Characterization of the stemphylium blight pathogens and their effect on lentil yield

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By

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ABSTRACT

The disease stemphylium blight has become common in lentil fields in Saskatchewan, but the effect of this disease on developing lentil plants, has not been studied under field conditions. Even though *Stemphylium botryosum* is suspected to be the pathogen causing stemphylium blight in lentil around the world, there is no confirmation of the pathogenic species involved in stemphylium blight of lentil in Saskatchewan. The objectives of this study were to determine the effect on lentil seed quantity and quality of *S. botryosum* infection at the seedling, the early-flowering, the mid-flowering or the podding stages, and to characterize the pathogen or pathogens causing stemphylium blight in Saskatchewan lentil fields.

Three field experiments were conducted over two years using green polyethylene low tunnels to create conducive environments and control the timing of infection. Cumulative disease severity, measured as area under the disease progress curve, in lentil treatments inoculated at the seedling stage was higher compared to treatments inoculated at later growth stages or to uninoculated control treatments, in which some stemphylium blight developed due to natural inoculum. Neither the amount of harvested seed, nor seed weight or seed size were reduced compared to the uninoculated control, even though disease severity was higher in inoculated *versus* uninoculated treatments. Seed infection levels of 2.6 to 3.4% in seed harvested from treatments that were inoculated at the seedling, early and mid-flower stages were significantly higher than those observed in seeds from uninoculated control treatments (0.6%) or from treatments inoculated at podding (1.2%).

Field isolates of *Stemphylium* spp. were compared to the ex-type isolate by morphology, and additionally to sequence data of five *Stemphylium* spp. and one *Alternaria* sp. obtained from GenBank by molecular phylogenetic analyses of the internal transcribe spacer (ITS) and the *glyceraldehyde 3 – phosphate dehydrogenase* (*gpd*) gene regions. Morphology of colony and conidia were not informative since features overlapped except for three isolates. Results of the molecular phylogenetic analyses revealed that *S. botryosum* is one of two possible *Stemphylium* spp. involved in the development of stemphylium blight in lentil. The three isolates with different morphology were also consistently clustered as a species distinct from the *Stemphylium* species.

Although yield loss could not be demonstrated here, further studies on the epidemiology of the pathogens causing stemphylium blight in Saskatchewan lentil fields are warranted in view of the fact that more than one candidate species was identified as the causal agent.

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DEDICATION

I want to dedicate this thesis to my dad Manuel Caudillo and my mom Dora Ruiz, to my beautiful sisters Manuela, Dora, Olympia and Edelmira and to my son Leonardo

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LIST OF ABBREVIATIONS

ACT	partial actin
AUDPC	Area under the disease progress curve
CAL	Calmodulin
CDC	Crop Development Centre
DAOM	Canadian Collection of Fungal Cultures in Ontario Canada
EF-1 alpha	Elongation Factor 1 Alpha
EGS	Edmore. G. Simmons, fungal collection from Mycological Services
gpd	glyceraldehyde – 3 phosphate dehydrogenase
GS	Glutamine synthetase
ICARDA	International Center for Agricultural Research in the Dry Areas
ITS	Internal transcribe spacer
ML	Maximum Likelihood method
NJ	Neighbor-Joining method
TSW	Thousand Seed Weight
TUB2	β-tubulin
UPGMA	Unweighted Pair Group Method with Arithmetic Mean method
vmaA-vpsA	intergenic spacer

1.   INTRODUCTION

Lentil (*Lens culinaris* Medikus) is a pulse crop species belonging to the family Leguminosae. It is widely cultivated worldwide, and similar to chickpea (*Cicer arietinum* L.) is endemic to the Middle East (Yadav et al., 2007; Muehlbauer, 2011). Lentil is an ancient crop that has been bred for thousands of years in its original location (Cubero et al., 2009). As a result of human movement, lentil was spread around the world, from Western Asia to north Africa, the Indian sub-continent, and more recently to the Americas (Bayaa, et al., 1995).

Lentil has gained in importance in the world for two major reasons. First, lentil is a nutritious food with 25 to 26% protein content (Yadav et al., 2007; Muehlbauer, 2011; Tewari et al., 2012). This is comparable to faba bean, higher than chickpea, and more than double that of wheat (Erskine et al., 1985). In addition, lentil is rich in vitamins A and B, and minerals such as phosphorus, iron, and calcium (Yadav et al., 2007). Likewise, lentil seedlings consumed as a fresh food, are rich in amino acids (Rozan et al., 2001). Lentil is a very versatile food, e.g. in India it is mainly prepared as dhal (Muehlbauer, 2011; Tewari et al., 2012; Yadav et al., 2007). Moreover, lentil crop residue is used to feed animals, providing a nutritious source of dry forage (Muehlbauer, 2011).In the textile and printing industry, lentil seed has been used as a dye (Yadav et al., 2007).

Lentil is produced on significant areas in many regions of the world. In 2013, nine countries including Australia, Canada, India, Turkey and the USA grew lentil on more than 100,000 ha each, whereas another 32 countries produced lentil on a smaller scale (FAO, 2013). In many countries, such as Turkey, the crop is consumed locally (Muehlbauer, 2011). Furthermore, major producers are also found in the Americas, from Chile and Argentina in the south, to Canada and the United States in the north, but in these countries lentil is primarily for export (Muehlbauer, 2011). In Australia, lentil production became popular at the end of the last century, and production increased gradually to more than four million metric tons by 2005, even though Australia is considered one of the countries with the lowest yield expectations, averaging 852 kg/ha. Lentil can be produced in semiarid conditions without irrigation systems or pest management (Muehlbauer, 2011). One of the benefits of lentil is the ability to tolerate dry, cool conditions; and production is well suited to

rain-fed conditions (Tewari et al., 2012). Inclusion of lentil in rotations benefits producers by contributing nitrogen to the soil, requiring less addition of nitrogen fertilizer, due to the ability of lentil to fix atmospheric nitrogen (Muehlbauer, 2011).

For these reasons lentil production has increased greatly in Canada since its introduction in 1970 (Morrall, 2003). Canada is one of the most important countries in pulse production, less as a consumer, and largely as an exporter. Canada is the top exporter of lentil in the world with an economic impact of more than CAD $ 980 million (FAOSTAT3, 2015). With more than one million tonnes produced in 2012, lentil ranked fifth among Canadian export commodities. Lentil production in Canada has risen from 142,800 to 1,537,900 tonnes during 1991 to 2012 (FAOSTAT3, 2015). In 2014, Saskatchewan produced 96% of Canadian lentil, of which 98% was exported contributing 65% of world lentil exports (Saskatchewan Ministry of Agriculture, 2015).

Lentil production is affected by biotic and abiotic factors (Tewari et al., 2012). To improve production, genetic variability of lentil cultivars is being exploited to develop varieties that offer increased yield. Some studies at the International Center for Agricultural Research in the Dry Areas (ICARDA) have focused on the phylogeny of wild lentil to evaluate their use in developing a high-yielding lentil for rain-fed field conditions (Bayaa et al., 1995). Additionally, biotic stresses such as diseases can impact productivity and consequently result in yield loss. In lentil, more than 19 fungi have been reported as disease-causing pathogens (Muehlbauer, 2011), of which stemphylium blight is one among several sighted in Saskatchewan. This disease is suspected to be caused by *Stemphylium botryosum* (Wallr.), a member of the family Pleosporacea.

In Bangladesh, the first report of stemphylium blight caused by *S. sarciformis* L. was published in 1986, where the disease has been reported to cause yield losses of more than 80% (Bakr and Ahmed, 1992). In 2009, lentil yields decreased in Bangladesh due to abiotic and biotic stresses, among which *S. botryosum* was the most important one (Rahman et al., 2010). This fungus can cause 100% yield loss under favorable conditions for stemphylium blight development (Hosen et al., 2009).

Stemphylium spp. warrant further investigation as lentil pathogens in Saskatchewan fields, in particular because the effect of this group of fungi on lentil plants has not been studied under field conditions. Furthermore, the particular species affecting lentil in Saskatchewan has not been confirmed. This project was based on two hypotheses. Firstly, it was hypothesized that

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stemphylium blight causes yield loss in Saskatchewan lentil when plants are infected at the reproductive stages. Secondly, it was hypothesized that stemphylium blight on lentil in Saskatchewan is caused by the species *S. botryosum*. To prove these hypotheses, experiments were initiated with the objectives to

1. assess the effects on lentil yield and seed quality in response to infection by *S. botryosum* at seedling, early-flowering, mid-flowering and podding stages in field trials, and to
2. characterize the species of *Stemphylium* infecting lentil in Saskatchewan by morphological descriptions and molecular phylogenetic analyses.

2.   LITERATURE REVIEW

2.1.   *Lens culinaris* Medikus

2.1.1   Origins

Lentil is an ancient pulse crop endemic to the Middle East (Yadav et al., 2007; Muehlbauer, 2011; Tewari et al., 2012), dating back to the Neolithic era, when the first humans cultivated, domesticated, and consumed this crop as an important source of nutrients. For thousands of years lentil was spread through human activities from western Asia to the Indian subcontinent, and later to Europe and some north African countries (Muehlbauer, 2011). Centuries later, during the colonization of the Americas, lentil arrived in South America and was recognized as a nutritious food source cooked in a variety of ways. In the last century, lentil arrived in North America, and has become an important economic pulse crop (Koike et al. 2001; Muehlbauer, 2011; Thomas et al. 2011; Misawa, 2012).

Molecular studies and breeding experiments with wild species have helped to determine the origins of the cultivated species *Lens culinaris*. Among the wild species in the genus *Lens*, there are two possible progenitors of *L. culinaris*: *L. orientalis* Boiss and *L. nigricans* Bieb. Both have the same chromosome number (2n =14) as *L. culinaris*, but differ in their eco-geographical origin. It has been demonstrated that *L. culinaris* is more closely related to *L. orientalis* than *L. nigricans,* which supports the conclusion that *L. culinaris* originated in the Middle East (Ladizinsky, 1979).

2.1.2   Taxonomic classification

The Leguminosae, also referred to as Papilionaceae of Fabaceae (Muehlbauer, 2011), has over 18,000 species in 700 genera, and ranks third among the largest taxonomic groups, hence it is an important family (Trinick, 1982). Within the Leguminosae, the tribe Viciaea comprises important genera such as *Cicer*, *Vicia*, and *Pisum* (Muehlbauer, 2011; Tewari et al., 2012), as well as the genus *Lens* Miller (Landizinsky, 1979). Some annual species belonging to this genus are *L. lamottei* (Czefr)*, L. ervoides* (Brign.)*, L. tomentosus, L. odemensis, L. nigricans* (Bieb.)*,* and *L.*

orientalis (Boiss.) (Muehlbauer, 2011). However the most cultivated species of the genus *Lens* is *L. culinaris* (Medik) (Landizinsky, 1979).

2.1.3 Morphology

Lentil is an annual herbaceous plant (Landizinsky, 1979). Depending on environmental conditions and cultivar, lentil plant height can vary from 15 to 75 cm. The morphology is described simply as a thin, erect stem with primary branches growing from the main stem with abundant secondary branches that give rise to flowers and eventually seeds (Muehlbauer, 2011).

Lentil has a taproot system that grows to about 60 cm in a shallow rhizosphere and forms several skinny roots (Saxena, 2009). Atmospheric nitrogen is fixed in root nodules, which develop in response to infection by the bacterium *Rhizobium leguminosarum* (Muehlbauer, 2011). Nitrogen fixation is one of the desirable features that make lentil a successful crop. The flower has papilionaceous features, is 4 to 8 mm long, and ranges in colour from white to shades of purple. Up to four flowers can be borne on each peduncle, a characteristic that facilitates self-pollination (Muehlbauer, 2011; Tewari et al., 2012). Lentil leaves are approximately 1 to 3 cm in length and compound with tendrils at the end. They are arranged in 14 leaflets, each one with a tiny stipule at the base of the stem. The pods are oblong and flattened, and each has one or two, but not more than three seeds (Muehlbauer, 2011).

Lentil seeds vary greatly in size and colour. Based on seed size, lentil cultivars can be classified into two groups: small-seeded Persian or microsperma lentil (Muehlbauer, 2011; Yadav et al., 2007) range from 2 to 4 mm in diameter with a thousand seed weight of approximately 40 g (Saskatchewan Pulse Growers. 2012.). On the other hand, large seeded or macrosperma lentil (Yadav et al., 2007) range from 4 to 9 mm in diameter (Muehlbauer, 2011) and are named Chilean with more than 50 g per thousand seeds (Saskatchewan Ministry of Agriculture. 2010). Lentil seeds are also grouped by testa colour into green or greenish-red, gray, black, brown, purple, black mottling and light tan (Muehlbauer, 2011). Varieties are grouped into ten market classes based on seed size and colour: small red, extra small red, large red, small green, extra small green, medium green, large green, French green, green cotyledon, and Spanish brown (Saskatchewan Ministry of Agriculture, 2013).

According to the Saskatchewan Ministry of Agriculture (2013), producers in Saskatchewan have the option to choose a lentil cultivar by relative maturity ranging from early to late maturing,

disease resistance (ascochyta blight and anthracnose Race 1), seed size and agro-ecological zone. This crop is adapted to the brown and dark soil zones of Saskatchewan (Saskatchewan Pulse Growers, 2012).

2.1.4 Life cycle

The lentil plant has an indeterminate growth habit, and when favourable environmental conditions are present, vegetative and generative growth overlap during the season. Lentil is highly influenced by temperature. For example, in the Mediterranean countries and North Africa where the environmental conditions are warm, vegetative and reproductive growth occurs over a shorter time (75-100 days after sowing) compared to cold regions (120-160 days after sowing) (Saxena, 2009). Even though lentil is moderately resistant to high temperature, it is considered a cool season crop (Saxena, 2009). In the Middle East - North Africa region it is cultivated as a winter crop as the rain accumulated during the rainy season is used to irrigate the crop (Tewari et al., 2012). A similar concept is applied on the Canadian prairies where the moisture accumulated from winter snow facilitates germination in spring, the cool spring conditions promote vegetative growth and the warm and dry conditions during the summer result in the maturation of the crop (Shrestha et al., 2009).

2.1.4.1 Flowering process

The papilionaceous lentil flowers can experience low levels of out-crossing by pollinators, but this is not common, and successful fecundity during anthesis relies on self-fertilization (Kaye, 1999). The flowering process may be affected by intrinsic constraints such as resource competition among plant structures during vegetative growth while flower development is taking place at the same time (Mondal, et al., 2013). It may also be affected genetically, controlled through single-gene and polygenic systems that determine the days to flowering. In lentil, for example, early-flowering is governed by the recessive gene *sn* (Sarker et al. 1999).

In addition, extrinsic factors such as the environment can affect flowering. Temperatures above 20ºC promote early flowering in lentil and reduced branching or vegetative growth, while post-flowering, dry-matter production and seed set is negatively affected (Summerfield et al. 1989).

2.1.4.2 Physiology of seed yield

The major factors that limit higher yields are similar to those that limit flowering, including biotic and abiotic stresses, poor genetic variability, and lack of genotypic adaptation (Tewari et al., 2012). Among abiotic stresses, low moisture availability is the most important since water deficits during flowering reduce plant height and leaf area, which results in reduced seed yield (Shrestha et al., 2009).

Lentil forms many flowers, yet few develop into pods. Nevertheless, long periods of flowering may lead to higher yield (Mondal, et al., 2013). On the other hand, harvest index is negatively affected by competition for plant resources between pod set and vegetative growth (Mondal et al., 2013; Malek et al., 2012). It has been suggested that increments in resources may lead to increases in seed yield; however, seed yield is determined by the interaction of genotype and environment (Hanlan et al., 2006).

Several physiological parameters are involved in the production of seed (Mondal et al., 2013). Traits such as leaf area, crop growth rate, net assimilation rate, relative growth rate, total biomass, maximum absolute growth rate and leaf area index, as well as the variability of these traits among genotypes have been described in lentil and other pulse crops such as soybean and mung bean (Mondal et al., 2013; Malek et al., 2012). High values in leaf area and high relative growth rate lead to high total biomass and therefore higher seed yields.

