isolates were cultured on all four media as per the procedure described in section 3.1.1. and the number of conidia produced from each Petri dish was recorded.

3.1.3 Determination of Appropriate Age of Plant for Inoculation

In order to determine the optimum age of lentil plants for inoculation with conidia of *S. botryosum*, two different experiments with Eston and CDC Milestone cultivars in each experiment, were conducted in the greenhouse using 4 different plant growth stages: 14 days after planting (DAP) (seedling), 28 DAP (vegetative), 42 DAP (flowering) and 56 DAP (pod filling). Eston was used as a resistant line in the experiment because it was found to be moderately resistant in preliminary screening. At the time this experiment was conducted, a limited amount of seed was available for Barimasur-4, an unadapted lentil cultivar with reported resistance to stemphylium blight (Erskine and Sarker, 1997), thus it was not included in this study.

3.1.3.1 Plant Material and Establishment

Lentil seeds were planted in 5" plastic pots filled with soilless medium (Redi-Earth® No. 43, W.R. Grace & Co. of Canada Ltd., Ajax, ON). The seeds were nicked with forceps before sowing to ensure imbibition. The plants were thinned to four per pot after emergence. Plants were fertilized one week after sowing using a solution of 20:20:20 fertilizer at 3 g L^{-1}. The pots were placed on a greenhouse bench in a randomized complete block design. Individual pots were wrapped with transparent polythene sheets to increase the humidity around the plants.

3.1.3.2 Inoculation, Incubation and Disease Scoring

An inoculation suspension was produced from SB-19 grown on V8P medium using the procedure described in section 3.1.1. The conidial concentration was adjusted to 2×10^5 conidia ml^{-1}. Two drops of Tween® 20 were added to facilitate conidium-plant tissue contact by reducing the surface tension of water. Plants of four different growth

stages, 14 DAP, 28 DAP, 42 DAP and 56 DAP, were inoculated using an air-brush to evenly apply the conidial suspension. Plants were incubated for 48 hours in a mist chamber at 95% humidity and 20°C following inoculation. Pots were then transferred to mist benches in the greenhouse and the plants were kept there for 3 weeks to promote disease development.

Disease levels were first recorded three days after inoculation (DAI) when the first symptoms of stemphylium blight appeared. Disease scores were recorded at three day intervals and a total of four ratings were used to determine the rate of disease development. In this experiment in the greenhouse, the disease was scored using the Horsfall-Barratt scale (Horsfall and Barratt, 1945) of twelve grades between 0 and 11. The scale was originally developed for powdery mildew of pea (*Pisum sativum*).

The grade number was converted into % disease severity according to the scale system, which was further used to calculate a value for Area Under the Disease Progress Curve (AUDPC) using the following formula.

$$AUDPC = \sum_{i=1}^{n-1} [(y_i + y_{i+1})/2 \, (t_{i+1} - t_i)] \qquad \text{(i)}$$

y = score assigned; t = days after inoculation; n = number of scores

3.1.4 Testing of Isolates SB-19 and SB-BAN in the Growth Chamber

Two isolates of *S. botryosum*, SB-19 and SB-BAN, were tested for their aggressiveness on the lentil cultivar Barimasur-4 (resistant) and CDC Milestone (susceptible). The experiment was conducted as a RCBD with 8 replications in a controlled environment growth chamber in the University of Saskatchewan phytotron when lentil plants were 42 DAP. The experiment was conducted and repeated a second time using the procedure described below.

3.1.4.1 Plant Material and Establishment

Lentil seeds of Barimasur-4 and CDC Milestone were planted in 5" plastic pots filled with soilless medium (Redi-Earth® No. 43). The seeds were nicked with forceps before sowing to ensure imbibition. The plants of each cultivar were thinned to four per

pot after emergence and were fertilized two weeks after sowing using 20:20:20 fertilizer at 3 g L^{-1}. The pots were wrapped with transparent polythene before inoculation to increase humidity. The treatment combinations of cultivar × isolate were randomized and placed in a controlled environment growth chamber in a RCBD.

3.1.4.2 Inoculation, Incubation and Disease Scoring

Conidial suspensions of both SB-19 and SB-BAN were produced as described in section 3.1.1. The treatment combinations of cultivar × isolate were sorted for one isolate kind and inoculated separately from the other isolate as per the procedure described in section 3.1.3.2. The treatment combinations of cultivar × isolate were placed back in the controlled environment growth chamber in previously randomized order after inoculation. Growth room benches equipped with intermittent flood irrigation were covered with boxes made of Plexiglas sheets. A fine water droplet humidifier (model 7075M manufactured by Herrmidifier, Sanford, NC, USA) was placed inside the box to achieve 95% and higher RH. Day and night temperatures were maintained at 20°C and 15°C, respectively, and monitored using a Hobo data logger (Onset Computer Corporation, MA, USA). Daylength was set for 16 hours with a night period set to 8 hours. Plants were placed inside the boxes and incubated for 12 days.

The Horsfall-Barratt scale is a logarithmic scale with unequal intervals between scores that makes it unsuitable for quantitative genetic analysis. To overcome this problem, a 0-10 linear semi-quantitative scale (Hashemi et al., 2005b) (Table 1) was used. The scale was tailored to follow the disease development pattern that consisted of appearance of chlorotic spots followed by gradual defoliation of plants.

Table 1. Rating scale for stemphylium blight of lentil.

Grade	Symptoms
0	Healthy plant; free of disease
1	Dull leaves or a few tiny tan spots
2	A few small to large chlorotic spots
3	Expanding lesions on leaves and leaf drop starting
4	$1/5^{th}$ / 20% nodes on main stem showing chlorotic / necrotic symptoms and / or leaf drop

5	$2/5^{th}$ / 40% nodes on main stem showing chlorotic / necrotic symptoms and /or leaf drop
6	$3/5^{th}$ / 60% nodes on main stem showing chlorotic / necrotic symptoms and / or leaf drop
7	$4/5^{th}$ / 80% nodes on main and lateral stems showing chlorotic / necrotic symptoms and leaf drop
8	100% leaves dried up/ defoliated but small green tip recovering
9	100% leaves dried up/ defoliated including tip but stem still green
10	Whole plant dried up and completely dead

(Hashemi et al., 2005b)

The first disease scoring date (DSD1) was 3 DAI when symptoms of stemphylium blight first appeared. Disease scores were recorded four times at three day intervals and a value for AUDPC was calculated from the formula given in section 3.1.3.4.

3.2 Genetic Study

3.2.1 Preliminary Screening of Parental Lines

3.2.1.1 Plant Material and Establishment

Lentil cultivars 'CDC Robin', 'CDC Milestone', 'Eston', 'CDC Glamis', 'CDC Redcap', 'CDC Blaze', 'CDC Vantage', 'Barimasur-4' and the parents involved in crosses developed by ICARDA, including ILL 5888-2, ILL 8007, ILL 8010, Ranjan, Precoz (ILL 4605-2), Asha, Subroto, ILL 8008 and ILL 8009 were screened for resistance to *S. botryosum* under greenhouse conditions. Lentil seeds of each cultivar were planted in 5" plastic pots filled with soilless medium (Redi-Earth® No. 43). The seeds were nicked with forceps before sowing to ensure imbibition. The pots were placed on greenhouse benches, equipped with intermittent flood irrigation. The plants of each cultivar were thinned to four per pot after emergence and were fertilized one week after sowing using a solution of 20:20:20 fertilizer at 3 g L^{-1}. The pots were wrapped with transparent polythene before inoculation. The ICARDA crosses (Appendix 26) were developed by Dr. A. Sarker, lentil breeder at ICARDA, Tel Hadya, Syria.

3.2.1.2 Inoculation, Incubation and Disease Scoring

Conidial suspensions were produced using the procedure outlined in section 3.1.1 and the conidial concentration was adjusted to 2×10^5 conidia ml^{-1}. Two drops of Tween® 20 were added to facilitate conidium-plant tissue contact. Plants were inoculated 14 DAP (5 nodes or 3 leaf stage) by evenly spraying the plants with the conidial suspension using an air-brush. Plants were incubated in a mist chamber at 95% RH and 20°C for 48 hours in the dark following inoculation. Plants were then transferred to mist benches in the greenhouse.

Disease scores were first recorded at 3 DAI when the first symptoms of blight appeared. Disease scores were recorded at intervals of three days and four ratings were performed to determine the rate of disease development. In preliminary screening in the greenhouse, the disease was scored using the 0-11 Horsfall-Barratt scale. The grade number was converted to % disease severity according to the scale system, which was further used to determine AUDPC using the formula given in section 3.1.3.4.

3.2.2 Screening of Lentil Genotypes and RILs in Growth Chamber

3.2.2.1 Plant Material and Establishment

A set of 20 lentil genotypes consisting of CDC Robin, CDC Rouleau, Precoz, ILL 8008, Eston, Crimson, CDC Viceroy, CDC Blaze, CDC Redberry, 1308M-7, 1254S-16, 1227S-28, 1207D-13, 1205M-5, 1296D-5, 1775S-12, Barimasur-4 (resistant check) and CDC Milestone (susceptible check) were grown in 38-cell seeding trays in a growth chamber. The trays were 21" long, 10" wide and 5" tall and filled with soilless medium (Redi-Earth® No. 43). Two seeds of each lentil genotype were seeded and one plant per cell was maintained after thinning. The randomized entries of lentil genotypes were planted with two plots of each resistant (Barimasur-4) and susceptible check (CDC Milestone) included after two randomized entries in RCBD with 6 replications.

A set of 150 $F_{6:7}$ RILS derived from the cross Barimasur-4 × CDC Milestone was grown in the field in the summer of 2004. The amount of seed per RIL was variable so seeds were hand planted in furrows prepared at the Preston Avenue plot

area in Saskatoon. Each RIL was harvested separately at maturity, then dried and threshed. All 150 lines were subsequently used in growth chamber experiments. Sufficient seed was available from 82 of the RILs to allow for planting of a field nursery in Bangladesh.

In the growth chamber, the 150 RILs were seeded in 38-cell trays with 4 replications. One resistant (Barimasur-4) and one susceptible (CDC Milestone) check were seeded after each randomized RIL plot in the trays to control spatial variability. A randomized complete block design was used. Seeds were nicked with forceps, sown at two seeds per cell and thinned to one plant per cell after emergence. Plants were fertilized 14 DAP using a solution of 20:20:20 fertilizer at 3 g L^{-1}.

3.2.2.2 Inoculation, Incubation and Disease Scoring

The set of 20 lentil genotypes was inoculated with the SB-19 and SB-BAN isolates in separate experiments at 14 DAP and 28 DAP. An inoculation suspension was produced following the procedure given in section 3.1.1 and plants were inoculated and incubated according to the procedure described in section 3.1.3.2.

The first disease scoring date (DSD1) was 3 DAI at the appearance of first symptoms of the disease. Disease scores were recorded 4 times using the scale described in section 3.1.4.2 at intervals of 3 days and AUDPC was calculated as described before.

3.2.3 Screening of Lentil Genotypes and $F_{6:7}$ RILs of Barimasur-4 × CDC Milestone under Field Conditions in Bangladesh

Through ICARDA's international support program and in collaboration with ICARDA's lentil breeder, Dr. Ashutosh Sarker, and the Bangladesh Agricultural Research Institute's (BARI) Director of Research, Dr. M. M. Rahman, arrangements were made to establish a stemphylium blight nursery at the Pulses Research Centre (PRC), Ishurdi, Bangladesh. The Pulses Research Centre had the required facility and technical help for setting up the nursery. Dr. Mohamed Harunor Rashid, Plant Pathologist at PRC supervised establishment of the lentil nursery and periodic assessment of severity of stemphylium blight.

Ishurdi is situated in the flat deltaic coastal region of Bangladesh. It has a tropical climate with a mild winter (October to March), a hot, humid summer (March to June) and a humid and warm monsoon season (June to October). January tends to be the coolest month with mean temperature of 16.6°C (maximum and minimum temperature of 23.7°C and 12.0°C, respectively) with mean RH of 78.2%. In February, the average temperature rises above 21°C with maximum and minimum temperature of 28.4 and 16.0°C, respectively. Lentil is grown in the cool, dry winter period from November to the end of February. During this period, conditions are favourable for stemphylium blight development in lentil.

The set of 20 lentil genotypes listed in the sections 3.2.1.1 and 3.2.2.1, plus 82 $F_{6:7}$ RILs of Barimasur-4 × CDC Milestone, including resistant and susceptible parents were screened for stemphylium blight resistance with natural inoculum. Utfala, a susceptible local check was repeatedly included after every two rows to promote uniform infection in the entire experiment. A single 2 m row of each genotype was grown in each of 3 replications arranged as a randomized complete block design. The date of seeding was 11 November 2004. Individual rows were scored on first incidence of the disease using a 0-10 scale as described in Table 1. All genotypes were scored 3 times at 15 day intervals and AUDPC was calculated (i) to assess the disease severity.

3.3 Statistical Analysis

All the data were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) using SAS version 9.1 (SAS Institute Inc., Cary, N.C., USA) before subjecting them to analysis of variance. Very slight differences from normality and homogeneity of variance were ignored and it was assured that data met at least one condition of the ANOVA.

In experiment 3.1.1, comparing the four isolates, and in experiment 3.1.2, comparing the four media, numbers of conidia were recorded and analyzed using PROC GLM in SAS and means were compared using Least Significant Difference (LSD) at the 0.05 % significance level.

In experiment 3.1.4 in which the aggressiveness of the two *S. botryosum* isolates SB-19 and SB-BAN was compared, data from both the repeats were pooled

(equal variances) and subjected to analysis of variance using the GLM procedure in SAS and means were compared using LSD at the 0.05% level of significance.

In preliminary screening of lentil genotypes for disease resistance (Experiment 3.2.1), the H-B scale was used to score disease severity and AUDPC was calculated using the procedure mentioned in section 3.1.3.2. An AUDPC value below 500 was considered a resistant reaction and above 500 was considered susceptible.

For data from the RILs grown in the phytotron and under field conditions, analysis of variance was done for disease severity scores of each date (DSD1 through DSD4) using PROC GLM of SAS, and means were compared using LSD at the 5% level of significance. In the field screening of the RILs in Bangladesh, disease scoring was done 3 times at an interval of 15 days. Scores for each date were plotted and assessed for segregation based on the phenotype of resistant and susceptible checks and parental lines. On the 0-10 scale, a score equal to or below 5 was considered resistant based on the scores given to Barimasur-4 (resistant parent) and a score above 5 was considered susceptible based on the reaction of CDC Milestone (susceptible parent). The frequency of RIL scores was plotted in graphs and observed for possible pattern of segregation based on one and two gene models. Correlation coefficients between the scores of different dates of field (DSD1 to DSD3) and indoor screening (DSD1 to DSD4) were calculated and tested for significance ($r \neq 0$) using a t-test at the 5 % level of significance and n-2 degrees of freedom by the following formula.

$$t = [\, r\, N - 2) \,] \,/\, 1 - r^2 \quad (df = (N - 2)) \qquad \text{(ii)}$$

Where t = t-test for significance of correlation coefficient; r = Pearson Correlation Coefficient and N = # of pairs of genotypes

4. Results

4.1 Identification of a Suitable Isolate of *Stemphylium botryosum* to be Used in Indoor Screening for Stemphylium Blight Resistance

Preliminary screening revealed considerable variability in sporulation level among many Saskatchewan isolates of *S. botryosum* (Hashemi et al., 2004). Isolates SB-16, SB-17, SB-19 from Saskatchewan and SB-BAN from Bangladesh, were selected for further screening, based on their superior ability to produce conidia in preliminary experiments. The results of further testing of the four isolates revealed significant differences in conidia production ($P < 0.0001$) (Appendix 1). SB-19 (mean = $77.2 \pm 6.2 \times 10^4$ conidia ml^{-1}) and SB-BAN (mean = $79.9 \pm 4.7 \times 10^4$ conidia ml^{-1}) produced significantly more conidia compared to SB-16 (mean = $8.0 \pm 2.8 \times 10^4$ conidia ml^{-1}) and SB-17 (mean = $11.6 \pm 3.2 \times 10^4$ conidia ml^{-1}).

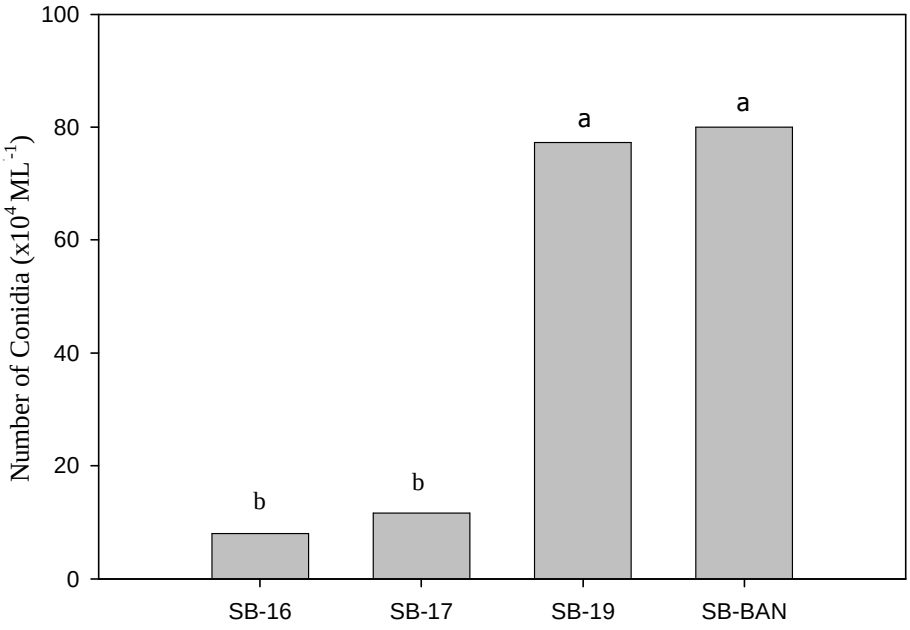

Fig. 1. Sporulation of four isolates of *Stemphylium botryosum* on V8P medium 15 days after inoculation. ($LSD_{0.05} = 12.3$).

No visible morphological differences were observed in the structure of the conidia of the four isolates. SB-19 from Saskatchewan and SB-BAN from Ishurdi, Bangladesh were not significantly different in their levels of conidia production (Fig. 1).

4.2 Identification of a Suitable Culture Medium

After several media were tested in preliminary screening (Hashemi et al., 2004), V8 Juice Agar (V8), V8 Juice with PDA (V8P) and two modified V8P media, V8PTD1 and V8PTD2, 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 ANOVA revealed significant differences among media (P = 0.001) (Appendix 2).

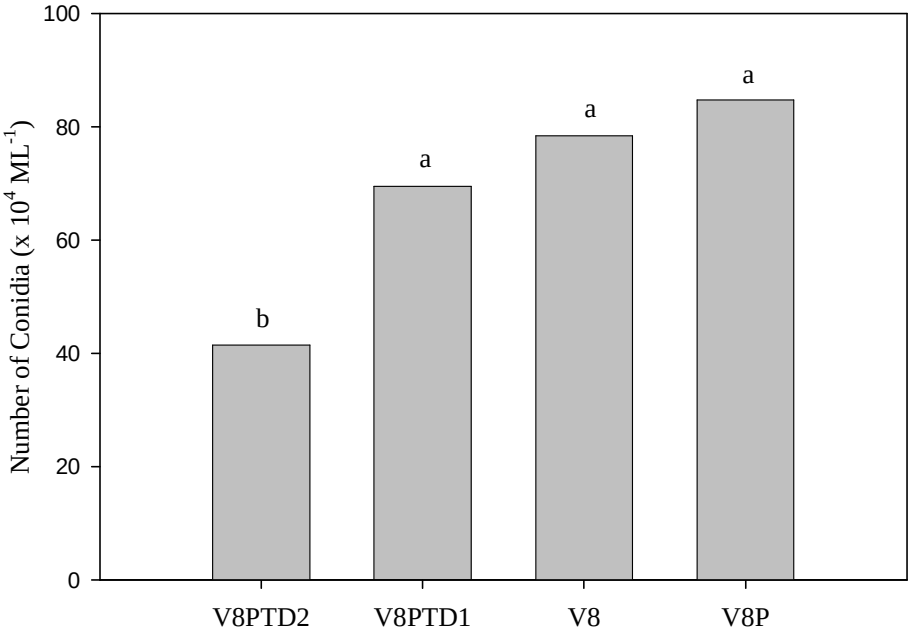

Fig. 2. Conidia production of isolate SB-19 of *Stemphylium botryosum* on four different media 15 days after inoculation ($LSD_{0.05}$ = 19.5).

The fungus produced the highest number of conidia (84.7 ± 6.0 × 10^4 conidia ml^{-1}) on V8P medium followed by V8 (78.5 ± 6.0 × 10^4 conidia ml^{-1}), V8PTD1 (69.5 ± 4.2 × 10^4 conidia ml^{-1}) and V8PTD2 (41.5 ± 6.1 × 10^4 conidia ml^{-1}).

The LSD comparison of mean conidia concentration showed that V8P, V8 and V8PTD1 were not different from each other ($P< 0.05$) but all three media resulted in significantly higher sporulation of *S. botryosum* than V8PTD2 (Fig. 2).

4.3 Determination of the Appropriate Age of Plant for Inoculation

The lentil cultivars Eston and CDC Milestone were inoculated at 14, 28, 42 and 56 DAP in order to determine the appropriate age of plant for inoculation with *S. botryosum* isolate SB-19. The data from both repetitions of the experiment were pooled (equal variances) and subjected to ANOVA.