2.1.5   Diseases

Throughout the growing period, lentil plants are affected by abiotic and biotic stresses (Muehlbauer, 2011; Tewari et al., 2012). Worldwide, pulses are attacked throughout their life cycle and in all plant parts by a wide range of plant pathogens, such as bacteria, nematodes, phytoplasmas, viruses, fungi and also some parasitic plants (Allen and Lenne, 1998). Fungi are the largest group of plant pathogens that cause disease in legumes (Bayaa and Erkskine, 1998). They can attack any part of the host plant, but there are also some fungal pathogens that specialize on specific plant parts (Punja, 2003).

Lentil crops in Canadian fields are affected by several fungal diseases at various life stages (Morrall, 2003). Root diseases commonly found on the Canadian prairies are root rot or damping-off caused by a group of fungi often including *F. avenaceum* Corda ex Fr. Sacc, *Pythium* spp. and *R. solani*, and more recently *Aphanomyces euteiches* Drechsl. (Banniza et al., 2013). Seedling

blight and damping-off can also be caused by *B. cinerea* and seed-borne *Sclerotina sclerotiorum* de Bary. From the seedling stage to maturity lentil can be affected by foliar diseases such as ascochyta blight, anthracnose, sclerotinia stem and pod rot or white mould caused by *Sclerotinia sclerotiorum*, botrytis stem and pod rot, septoria leaf spot caused by *Septoria* spp. and stemphylium blight (Morrall, 2003). Other diseases of minor importance reported elsewhere in North America are leaf spot (*Alternaria alternate* Fr. Keisser) and leaf mould (*Cladosporium herbarum* Pers. Link), (Bayaa and Erkskine, 1998).

Bacterial diseases are considered unimportant in Canadian fields, whereas those caused by viruses have the potential to affect seed yield and quality. However, symptoms other than stunting of plants, leaf malformation and yellow or red discolorations in lentil have not been observed (Morrall, 2003). Five species of nematodes were reported in Syria and India as pathogens of lentil (Bayaa and Erskine, 1998), whereas specific data from western Canada is currently not available.

2.2. Importance of *Stemphylium* species

Several species of the genus *Stemphylium* have recently gained in importance as causal agents of plant diseases around the world. For example, *S. vesicarium* and *S. botryosum* were isolated from Chinese chive (*Allium fistulosum* L.) leaves in Hokkaido, the northernmost island of Japan in 2012 (Misawa and Yasuota, 2012). Between these two *Stemphylium* spp., the latter was identified as the causal agent of brown leaf blight inducing considerable damages in Chinese chive crops (Misawa, 2012). Moreover, *S. botryosum* can cause a serious defoliation of kiwi (*Actinidia deliciosa* C.F. Liang & A.R. Ferguson.) in Greece (Thomidis and Michailides, 2008), and this species was reported to infect spinach (*Spinacia oleracea* L.) in the United States; an incidence of approximately 29% was reported (Koike et al., 2001). There are more than 33 species of *Stemphylium* described as saprophytes or pathogens, some of which have a wide range of hosts (Camara et al., 2002) over a large geographical distribution (Table 2.1).

Table 2.1. Distribution and hosts of *Stemphylium* species

Stemphylium species	Host	Location	Reference
Stemphylium botryosum	*Allium cepa*	USA	Meredith, 1965
S. botryosum f. sp. *lycopersici*	*Lycopersicum esculentum*	Israel	Bashi and Rotem, 1974
S. botryosum	*Medicago sativa*	USA	Cowling et al., 1981
S. botryosum *S. herbarum* *S. alfalfa*	*Medicago sativa*	Austria and USA	Simmons, 1985
S. botryosum *S. vesicarium* *S. globuliferum*	Isolated from soil *Echium sp.* *Beta vulgaris* *Medicago sativa* *Simmondsia chinensis*	Austria	Irwin et al., 1986
S. vesicarium	*Asparagus officinalis*	USA	Fallon et al., 1987
S. botryosum	*Lactuca sativa*	USA	
S. majusculum	*Asparagus officinalis*	England	
S. botryosum	*Lens culinaris*	Hungary	Simay, 1990
S. botryosum	*Lycopersicum esculentum*	India	Mathur and Bhatnagar, 1992
S. vesicarium	*Pyrus communis*	Spain	Montesinos and Vilardell, 1992
S. sarciniformis	*Lens culinaris*	Bangladesh, India	Bakr and Ahmed, 1992
S. vesicarium	*Allium cepa*	South Africa	Aveling and Snyman, 1993
S. botryosum *S, majusculum* *S. botryosum*	*Brassica napus*	Italy	Solfrizzo et al., 1994
S. herbarum	*Brassica napus*	Italy	Andersen et al., 1995
S. alfalfa	*Brassica napus*	Italy	
S. vesicarium	*Brassica napus* *Pyrus communis*	Italy Denmark	
S. botryosum	*Lens culinaris*	Bangladesh	Chowdhury et al., 1997
S. vesicarium	*Pyrus communis*	Italy	Singh et al., 2000
S. vesicarium	*Allium sativum*	Spain	Basallote-Ureba et al., 1999
S. vesicarium	*Pyrus communis*	Spain	Llorente et al., 2000
S. solani	*Gossypium hirsutum*	Brazil	Metha and Brogin, 2000
S. vesicarium	*Asparagus officinalis*	Michigan, USA	Meyer et al., 2000
S. vesicarium	*Allium sativum*	Australia	Suheri and Prince, 2000

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Table 2.1: Continued.

Stemphylium species	Host	Location	Reference
S. vesicarium	*Pyrus communis*	Spain	Llorente and Montesinos, 2001
S. vesicarium	*Pyrus communis*	Italy	Rossi et al., 2005
S. botryosum	*Lens culinaris*	India	Huq and Khan, 2008
S. phaseolina	*Phaseolus vulgaris*	China	Wang et al., 2010
S. variabilis	*Allium sativum*	France	
S. botryosum	*Lupinus angustifolious*	Australia	Thomas et al., 2011
S. solani	*Solanum lycopersicum*	Malaysia	Nasehi et al., 2012
S. solani	*Cucumis sativus*	Greece	Vakalounakis and Markakis, 2013

Stemphylium species may also be pathogenic to animals and humans, and allergens produced by *A. alternate* were also reported to be produced by *S. botryosum* (Gutierrez–Rodriguez et al., 2011). The mycotoxin stemphol, exuded by *S. botryosum* and *S. majusculum,* may cause cell death in mammals as was observed in plant cells (Solfrizzo et al., 1994).

Phytopathogenic microorganism may directly affect plants by physical interference with, and obstruction or destruction of plant tissue, and / or indirect by causing different levels of stress as a result of exuded toxins (Heiny and Gilchrist, 1991). Susceptibility to a toxin-producing pathogen may differ among plant species. Phytotoxins can be host-specific, which means that a fungus produces toxins that only affect the principal host and their cultivars, and are referred to as host-specific toxins (Mehta and Brogin, 2000). Alternatively, they can be non-host-specific and affect a wide range of species (Heiny and Gilchrist, 1991). *Stemphylium botryosum* produces a toxin called stemtoxin (Heiny and Gilchrist, 1991), and some research has been done to study the role of this toxin in the context of disease development and its relationship with resistance and susceptibility. During the infection of alfalfa plants with *S. botryosum* a hypersensitive reaction was observed in resistant plants, which restricted mycelium growth compared with extensive growth on susceptible plants (Borges et al., 1976), leading to the conclusion that the toxin is a pathogenicity factor (Mehta and Brogin, 2000).

2.2.1 Yield loss due to stemphylium blight in lentil

The effect of stemphylium blight on lentil seed yield has been reported from other countries, but the potential effect in Canadian fields has not been assessed (Morrall, 2003). Reports from Bangladesh have described this fungal disease as a major problem that has caused up to 80% yield loss in lentil fields (Bakr and Ahmed, 1992; Sinha and Singh, 1993). In yield loss studies, the epidemiology of the disease was also described as highly affected by environmental conditions. Under optimal conditions and in the cases of susceptible lentil genotypes, yield loss may be 100% (Hosen et al., 2009).

Surveys in the central and northern crop districts of Saskatchewan in 2006 revealed high levels of infection of lentil seed with *Stemphylium* spp. (Morrall, et al. 2006). In more recent years stemphylium blight has been observed more frequently in lentil fields in Canada (Mwakutuya and Banniza, 2010).

The economic impact of this disease depends on the potential yield loss through reductions in seed quality and quantity harvested from highly infected fields (Banniza et al., 2006). As long as the destructive potential of stemphylium blight is unknown, the benefit of control is uncertain (Dokken-Bouchard, 2010; Morrall, 2003).

2.2.2 *Stemphylium botryosum*

2.2.2.1 Classification, taxonomy and identification

The genus *Stemphylium* belongs to the phylum Ascomycota. This phylum contains the class Loculoascomycetes, the principal feature of which is a bitunicated ascus. Grouped within the class is the large order Pleosporales, which contains numerous families and genera, many of which are important plant pathogens (Dugan, 2006; Agrios, 1997). *Stemphylium* belongs to the Pleosporacea, which is characterized by medium to large pseudothecia that do not have a compressed apex, but a round ostiole, and usually multi-septate ascospores without germ slits (Dugan, 2006). The identification of a fungal pathogen often involves the description of symptoms on the host and the morphological characteristics of the isolated pathogen (Punja, 2003). The first description of the genus *Stemphylium* was done by Wallroth in 1833. The species *S. botryosum* was described based on an isolate from asparagus (*Asparagus officinalis* L.) using the morphology of conidia,

conidiophores and mycelia (Wallroth, 1833; Simmons, 1967). More than one hundred years later, the description of *S. botryosum* by morphology as well as morphometry of conidia and conidiophores was done by Wilshire (1938). From these initial descriptions based only on morphology of the asexual state of the pathogen a common approach was developed for further species descriptions. The sexual state of *S. botryosum* was first, incorrectly, referred to as *Pleospora herbarum* Rabenh. by Wiltshire (1938). In 1838 a description of *Macrosporium sarcinula*, the asexual state of *P. sarcinulae* (= *P. herbarum*) was published by Berkeley and the conidial description matched that of *S. botryosum* by Wiltshire (1938). It was only in 1985 that *S. botryosum* was associated with *P. tarda*, whereas *P. herbarum* was found to be the sexual state of *S. herbarum* (Simmons, 1985).

Alternaria and *Ulocladium* are other genera that have been confused with *Stemphylum.* Simmons (1967) addressed this matter by describing sexual and / or asexual states of *Alternaria*, *Ulocladium* and *Stemphylium* with type specimens, which are individuals designated as representative of species. The fact that some *Stemphylium* species lost the ability to reproduce sexually has complicated their identification of species (Camara et al., 2002).

The morphology of conidium and conidiophores is commonly used for the classification of species. According to Thomidis and Michailides (2008), conidia of *S. botryosum* are about 20 to 29 µm long and 14 to 21 µm wide, with a mean length/width radio of 1.42. Koike et al. (2001) reported the dimensions of *S. botryosum* conidia as 19 to 28 x 14 to 19 µm, with a mean length/width radio of 1.43. On the other hand Bayaa and Erksine (1998) gave measurements of 24 to 40 x 14 to 25 µm length and width, respectively, for conidia of *S. botryosum*, and described them as olive brown, with a muriform and echinulated shape, with three to four septae and as being constricted in the middle. Misclassification of pathogenic species within the genus *Stemphylium* has been common since descriptions of morphological characters often overlap (Wang et al., 2010; Camera et al., 2002).

Phylogenetic analysis has developed into a major tool for the study of evolutionary reconstruction and species identification. However a phylogenetic analysis that is morphology-based tends to be limited by the lack of phenotypic characters that most microorganisms display compared with molecular phylogenetic analysis, which is more powerful and is becoming a common approach in evolutionary reconstruction and identification of species (Sleator, 2011).

Molecular phylogenetic analysis helps to describe genetic diversity such as in the case of the species *S. solani*, for which host specialization of isolates from tomato and cotton in Brazil were differentiated by the internal transcribed spacer (ITS) (Mehta, 2001). A group of *Stemphylium* spp. including *S. botryosum* pathogenic on alfalfa segregated into two clusters based on the ITS and *glyceraldehyde – 3 phosphate dehydrogenase* (*gpd*), indicating that they may represent two species, which was not supported by morphological data (Camara et al., 2002). Inderbitzin et al. (2009) studied the relationship among sexual and asexual states of *Stemphylium* spp., and showed that in some cases the sexual and its respective asexual state were grouped separately, indicated that one or the other had been misclassified.

The use of type specimens as a reference for comparison is a critical feature in the classification and identification of a pathogen. However, some type specimens were described more than one hundred years ago and have since been lost, or they are not available for molecular analyses. In such cases, designation of an epitype based on the original descriptions is necessary. In the last decade, fungal taxonomy has been increasingly based on DNA sequence data, and less on morphology or other biological parameters. Crous et al. (2014) recommended the deposition of cultures and DNA sequence data of the ex-type linked to holo-, lecto-, neo- or epitype specimens into the public database www.GeneraofFungi.org (GoF).

It has been estimated that only about 8% of the fungal species described are reliably supported with molecular data (Crous et al., 2014). Nevertheless, very conserved genic regions such as the ITS of the nuclear ribosomal DNA are now used as barcodes for fungi, but other loci are also recommended and tested for high resolution in the identification of, or differentiation among species, including the genus *Stemphylium* (Crous et al., 2014; Wang et al., 2010; Inderbitzin et al., 2009; Camara et al., 2002).

2.2.2.2 Biology and life cycle

The life cycle of *S. botryosum* on lentil is not well understood. The pathogen possesses a wide host range in the *Leguminosae*, but also in other plant families (Bayaa and Erksine, 1998). The first symptoms on lentil plants observed in Canadian fields are small beige spots on leaves and flowers (Mwakutuya and Banniza 2010). Similar symptoms are reported from Bangladeshi fields where initial symptoms begin with tiny pin-headed light brown spots that increase rapidly in size until they cover all of the tissue, turning the foliage dull yellow that gives the plants the blighted

appearance. As a result, leaflets drop, branches become bare and finally die (Bayaa and Erskine, 1998). The fungus can also cause flower abortion and consequently yield loss.

On spinach, *S. botryosum* is recognized by the early presence of leaf spots of 2 to 5 mm in diameter that are oval or circular. Spots are grey-green on immature leaves, and after the spots expand, plants decay and eventually die (Koike et al., 2001). In alfalfa, *S. botryosum* penetrates via stomata three hours after inoculation and forms bulbous sub-stomatal primary hyphae followed by intercellular secondary hyphae after 15 to 18 h. Infection spreads rapidly and symptoms become obvious. The first conidia appear five days after inoculation (Pierre and Millar, 1965).

A more complete picture of the life cycle is available for *S. vesicarium* on Chinese chive (*Allium fistollum* L.). The first stage of the life cycle occurs during the cropping season, when conidia are released from leaves with symptoms. Pseudothecia arise at the end of the cropping season. During the third stage, the pathogen overwinters in pseudothecia on the leaves. The last stage is characterized by the presence of ascospores of *Pleospora* sp., which form the primary source of inoculum for the newly grown *A. fistollum* crop (Misawa, 2012). Two types of symptoms were described, the first one a yellow mottled lesion with a diameter of 0.5 to 4 cm, and the second, brown oval lesions of 7 cm in diameter (Misawa and Yasuoda, 2012).

Environmental conditions have been recognized as a major factor in the development of stemphylium blight. For example, in the United States it was demonstrated that two biotypes of *S. botryosum* on alfalfa (*Medicago sativa*) were present. One biotype develops symptoms at temperatures of about 16 to 20°C (cool temperature biotype), whereas a second biotype develops symptoms at a temperature range of 23 to 27°C (warm temperature biotype) (Heiny and Gilchrist, 1991). Infection by *S. botryosum* on lentil can occur during a wide range of temperature from 5 to 30°C, but optimal infection was observed at temperatures of 25 to 30°C (Mwakutuya, 2006). Humidity is another important factor as Hernandez-Perez and du Toit (2006) reported that leaf spots on spinach caused by *S. botryosum* occurred more frequently when conditions were cool and moist. Intermittent foggy conditions with temperature from 25 to 27°C are favourable conditions for the development of stemphylium blight in lentil (Chowdhury et al., 1997). Similarly, Mwakutuya (2006) observed that a leaf wetness period exceeding 48 h at optimal temperatures of 25 to 30°C was conducive to infection of lentil. Disease severity of alfalfa plants can be influenced by light exposure pre- and post-inoculation with the cool-temperature biotype of *S. botryosum*.