Highly significant effects due to plant age at the time of inoculation were observed for both Eston ($P = 0.0001$) and CDC Milestone ($P = 0.0001$). The ANOVA for DAP at the time of inoculation indicated no effect of repetitions on the model for both Eston ($P = 0.12$; Appendix 3) and CDC Milestone ($P = 0.87$; Appendix 4). Non-significant interactions between replication and DAP at the time of inoculation were observed for both Eston ($P = 0.44$) and CDC Milestone ($P = 0.99$).

Non-inoculated plants showed no symptoms of the disease. CDC Milestone consistently developed more disease than Eston at all inoculations. Comparison of disease scores in relation to time of inoculation using LSD comparison revealed differences for Eston ($LSD_{0.05} = 105.6$) and CDC Milestone ($LSD_{0.05} = 33.5$) (Appendix 4.1). CDC Milestone showed significant differences in disease severity (AUDPC) when inoculated at 14, 28 and 42 DAP but the severity of disease for 42 and 56 DAP was not significantly different (Fig. 3). Disease severity ratings for Eston were generally more variable and showed a progression towards more severity with increasing age at which plants were inoculated (Fig. 3).

Although there were differences between Eston and CDC Milestone in levels of disease severity for all four inoculation dates in two experiments, an experiment consisting both resistant and susceptible genotypes will reveal the maximum differential between cultivars (Fig. 3). The first disease scores were recorded 17 DAP (3 DAI) on 2-week-old plants. However, rapid regrowth after defoliation at juvenile growth stages complicated the disease scoring. In order to deal with this problem, 42

DAP was chosen for further screening to avoid the complexity of scoring and subjectivity.

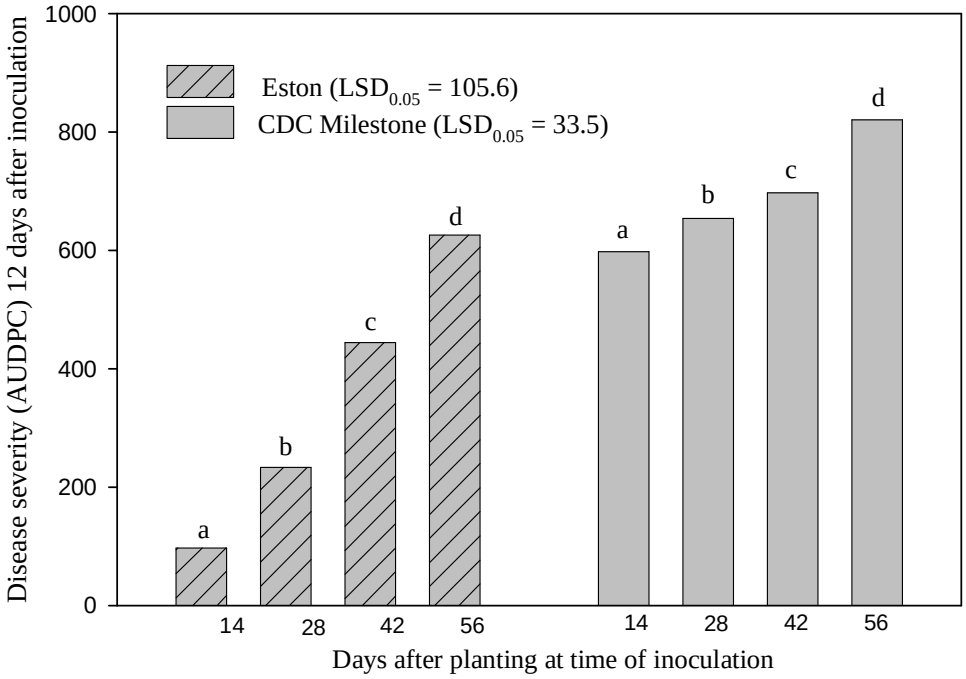

Fig. 3. Stemphylium blight severity in Eston and CDC Milestone lentil inoculated at 14, 28, 42 and 56 days after planting under greenhouse conditions.

4.4 Comparison of the Aggressiveness of Saskatchewan and Bangladesh Isolates of *Stemphylium botryosum*

CDC Milestone (susceptible) and Barimasur-4 (resistant) were used to characterize the aggressiveness of two isolates of *S. botryosum*: SB-19 and SB-BAN. SB-BAN was found to be more aggressive than the local isolate SB-19, but both reacted similarly on the differential cultivars. Significant differences in aggressiveness between isolates were evident from the ANOVA ($P < 0.001$; Appendix 5). The interaction between isolate and cultivar was not significant ($P = 0.23$; Appendix 5). Disease severity was higher on both CDC Milestone (mean = 73.6 ± 1.8) and Barimasur-4 (mean = 54.5 ± 1.9) when inoculated with SB-BAN, than when inoculated with SB-19 (63.0 ± 1.8 and 41.4 ± 1.8, respectively) (Fig. 4).

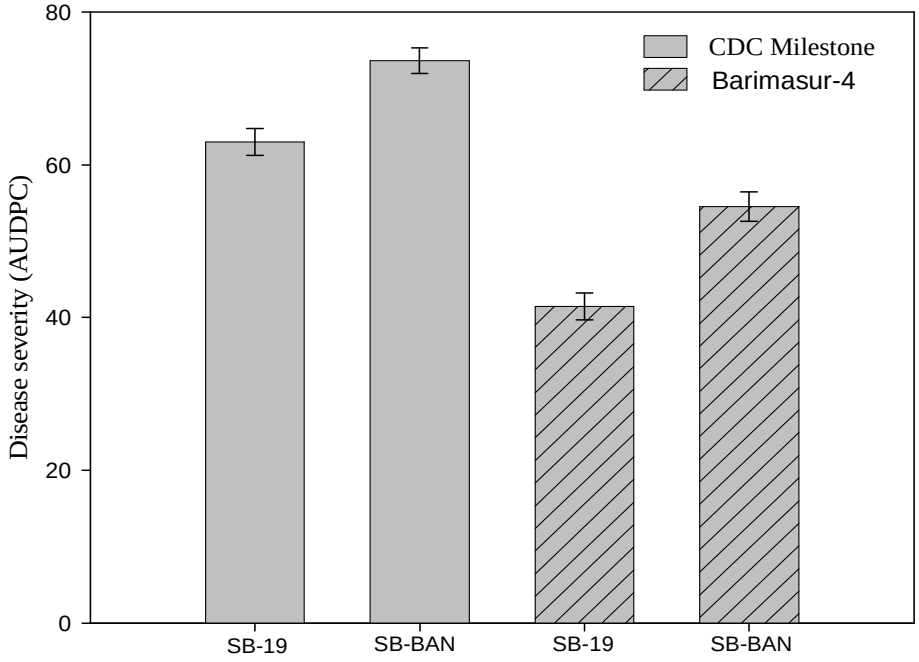

Fig. 4. Aggressiveness of isolates SB-19 and SB-BAN of *Stemphylium botryosum* on CDC Milestone and Barimasur-4 lentil inoculated 42 days after planting under phytotron conditions. Y-bars are the standard errors of means.

4.5 Screening of Lentil Germplasm for Sources of Resistance

Selected Canadian cultivars and the parents of several RIL populations developed by ICARDA were inoculated 14 DAP and evaluated for their level of resistance to *S. botryosum* isolate SB-19. Preliminary screening showed that lentil germplasm could be classified into resistant and susceptible groups (Fig. 5). Eston and CDC Blaze showed less disease severity compared to Barimasur-4. CDC Robin, CDC Glamis, CDC Milestone, CDC Redcap and Crimson were susceptible Canadian cultivars. Exotic germplasm with South Asian or Mediterranean adaptation also showed a wide variation and could easily be separated into resistant and susceptible groups. Precoz and ILL 8008 showed resistant reactions, whereas, ILL 5888-2, ILL 8007, ILL 8010, Ranjan, Asha, Subroto and ILL 8009 were susceptible. Barimasur-4 is adapted to Bangladesh and the relative difference in AUDPC between this cultivar and

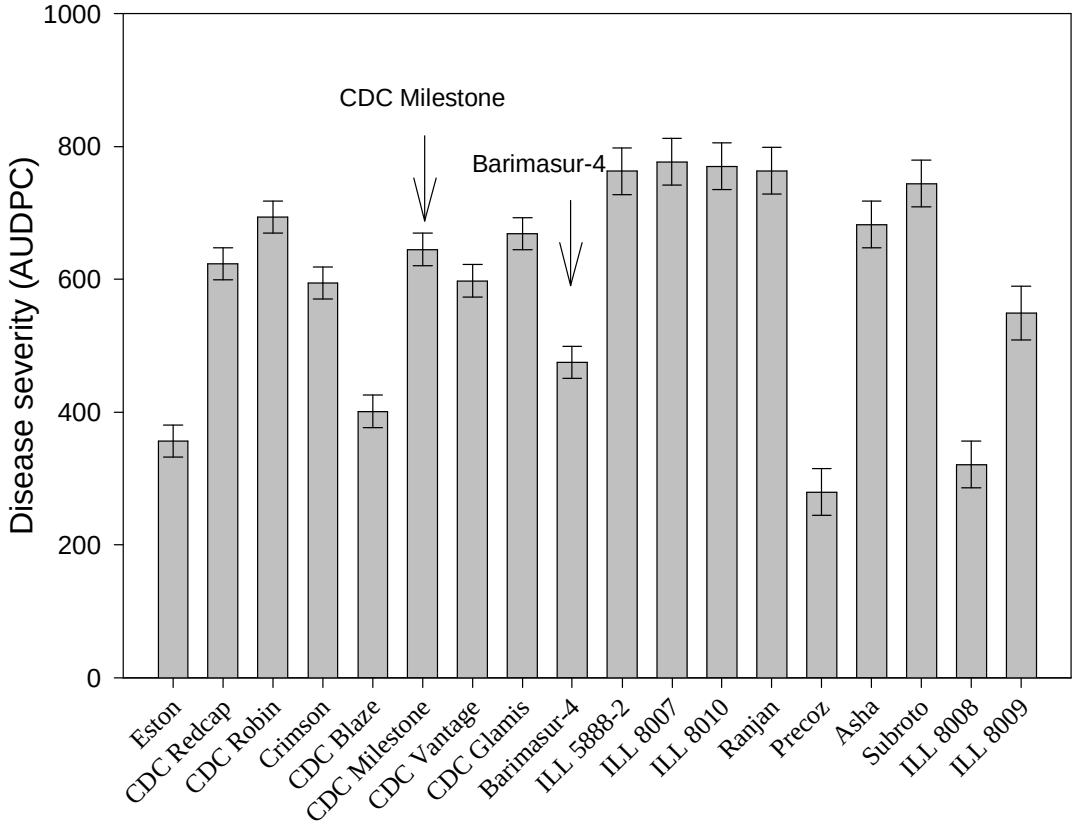

Fig. 5. AUDPC values for lentil genotypes inoculated with *Stemphylium botryosum* isolate SB-19 14 days after planting under greenhouse conditions. Y-bars are the standard errors of the means

the other Bangladesh cultivars Ranjan, Asha and Subroto is similar to that observed between AUDPC for Eston and susceptible Canadian cultivars.