Alfalfa plants with a 12 h pre-inoculation light exposure showed higher disease development than those with a 12 h dark expose before inoculation, and inoculated plants exposed to a 12 h light period after inoculation had higher disease severity than plants inoculated before a 12 h dark period. Plants incubated under continuous light after inoculation developed no symptoms, so light periods before and after inoculation followed by dark periods were important for disease development (Cowling and Gilchrist, 1982).

In Bangladesh *S. botryosum* was detected on lentil debris during the non-crop season where it multiplied and from where it easily spread by wind (Huq and Khan, 2008). The pathogen in Canada was reported as seed- and stubble-borne, and as a saprophyte (Morrall, 2003).

2.2.3 Control options and agronomic management

Compared to many crops, lentil is a low input crop, in particular in developing countries, so it is desirable to develop low cost agronomic and disease management methods. For this reason, recent studies have focused on the biology of the pathogen and its interaction with the host plant lentil. Moreover, the development of resistant varieties has been recognized as one feasible option (Bayaa et al., 1995). However, chemical control is the most common option to control lentil diseases.

In a Bangladeshi study on stemphylium blight control, the four foliar fungicides iprodione, sulfur, propineb, and mancozeb were applied three times at seven-day intervals after the first visible stemphylium blight symptoms appeared on lentil. All fungicides had positive effects on disease control. However treatments with iprodione had the highest seed yield followed by those sprayed with propineb and mancozeb (Bakr and Ahmed, 1992). In Bangladesh the application of iprodione at intervals of seven days from the initial symptoms is recommended. Additional cultural practices, such as delaying sowing are also recommended, although a delay in sowing can also have a negative impact on lentil seed yield (Bayaa and Erskine, 1998).

Another example is the control of brown spot of pear caused by *S. vesicarium* (Wallr.) in Europe. By using the preventive fungicides carbamates and carboximides at seven-day intervals the disease can be controlled successfully, however multiple applications are required, which increase production costs and has negative environmental effects (Llorente et al., 2000).

In Canadian fields, foliar fungicides used to control other fungal diseases such as ascochyta blight or anthracnose can also reduce the severity of stemphylium blight (Morrall, 2003). The

efficacy of the fungicides chlorothalonil, pyraclostrobin, azoxystrobin, and boscalid registered in Saskatchewan for disease control in lentil (Government of Saskatchewan, 2016) was already assessed for the control of stemphylium blight in 2005. This revealed that the fungicides boscalid followed by azoxystrobin, were more effective than chlorothalonil, or pyraclostrobin (Banniza et al., 2006). However, there are no fungicides registered in Canada to control stemphylium blight on lentil (Dokken-Bouchard, 2010; Government of Saskatchewan, 2016).

Other options to avoid losses due to diseases are based on the development of new lentil cultivars with morphological features that may influence resistance or disease escape. For example, an analysis of aerial plant structures of susceptible and resistant cultivars of lentil demonstrated significant differences in the thickness of the cuticle with more than three times thicker for resistant cultivars (0.00092 ± 0.00077 mm) compared to susceptible cultivars (0.00028 ± 0.00094 mm) (Chowdhury et al., 1997). Also, the number of stomata in resistant cultivars (20 ± 3.42) was less compared with that of the susceptible cultivar (31 ± 2.81). Fewer stomata and greater thickness of the cuticle were thought to help reduce fungal penetration.

Genetic resistance is the most economical, efficient, and environmentally acceptable options in pest control (Bailey, 2003). The exploration and exploitation of the different lentil genepools may reveal a wide range of resistance genes that can be used for the development of resistant cultivars to specific diseases (Turkington, 2003; Bayaa et al., 1995; Ladizinsky, 1979). Resistance to stemphylium blight was observed by Podder (2012) in the wild species *L. lamottei* and *L. ervoides* after screening several accessions of each species.

3.   STEMPHYLIUM BLIGHT INDUCED YIELD LOSS IN LENTIL

3.1   Introduction

Lens culinaris Medikus is the cultivated species of lentil that is widely grown as a crop because of its nutritional value and the low cost of production (Muehlbauer, 2011; Yadav et al., 2007). Well adapted to cold and dry conditions, lentil cultivation has spread from its origin in the Middle East to America in the last decades (Muehlbauer, 2011; Koike et al. 2001).

Lentil has become one of the most important crops in Canada since its introduction in the 1970's (Morrall, 2003). It is the fifth largest crop produced in Canada, and Canada ranks first as an international lentil exporter (FAOSTAT3, 2015). Moreover, the province of Saskatchewan produces 98% of the exported lentil (Saskatchewan Ministry of Agriculture, 2015), which makes Saskatchewan the dominant province in lentil production (Canadian Grain Commission, 2014).

Similar to other crops, lentil production is constrained by biotic and abiotic factors. Environmental conditions are one of the major factors that affect lentil production since lentil is highly influenced by temperatures and precipitation (Tewari et al., 2012; Saxena, 2009). Lentil genotypes adapted to warm conditions develop reproductive stages earlier than those adapted to cold weather (Saskatchewan Ministry of Agriculture. 2010). Therefore, the potential for seed yield is the result of a combination of genotypes and environment components (Hanlan et al., 2006).

Biotic stresses caused by diseases such as stemphylium blight can also have negative effects on seed yield (Banniza et al., 2006; Rahman et al., 2010; Muehlbauer, 2011). In addition to reduction in the seed yield of lentil, seed quality can also deteriorate due to diseases, both of which have direct negative effects on the value of the lentil crop (Bailey, 2003).

More than a dozen fungal pathogens of lentil have been reported around the world, of which *Ascochyta lentis* Vass., has been identified as important in Canada and South Asia, *Botrytis cinerea* Pers. ex Fr. and *Uromyces viciae – fabae* Schoret. are widely distributed, and *Stemphylium botryosum* Wallroth is a major pathogen on the Indian Subcontinent (Bayaa and Erksine, 1998). The latter, is well described as one of the major factors in seed yield reductions in Bangladesh

where it was reported to cause up to 100% yield loss (Hosen et al., 2009; Bayaa and Erksine, 1998). Likewise stemphylium blight has also been observed in Canadian lentil fields in recent years (Mwakutuya and Banniza, 2010; Morrall, et al. 2006), but investigation of the pathogen in Canada has been limited to date (Mwakutuya and Banniza, 2010).

Research into the effects of several factors involved in the infection process and disease development of stemphylium blight, such as wetness period, susceptible plant age, optimal environmental conditions, optimal conidial concentration for artificial inoculations, disease severity and efficiency of fungicides on lentil plants infected artificially under controlled conditions have been conducted in Canada (Banniza et al. 2006). However, the effect of this disease on lentil plant development has not been studied under Canadian field conditions (Morrall, 2003; Banniza et al., 2006).

Stemphylium botryosum warrants further research because this lentil pathogen is common in lentil fields in Saskatchewan. The objective of this study was to assess the effect of stemphylium blight infection at the seedling, early-flowering, mid-flowering and podding stages on disease severity, seed yield and seed quality.

3.2   Materials and Methods

3.2.1   Plant material

The Canadian lentil cultivar CDC Robin was used for this project, originating from supplies of the Crop Development Centre (CDC) of the University of Saskatchewan from production in Saskatchewan in 2002. This variety was developed at the CDC from CDC Matador / Eston / ESOR – 3 – 6 – 1 in 1992 and was registered in 1999. The line ESOR 3 – 6 – 1 is derived from a cross of Eston and a line of *Lens orientalis*, and Matador is derived from a cross of Indianhead and Eston. Partial resistance to *Ascochyta lentis* and to *Colletotrichum lentis* Damm was transferred from Indianhead to CDC Robin (Chongo et al., 1999; Buchwaldt et al., 1999). CDC Robin has a brown seed coat color and red cotyledons and fits into the small red market class of lentil. It was bred for lentil production in western Canada as a high yielding variety (Vandenberg et al., 2002).

3.2.2 Determination of the optimal conidial concentration under greenhouse conditions

A preliminary experiment was carried out to determine the optimal conidial concentration for field inoculations. Using a randomized complete block design, conidial concentrations of 1 x 10^3, 1 x 10^4, and 1 x 10^5 conidia mL^{-1} were assessed. The experiment was established in the greenhouse of the Department of Plant Sciences, University of Saskatchewan at Saskatoon during May and June of 2013. The experiment was repeated once.

Isolate SB19 of *Stemphylium botryosum* from the culture collection of the Pulse Crop Pathology Research Group of the CDC was selected to be used as inoculum for all experiments in this project. It was isolated from infected lentil plant material collected in a field near Bladworth, Saskatchewan, in 2002. This isolate had high spore production and high virulence on plants of lentil cultivar CDC Milestone when inoculated under laboratory conditions (Mwakutuya and Banniza, 2010; Podder, 2012).

Conidial suspensions were prepared with conidia previously produced in 2012 by the Pulse Crop Pathology Research Group of the CDC (Klassen et al., 2012). Conidial suspensions were prepared by adding 1 g of conidia to 1000 mL of distilled water in continuous agitation for at least 30 min. Dilutions to achieve the required spore concentrations were determined by counting four preparations of the suspensions on a Neubauer hemocytometer and diluting to obtain the desired conidial concentrations of 1 x 10^3, 1 x 10^4, and 1 x 10^5 conidia mL^{-1}. Before inoculation two drops of Tween® 20 surfactant per 1000 mL of suspension were added.

Pots of 10 x 10 cm were seeded with CDC Robin at a density of six seeds per pot into Sunshine® No. 4 mix mixed with perlite (3:1). Four replicate pots per treatment were planted. After seedling emergence, plants were fertilized with fertilizer solution prepared with PlantProd® (20–20–20 plus micronutrients) once a week.

Three weeks after planting, plants were thinned from six to four plants per pot and were inoculated by spraying each conidial suspension with an air brush until run off using approximately 3 mL of conidial suspension per plant. Plants were incubated for 48 h in high humidity in a misting chamber before being returned to the greenhouse bench. Lentil plants were grown in the greenhouse with misting irrigation at 22°C during the day and 20°C at night, with a photoperiod of 16 h under natural light supplemented with 400 watts high pressure sodium lamps at light intensity of 300 - 1100 µmol $m^{-2} s^{-1}$.

3.2.3   Inoculum production for field experiments

A stock of about 40 g of *S. botryosum* SB19 conidia with above 90% spore germination was available from previous production to use for the first experiment in 2013. However, it was necessary to continue with the mass production of conidia to have enough inoculum for the second field experiment in 2014. The inoculum was produced from 2013 to 2014 at the Crop Science Field Laboratory facility of the CDC following the procedures developed by Klassen et al. (2012) with further adaptation (Appendix 1). Conidial suspension for field experiments were prepared following the same procedures as described in section 3.2.2 and each field plot was inoculated with 1.5 L of 1×10^3 conidia mL^{-1} of the conidial suspension.

3.2.4   Field experiments

Three field experiments were conducted to estimate lentil seed yield loss in response to stemphylium blight infection at different growth stages using a randomized complete block design with two factors. In 2013 and 2014, experiments were established at the Seed Farm at Saskatoon (52$^\text{o}$ 08' 08.5" N, 106$^\text{o}$ 37' 13.5" W), and in 2014 a second experiment at the Preston Avenue experimental site (52$^\text{o}$ 07' 35.5" N, 106$^\text{o}$ 37' 19.6" W) was added.

The first experimental factor was the timing of inoculation at four growth stages: seedling, early-flowering, mid-flowering and the podding stages (Table 3.1). An uninoculated control was also included. A second experimental factor consisting of low tunnels was added to limit exposure to natural infection by *S. botryosum*, and to allow for inoculation at specific growth stages of lentil plants. Low tunnels also created a microclimate conducive to infection. To estimate the effect of cover materials (low tunnels) on lentil development and yield, all inoculation treatments were also applied to non-covered treatments.

Table 3.1: Inoculation dates for the four lentil growth stages in field experiments at the Seed Farm 2013 and 2014, and at the Preston Avenue site in 2014.

Lentil growth stages	2013 Seed Farm	2014 Seed Farm	Preston field
Seedling	12-June (26 d.a.s.)	12-June (30 d.a.s.)	12-June (30 d.a.s.)
Early-flowering	02-July (46 d.a.s.)	02-July (50 d.a.s.)	02-July (50 d.a.s.)
Mid-flowering	18-July (62 d.a.s.)	17-July (65 d.a.s.)	17-July (65 d.a.s.)
Podding	02-August (77 d.a.s.)	06-August (85 d.a.s.)	06-August (85 d.a.s.)

d.a.s: Days after seeding; Seedling: 12 nodes; Early-flowering: 15 nodes; Mid-flowering: 24 nodes, from 20 to 24 nodes with new flowers and from 13 to 16 nodes with pods in the filling process; Podding: 24 nodes and 80% pods in the filling process.

In 2013, the two cover materials green polyethylene and Novagryl® were included. Novagryl® is a highly stretchable three layer fleece made of polypropylene. Novagryl® filters out 20% of incoming light. It is supplied in rolls of 1.9 m width (Crop Solutions Ltd, 2007). Perforated green polyethylene filters out 41% of incoming light, and also induces cool conditions (Waterer et al., 2011) (Fig. 3.1).

Figure 3.1: Low tunnels made with Novagryl® (front left) and green polyethylene (front right) materials in the field experiment at the Seed Farm in 2013.

Although both materials were similarly successful in creating conducive infection conditions in 2013, the plants under green polyethylene low tunnels developed higher disease severity and were less severely infested by aphids. Therefore, only green polyethylene was used in experiments in 2014.

3.2.4.1 Experimental procedures

In all experiments plot size was approximately 4 x 1 m (4 m^2) with three rows at 30 cm spacing. Pre-seeding and post-seeding chemicals applied in the field and the agronomic management procedures are summarized in Appendix 2. Field plots were inoculated when plants had reached the respective growth stage by spraying 1.5 L conidial suspension of 1 x 10^3 conidia mL^{-1} of isolate SB19 per plot with a 3.8 L Gilmore$^®$ hand sprayer, which was equivalent to run off.

Low tunnels were established immediately after inoculation, which was 25 and 29 days after seeding in 2013 and 2014, respectively. Four supportive wire hoops per plot were used to create low tunnels in plots treated as covered, and each cover material was tightened at ground level with six sand bags per plot to facilitate easy opening of tunnels for inoculation and assessment. The tunnel material was temporarily removed once a week at the time of disease assessment and when an inoculation took place. Plots were covered again right after inoculation. All plots were then exposed to overhead misting irrigation for 1 h to ensure high humidity for infection.

3.2.4.2 Data collection and analyses

Stemphylium blight severity for the preliminary experiment under greenhouse conditions was recorded at seven days after inoculation followed by three successive ratings at three-day intervals. A semi-quantitative scale developed for stemphylium blight assessment by Banniza et al. (2006) was used (Table 3.2).

Severity of stemphylium blight in the field experiments was assessed prior to each inoculation in all treatments (except for the first rating in 2013) on a weekly basis in all field experiments. A quantitative scale ranging from 0 to 10 with 10% increments was used (Table 3.3)

Table 3.2: Semi-quantitative rating scale to assess stemphylium blight on lentil plants under greenhouse conditions

Scale	Symptoms
0	healthy plants
1	few tiny lesions
2	a few chlorotic lesions
3	expanding lesions on leaves, onset of leaf drop
4	1/5th of nodes affected by lesions and leaf drop
5	2/5th of nodes affected
6	3/5th of nodes affected
7	4/5th of nodes affected
8	all leaves dried up
9	all leaves dried up but stem green
10	plant completely dead

Table 3.3: Quantitative rating scale to assess stemphylium blight severity of each plant in field experiments.