A set of 20 lentil genotypes including a few advanced breeding lines was screened for resistance following the preliminary screening. Screening in the field in Bangladesh and in the growth cabinet with SB-BAN showed consistent reaction to the disease (Table 2).

Table 2: Response of lentil genotypes to *Stemphylium botryosum* inoculation when screened under three different environments.

Genotypes	Natural inoculation under field conditions in Bangladesh	Preliminary screening with isolate SB-19 in greenhouse	Phytotron with SB-BAN
CDC Milestone	S†	S	S
CDC Robin	S	S†	S†
CDC Rouleau	S	×	S
Crimson	S	S	S
CDC Viceroy	S	×	S
CDC Blaze	S	S	S
Barimasur-4	R*	R	R*
Precoz	S	R*	R*
ILL 8008	S	R	S
Eston	S	R	S

† Most susceptible genotype in the given screening condition
* Most resistant genotype in the given screening condition
× Not included in the screening

Barimasur-4 was consistently resistant in all environments, whereas CDC Milestone was consistently susceptible (Figs. 5, 6). Precoz was resistant in the controlled environments but shifted towards susceptibility in the field. CDC Blaze and Precoz were resistant in the controlled environments but shifted towards susceptibility in the field. CDC Blaze, CDC Viceroy, 1308M-7, 1227S-28, 1207D-13, 1205M-5, 1196D-5 and 1175S-12 were susceptible in all environments. For Precoz in the growth chamber and for 5 highly susceptible lines under field conditions in Bangladesh, ratings were identical for all replications, therefore no standard error bar was presented (Fig. 6).

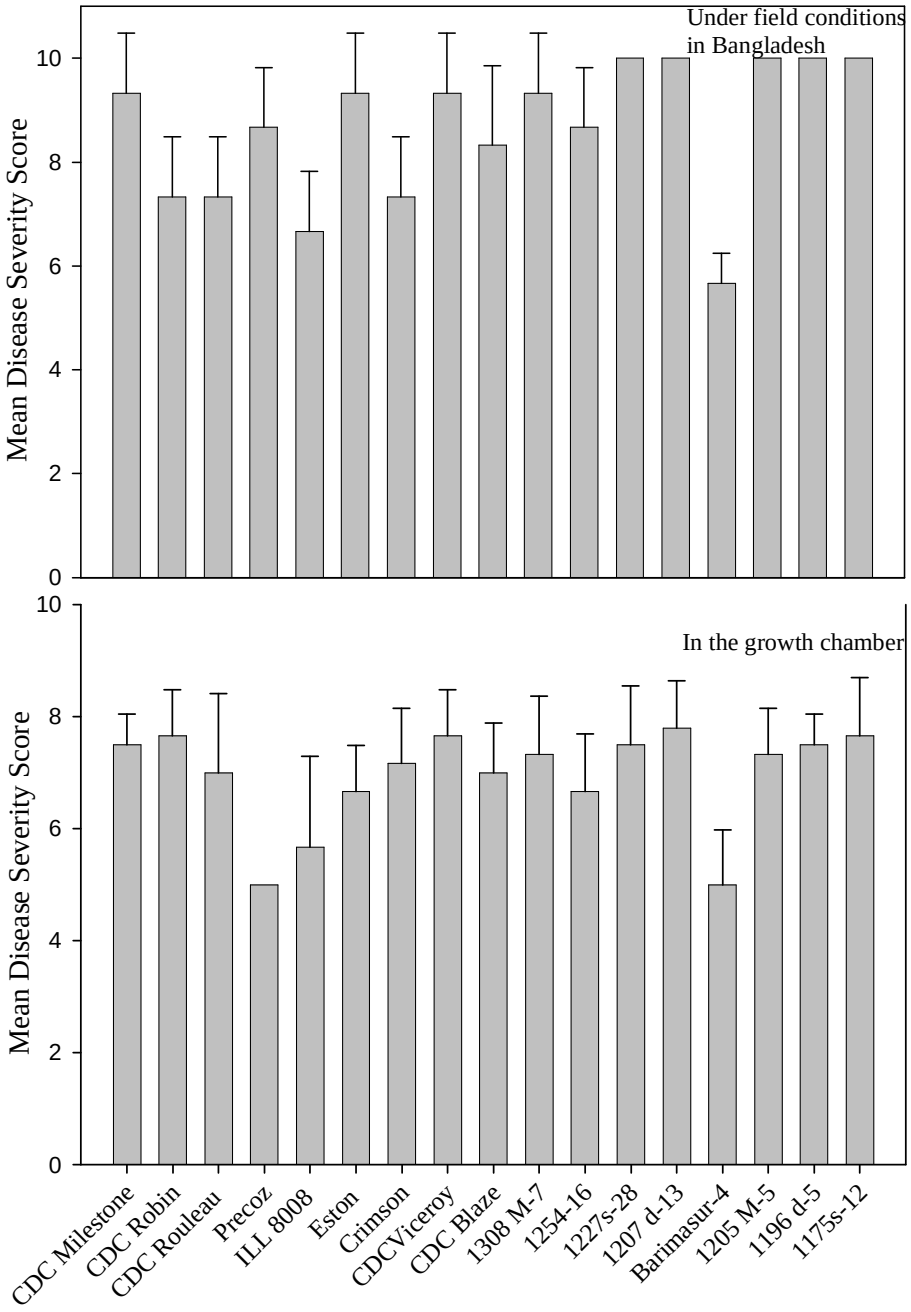

Fig. 6. Screening of lentil genotypes for resistance to *Stemphylium botryosum* under field conditions in Bangladesh with natural inoculum and in the growth chamber when inoculated with Isolate SB-19 42 days after planting. Y-bars are the standard errors of the means.

4.6 Screening of RILs under Field and Indoor Conditions

The RIL population developed from the cross CDC Milestone × Barimasur-4 showed variation in disease severity level under all screening conditions except in the growth chamber when the population was inoculated with SB-BAN isolate at 14 DAP. All the RILs plus checks died before DSD1 at 3 DAI when inoculated with SB-BAN isolate at 14 DAP in the growth chamber. Distribution of stemphylium blight severity of the RILs was compared for four different regimes (Fig. 7) and all the conditions showed differential reaction of resistant and susceptible parents except when the population was inoculated by SB-19 isolate at 14 DAP in the growth chamber (Fig. 7).

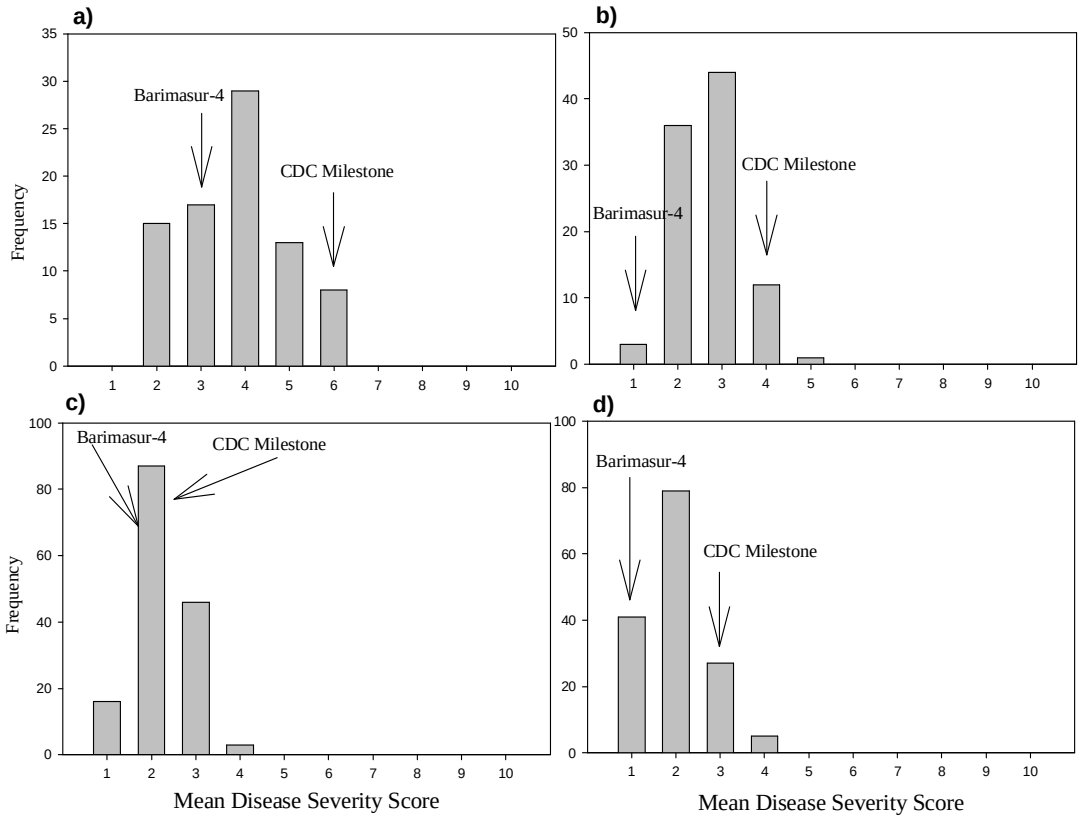

Fig. 7. Distribution of stemphylium blight severity mean disease scores for the RILs developed from the cross CDC Milestone × Barimasur-4 when scored in four different screening environments. Scores are from disease scoring date 1 (3 days after inoculation) in growth chamber experiments and 68 days after planting at the first incidence of the disease in the field under natural inoculum. a) Field at Ishurdi, Bangladesh; b) In the growth chamber inoculated with isolate SB-BAN at 42 days after planting; c) In the growth chamber inoculated with isolate SB-19 at 14 days after planting and d) In the growth chamber inoculated with isolate SB-19 at 42 days after planting.

The RIL population exhibited near normal distribution of frequency of mean disease scores under field conditions in Bangladesh for the DSD2 and DSD3 (Figs. 8a, 9a). A normal distribution of frequency of mean disease scores was also observed for DSD2, DSD3 and DSD4 when plants were inoculated with SB-BAN in the growth cabinet at 42 DAP (Figs. 8b, 9b, 10a).