Scale	Percentage of plant damaged
0	0
1	1 - 10 %
2	11 – 20 %
3	21 – 30 %
4	31 – 40 %
5	41 – 50 %
6	51 – 60 %
7	61 – 70 %
8	71 – 80 %
9	81 – 90 %
10	91 – 100 %

Five arbitrarily selected plants were rated per treatment. Eight ratings were done in 2013 and 10 in 2014 (Table 3.4). Quantitative data were transformed to percentage disease severity using the class mid points. The average rating per treatment was used to calculate the Area Under the

Disease Progress Curve (AUDPC) as a measure of repeated quantitative disease ratings (Shaner and Finney, 1977):

$$AUDPC = \sum_{i=1}^{n} [(Y_{i+1} + Y_i)/2] * [X_{i+1} - X_i]$$

Where Y_i is disease severity in percentage at the ith observation, X_i is number of days after inoculation at the ith observation, and n is the total number of observations (Shaner and Finney, 1977).

Table 3.4: Dates of disease severity assessments in field experiments at the Seed Farm in 2013 and 2014, and the Preston Avenue site in 2014.

Ratings	2013 Seed Farm	2014 Seed Farm	2014 Preston field
1st	26-Jun	11-June	11-June
2nd	02-Jul	18-June	18-June
3rd	10-Jul	25-June	25-June
4th	17-Jul	01-July	01-July
5th	23-Jul	9-July	9-July
6th	31-Jul	16-July	16-July
7th	08-Aug	22-July	22-July
8th	13-Aug	30-July	30-July
9th	---	05-August	05-August
10th	---	11-August	11-August

Seed harvest was performed with a combine for each plot / treatment. Harvested seeds were dried and weighed. Lentil seed yield was calculated based on the harvested plot area of 4 m². Randomly selected seeds from the original sample recovered from each plot / treatment were assessed for Thousand Seed Weight (TSW), percent seed infected by *S. botryosum*, percent seed staining, and seed size (diameter and thickness). TSW per plot / treatment was estimated by weighing 200 seeds and multiplying by five. The percentage of stained (deviation from the

characteristic mottling of CDC Robin seeds) or wrinkled seeds were visually estimated from 100 randomly selected seeds (Canadian Grain Commission, 2014).

To determine the percentage of seed infection with *S. botryosum*, 100 randomly selected seeds from each original sample were placed into ten Petri dishes of 90 mm diameter filled with potato dextrose agar (PDA) medium. The Petri dishes were incubated under continuous light at 25°C, and the assessments were made after seven days of incubation by counting the number of infected seeds. Then glass slides of fungal structures were prepared to confirm the identity of the pathogen based on morphology by microscopy (International Seed Testing Association, 2014).

For seed sizing, 250 g of seeds from the original sample were passed through a set of five round-holed screens with diameters ranging from 5.16 mm (13/64") to 3.57 mm (9/64"), and six slotted screens with holes from 2.87 mm (7.5/64") to 1.98 mm (5/64") in increments of 0.2 mm (1/64") to estimate diameter and thickness, respectively. Percentage of seeds retained in each screen was used to calculate average seed size as follows (Fedoruk, 2013):

$$\text{Seed size} = \Sigma \ (\% \ \text{of seed on screen} * \text{screen hole size (mm)}) \ / \ 100$$

Data were tested for normality of residuals with the Shapiro-Wilk test. In some cases it was necessary to transform data to meet the assumptions of ANOVA. Homogeneity of variances was tested with the Levene's test. Heterogeneous variances were modeled with the repeated statement in the mixed model procedure. All statistical analyses were carried out with the Statistical Analysis Software (SAS, Institute Inc.2013). Repeated measures analyses of data from the preliminary experiment under greenhouse conditions were conducted with the mixed model procedure. Conidial concentrations and time (measured in days after inoculation) were treated as fixed factors, whereas blocks and repeats were considered random factors. Time was considered the repeated measure.

For field experiments, trends in individual experiments were similar, so only combined results for the three site-years are presented. Data collected from the low tunnels covered with Novagryl® for 2013 were excluded for this purpose, but results of individual experiments (including Novagryl®) are presented in Appendix 4.

For combined analyses of field data, site-years and blocks were treated as random factors and the inoculation timings and tunnels were considered fixed factors. As an initial step, covered

(low tunnels) and non-covered plant responses were compared by simple linear contrasts. Then the efficacy of inoculations in low tunnels was determined with simple linear contrasts between the uninoculated control and the inoculation treatments in tunnels. Treatment means among covered plots were also compared with Fisher's least significant difference or simple linear contrasts.

3.3 Results

3.3.1 Greenhouse experiments

Repeated measures analysis indicated a very highly significant effect of conidial concentration, time and their interaction on stemphylium blight severity ($P < 0.0001$). For all concentrations, stemphylium blight severity increased with time. Disease severity increased incrementally with higher conidial concentrations, and reached 7.4 on the rating scale after inoculation with 1×10^5 compared to 3.7 after inoculation with 1×10^3 conidia mL^{-1} (Fig. 3.2).
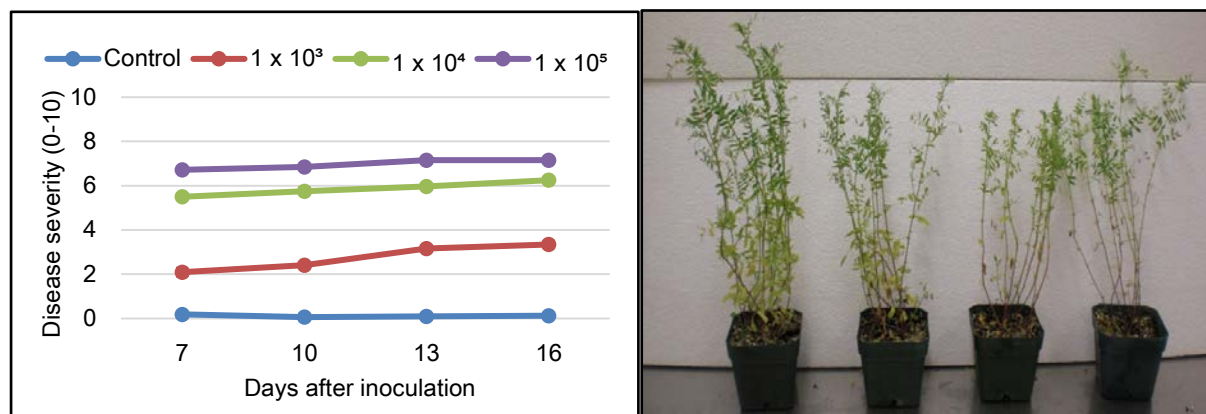
Figure 3.2: Left: Stemphylium blight severity at seven, ten, thirteen, and sixteen days after inoculation on lentil cultivar CDC Robin inoculated with three different conidial concentrations of *Stemphylium botryosum*. Right: Plants of CDC Robin inoculated with *Stemphylium botryosum* under controlled conditions. From left to right: Control, 1×10^3, 1×10^4, 1×10^5 conidia mL^{-1}

3.3.2   Field experiments

3.3.2.1 Stemphylium blight severity and yield loss

Rainfall from May to August in 2013 and 2014 reached 180 and 222 mm, respectively. The maximum temperatures in the crop season 2013 and 2014 were 32°C and 31°C, respectively, and the minimum temperature in both years was -5°C (Environment Canada, 2015).

Stemphylium blight was observed in treatments in low tunnels and in the non-covered treatments, including the control treatments at both sites and in both years. At the Seed Farm in 2013, plants under low tunnels had on average 53% stemphylium blight severity (mean of all treatments at last rating date) compared to 42% in the non-covered treatments. Similar effects were observed in experiments in 2014, where disease levels of 72% and 83% developed on plants in low tunnels compared to 60% and 61% disease severity on plants in non-covered treatments at the Seed Farm and at the Preston Avenue site, respectively.

Stemphylium blight was relatively uniform among non-covered treatments as expected due to infection through natural inoculum. Disease in control treatments in low tunnels was likely caused by natural infection during the weekly assessment of disease severity when the cover material was removed for disease assessments. Furthermore, low tunnel materials are permeable to air and possibly conidia. To assess the effect of tunnels on lentil plants and disease, their development in tunnels and in non-covered treatments was compared. The effect of stemphylium blight infection at different growth stages was then evaluated in detail for plants grown in low tunnels only where infection timing could be controlled to a certain degree.

3.3.2.2 Plant responses in low tunnels vs. non-covered treatments

Analysis of AUDPC data including the non-covered treatments revealed significant effects of inoculation timing ($P = 0.0001$) and cover treatments ($P = 0.0001$). The interaction of cover treatment and inoculation timing was not significant ($P = 0.0507$). Contrast analysis between covered and non-covered treatments revealed a highly significant difference ($P = 0.0001$). Data collected from covered treatments had an average of 69% higher cumulative disease compared to non-covered treatments.

The analysis of seed yield data including the non-covered treatments revealed non-significant effects of inoculation timing and the interaction between cover treatment and

inoculation timing (P = 0.9346). Covering treatments had significant effects on seed yield (P = 0.0001) and the linear contrast between covered and non-covered treatments revealed a highly significant difference in seed yield (P = 0.0001). Seed yield was 43% lower in tunnels compared to non-covered treatments.

Analysis of thousand seed weight (TSW) data including the non-covered treatments revealed that inoculation timing (P = 0.3171) and its interaction with covered treatments (P = 0.381) had no effect on TSW, whereas the covered treatment had a significant effect (P = 0.0021). Contrast analysis revealed a highly significant difference between covered and non-covered treatments (P = 0.0021) with on average a 4% reduction in TSW in low tunnels compared to non-covered treatments.

The percentage of seed infected with *Stemphylium* spp. was neither affected by inoculation timing (P = 0.178) nor by the covering treatment (P = 0.0831) when analyzing data from covered and non-covered treatments. Inoculation timing had an effect on seed staining (P = 0.0001), but not the covered treatments or the interaction (P = 0.0915). Inoculation timing or its interaction with the cover treatments had no effect on seed diameter (P = 0.8668) or seed thickness (P = 0.7861), yet the cover treatments had a highly significant effect on diameter (P = 0.0001) and thickness (P = 0.0045). Linear contrast analysis revealed that tunnels reduced seed diameter by 3% (P = 0.0001) and seed thickness by 3 to 5% (P = 0.0045) compared with non-covered treatment. In summary, inoculation timing had no effect on plants in non-covered treatments, but lentil plants grown in tunnels had higher disease levels, lower seed yields, lower TSW, higher percentage of seed infected with *Stemphylium* spp. and higher percentage of seed staining, which confirmed that low tunnels were effective in creating a more conducive environment for infection and that inoculations were successful. In addition to the impact of higher disease levels, cover materials themselves probably had a negative effect on yield. However, since yield loss was assessed in relative terms by comparing inoculated with uninoculated covered control treatments, further analyses to determine the effect of infection at different growth stages on stemphylium blight severity (AUDPC), seed yield and seed quality were conducted on data from treatments under low tunnels only.

3.3.2.3 Disease development in low tunnels in field experiments

The analysis of AUDPC data collected from green polyethylene low tunnels revealed a highly significant effect of inoculation timing (P = 0.0001). The effect of inoculation timing on

AUDPC was confirmed by simple linear contrast analyses between the uninoculated control treatments *versus* the inoculated treatments. AUDPC in control treatments were significantly lower compared to treatments inoculated at various growth stages. Means comparison revealed that the level of stemphylium blight on plants inoculated at the seedling stage was higher than on uninoculated plants or plants inoculated at later growth stages. Cumulative stemphylium blight severity of the treatments inoculated at the seedling stage was increased by 42% compared to the uninoculated treatments, and by on average 13% compared to treatments inoculated later, which developed similar levels of disease (Fig. 3.3).
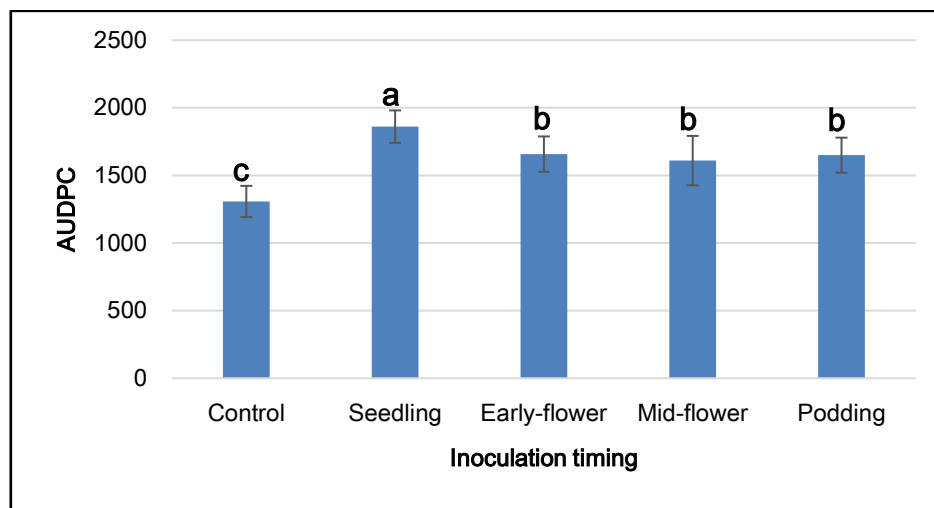
Figure 3.3: Area under the disease progress curve (AUDPC) of stemphylium blight on lentil cultivar CDC Robin grown under green polyethylene low tunnels in three field experiments at Saskatoon in 2013 and 2014. Treatments were inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four growth stages. Separation of means by Fisher's LSD ($P <$ 0.05) represented by different letters above bars.

3.3.2.4 Lentil seed yields harvested from low tunnels

Seed yields ranged from 1642 to 1808 kg / ha (Fig. 3.4). There was no effect of inoculation timing on seed yields ($P = 0.7687$).
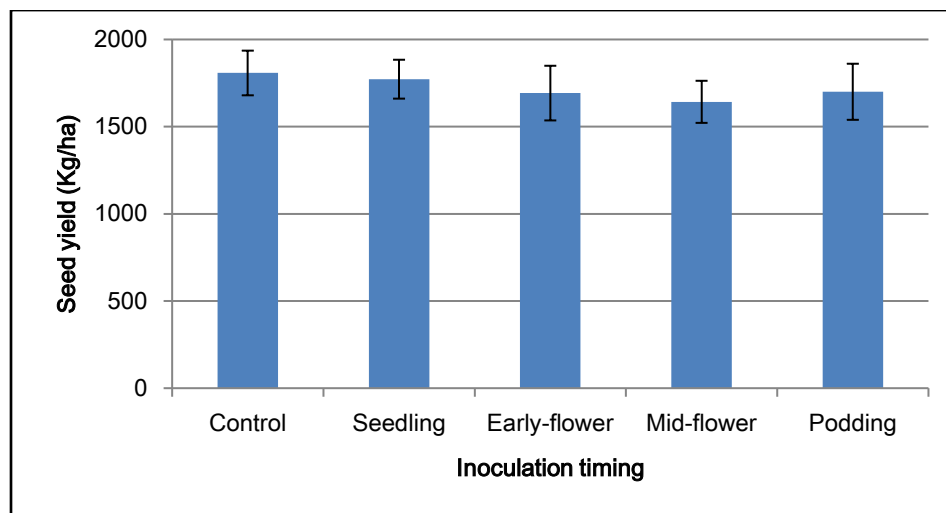
Figure 3.4: Seed yield of lentil cultivar CDC Robin grown in green polyethylene low tunnels in three field experiments at Saskatoon in 2013 and 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four growth stages.