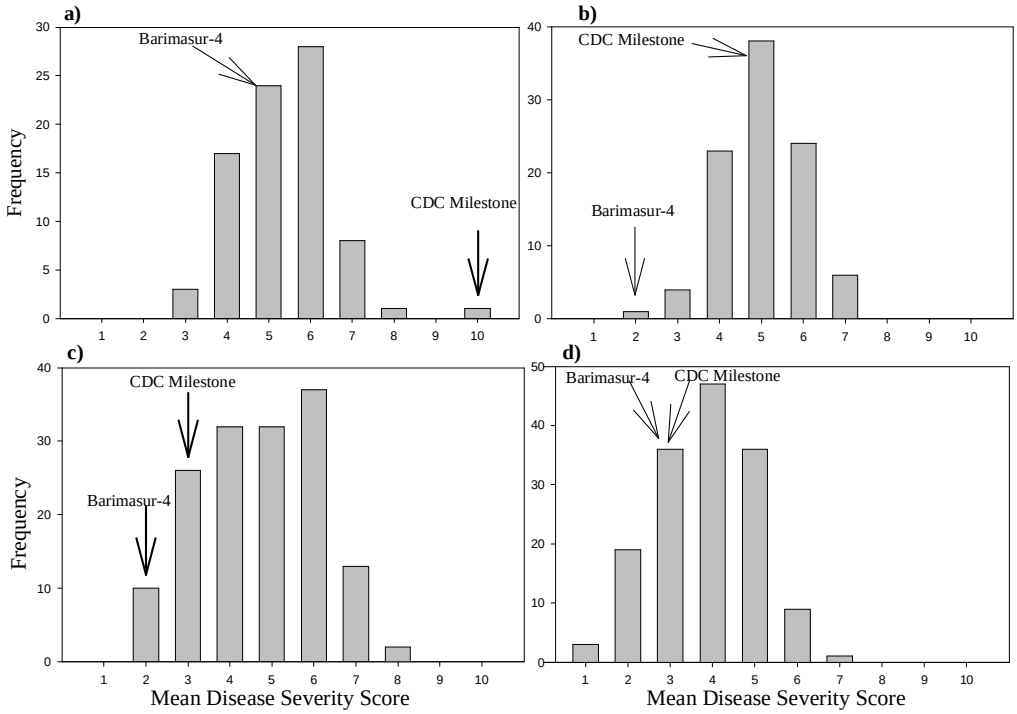

Fig. 8. Distribution of mean stemphylium blight severity scores for the RILs developed from the cross CDC Milestone × Barimasur-4 when screened in four different environments. Scores are from disease scoring date 2, 6 days after inoculation in growth chamber experiments and 83 days after planting at the first incidence of the disease in the field with natural inoculum. a) Field at Ishurdi, Bangladesh; b) In the growth chamber inoculated with isolate SB-BAN at 42 days after planting; c) In the growth chamber inoculated with isolate SB-19 at 14 days after planting and d) In the growth chamber inoculated with isolate SB-19 at 42 days after planting.

The frequency distributions (Figs. 7-10) of mean disease scores of the RILs were continuous. A bimodal distribution with two phenotypic classes would be expected if a single gene was controlling resistance. Differences among RILs for mean

disease scores and AUDPC were highly significant (P <0.05) for all disease scoring dates (DSD1 to DSD4) (Appendices 6-24).

The isolate SB-BAN provided clear separation of reaction for resistant and susceptible checks at all dates of disease scoring under field and growth chamber conditions (Figs. 7-9). In contrast, inoculation with SB-19 did not provide wide separation of these checks in the growth chamber test, though it initially provided good separation in preliminary screening of genotypes under similar conditions.

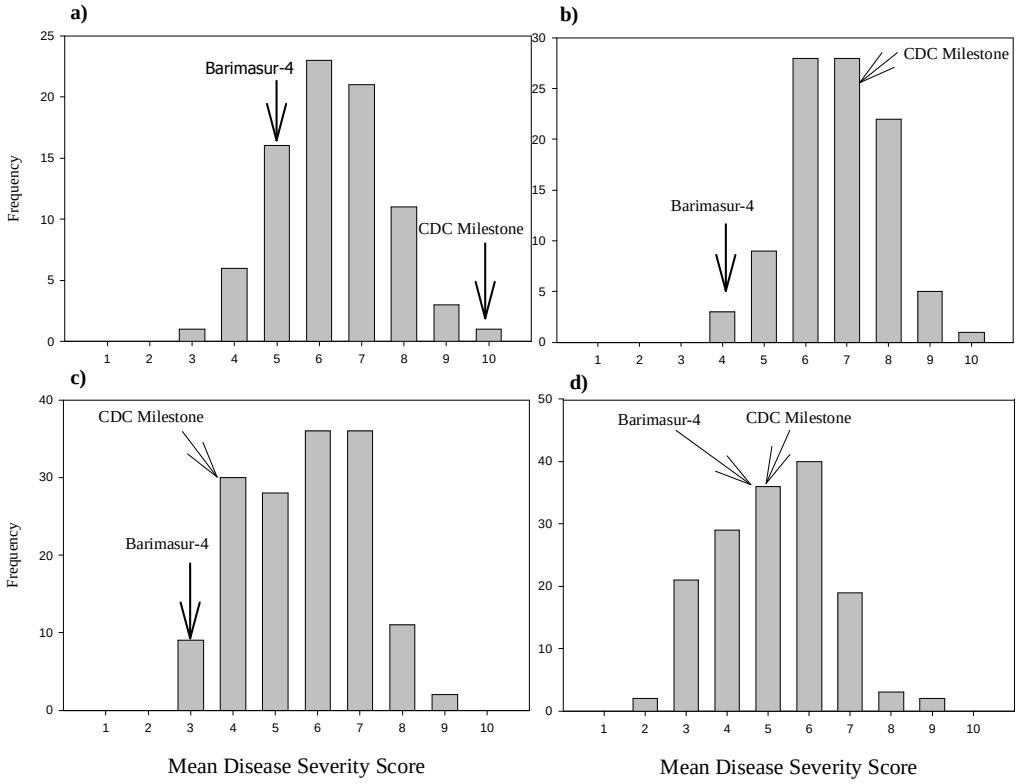

Fig. 9. Distribution of mean stemphylium blight severity scores for the RILs developed from the cross CDC Milestone × Barimasur-4 when screened in four different environments. Scores are from disease scoring date 3, 9 days after inoculation in growth chamber experiments and 98 days after planting at the first incidence of the disease in the field with natural inoculum. a) Field at Ishurdi, Bangladesh; b) In the growth chamber inoculated with isolate SB-BAN at 42 days after planting; c) In the growth chamber inoculated with isolate SB-19 at 14 days after planting and d) In the growth chamber inoculated with isolate SB-19 at 42 days after planting.

Disease scores of some RILs were higher and lower than those of the resistant and susceptible parents, indicating transgressive segregation for resistance and susceptibility. For DSD2, three lines were observed with the score of 3 compared to a score of 5 for the resistant parent under field conditions (Fig. 8a).

Seven genotypes were observed with the mean disease severity score of 7 when the susceptible parent was rated as 5 in the phytotron after inoculation with the isolate SB-BAN at DSD2 (Fig 8b). Two highly resistant lines with the score of 1 were observed when the same population was inoculated with SB-19 under growth chamber

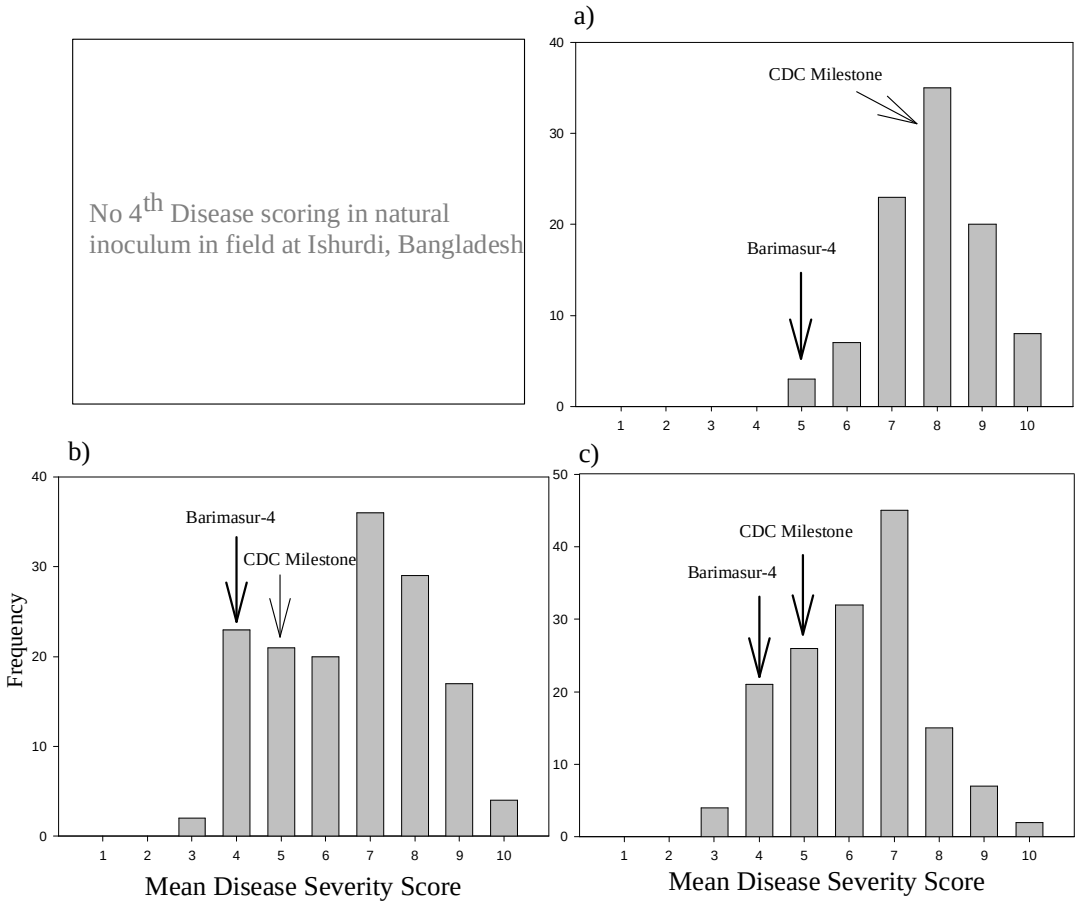

Fig. 10. Distribution of mean stemphylium blight severity scores for the RILs developed from the cross CDC Milestone × Barimasur-4 when screened in three different environments. Scores are from disease scoring date 4, 12 days after inoculation in growth chamber experiments. There was no disease scoring date 4 in the field. a) In the growth chamber inoculated with isolate SB-BAN at 42 days after planting; b) In the growth chamber inoculated with isolate SB-19 at 14 days after planting and c) In the growth chamber inoculated with isolate SB-19 at 42 days after planting.

conditions at DSD2, although no separation of the resistant and susceptible parents was observed (Fig 8d).

At DSD4, the whole RIL population, including the parents, shifted towards susceptibility under all testing conditions (Fig. 10).