3.3.2.5 Thousand seed weight of seed harvested from low tunnels

The TSW ranged from 25.4 to 26.4 g (Fig. 3.5) and analysis revealed a non-significant effect of inoculation timing ($P = 0.1762$). Based on observations for disease development, linear contrast analyses were also conducted to compare the effects of each inoculation timing with that of uninoculated control treatments for TSW, revealing that TSW of the early-flower inoculation treatment was lower than that of the control treatment ($P = 0.035$).
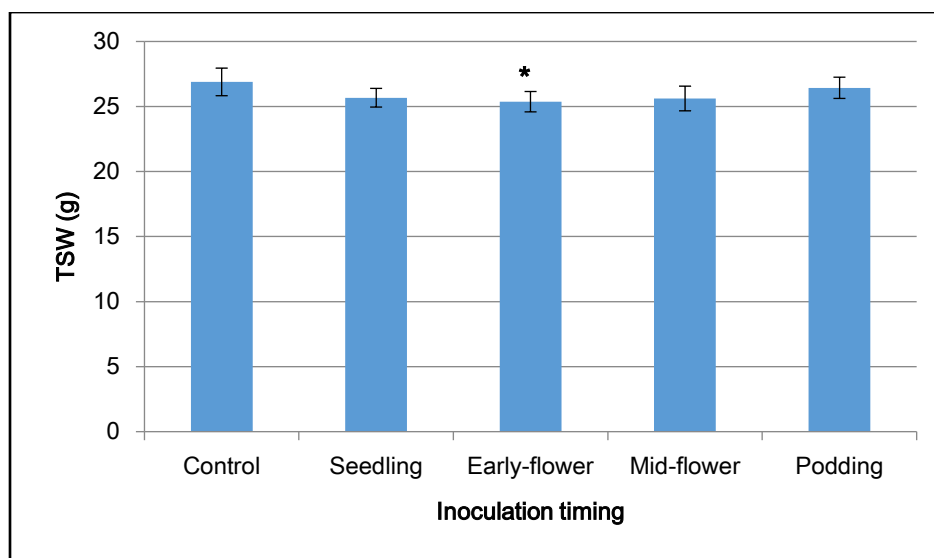
Figure 3.5: Weight of 1000 seeds (TSW) of lentil cultivar CDC Robin grown in low tunnels in three field experiments at Saskatoon in 2013 and 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four growth stages. * indicates the treatment is significantly different from the control based on simple linear contrasts.

3.3.2.6 *Stemphylium* infected lentil seed grown under low tunnels

The incidence of seed infected with *Stemphylium* spp. ranged from 0.7 to 3.4% (Fig. 3.6). Data analysis revealed an effect of inoculation timing on the percentage of seed infected ($P = 0.0025$). Linear contrasts analyses between uninoculated and inoculated treatments were significant ($P \leq 0.003$).

Means comparisons indicated that the level of seed infected with *Stemphylium* spp. in the treatments inoculated at the seedling, early-flower, mid-flower stages were similar and higher than those for seed from treatments inoculated at the podding stage and from the control. Infection levels were on average 2.8 times higher in those treatments (Fig. 3.6).
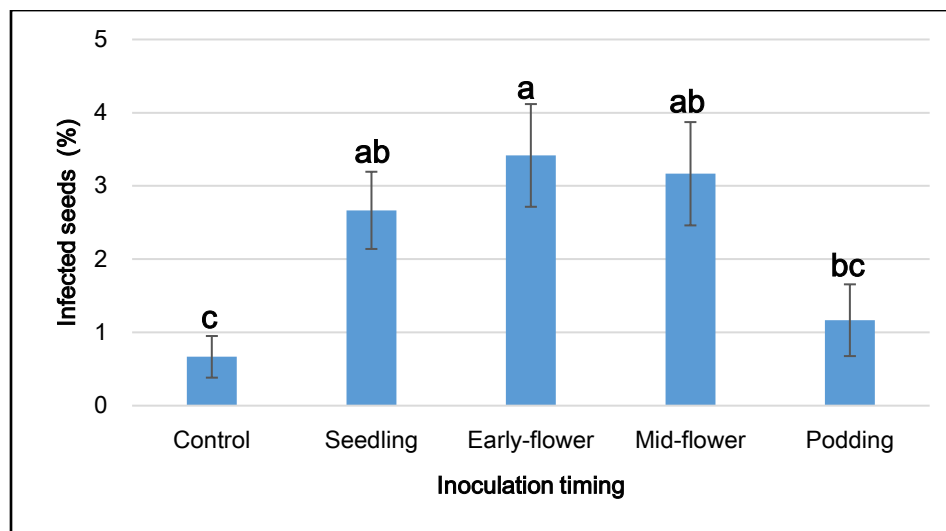
Figure 3.6: Percentage of seed infected with *Stemphylium* spp. of lentil cultivar CDC Robin grown in green polyethylene low tunnels in three field experiments at Saskatoon in 2013 and 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four growth stages. Separation of means by Fisher's LSD ($P < 0.05$) represented by different letters above bars.

3.3.2.7 Percentage staining of seed harvested from low tunnels

Seed staining ranged from 6 to 13% (Fig. 3.7) and analysis of data revealed that inoculation timing had no effect ($P = 0.2267$). However, linear contrast analyses revealed differences between the uninoculated control treatments *versus* inoculated treatments ($P = 0.0013$).
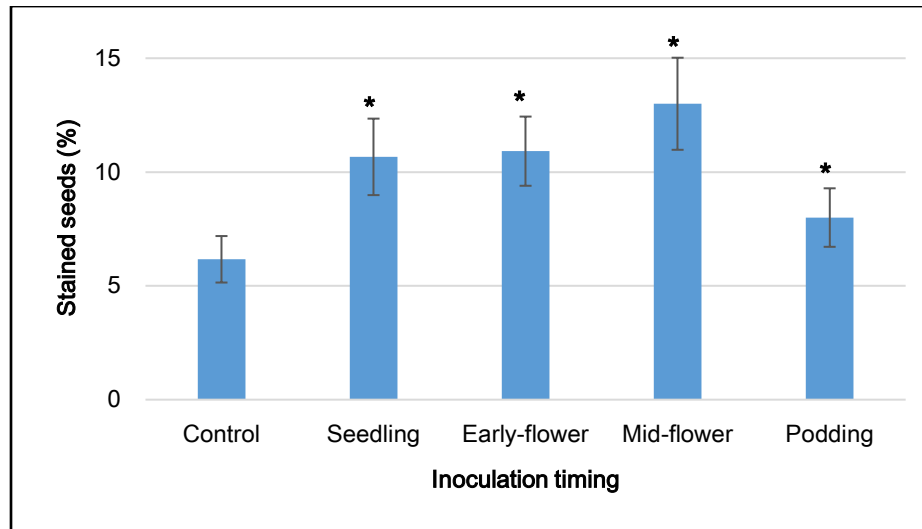
Figure 3.7: Percentage of seed staining on seeds harvested at Saskatoon from lentil cultivar CDC Robin grown in green polyethylene low tunnels in three field experiments at Saskatoon in 2013 and 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four growth stages. * indicates treatments are significantly different from the control based on simple linear contrasts.

3.3.2.8 Seed diameter and thickness

Seed diameter ranged from 3.8 to 3.9 mm and seed thickness from 2.1 to 2.2 mm (Fig. 3.8). Data revealed an effect of inoculation timing on diameter ($P = 0.04$), but not on thickness ($P = 0.72$). Further exploration of seed diameter through linear contrast analysis revealed that seed from treatments inoculated at seedling, early- and mid-flower growth stages were smaller than the uninoculated control and podding stage.
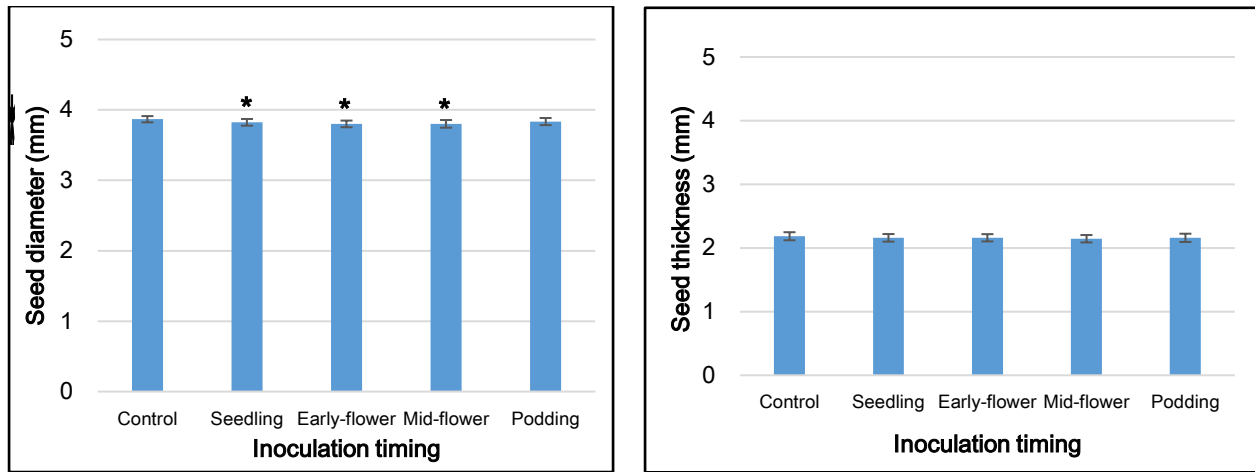
Figure 3.8: Seed diameter (left) and thickness (right) of lentil cultivar CDC Robin grown in green polyethylene low tunnels in three field experiments at Saskatoon in 2013 and 2014. Treatments were inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four growth stages. * indicates treatments are significant different from the control based on simple linear contrasts.

3.4   Discussion

The greenhouse experiment showed increasing disease severity on lentil plants as they were inoculated with more conidia. The lowest conidial concentration (1 x 10^3 conidia mL^{-1}) was selected for field inoculations because higher conidial concentrations may have led to premature plant death with the result that seed yield and quality effects could not have been assessed.

Assessment of disease symptom of cv. CDC Robin lentil in field experiments indicated that infection was due to *S. botryosum* isolate SB19 and natural inoculum of *Stemphylium* spp. This occurred even on plants in low tunnels, where stemphylium blight symptoms were detected in the uninoculated control treatments. However, lentil plants had higher disease severity in low tunnels compared to non-covered plants, and showed responses to inoculation timings, which confirmed the effectiveness of artificial inoculation under low tunnels. Stemphylium blight levels from 53 to 83% in low tunnels were higher compared with 25 to 62% reported by Podder (2012) for plants grown under such conditions.

The optimal temperature for development of stemphylium blight on lentil plants was reported as 25 to 30°C by Mwakutuya and Banniza (2010). This is similar to the temperatures recorded in low tunnels in this study. Longer duration of temperatures above 25°C, as well as longer periods of high relative humidity compared with the non-covered treatments were observed in low tunnels (Appendix 3).

Describing the specific plant stage at the time of inoculation helped to relate the effect of the disease to specific growth stages (Table 3.2). Environmental conditions as well as light conditions varied between years, therefore description of plants in terms of number of nodes, percentage of flowering plants and percentage of pods filled throughout the field was more informative compared with plant age expressed in number of days after seeding only.

Assessing AUDPC captures physiological and environmental effects that have direct influence on final disease severity (Shaner and Finney, 1977). It is considered an indirect measurement of pathogen population (Brooks, 2000) and is an easy method to summarize the development of a polycyclic disease such as stemphylium blight.

The differences in disease severity observed in response to inoculation timing revealed that plant age at the time of infection has an effect on disease development over time, which was similar to earlier observations in the experiments under controlled conditions in the greenhouse (Banniza

et al., 2006; Kumar, 2007). Lentil plants infected with *S. botryosum* as seedlings (26 days after seeding) are likely to develop higher disease severity compared with plants inoculated at early-flowering, mid-flowering and at the podding stage. Inoculation at the podding stage occurred one week before plants were desiccated in all three experiments, with the result that only one disease assessment was made after podding inoculation. This late inoculation may therefore have been too late to determine its effect on stemphylium blight development as compared with the other inoculation timings using AUDPC.

The cumulative disease level observed in the uninoculated control treatments suggested that the field was exposed to background inoculum that likely infected all covered and non-covered treatments before and after artificial inoculation. Therefore, the differences between the uninoculated control and the inoculation treatments may have been smaller than would have been the case if the uninoculated control had been disease-free. Stemphylium blight as a polycyclic disease produces several generations of conidia that easily may have moved by wind and passed through the holes in the cover material increasing the level of infection and reducing the amount of disease-free foliage. This likely affected treatments inoculated in the later stages more so than those inoculated at the seedling or early flowering stages, as the impact of inoculum was reduced by the higher levels of diseased tissue at later growth stages. Indeed, averaged across all three experiments, stemphylium blight severity in uninoculated, covered control treatments increased from just over 1% at the first assessment date to 19% at the final assessment date.

In Saskatoon in 2013, seed yields were marginally reduced in treatments inoculated at the early flowering stage compared to the uninoculated control, however analysis of combined yield data revealed that inoculation timing had no effect on yield compared with the control. Findings here are in contrast to the strong negative relationship between stemphylium blight and seed yield described by Bakr and Ahmed (1992), Hosen et al. (2009) and Rahman et al. (2010). Sustaining seed yields on diseased plants may have been the result of intermediate levels of resistance of CDC Robin (Banniza et al., 2006). More likely, yield differences between the uninoculated control and inoculation treatments were diminished due to the effect of background inoculum that resulted because the uninoculated control treatments were not disease-free as mentioned before for AUDPC.

The cultivar description of CDC Robin lists a TSW of 29 g under field conditions (Vandenberg et al., 2002), and was similar to the 30 g per 1000 seeds obtained in field experiments here in low tunnels and non-covered treatments. Overall, inoculation timing did not affect TSW of CDC Robin. However, simple linear contrast analyses revealed that TSW was reduced when lentil plants were infected at the seedling, early- and mid-flowering stages compared with the uninoculated control treatments in the experiment at the Seed Farm in 2014 (Appendix A4.4.3). Nevertheless this could not be confirmed in the other two field experiments.

High levels of *Stemphylium* spp. seed infection in treatments inoculated with *S. botryosum* compared with the uninoculated treatments were observed, and inoculations at the early-flowering stage resulted in the highest infection level. The level of seed infection negatively affected seed quality. According to the guidelines for seed-borne diseases of pulse crops (Government of Saskatchewan, 2016), the importance of seed quality is dependent on the final purpose of the seeds produced. If harvested seeds will be used for crop production then features such as high germination, and low levels of seed-borne diseases close to zero are recommended. If the seeds are designated for consumption, the size, color and shape of lentil seeds are the most important features. According to the official grain grading guide, lentils have to meet commercial specifications for grading factors such as colour, seed damage, stained seed, wrinkled seed, as well as seed infection with seed-borne pathogens (Canadian Grain Commission, 2014).

The amount of seed coat staining in the red lentil varieties does not apply for marketing purposes (Canadian Grain Commission, 2014) since red lentils are mostly decorticated before sale. However, the level of seed staining in the seed harvested from inoculated treatments compared with the uninoculated control treatments was significant based on contrast analysis, hence for other lentil classes seed staining due to stemphylium blight could result in downgrading. Therefore, irrespective of whether lentil seeds are used for planting or consumption, export or domestic use, a reduction in seed quality can have a negative impact on Saskatchewan growers as the increased treatment cost affects the total input costs (Erskine et al., 1985; Saskatchewan Ministry of Agriculture, 2015).

The assessment of lentil cv. CDC Robin for infection with *S. botryosum* isolate SB19 at the seedling, early-flowering, mid-flowering and podding stages under green polyethylene low tunnels in field conditions revealed that lentil plants inoculated at the seedling stage developed higher

disease severity, whereas seed yield overall was not affected by inoculation timing. Seed quality was compromised when plants were inoculated at the reproductive stages (early-flower and in some degree at mid-flower) compared with other growth stages. Reduction in seed weight, increased seed infection with *Stemphylium* spp., and reduction in seed diameter from the treatments inoculated at early-flowering stage were observed. The latter parameter was also reduced when inoculation was at the mid-flowering stage. These observations lend some support to the hypothesis that infection during reproductive stages impacts seed quality, but does not reduce yield.

4. ETIOLOGY OF STEMPHYLIUM BLIGHT IN SASKATCHEWAN

4.1 Introduction

Many species have been described in *Stemphylium* since Wallroth (1833) described this genus, naming the type species of the genus *S. botryosum*. However, the description of *S. botryosum* by Wallroth relied on a limited number of traits (Wiltshire, 1938; Simmons, 1967). Wiltshire (1938) published a detailed description of *Stemphylium* species based on conidium and conidiophore morphology. He also described the association of *S. botryosum* with *Pleospora herbarum* Rabenhorst as the perfect stage. Later on, Simmons (1967) addressed the problem of misclassification and misidentification of pathogenic species with a description and discussion of similarities among *Stemphylium*, *Alternaria* and *Ulocladium*. He also emphasized that only for *S. botryosum* was there solid evidence for its sexual state in *P. herbarum*. The association of *Pleospora* spp. with at least five other *Stemphylium* species, *S. vesicarium* (Wallroth), *S. majusculum* (Simons), *S. triglochinicola* (Sutton and Pirozynski), *S. lancipes* (Ellis and Everhart), and *S. globuliferum* (Vestergren) was subsequently made, of which the last was commonly misidentified as *S. sarciniforme* or *S. botryosum* (Simmons, 1969). Finally in 1985, the associations of *P. herbarum* and *S. herbarum*, *P. tarda* and *S. botryosum*, and *P. alfalfa* (Simmons) and *S. alfalfae* (Simmons) were made (Simmons, 1985).

Lately, studies to differentiate species in the genus *Stemphylium* have repeatedly demonstrated overlap of morphological characters making the identification and description of species difficult (Camera et al., 2002; Wang et al., 2010). Molecular phylogenetic analysis has been increasingly used as an additional tool to classify species (Sleator, 2011). The use of morphological characters in conjunction with other characters such as conserved gene sequences may lead to more solid descriptions of species that improve understanding of relationships among species (Cai et al., 2009).

A common approach to phylogenetic studies in eukaryotes is the description of multiple genes such as in the case of the genus *Colletotrichum* where a combination of the nuclear rDNA internal transcribed spacer (ITS) region, *glyceraldehyde – 3 phosphate dehydrogenase* (*gpd*),

calmodulin (CAL), partial actin (ACT), glutamine synthetase (GS), and β-tubulin (TUB2) was found to be more informative than analysis of a single locus or a few loci (Cai et al., 2009).While the ITS sequence is the most frequently used locus in phylogenetic studies of fungi based on its very conserved regions, the *gpd* region possesses relatively high variability that makes this gene more suitable for phylogenic analysis at the species level. Both these genes have been used to explore relationships among *Stemphylium* spp. (Wang et al., 2010).

The objectives of this study were to identify the species that causes stemphylium blight of lentil in Saskatchewan, and to determine the phylogenetic relationships among *Stemphylium* species based on morphology, morphometry and phylogenetic analyses.

4.2   Materials and Methods

4.2.1   Isolates

Eleven isolates of *Stemphylium* spp. were selected from the culture collection of the Pulse Crop Pathology Research Group of the Crop Development Centre (CDC) at the University of Saskatchewan. The isolates were collected between 2002 and 2013 from lentil plants with stemphylium blight symptoms. The selection was based on geographic origin and different soil-climatic zones in Saskatchewan. Four additional isolates were collected from lentil plants from a field experiment at the Seed Farm of the Department of Plant Sciences in 2013, three isolates were collected from pea at Swift Current in 2013, one isolate from faba bean collected in Saskatchewan in 2012 and one isolate originated from alfalfa collected in Saskatchewan in 2014 (Table 4.1). In addition to these field isolates from Saskatchewan, the ex-type specimen of *S. botryosum* was obtained from the Canadian Collection of Fungal Cultures in Ontario Canada (DAOM), two isolates from Bangladesh and one isolate of *Alternaria* sp. isolated from lentil in 2013 were included to add diversity and to determine whether lentil isolates belong to *S. botryosum* (Table 4.1). The working collection of these isolates was stored cryogenically. Additionally, in the phylogenic analysis, sequences of the ITS and *gpd* regions of five named *Stemphylium* spp. and one *Alternaria* sp. were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 4.2).

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Table 4.1: Isolates of *Stemphylium* species, ex-type specimen of *Stemphylium botryosum,* and *Alternaria* sp. selected for morphological characterization and phylogenetic analysis

Culture collection ID	Collected	Soil type	Geographic location in SK	Origin	Host
SB11	2003	Brown	SW	Cabri SK/168	*Lens culinaris* Med.
SB17	2004	Brown	SW	Hodgeville SK /135	*Lens culinaris* Med.
SB19	2002	Dark brown	SE	Bladworth SK /282	*Lens culinaris* Med.
SB20	2003	Dark brown	SE	Briercrest SK /130	*Lens culinaris* Med.
SB27	2004	Dark brown	SW	Wiseton SK /286	*Lens culinaris* Med.
SB31	2004	Dark brown	NW	Clavet SK/343	*Lens culinaris* Med.
SB32	2004	Brown	SW	Eston SK/259	*Lens culinaris* Med.
SB40	2004	Dark brown	SE	Lajord SK/128	*Lens culinaris* Med.
SB44	2004	Dark brown	NW	Scott SK/380	*Lens culinaris* Med.
SB49	2004	Black grey	NE	Tisdale SK/427	*Lens culinaris* Med.
SB85	2004	Black grey	NW	Rosthern SK/403	*Lens culinaris* Med.
SB126	2013	-	-	Bangladesh	*Lens culinaris* Med.
SB131	2013	-	-	Bangladesh	*Lens culinaris* Med.
SB133	2013	Dark brown	NW	Saskatoon SK/344	*Lens culinaris* Med.
SB134	2013	Dark brown	NW	Saskatoon SK/344	*Lens culinaris* Med.
SB135	2013	Dark brown	NW	Saskatoon SK/344	*Lens culinaris* Med.
SB136	2013	Dark brown	NW	Saskatoon SK/344	*Lens culinaris* Med.
SB137	2012	-	-	-	*Vicia faba* L.
SB138	2013	-	-	Saskatchewan	*Medicago sativa* L.
SB139	2013	Dark Brown	SW	Swift Current SK/165	*Pisum sativum* L.
SB140	2013	Dark Brown	SW	Swift Current SK/165	*Pisum sativum* L.
SB141	2013	Dark Brown	SW	Swift Current SK/165	*Pisum sativum* L.
DAOM195299	-	-	-	*Stemphylium botryosum* ex-type	*Medicago sativa* L.
Alternaria sp.	2013	Dark brown	NW	Saskatoon SK/344	*Lens culinaris* Med.

Table 4.2. Accession numbers of partial sequences retrieved from GenBank for the ITS and *gpd* gene regions of six isolates included in the phylogenetic analysis of *Stemphylium* species.

Species	Original ID[1]	Accession number	
		ITS[2]	*gpd*[3]
Alternaria alternata	EGS 34-016	AF071346	AF081400
Stemphylium alfalfae (Ex-type)	EGS 36-088	AF442775	AF443874
Stemphylium gracilariae (Ex-type)	EGS 37-073	AF442784	AF443883
Pleospora herbarum / *Stemphylium herbarum* (Ex-type)	EGS 36-138	AF442785	AF443884
Stemphylium vesicarium (Type)	EGS 37-067	AF442803	AF443902
Pleospora tarda / *Stemphylium botryosum*(Ex-type/Type)	EGS 08-069	AY329168	AY316968

1 EGS = E. G. Simmons, Mycological Services, Crawfordsville, Indiana;
2 Internal transcribe spacer (18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence) gene region;
3 Partial *glyceraldehyde-3-phosphate dehydrogenase* gene.

4.2.2   Morphological and morphometric isolate description

The qualitative description of each isolate and the description of the shape and size of conidia were conducted for all isolates listed in Table 4.1, with the exception of *Alternaria* sp. The experiment was a randomized complete block design with four replicate Petri dishes (90 mm) for each isolate and the experiment was repeated once. Each isolate was cultured from a single conidium on potato dextrose V8 juice (V8-PDA) medium (10 g potato dextrose agar, 10 g granulated agar, 3 g $CaCO_3$, 150 mL V8-juice and 850 mL distilled water) (Camara et al., 2002) and was incubated at 25°C under continuous light. Culture color, shape, texture and diameter were described and/or measured.

Morphometric studies of conidial shape and size were done with sixty conidia arbitrarily picked from the four replicates (about 20 conidia per Petri dish), for which the length and width were measured.

4.2.3   Molecular phylogenetic analysis

The internal transcribe spacer (ITS) and a partial region of the *glyceraldehyde-3-phosphate dehydrogenase* gene (*gpd*) were sequenced to establish monophyletic relationships. As an initial step, all 24 isolates listed in Table 4.1 were grown on PDA medium as monoconidial cultures to

produce fungal tissue. A plug of the mycelium was transferred into liquid potato dextrose medium (4 g potato extract, 20 g dextrose, 876 mL of distilled water) and incubated at 25°C under 12 h light/dark (Mehta, 2001). The mycelia produced after three days of incubation were filtered through sterile miracloth and transferred into 2 mL vials. Vials with mycelia were frozen at -80°C for 24 h, before being lyophilized for another 24 h. Freeze-dried mycelia were pulverized by introducing two to three glass pearls into each tube and shaking them for five minutes in a mechanical shaker at approximately 1000 r.p.m. Genomic DNA was extracted with the DNeasyTM Plant Mini Kit-QIAGEN, (Chou and Wu, 2002; QIAGEN, 2012) following the manufacturer's instructions. The genomic DNA from all isolates were stored in deionized RNA-free water at -20°C.

The amount of genomic DNA of each isolate was determined by mixing 2 µL of DNA solutions with 6 µL of RNA-free deionized water and 2 µL of Tracklt™ Cyan\Orange loading buffer (Invitrogen$^{®}$, Life Technologies Corporation, www.lifetechnologies.com). The samples were loaded into a 1% agarose gel and run at 90 volts for 1 hour. A low DNA mass ladder (Invitrogen$^{®}$, Life Technologies Corporation, www.lifetechnologies.com) was included to compare and estimate the amount of DNA. DNA samples were subsequently diluted to achieve a final concentration of 20 ng µL^{-1} for polymerase chain reaction (PCR).

ITS and *gpd* primers (Table 4.3) for amplification and sequencing were described by Wang et al. (2010). The reaction mixture consisted of 2 µL of 10 X buffer (MgCl$_2$), 0.25 µL of 10 mM of dNTP mixture, 0.2 µL of 10 pmol of each primer, 0.2 µL of Taq DNA Polymerase (GenScript, USA Inc. www.genscript.com), 20 ng of DNA extracted, and 16.15 µL of sterile water. PCR was performed on a C1000 Thermal Cycler$^{®}$ with 35 cycles at 94°C for 30 s of denaturation, 57°C for 1 min, 72°C for 1.5 min of annealing, and a final extension at 72°C for 7 min (Wang et al. 2010; and Camara et al., 2002).

DNA bands were excised from the agarose gel with a sterile scalpel. The pieces of gel were weighed and purified with the Bio Basic Inc EZ-10 Spin Column kit. Aliquots of 50 ng µL^{-1} of the purified ITS bands (above 2000 bp), and 10 ng µL^{-1} of the purified *gpd* bands (below 2000 bp) and the respective primers (Table 4.3) were used for sequencing at the National Research Council of Canada (NRC), Saskatoon, Saskatchewan.

Table 4.3: Primers used for phylogenetic analysis of *Stemphylium* species (Wang et al., 2010; Camara et al., 2002; and White et al., 1990)

Primer	Sequence 5'- 3'
NS 1 (PCR amplification)	GTAGTCATATGCTTGTCTC
ITS 3 (sequencing)	GCATCGATGAAGAACGCAGC
ITS 4 (PCR amplification, sequencing)	TCCTCCGCTTATTGATATGC
ITS 5 (sequencing)	GGAAGTAAAAGTCGTAACAAGG
ITS 2c (sequencing)	CAGTAAACATGGAAGTTCGA
*gpd*_f (PCR amplification)	GCACCGACCACAAAAATC
*gpd*_r (PCR amplification)	GGGCCGTCAACGACCTTC
*gpd*_ef (sequencing)	CGGCTTCGGTCGCAT
*gpd*_er (sequencing)	GCCAGGCAGTTGGTTGTG
*gpd*_if (sequencing)	CACGGCCAGTTCAAG
*gpd*_ir (sequencing)	GGCGGGGTCCTTCTCC

4.2.4 Data collection and analysis

The morphological and morphometric descriptions of each isolate colony (except *Alternaria* sp.) were done after 7 days of incubation. The diameter of each culture colony was measured in millimeters. Analysis of variance was conducted with the mixed model procedure in SAS. Blocks and repeats of the experiment were considered random factors, and isolates were the fixed factor. Linear contrast analyses were conducted to compare the ex-type *S. botryosum* (DAOM195299) and other isolates. Means and standard errors of the means were generated with the means procedure in SAS.

The length and width of the 60 conidia per isolate arbitrarily picked from 14-day old cultures were measured on glass slides with distilled water with a Carl Zeiss compound microscope at 20x magnification. Representative pictures of conidia were taken for each isolate with the ZEN Carl Zeiss camera. The size and width of conidia were compared with those of the ex-type of *S. botryosum* by single degree of freedom contrasts.

Sequences of the ITS (18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence) gene region and the partial *gpd* gene of each isolate were analyzed and edited with the DNA Baser Sequence Assembler v4 (2013) (Heracle BioSoft, www.DnaBaser.com). The edited sequences of each target fragment were pooled and a consensus sequence was generated. Sequence data for the two gene regions (ITS + *gpd*) were concatenated

(Gadagkar et al., 2005) for the 24 isolates sequenced, and the six sequences of species belonging to the Pleosporales retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Pairwise and multiple alignments were done for each gene separately and for the concatenated data set with the ClustalW algorithm. Alignments and the phylogenic analyses were carried out using Molecular Evolutionary Genetics Analysis Version 6.0 (Tamura et al., 2013).

The first approach in the phylogenetic analysis was through the Neighbor-Joining (NJ) (Saitou and Nei, 1987) method as it is considered to be the fastest and most efficient method (Gadagkar et al., 2005). Additionally, the Maximum Likelihood (ML), and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath and Sokal, 1973) methods were explored to obtain further evidence of the evolutionary history of *Stemphylium* spp.

Branch topologies of phylogenetic trees constructed with the NJ and UPGMA methods were tested with the bootstrap test with 1000 replicates. The evolutionary distances were calculated with the p-distance method including the 1st, 2nd, 3rd and noncoding codon positions. Gaps and missing data were completely deleted.

The Tamura-Nei model (Tamura and Nei, 1993) was used to construct the highest log likelihood (ML) phylogenetic tree. The NJ and BioNJ algorithms with the Maximum Composite Likelihood (MCL) were applied to estimate the pairwise distances. The 1st, 2nd, 3rd and noncoding codon positions were included; all gaps were treated as missing data and were eliminated. The Bayesian inference method using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) was used to generate the species clusters in the ML tree of the concatenated gene regions. This analysis ran for 10 million generations in place of 1 million, and trees were sampled at every 1000 generation in place of 100 generations (Bhadauria et al., 2015).

Additionally, similarity analyses and estimation of the number of base pair differences per site, and estimation of the percentage identity among concatenated sequences of each isolate were carried out using Basic Local Alignment Search Tool logarithm (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and pairwise analyzes (MEGA6, Tamura et al., 2013), respectively. A percent identity matrix was created using multiple sequence alignment by Clustal2.1 Omega (EMBL-EBI, www.ebi.ac.uk).