There was no 4th scoring date in the field because the population reached maturity less than 2 weeks after DSD3. This rapid onset of maturity is typical of the lentil cropping system in Bangladesh where a rapid rise in temperature occurs in March. Parental disease scores at DSD3 were similar when inoculated with isolate SB-19 in the growth chamber conditions regardless of the age of plants at the time of inoculation.

Based on the plotted distribution of the frequency plots of the RIL mean disease severity scores, resistance to stemphylium blight appeared to be quantitatively inherited. Therefore, the data were not tested for the phenotypic segregation ratio of 1:1 as originally hypothesized based on the report of a single dominant gene controlling the resistance to *S. vesicarium* in onion (Pathak et al., 2001).

The correlation between individual mean disease severity scores for the RILs of different scoring dates (DSD1 to DSD4) of indoor and field screenings ranged from -0.16 to 0.12 (Appendix 25). The results of t-tests revealed non-significant correlations ($P > 0.05$) between mean disease severity scores of all the scoring dates of all environmental conditions.

5. Discussion

Crop diversification efforts have led to increased area and production of pulse crops in western Canada. Appearance of new diseases may cause instability of production and eventual exclusion of lentil from crop rotations. Cultivars that are resistant to ascochyta blight and anthracnose may become a less desirable option for farmers if they are susceptible to stemphylium blight. It is possible that changes in cultural practices may favour foliar diseases. Zero or minimum tillage certainly would increase the potential severity of infection for diseases like stemphylium blight which overwinters on plant debris.

Research on genetic resistance to fungal pathogens of crop plants requires the availability of reliable and robust indoor screening techniques. Sporulation potential could be a parameter used to test the suitability of fungal isolates for use in indoor screening techniques. The development of resistant cultivars requires a good understanding of pathogen variability and sources of resistance. The pathogenic variability, racial structure and sporulation potential of *S. botryosum* has not been previously reported in lentil. In a study by Choudhury et al. (1997), isolates of *S. botryosum* of lentil from only a small region in Bangladesh were studied for their sporulation on different media, but the results were not fully comparable as the author did not quantify the amount of sporulation and used a four category relative scale to assess amount of conidia production. The current study considered 3 out of 23 isolates previously studied (Hashemi et al., 2004) from different regions of Saskatchewan and one isolate from Bangladesh. Conidia concentration was quantified to assess suitability of these isolates for use in indoor screening of disease resistance in lentil.

Isolate SB-19 from Saskatchewan sporulated abundantly compared to others and was not significantly different from the isolate SB-BAN from Bangladesh. Therefore both isolates could be considered for use in screening for resistance. Although results from this experiment suggested that there are differences in sporulation of the isolates from different regions, a single isolate from Bangladesh cannot truly represent the pathogen population prevalent in the region. More isolates from Bangladesh could also

be evaluated for use in indoor screening. Testing their aggressiveness may also reveal their usefulness in screening for resistance. Although the results of *in vitro* experiments may not be the same as *in situ*, a comparative study under indoor conditions and in the field under natural inoculum was helpful to assess the reliability of the indoor screening technique.

Cultural conditions including temperature, light, and pH of the media cause variation in sporulation of fungal cultures (Mussi and Kurozawa, 1996; Kim and Xiao, 2005). Alternation of light and dark period should be studied more precisely to determine if these conditions have complementary or inhibitory effects on sporulation of *S. botryosum*. It was observed in this study that *S. botryosum* isolates changed their ability to sporulate after storage and subculturing. Further studies are needed to compare different *S. botryosum* isolates for their aggressiveness on the lentil host and their ability to remain aggressive if subjected to repeated subculturing. Molecular analysis may better reveal the genetic diversity and relatedness of *S. botryosum* populations from different parts of the world. A representative collection of isolates from different regions could be tested to determine if races or pathotypes exist. This would be possible through development of an international consortium that might collect and maintain a diverse isolate inventory of *S. botryosum*.

Many semi-synthetic media used to culture *S. botryosum* isolates from lentil in previous studies were not effective for inducing conidia production with the exception of wheat straw agar (Choudhury et al., 1996). Wheat straw agar tested in preliminary experiments with many Saskatchewan isolates resulted in little or no conidia production (Hashemi et al., 2004). In a disease resistance study in alfalfa, conidia of *S. botryosum* were harvested from V8-juice agar cultures and the concentration of conidial suspension (7×10^4 conidia ml^{-1}) was optimized by filtering the suspension through several layers of cheese cloth (Borges et al., 1976). Salter and Leath (1991) also used V8-juice agar and achieved 1×10^4 to 5×10^4 conidia ml^{-1}. In our preliminary testing, low levels of conidia production were observed for all media treatments, but all showed increased conidia production compared to wheat straw agar. Significantly higher sporulation was later achieved with V8 and V8P media. The isolate SB-BAN had higher conidia production than SB-19 on both V8 and V8P media.

Washing conidia from hundreds of Petri dishes is cumbersome and time consuming. Therefore, other fungal culture protocols for mass scale culture are needed to overcome the limitation for inoculation of large experiments. Inoculation using mycelia (w/v), instead of conidia was found to be effective (Hashemi et al., 2005a).

Use of toxins from culture filtrates may also show promise as a simpler method for screening large numbers of lentil genotypes. *Stemphylium* spp. produce stemphol (Solfrizzo et al., 1994), a toxin that facilitates infection of the host. Developing a bioassay to screen for resistance may be possible using culture filtrates of *S. botryosum*. Culture filtrates of *S. vesicarium* isolates were successfully used in screening for brown spot resistance in pear (Singh et al., 1999).

The current study provided a protocol by standardizing culture media and identifying suitable isolates. A previous study on *S. botryosum* (Chowdhury et al., 1996) in lentil was limited to culture method and tests of sporulation did not lead to an artificial inoculation technique. The technique developed in this series of experiments may be useful when natural disease pressure is ineffective or unreliable. In this situation it was possible to explore the variation available for resistance to *S. botryosum* in lentil germplasm using local lentil cultivars and unadapted germplasm accessions. Although the results of the disease scorings were not consistent, a basis for rapid screening of lines is now available. Once phenotyping methods become more consistent and reliable, marker assisted selection may eventually be introduced into breeding programs. More intense screening of larger collection of genotypes including wild germplasm should be done in order to determine if complete resistance can be identified. It would also be useful to use RIL populations derived from parents of similar adaptation as a mean of reducing phenotypic error in scoring disease resistance.

In determining the appropriate age of plants for inoculation with *S. botryosum*, higher disease severity was observed when inoculation was performed 42 or 56 DAP. This suggests that susceptibility of plants to stemphylium leaf blight increases as the plants reach the reproductive phase. This is possibly due to the influence of a dense canopy on disease severity at the late vegetative growth stage. Plant age at the time of inoculation had less of an effect on the development of disease on CDC Milestone (susceptible) compared to Eston (resistant). This may indicate that CDC Milestone

neither exhibited any kind of juvenile resistance nor any defense response at the later stage of development. It was evident from the experiment that a maximum separation of resistant and susceptible genotypes could be achieved when lentil plants were inoculated at 14 DAP (seedling stage) and 28 DAP (vegetative stage). This may be due to higher expression of resistance genes at these times. Chongo and Gossen (2001) also reported similar findings for ascochyta blight severity in western Canadian chickpea cultivars.

The first disease scores were recorded 17 DAP (3 DAI). In subsequent scoring of these plants, regrowth after defoliation complicated the disease scoring procedure. In order to deal with this problem, 6 week old plants were chosen for further screening of disease reactions to avoid the complexity of scoring due to regrowth. The increased variation in disease severity level observed between replications in Eston at 42 DAP may be due to variation within the cultivar. More genotypes from resistant and susceptible groups should be screened to determine and to confirm the optimum time for inoculation.

Comparing disease severity data from field studies and correlating the data with those recorded under controlled conditions may provide some idea of optimum plant age for inoculation. The wide range of adaptation of genotypes included in the current study revealed phenological differences for plants of similar age. Ignoring the actual plant age and classifying lentil genotypes based on their phenological stages at inoculation or grouping genotypes on the basis of phenology prior to inoculation could help to improve precision.

In order to make logical associations between indoor and field screening techniques for characterizing resistance to *S. botryosum*, a comparison was made between the aggressiveness of isolates SB-19 from Saskatchewan and SB-BAN from Bangladesh. Results showed that SB-19 was less aggressive than SB-BAN. Stemphylium blight is the major disease of lentil in Bangladesh, and it is very likely that more aggressive isolates exist in that environment. The isolates collected from different regions of Saskatchewan in the year 2003 might also have declined in aggressiveness due to repeated sub-culturing. Although no adverse effect of continual sub-culturing on production of stemphol has been reported for *Stemphylium* spp., changes in the

secondary metabolite profile of *Fusarium oxysporum* isolates were observed in response to continual sub-culturing (Ryan et al., 2003).

The present results do not provide an in-depth assessment of the range of aggressiveness of *S. botryosum* because they included the effect of only two isolates on two differential parents. A differential set of genotypes should be used in conjunction with a larger number of isolates from different regions to determine possible interaction. A change in ranking of genotypes may also reveal racial structure in the pathogen population. Close monitoring of the disease in the field, combined with the analysis of a collection of isolates from different regions, should be performed to evaluate the aggressiveness of the pathogen over the years. It would be useful to include more isolates from Bangladesh to determine the relatedness among populations from different regions. In Bangladesh, the pathogen population has co-existed and evolved with the host for a relatively long period compared to Saskatchewan where lentil has been grown only since the early 1970s.

Based on observations in the field in Bangladesh in 2007, *S. botryosum* is now beginning to appear in Barimasur-4 while Barimasur-5, a newly released cultivar, remains unaffected by the pathogen (A. Vandenberg, pers. com.). In Saskatchewan, cool and moist weather at later stages of crop growth and development may lead to rapid development of foliar diseases. The main concern is whether the appearance of *S. botryosum* is due to conducive weather conditions in a particular year or if the pathogen has become a part of the agro-ecosystem.

Other research initiated at the CDC at the University of Saskatchewan on the biology of *S. botryosum* should reveal the population structure of the pathogen. This information could provide a basis for assessment of the potential economic damage caused by *S. botryosum* and could also help determine strategies for the prevention of the disease by chemical and cultural control.

Screening of lentil germplasm from around the world enables breeders to identify specific geographical sources of germplasm with improved disease resistance. This approach has proven successful in Saskatchewan for improving the resistance of lentil cultivars to both ascochyta blight and anthracnose (Tullu et al., 2006). Assessment of advanced breeding lines helps breeders prepare for future threats. In this project,

screening of some of the available advanced lentil germplasm provided some indication of the range of resistance to *S. botryosum* in the CDC breeding program.

Testing of lines under natural pathogen populations, possibly in Bangladesh, could be used to screen for sources of resistance to stemphylium blight for Canadian lentil germplasm provided that altered phenology in the short day environment does not present a biological barrier to effective screening. This method was used to identify lentil lines with stemphylium blight resistance in Bangladesh, leading to release of Barimasur-4 as a resistant cultivar (Sarker et al., 1999).