4.3   Results

4.3.1   Morphological and morphometric isolate description

Colony diameter varied among isolates ($P < 0.0001$). Colony diameters after seven days incubation ranged from 33 mm to 80 mm among isolates (Fig. 4.1). The comparison of means revealed a continuum of increasing diameters. The colony diameter of the ex-type (DAOM 195299) was 45.5 ± 2.59 mm and was similar to SB17, SB20, SB31 and SB134. This was confirmed through linear contrast analyses between the ex-type and those four isolates ($P > 0.05$), whereas contrasts between the ex-type and the other isolates were significant ($P < 0.05$).
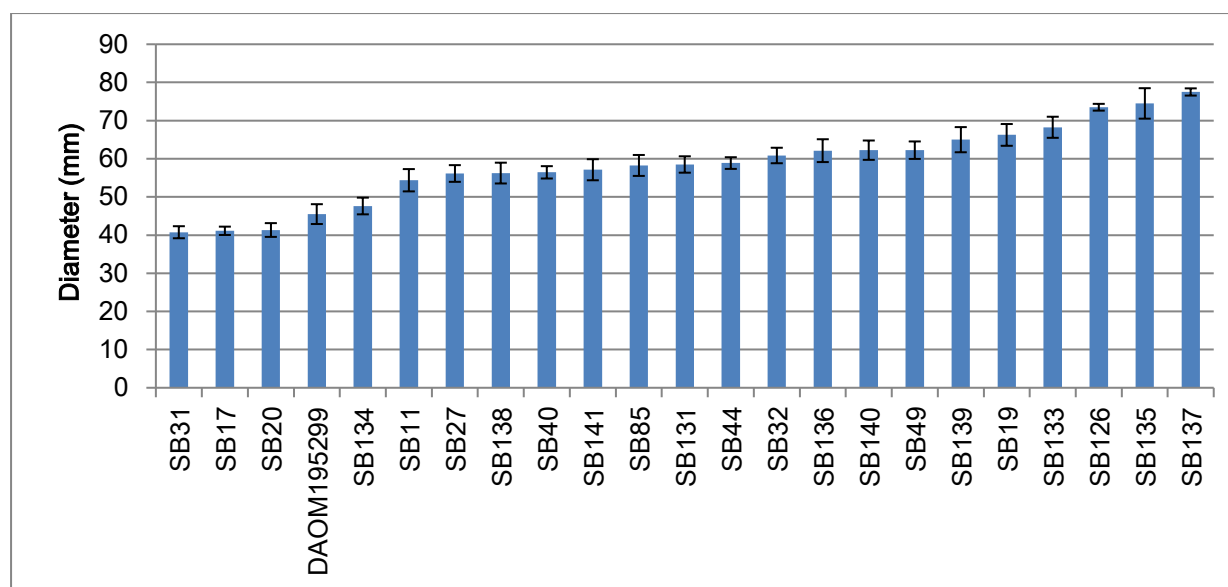
Figure 4.1: Colony diameter of field isolates of *Stemphylium* spp. and the *S. botryosum* ex-type (DAOM195299) after seven days incubation on V8-PDA medium at 25°C under continuous light.

The qualitative description of the 23 isolates after seven days incubation revealed four distinctive groups sharing color, texture, and shape of cultures. The first group included SB126, SB137 and SB138. Cultures of this group were dark green, and the colony surface was covered with white mycelia surrounded with yellow concentric rings; the texture was cottony (Fig. 4.2 Group 1). The second group consisted of SB27, SB44, SB49, SB85, SB133, SB136, SB139 and SB140. The colonies were greyish with circular concentric rings that turned from dark grey in the center of the colony to light grey to white towards the edge of colony; the texture was velvety (Fig.

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4.2 Group 2).The third group consisted of SB11, SB19, SB32, SB40, SB131, SB135, SB141 and the ex-type DAOM195299. The colonies were uniformly grey and had slightly circular, concentric rings that turned to light grey from the center of the colony to the outer edge. It also had a velvety texture (Fig. 4.2 Group 3). The fourth group with similar cultural features included SB17, SB20, SB31 and SB134. The colonies were greyish with irregular concentric rings ranging from dark grey in the center of colony to light grey and white at the outer edge (Fig.4.2 Group 4).
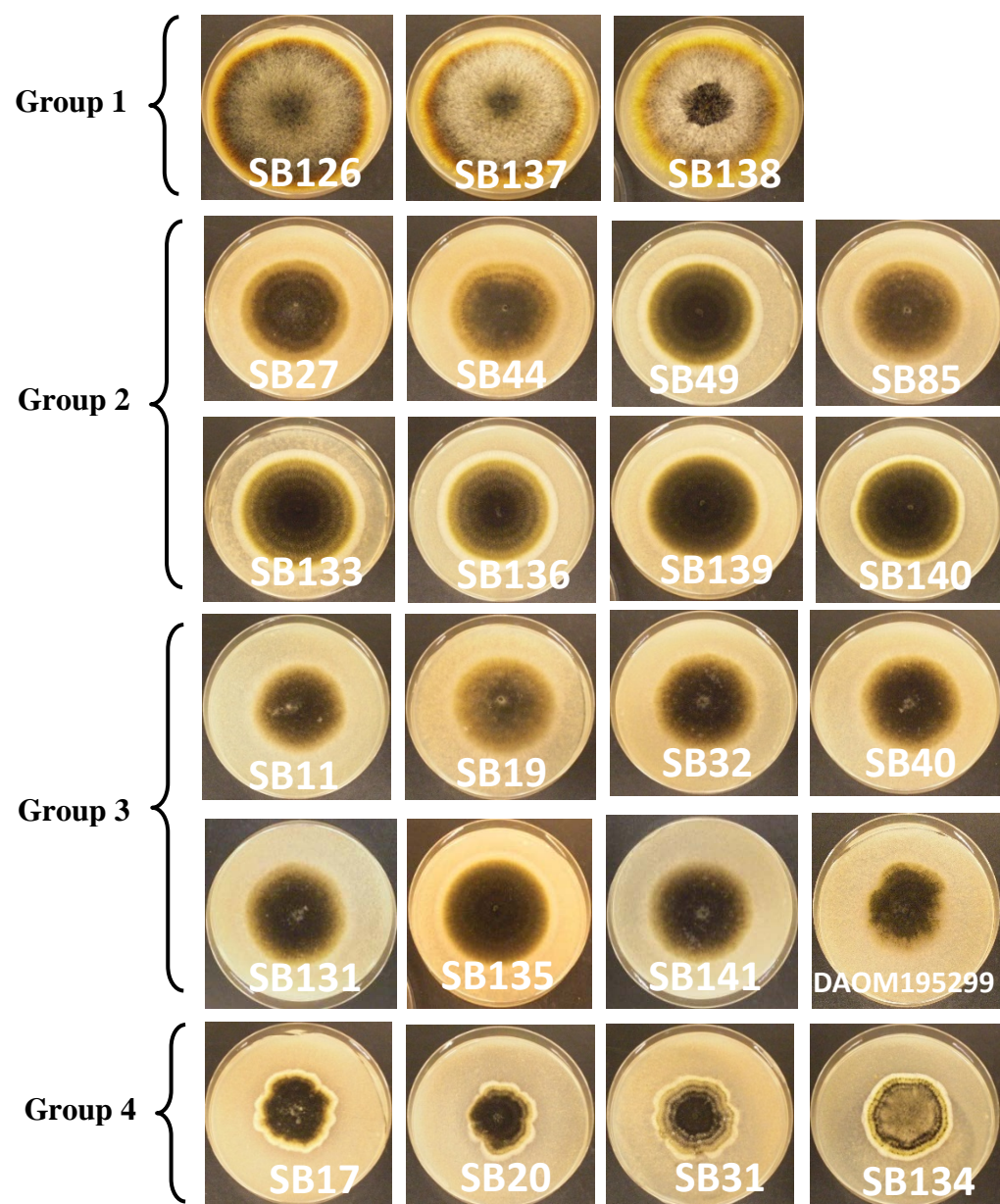
Figure 4.2. Cultures of field isolates of *Stemphylium* spp. and *S. botryosum* ex-type DAOM195299 after seven days incubation on V8-PDA medium at 25°C under continuous light.

The length and width of conidia of the 23 isolates ranged from 17 to 37 µm and 16 to 24 µm, respectively. Only slight variation in conidial colour, shape and septation were observed among isolates except for SB126, SB137 and SB138, which were smaller (Fig. 4.3). Analysis of conidial size (length and width) revealed large differences among isolates ($P < 0.0001$); however in some isolates the length or width overlapped (Table 4.4). Contrast analyses between the ex–type of *S. botryosum* and the 22 isolates revealed that only the length of SB131 was similar to the ex–type ($P = 0.26$), whereas the width of conidia of isolates SB19, SB32, SB131, SB135 were similar to that of the ex-type ($P \geq 0.1$).

Figure 4.3. Conidia of field isolates of *Stemphylium* spp. and the *S. botryosum* ex-type DAOM195299 after 14 days incubation on V8-PDA medium at 25°C. Photographed at 20x with the ZEN Carl Zeiss camera.

Table 4.4. Conidial length and width of field isolates (N = 60) of *Stemphylium* spp. and the *S. botryosum* ex-type (DAOM195299) after 14 days incubation on V8-PDA medium at 25ºC.

Isolate	Conidia size (µm)					
	Length			Width		
DAOM195299	35.7[a]	±	0.5[b]	22.2	±	0.3
SB11	32.9	±	0.6	20.2	±	0.3
SB17	27.8	±	0.5	19.1	±	0.4
SB19	26.3	±	0.7	22.8	±	0.8
SB20	30.4	±	0.6	19.6	±	0.3
SB27	30.4	±	0.5	21.1	±	0.3
SB31	29.2	±	0.6	19.2	±	0.3
SB32	33.7	±	0.5	21.7	±	0.3
SB40	30.7	±	0.6	20.8	±	0.4
SB44	30.8	±	0.5	21.1	±	0.3
SB49	29.0	±	0.5	19.8	±	0.3
SB85	30.9	±	0.7	24.1	±	0.7
SB126	17.0	±	0.2	16.8	±	0.2
SB131	36.5	±	0.5	22.4	±	0.3
SB133	26.0	±	0.8	23.2	±	0.8
SB134	30.6	±	0.5	16.6	±	0.3
SB135	27.5	±	0.6	21.2	±	0.6
SB136	32.1	±	0.5	20.8	±	0.3
SB137	16.9	±	0.3	16.3	±	0.2
SB138	18.5	±	0.3	18.3	±	0.2
SB139	29.5	±	0.5	19.5	±	0.3
SB140	27.7	±	0.4	18.5	±	0.3
SB141	28.9	±	0.4	19.2	±	0.3

[a] Mean.
[b] Standard deviation.

4.3.2   Molecular phylogenetic analysis

The systematic relationships among the 24 isolates and the six sequences retrieved from GenBank were estimated by comparisons of DNA sequences of the ITS and *gpd* gene regions, and the concatenated DNA sequences (ITS + *gpd*).

Comparison between the ITS and *gpd* phylogenetic trees and among NJ, UPGMA and ML methods revealed similarities in the tree topology. The trees constructed, based on ITS and *gpd* had a strong monophyletic group (bootstrap value = 99 to 100%) that included 20 of the 23 isolates of *Stemphylium* spp. studied. This group also included the *Stemphylium* and *Pleospora* species retrieved from GenBank. On the other hand, three of the 23 isolates under study were outside of

the monophyletic group in the ITS and *gpd* trees (Appendix 5 - 7. The phylogenetic trees of the concatenated DNA sequences built by using the NJ (Appendix 8), UPGMA (Appendix 9) and ML (Appendix 10) methods revealed topology similarities as well.

The evolutionary history of the concatenated DNA sequences inferred by using the ML method and clade support using the Bayesian posterior probability confirmed the inference obtained in the trees built with single gene regions (Fig. 4.4). This approach also revealed a monophyletic group with 20 of the 23 isolates sequenced and the five species of *Stemphylium* and *Pleospora*. Furthermore, two well supported groups (bootstrap values of 87 and 99%) within the monophyletic *Stemphylium* group were consistently clustered in every tree constructed (Fig. 4.4. Cluster A and Cluster B).

Similarity values from 99 to 100% among sequences within Cluster A (13 isolates and *S. botryosum* ex-type) were obtained through estimation of base pair differences per site. The number of different nucleotides per site sequences within Cluster A ranged from zero to two out of 1064 nucleotides. The pairwise analyses estimated from 922 positions after elimination of gaps and missing data revealed that the number of base pair differences per site after averaging all sequence pairs within sequences in Cluster A was 0.002. Similar values (> 99%) of similarity were also observed within sequences in Cluster B (six isolates and the sequences of *S. gracilariae*, *P. herbarum*, *S. vesicarium* and *S. alfalfae* retrieved from GenBank. The number of different nucleotides per site within Cluster B ranged from zero to 10 out of 1064 nucleotides and the pairwise analyses estimated from 922 positions after elimination of gaps and missing data revealed that the number of base pair differences per site after averaging all sequence pairs was 0.027. The percentage identity estimated within sequences in Cluster A and within sequences in Cluster B ranged from 96.8 to 100% and from 97.8 to 100%, respectively (Appendix 11).

Additionally, sub-group B.1 (belonging to Cluster B) with four isolates from lentil and two isolates from pea, all collected in Saskatchewan, as well as the accessions of the type species of *P. herbarum* EGS 36-138, *S. vesicarium* EGS 37-067 and *S. alfalfa* EGS 36-088 clustered together as well (Fig. 4.4.). The similarity values within sequences in B.1 ranged from 99 to 100% with zero to one out of 1061 nucleotide differences. The number of base pair differences per site after averaging all sequence pairs within B.1 was zero. The percentage identity estimated within sequences in Cluster B.1 ranged from 99.9 to 100% (Appendix 11).

Individual comparison between *P. tarda* EGS 08-069 *vs.* Cluster B.1 and *S. gracilariae* EGS 37-073 *vs.* Cluster B.1 were carried out. Similarity of *P. tarda* and *S. gracilariae* with isolates in Cluster B.1 was 99% for both species, and the number of different nucleotides per site sequences ranged from six of 988 to 10 of 1061, respectively. The number of base pair differences per site averaged over all sequence pairs between *P. tarda* and Cluster B.1, and between *S. gracilariae* and Cluster B.1 estimated by the pairwise analyses was 0.016 and 0.005 respectively. The percentage identity averaged in sequences within Cluster B.1 compared with *P. tarda* was 98.04% and compared with *S. gracilariae* 99.4% (Appendix 11).

Meanwhile, lower similarity values from 96 to 97% between Clusters A and B were revealed through the estimation of base pair differences per site. In addition, the number of different nucleotides per site between the two clusters ranged from 13 to 43 out of 1064 nucleotides, and the number of base pair differences per site averaged over all sequence pairs between Clusters A and B estimated by the pairwise analyses was 0.027. The percentage identity between sequences in Cluster A and Cluster B ranged from 94.1 to 96.8% estimated using multiple sequence alignments (Appendix 11).