It was apparent from screening in all three environments that most lentil genotypes showed consistent reactions to stemphylium blight infection. The questions arise as to whether or not races prevail in the pathogen population. This could be a source of variability in genotypic reaction. CDC Milestone, CDC Robin, Crimson and CDC Viceroy were consistently susceptible in all environments. Precoz was resistant under growth cabinet conditions but showed susceptibility in the field. Precoz is a cultivar from Argentina (Riva, 1975) and the inconsistent reaction may simply be due to poor adaptation of the line in Bangladesh. Precoz is not winter hardy and has plant height ranging from 11-21 cm. It is a green seeded, yellow cotyledon variety with test weight of 4.5 g/100 seeds (Kahraman et al., 2004). It was also registered with ICARDA as ILL 4605 and was used as one of the parents of Barimasur-4 (Sarker et al., 1999). Slightly late maturity and retention of green tissue for a longer period might possibly explain the susceptibility of the line in the field in Bangladesh. On the other hand, the pathogen population in Bangladesh may have become more aggressive. Precoz showed a higher level of resistance with both SB-19 and SB-BAN under the growth cabinet conditions. Several RILs developed from crosses with Precoz will become available within two years. These additional RILs may be useful for further investigation of inheritance of resistance to stemphylium blight.

Eston was moderately resistant when screened with isolate SB-19, but proved to be susceptible in field and under the growth cabinet conditions with isolate SB-BAN. Additional screening of germplasm including wild species could be used to find additional sources of resistance, a similar method to that used to discover genetic

resistance to anthracnose Race Ct_0 in *Lens ervoides*, *L. lamottei* and *L. nigricans* (Tullu et al., 2006).

The results of the first genetic investigation into the inheritance of stemphylium blight resistance in lentil were obtained by screening the RILs developed from the cross CDC Milestone × Barimasur-4. In general, at the initiation of disease at DSD1 (3 DAI), most RILs were scored as resistant, but as the disease progressed, the population shifted towards susceptibility (Figs. 7-10). Screening under natural inoculation conditions in Bangladesh and using the SB-BAN isolate under indoor conditions provided wide separation of disease scores for resistant and susceptible parents. Inoculation with the Saskatchewan isolate SB-19 did not result in differential reactions in the parents. This could be due to experimental error or lack of appropriate environmental conditions or they simply may not differ in their reaction to isolate SB-19. In all environments, resistance in this cross seemed to be quantitatively inherited, possibly involving many genes with minor effects. Further investigation will be required to determine the inheritance in other genetic backgrounds. The possibility exists that disease reactions were affected by phenological differences because Barimasur-4 is adapted to a sub-tropical short day environment and CDC Milestone is adapted to a temperate long day environment.

No correlation between mean disease severity scores from RILs for each scoring date could be established when experiments were compared. It was difficult to interpret and compare the results of indoor screening versus the field, possibly due to the interaction of the many other potential factors involved. Subjectivity of scoring as well as single plant scoring under indoor conditions vs. whole plot scoring in the field may also lead to inconsistent results. The field environment had entirely different conditions including differences in day length and temperature. Another factor is the presence of other major diseases like rust, caused by *Uromyces fabae*. Although Bangladesh is an endemic location for stemphylium blight, the disease appeared relatively late in the cropping season of 2004-2005. Screening of RIL populations could also be conducted at other locations such as at Dholi, India to cover the risk of poor or late disease development in Bangladesh.

Higher doses of single isolates may result in the detection of major gene resistance, but mixtures of races and pathotypes should be used to identify partial resistance. Use of lower doses of inoculum on differential genotypes may provide better results. In the current study, 2-week-old RILs and checks inoculated with the aggressive isolate SB-BAN died at DSD1 and were subsequently dropped from scoring. This result might provide a key for indoor screening if reduced conidial concentrations were applied at 14 DAP. Inoculation with half the concentration of conidial suspension of the isolate SB-BAN used in the experiment may provide better separation of resistant and susceptible groups of genotypes. The cost of screening would certainly be reduced if lentil genotypes could be screened for resistance at 14 DAP.

Determining the level of resistance to *S. botryosum* in new lentil cultivars will allow breeders to respond more quickly when the disease appears in more severe form. Screening of the RIL population developed from the cross Barimasur-4 × CDC Milestone in different environments, revealed resistance to be quantitatively inherited. A single dominant gene (Sm) was reported for resistance to leaf spot in tomato caused by *S. solani* (Behare et al., 1991; Laterrot, 1998). Resistance to stemphylium blight of onion caused by *S. vesicarium* has been reported to be under single dominant gene control (Pathak et al., 2001). In lettuce, resistance to *S. botryosum* proved to be controlled by two genes, a dominant gene Sm1 and a recessive gene sm2 (Netzer et al., 1985). The inheritance of resistance to *S. solani* in cotton was reported to be controlled by two genes with epistasis (Mehta and Arias, 2001).

The frequency distribution of mean disease severity scores for RILs of the lentil cross Barimasur-4 × CDC Milestone used in this study did not indicate involvement of a major gene for resistance. A significant correlation between indoor and field disease severity scores could not be established possibly due to subjectivity of disease scoring. The results from this study also indicated that Saskatchewan isolate SB-19 did not provide a differential response in the RIL population despite providing good separation in the disease severity scores of resistant and susceptible parents in the initial screening. A reason for this could be the loss in aggressiveness of the isolate due to repeated sub-culturing.

The current study considered the cross of Barimasur-4 (resistant) and CDC Milestone (susceptible) which had shown wide separation in the preliminary study. This was the only RIL population available with the seed in an advanced generation at the time of study. Additional crosses will soon become available for studying the genetics of stemphylium blight. These populations may have more distinct disease reactions for parents in terms of their level of resistance based on preliminary screening (appendix 26; Fig. 5 & 6). It was clearly evident from the results of preliminary screening (Fig. 5) that the crosses Precoz × ILL 8010, Precoz × Subroto, Precoz × ILL 8007, Precoz × ILL 5888-2 involve more diverse parents and may produce clearer segregation patterns based on a single gene hypothesis. Therefore use of populations derived from these crosses in further genetic investigations may be very useful.

Prevalence of phenological differences between the parents and among the RILs was a major challenge in this study. These phenological differences in lentil germplasm are primarily due to adaptation to photoperiod regimes. Significant differences from emergence to maturity were observed between Canadian and exotic lentil cultivars. Since the individual genotypes were not specifically characterized for their phenology it was difficult to synchronize the growth stages at the time of inoculation. The Canadian germplasm screened in Bangladesh did not show symptoms of senescence even at the onset of higher temperature in March, while the local Bangladesh cultivars matured quickly. The lentil germplasm adapted to Saskatchewan produced more biomass compared to the unadapted Bangladeshi germplasm. Adaptability itself is controlled by multiple genes and may possibly interact with the gene(s) for stemphylium blight resistance. Future inheritance studies using RIL populations of adapted × adapted crosses might reveal a major gene controlling resistance to stemphylium blight. An $F_{6:8}$ RIL population developed from cross ILL8008 × ILL 8009 is available immediately for further genetic study in short daylength environments.

The findings of this study provided a basis for developing a more robust indoor screening technique for breeders wishing to screen lentil germplasm for stemphylium blight resistances. The inoculation technique developed proved to be useful for small scale inoculation limited to small experiments. At the moment, field screening in Bangladesh or India appears to be a possible option for lentil breeders until a good

correlation between indoor and field screening technique is obtained. However, field screening techniques may require modifications to adjust for phenological variation. It may also be possible and ultimately more desirable to develop a marker-assisted method if accurate phenotyping of additional RILs segregating for stemphylium can be used to demonstrate qualitative inheritance or the existence of major QTLs for stemphylium resistance in lentil.

6. Summary and Conclusions

This was the first investigation of the inheritance of resistance to *S. botryosum* in lentil. The study required preliminary optimization of conidia production and inoculation protocols to meet the requirements of indoor screening as there was almost no published literature on the pathogen in lentil. Studies conducted on other host species were used as the basis for experiments. The following are the general conclusions of the current study.

1. Based on ability to sporulate, the isolate SB-19 was chosen for use in indoor screening. It was selected from a group of available isolates collected from Saskatchewan. The Bangladesh isolate SB-BAN was equal to SB-19 in sporulating ability. The SB-BAN produced more disease on lentil under indoor screening conditions.
2. V8P proved to be the best medium for sporulation of *S. botryosum* under appropriate incubation conditions. Modification of V8P medium with tamarind paste, a natural source of tartaric acid, did not facilitate release of conidia from the mycelium.
3. Maximum separation of partially resistant (Eston) and susceptible (CDC Milestone) cultivars could be achieved at 14 DAP using the SB-19, but results were not same with the parents in the RIL population. But when 2 weeks old plants were inoculated with Bangladesh isolate SB-BAN using the same conidia concentration (2×10^5 conidia ml^{-1}), all RILs studied and the checks died by the first date of scoring. This restricted the comparison with other screening methods. Therefore 42 DAP was considered the most appropriate for inoculation under indoor conditions in order to compare experiments. However, the study should be repeated with more differential genotypes using the isolate SB- BAN and reduced conidial concentration.
4. Resistance to *S. botryosum* appeared to be quantitatively inherited in the cross Barimasur-4 × CDC Milestone in both field and indoor screening. Refining the

pathological techniques and minimizing experimental error could provide better correlation between indoor and field screening.

Future Work

1. More isolates of *S. botryosum* from Saskatchewan and Bangladesh should be tested for their aggressiveness and suitability for indoor screening of genetic resistance to stemphylium blight in lentil. Understanding the genetic diversity and population structure of the fungus will provide a basis for improved screening techniques.
2. A mass-scale culture protocol for *S. botryosum* should be developed to facilitate large scale inoculation. A protocol for inoculation using a mycelial suspension of the fungus is being developed.
3. In the current screening protocol, growing plants for 42 days is time consuming. The experiment should be repeated using 14-day-old plants inoculated with reduced conidial concentrations of the aggressive isolate SB-BAN. This may provide better separation between resistant and susceptible lentil genotypes. It might be possible to compare this technique with the possible alternative of developing a bioassay protocol using the fungal toxin stemphol.
4. Barimasur-4 showed resistance to *S. botryosum* in all screening experiments. Genetic resistance to stemphylium blight was quantitatively inherited in progeny of the cross Barimasur-4 × CDC Milestone. Genetics of resistance should be determined in other genetic backgrounds with other sources of resistance to gain further insight into genetic resistance in Canadian germplasm.
5. Breeders may consider screening for stemphylium blight resistance in a disease nursery in Bangladesh, where field screening produced reliable results. If resistance genes can be identified based on consistent phenotypic reactions, developing molecular markers and employing QTL marker assisted selection may be an alternative to field screening.

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Appendix

Appendix 1. ANOVA for conidia production (10^4 ml^{-1}) of different isolates of *S. botryosum.*

Sources of variation	d.f.	M.S.	F-value	P-value
Isolates	3	18969.91	504.07	<0.0001
Replications	5	47.23	1.26	0.038
Isolate*Replications	15	37.63	7.69	0.74
Error	24	4.89		
Corrected total	47	57827.87		

R^2 = 0.98, CV = 6.90 %

Appendix 2. ANOVA for conidia production (10^4 ml^{-1}) of *S. botryosum* on different media.

Sources of variation	d.f.	M.S.	F-value	P-value
Replication	5	100.45	0.92	0.501
Media	3	1457.66	13.41	0.001
Error	15	108.66		
Corrected total	23			

R^2 = 0.92, CV = 7.60 %

Appendix 3. ANOVA for stemphylium blight severity (AUDPC) for Eston lentil inoculated with isolate SB-19 at 14, 28, 42 and 56 DAP.

Sources of variation	d.f.	M.S.	F-value	P-value
Repeat	1	133627.89	2.47	0.122
Age	3	650665.64	48.73	0.0001
Replication	5	6021.4 1	0.45	0.80
Replication*Age	15	12265.86	0.92	0.44
Error	23	13353.14		
Corrected total	47			

R^2 = 0.82, CV = 32.98 %

Appendix 4. ANOVA for stemphylium blight severity (AUDPC) for CDC Milestone lentil inoculated with isolate SB-19 at 14, 28, 42 and 56 DAP.

Sources of variation	d.f.	M.S.	F-value	P-value
Repeat	1	49.61	0.03	0.87
Age	3	107804.03	58.81	0.0001
Replication	5	7611.78	4.15	0.005
Replication*Age	15	28.23	0.02	0.99
Error	23	1832.95		
Corrected total	47			

$R^2 = 0.84$, CV = 6.18 %

Appendix 4.1. Mean AUDPC for stemphylium blight on Eston and CDC Milestone lentil inoculated at four plant ages.

Treatment	Eston	CDC Milestone
14 DAP	97.3	597.9
28 DAP	233.6	654.2
42 DAP	443.9	697.3
56 DAP	626.51	821.0
$LSD_{(0.05)}$	105.6	33.5

Appendix 5. ANOVA for disease severity caused by two isolates of *S. botryosum* on Barimasur-4 and CDC Milestone (resistant and susceptible parents).

Sources of variation	d.f.	M.S.	F-value	P-value
Repeat	1	352.88	8.98	0.004
Replication	7	56.17	1.43	0.214
Isolate	1	5862.96	149.21	<0.0001
Cultivar	1	1496.14	38.08	<0.0001
Isolate*Cultivar	1	58.18	1.48	0.23
Error	51	39.29		
Corrected total	63			

$R^2 = 0.80$ CV = 11.31

Appendix 6. ANOVA for stemphylium blight at DSD1 on RILs inoculated with isolate SB-BAN at 42 DAP.

Source	df	MS	F-Value	P-value
Rep	3	9.96	5.21	0.0016
Entry	95	2.12	1.11	0.2495
Error	284	1.91		
Corrected Total	382			

$R^2 = 0.30$ CV = 58.78

Appendix 7. ANOVA for stemphylium blight at DSD2 on RILs inoculated with isolate SB-BAN at 42 DAP.

Source	df	Mean Square	F-Value	P-value
Rep	3	17.18	5.63	0.0009
Entry	95	4.05	1.33	0.0396
Error	284	3.05		
Corrected Total	382			

$R^2 = 0.34$ CV = 37.61

Appendix 8. ANOVA for stemphylium blight at DSD3 on RILs inoculated with isolate SB-BAN at 42 DAP.

Source	df	Mean Square	F-Value	P-value
Rep	3	23.01	6.05	0.0005
Entry	95	5.16	1.35	0.0317
Error	284	3.82		
Corrected Total	382			

$R^2 = 0.34$ CV = 30.36

Appendix 9. ANOVA for stemphylium blight at DSD4 on RILs inoculated with isolate SB-BAN at 42 DAP.

Source	df	Mean Square	F-Value	P-value
Rep	3	21.17	6.10	0.0005
Entry	95	5.08	1.46	0.0090
Error	284	3.47		
Corrected Total	382			

$R^2 = 0.36$ CV = 24.77

Appendix 10. ANOVA for stemphylium blight severity (AUDPC) at DSD4 on RILs inoculated with isolate SB-BAN at 42 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	1466.58	5.96	0.0006
Entry	95	324.32	1.32	0.0437
Error	284	246.12		
Corrected Total	382			

$R^2 = 0.33$ CV = 32.73

Appendix 11. ANOVA for stemphylium blight at DSD1 on RILs at 68 DAP under field condition in Bangladesh.

Source	df	Mean Square	F-Value	P-value
Rep	2	15.14	14.01	<0.0001
Entry	81	3.71	3.43	<0.0001
Error	162	1.08		
Corrected Total	245			

$R^2 = 0.65$ CV = 29.74

Appendix 12. ANOVA for stemphylium blight at DSD2 on RILs at 83 DAP under field condition in Bangladesh.

Source	df	Mean Square	F-Value	P-value
Rep	2	47.92	23.28	<0.0001
Entry	81	3.86	1.87	0.0004
Error	162	2.06		
Corrected Total	245			

$R^2 = 0.55$ CV = 28.88

Appendix 13. ANOVA for stemphylium blight at DSD3 on RILs at 98 DAP under field condition in Bangladesh.

Source	df	Mean Square	F-Value	P-value
Rep	2	57.12	19.11	<0.0001
Entry	81	5.74	1.92	0.0002
Error	162	2.99		
Corrected Total	245			

$R^2 = 0.54$ CV = 28.91

Appendix 14. ANOVA for stemphylium blight severity (AUDPC) at DSD3 on RILs at 98 DAP under field condition in Bangladesh

Source	df	Mean Square	F-Value	P-value
Rep	2	47.92	24.45	<0.0001
Entry	81	3.86	2.32	<0.0001
Error	162	1313.10		
Corrected Total	245			

$R^2 = 0.594$ CV = 26.19

Appendix 15. ANOVA for stemphylium blight at DSD1 on RILs inoculated with isolate SB-19 at 42 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	2.19	2.85	0.0371
Entry	151	2.24	2.90	<0.0001
Error	449	0.77		
Corrected Total	603			

$R^2 = 0.50$ CV = 53.92

Appendix 16. ANOVA for stemphylium blight at DSD2 on RILs inoculated with isolate SB-19 at 42 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	6.88	4.52	0.0039
Entry	151	5.56	3.65	<0.0001
Error	449	1.52		
Corrected Total	603			

$R^2 = 0.56$ CV = 35.47

Appendix 17. ANOVA for stemphylium blight at DSD3 on RILs inoculated with isolate SB-19 at 42 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	2.73	1.36	0.2555
Entry	151	7.78	3.87	<0.0001
Error	449	2.01		
Corrected Total	603			

$R^2 = 0.57$ CV = 30.00

Appendix 18. ANOVA for stemphylium blight at DSD4 on RILs inoculated with isolate SB-19 at 42 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	1.30	0.65	0.5817
Entry	151	8.77	4.38	<0.0001
Error	449	1.99		
Corrected Total	603			

$R^2 = 0.60$ CV = 24.51

Appendix 19. ANOVA for stemphylium blight severity (AUDPC) at DSD4 on RILs inoculated with isolate SB-19 at 42 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	346.62	2.72	0.0441
Entry	151	484.96	3.80	<0.0001
Error	453	127.49		
Corrected Total	607			

$R^2 = 0.56$ CV = 31.83

Appendix 20. ANOVA for stemphylium blight at DSD1 on RILs inoculated with isolate SB-19 at 14 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	6.93	9.33	<0.0001
Entry	149	1.40	1.88	<0.0001
Error	440	0.74		
Corrected Total	592			

$R^2 = 0.41$ CV = 45.07

Appendix 21. ANOVA for stemphylium blight at DSD2 on RILs inoculated with isolate SB-19 at 14 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	46.89	17.26	<0.0001
Entry	149	7.55	2.78	<0.0001
Error	440	2.72		
Corrected Total	592			

$R^2 = 0.52$ CV = 37.65

Appendix 22. ANOVA for stemphylium blight at DSD3 on RILs inoculated with isolate SB-19 at 14 DAP under growth chamber conditions

Source	df	Mean Square	F-Value	P-value
Rep	3	10.76	4.32	0.0051
Entry	149	8.06	3.23	<0.0001
Error	440	2.49		
Corrected Total	592			

$R^2 = 0.53$ CV = 29.46

Appendix 23. ANOVA for stemphylium blight at DSD4 on RILs inoculated with isolate SB-19 at 14 DAP under growth chamber conditions.

Source	df	Mean Square	F-Value	P-value
Rep	3	6.05	2.01	0.1121
Entry	149	11.29	3.75	<0.0001
Error	440	3.01		
Corrected Total	592			

$R^2 = 0.56$ CV = 27.56

Appendix 24. ANOVA for stemphylium blight severity (AUDPC) at DSD4 on RILs inoculated with isolate SB-19 at 14 DAP under growth chamber conditions.

Source	df	Mean Square	F-Value	P-value
Rep	3	3220.97	8.39	<0.0001
Entry	149	1084.29	2.82	<0.0001
Error	447	383.97		
Corrected Total	599			

$R^2 = 0.50$ CV = 33.60

Appendix 25. Correlation of mean stemphylium blight severity scores of different dates of scorings (DSD1 to DSD4) under field and growth chamber conditions.

	Indoor scoring dates			
Field scoring dates	DSD1	DSD2	DSD3	DSD4
DSD1	-0.16 (0.502)	-0.10 (0.898)	-0.11(0.580)	
DSD2	-0.03 (0.243)	0.10 (0.871)	0.06 (0.521)	0.12 (0.077)
DSD3	-0.06 (0.522)	0.04 (0.392)	0.01(0.064)	0.06 (0.525)

P-values given in parentheses. No 4th disease scoring (DSD4) in field.

Appendix 26. Populations developed for genetic studies of stemphylium blight resistance in lentil by CDC and ICARDA.

Population	Pedegree	# of seeds/ lines	Stage
2497s	ILL8006-BM4 × CDC Milestone	157	$F_{6:8}$ seed
X2003S-71	Precoz × ILL 5888	99	F_7 seed
X2003S-73	Precoz × ILL 8007	129	F_2 seed
X2003S-73	Precoz × ILL 8007	165	F_2 seed
X2003S-74	Precoz × Subroto	145	F_7 seed
X2003S-75	Asha × Precoz	132	F_2 seed
X2003S-76	Ranjan × Precoz	160	F_2 seed
X2003S-86	ILL 8008 × ILL 8009	180	F_7 seed
X2003-96	Precoz × ILL 8010	128	F_2 seed
X2003-96	Precoz × ILL 8010	162	F_2 seed