The *Alternaria* sp. isolate collected from the field, the *A. alternata* EGS 34-016 sequences retrieved from GenBank, as well as three isolates isolated from lentil with stemphylium blight symptoms, (SB126 from Bangladesh, faba bean isolate SB137, alfalfa isolate SB138 from Saskatchewan) presumed to be *Stemphylium* spp., were positioned as out-groups. Comparisons among the sequences in the monophyletic *Stemphylium* group with the *Alternaria* cluster and with the other three isolates in the out-group had 86% similarity (in both comparisons) and 147 and 392 different nucleotides per site out of 1078 and 1064 nucleotides, respectively. The number of base pair differences per site averaged over all sequence pairs between the monophyletic *Stemphylium* group and *Alternaria* spp. cluster and with the three isolates in the out-group estimated by the pairwise analyses was 0.123 and 0.380, respectively. The percentage of identity between the monophyletic *Stemphylium* group *vs*. *Alternaria* ranged from 85.9 to 87%, and among the three isolates out-grouped ranged from 58.7 to 59.2% (Appendix 11).
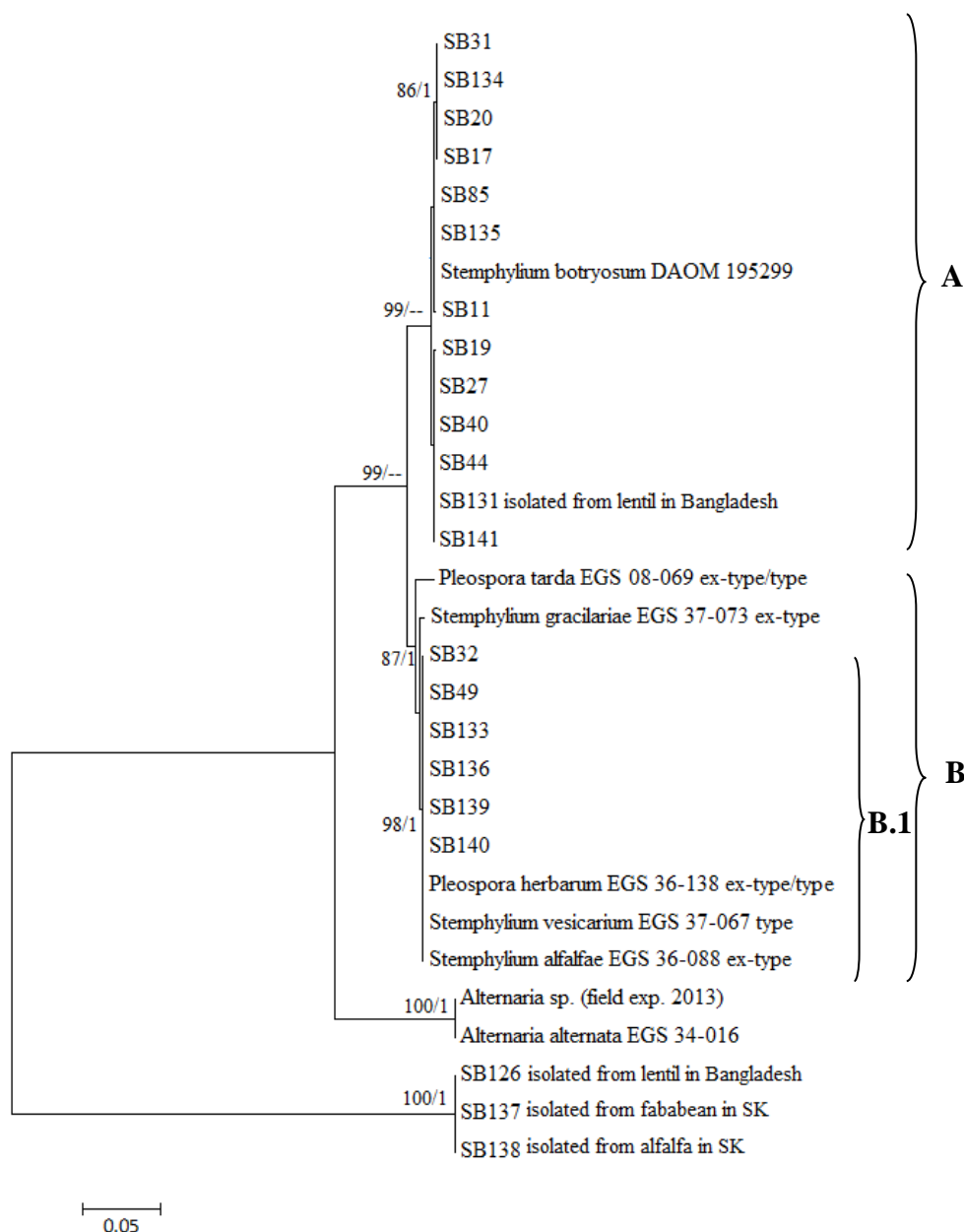
Figure 4.4: Most likely phylogenetic tree obtained with the Maximum Likelihood method (log likelihood = -2936.67) from the concatenated DNA sequences of the ITS and *gpd* of 23 isolates of *Stemphylium* spp. and an *Alternaria* sp. sequenced in this study, and sequences of ex-types/types of five *Stemphylium* species and one isolate of *Alternaria alternata* retrieved from GenBank. Bootstrap values ≥ 75% and Bayesian posterior probability values above 0.73 are shown above or below branches. The evolutionary analyses were conducted in MEGA6, and the Bayesian clade support was conducted in MrBayes 3.1.

4.4   Discussion

The colony diameter of the 23 isolates under study was not a feature that differentiated potential species of *Stemphylium* since there was a continuum of increasing diameters. On the other hand the qualitative culture description was more informative since four distinctive groups with similar features were distinguished. However, under the same temperature conditions (25℃), but on PDA medium, Rahman et al. (2010) observed that several isolates of *S. botryosum* can develop slight differences in colony features. Similarly, Hosen et al. (2009) described significant variation in colony features among four isolates of *S. botryosum* obtained from lentil with stemphylium blight symptoms when those isolates were cultured at 25℃ on lentil dextrose agar. Both groups of authors described an average colony of *S. botryosum* as greenish brown to black with a peripheral white ring, with velvety texture, which was similar to the description of the colonies of 20 isolates in this study including the ex-type (DAOM195299). Meanwhile, in this study only four isolates showed the irregular shape of the colony described by both authors.

The analyses of conidial size measurements revealed that only isolate SB131 shared the same length and width with the ex-type, whereas the ex-type was larger compared to the rest of the isolates. No qualitative differences were observed in terms of shape, color, or septation. Three isolates, SB126 from lentil in Bangladesh, SB137 from infected faba bean in Saskatchewan, and SB138 from alfalfa in Saskatchewan shared similar colony diameters, colony features and conidial sizes, but were different compared to the published *S. botryosum* descriptions and the rest of the isolates studied.

The molecular phylogenetic analyses of the 23 presumed *Stemphylium* species, irrespective of the approach for analysis, revealed one strongly supported *Stemphylium* group with 20 presumed *Stemphylium* isolates studied here as well as the five accessions of *Stemphylium* species included from GenBank. A similar grouping of *Stemphylium* species in a monophyletic group was reported previously (Wang et al., 2010; Inderbitzin et al., 2009; Camara et al., 2002). While the ITS is considered the barcoding region for fungi (Crous et al., 2014), *gpd* is one of at least three additional genes (*gpd*, *EF-1 alpha* and *vmaA-vpsA*) (Inderbitzin et al., 2009) already used for evolutionary reconstruction of *Stemphylium* species (Graf et al., 2016; Kurose et al., 2015; Pei et al., 2011; Deng et al., 2014).

The three methods of phylogenetic analyses used here (NJ, UPGMA and ML) were consistent in separating the group of *Stemphylium* species, the out-group of *Alternaria,* and the out-group of three misidentified isolates with strong cluster support through bootstrap values from 99 to 100%. Similarly, bootstrap values of 100 and 97% in the cluster of *Stemphylium-Pleospora* and *Alternaria alternate*, respectively, using combined ITS and *gpd* sequences were also reported by Wang et al. (2010). This clade support, based on bootstrap values, was higher than those reported to support the separation of the genera *Alternaria, Ulocladium* and *Stemphylium* (73, 82 and 100%, respectively) using ITS sequences only (Chou and Wu, 2002). Lower bootstrap values of 58 to 100% for cluster support to separate *Alternaria* spp. and *Setosphaeria* spp. from *Stemphylium-Pleospora* spp. using combined ITS and *gpd* sequences were reported by Camara et al. (2002). Tree topology and bootstrap values from 79 to 100% also supported the separation of the genera *Ophiosphaerella* and *Phaeosphaeria* and *Pleospora* using phylogenetic analyses of the ITS sequences (Camara et al., 2000).

The separation among *Stemphylium* species and from other genera has consistently been supported by the bootstrap test or the Bayesian clade support (Camara et al., 2000; Camara et al., 2002; Inderbitzin et al., 2009; Wang et al., 2010). Based on results here, the three out-grouped isolates misidentified as *Stemphylium* appear to belong to another group distinct from *Alternaria* spp. and *Stemphylium* spp., which is also supported by differences in colony and conidial morphology. *Alternaria* was used as an out-group since it is also a monophyletic genus (Barbee et al., 1999) with many morphological similarities, but which was previously shown to be distinct from *Stemphylium* (Yanez, 2001).

The grouping of the *Stemphylium* isolates into Clusters A and B was observed in the topology of each tree based on a single gene or with the concatenated sequences. Clusters were supported with strong bootstrap values of 87 and 99% for Cluster A and B, respectively. Similarities among isolates within cluster A were from 97 to 100% and similarities among isolates within Cluster B were from 98 to 100%. These values were similar to, or higher than values used to separate isolates of *Ophiosphaerella agrotis* (similarity among isolates from 87% to 100%) from *Ophiosphaerella* spp. (similarity among isolates from 95% to 97%) by Camara et al. (2000). However, the similarity values are lower than 99.1% used to separate species of *S. solani* and *S. globuriferum* using ITS sequences only (Hanse et al., 2015). Furthermore, the number of different

nucleotides (13 to 43 out of 1064 nucleotides among isolates within Cluster A and among isolates within Cluster B, respectively) and base pair differences per site (0.027) between the two clusters was larger than the seven nucleotide differences out of 529 nucleotides (0.013) used to separate *S. lycopersici* from *S. vesicarium* by Yanez (2001). Therefore, the similarity shared among isolates within each cluster and the difference in the nucleotide differences between clusters may support the assumption that Clusters A and B represent two different species of *Stemphylium*. However, even the use of multiple loci does not guarantee that species are accurately distinguished as pointed out by Inderbitzin et al. (2009), who found that *P. herbarum, P. alfalfa, P. tomatonis, P. sedicola* and *S. vesicarium* clustered together as one species in the phylogenetic analyses based on four loci.

The location of *P. tarda* EGS 08-069 in the phylogenetic tree revealed that it is more closely related to *S. gracilariae* and the species representing isolates in B.1 than to *S. botryosum*. The comparison of sequences by the BLAST algorithm in the GenBank data base revealed that the identity of the sequences of *P. tarda* EGS 08-069 and *P. herbarum* var. *herbarum* CBS 191.86 (data not shown) was the same. On the other hand, the unexpected relationship of *P. tarda* isolate EGS 08-069, listed as ex – type / type by Inderbitzin et al. (2009), but not according to Simmons (1985), indicated that it was distinct from the ex-type specimen DAOM 195299. The ex-type specimen of *S. botryosum* originates from the type specimen EGS 04-118C designated by Simmons (1985), whereas *P. tarda* EGS 08-069 was another isolate studied but not designated as type (Simmons,1985). Based on results here, *P. tarda* EGS 08-069 is not suitable for the identification of *S. botryosum*.

Whereas isolates in Cluster A can be confirmed as *S. botryosum* with some confidence based on the comparison with the ex-type for *S. botryosum* DAOM 192599, the species of the four isolates collected from lentil and two from pea in Cluster B, is not clear since three validly described species of *Stemphylium* had identical sequences (as retrieved from GenBank) suggesting that they are in fact the same species. The possibility of detecting more than one species of *Pleospora / Stemphylium* in Canadian fields was mentioned previously by Inderbitzin et al. (2009) who collected three *Pleospora* spp. from nonagricultural hosts in British Columbia, Canada, that were recognized as three different species based on ITS, *gpd*, *EF-1 alpha* and / or *vmaA-vpsA*.

The use of multiple loci in phylogenetic species recognition does not always differentiate between species that are closely related (Inderbitzin et al., 2009). Nevertheless, identical sequences

for the ITS and *gpd* found for different (morphology-based) species of *Pyrenophora* / *Drechslera* in public data banks highlights the limitation of morphology only-based species delineation (Zhang and Berbee, 2001). Results here suggest that an extensive review of the genus *Stemphylium* is necessary to clarify the number of species in this genus, so that an accurate identification of the pathogens causing stemphylium blight in Saskatchewan's lentil fields can be accomplished.

5. GENERAL DISCUSSION

Field experiments on stemphylium blight infection at different growth stages of lentil did not result in yield loss. This is in contrast to reports from Bangladesh where more than 80% yield loss was reported (Hosen et al., 2009). Yield comparisons here were probably compromised by the relatively high amount of stemphylium blight that developed in control treatments. Therefore, the question of yield loss due to stemphylium blight in Canadian lentil remains inconclusive. However, higher seed infection levels and seed staining of lentils from the early and mid-flower inoculation treatments may indicate that the quantity of seeds could also be affected if compared to a truly disease-free control.

Based on greenhouse experiments, it was previously observed that seed yield of a susceptible, but not a partially resistant cultivar steadily declined when plants were inoculated at the seedling, pre-flower or pod setting stage (Banniza, et al. 2006). This consistent decline in seed yield due to stemphylium blight was not observed under field conditions here, even though the level of disease was higher in the treatments artificially inoculated with *S. botryosum* compared with control treatments. It is possible that the biological threshold for yield loss was not achieved, that CDC Robin has some level of resistance to stemphylium blight, or that stemphylium blight may be one of those diseases where the relationship between disease severity and seed yield is not very strong. This was observed in field pea in Saskatchewan where disease resulting from *Mycosphaerella pinodes* infection, did not reduce pea yields regardless of fungicide applications (Gossen et al., 2001) or tillage system used (Bailey et al., 1999).

The empirical model used to study yield loss here focused on the relationship between cumulative disease over time and seed yield at harvest, which is a common approach to establish a disease - yield loss curve (Cooke, 2006). A model that captures all explanatory variables would likely be better to determine an economic threshold. Temperature and humidity are two important variables whose effects on disease initiation have been studied (Huq and Khan, 2008). For example, leaf wetness is a key factor for infection and disease severity as was observed with *S. vesicarium* infecting onion leaves (Llorente and Montesinos, 2001). Leaf wetness period and optimal

temperatures were also studied for the development of stemphylium blight in lentil under controlled greenhouse conditions (Mwakutuya and Banniza, 2010). Use of low tunnels optimized conditions for *S. botryosum* infection, promoted stemphylium blight and host growth. However, the complexity of the disease-yield loss relationship increases with multiple diseases, or when environmental variables are included (Teng, 1987).

Whereas the effects of environmental conditions on the development of stemphylium blight are relatively well studied (Mwakutuya and Banniza, 2010), the interaction of multiple pathogens on lentil is not understood. In addition to stemphylium blight, botrytis grey mold and sclerotinia were observed with higher incidence in low tunnels during the first year compared to the second year experiments. It is possible that these diseases in addition to stemphylium blight in 2013 contributed to the yield loss that was observed in that year. Pathogens such as *Epicoccum* sp., *Alternaria* sp., *Botrytis* sp., and *Fusarium* sp. were also detected in seed samples, but at lower levels than *Stemphylium* spp. A report of *S. botryosum* and *Epicoccum purpurascens* Ehrenb. ex Schlecht. causing leaf spots on lentil in Hungary revealed that both species were pathogenic causing similar reddish spots in early infections. Symptom differentiation occurred on older leaflets when the colour of spots turned to grey in the case of *S. botryosum*, and to black in the case of *E. purpurascens* (Simay, 1990). *S. vesicarium* and *Alternaria porri* cause severe epidemics in onion fields in the United States with up to 90% crop loss due the two pathogens that produce similar purple spot symptoms on onion leaves (Suheri and Prince, 2000; Yanez, 2001). In both examples the two pathogens had overlapping symptoms that could not be distinguished in the field, at least initially in the case *S. botryosum* and *E. pupurascens*. Co-infection by several pathogens may therefore, contribute significantly to yield loss.

Direct losses caused by a disease can be primary or secondary. The direct primary loss is measured at seed harvest and involves reduction in quantity and quality in addition to the economic loss caused by the expenses for control measures (Cooke, 2006). Although yield loss at harvest is the most immediate effect a producer may experience due to stemphylium blight, additional consequences may be encountered in the following year when diseased seed is used for seeding the next lentil crop. Seed infection can affect seed germination and seedling vigour, depending on the level of seed-to-seedling transmission, and can represent the initial sources of inoculum in a field. It is known that infected lentil seeds with *S. botryosum* have reduced germination, and seed staining

levels are inversely proportional to germination rates (Banniza et al., 2006). The highest levels for seed infection of 3.4% and for seed staining of 13% obtained in this study were not as high as the 25% for infected seed and 75 to 100% for stained seeds reported previously (Banniza et al., 2006). However, it will be necessary to determine the seed-to-seedling transmission rate since this is required to estimate the risk of seed infection for subsequent crops. Seed infection as the main source of inoculum was reported for *S. vesicarium* in onion fields (Yanez, 2001).

The direct secondary loss is long term through the accumulation of inoculum in the soil and in stubble that reduces the expected future yield (Cooke, 2006). This is of particular importance when crop rotations are shortened as is currently the case for lentil in Saskatchewan (Madden, 2016). In addition to the accumulation of inoculum from residue, there is also a chance that this residue contains the sexual stage, which is considered the overwintering stage as reported for *S. vesicarium* on asparagus (Meyer et al., 2000; Basallote-Ureba et al., 1999), and pear (Rossi et al., 2005).The presence of the sexual stage of a pathogen implies genetic recombination that could change the population over generations, particularly with regard to the development of new pathogen virulence that could affect resistance genes in the host.

Although breeding programs can improve the performance of lentil varieties by breeding for disease resistance and yield, this is a costly and time consuming process, thus it is important to determine the potential for stemphylium blight to cause economic loss to lentil producers. For Saskatchewan, the most important lentil diseases are ascochyta blight and anthracnose (Morrall, 1997). An example of the successful lentil breeding is CDC Robin, one of the first cultivars with anthracnose (Race 1) and ascochyta blight resistance that was developed at the Crop Development Centre of the University of Saskatchewan without compromising seed yield (Vandenberg et al., 2002). The introduction of resistant varieties can lead to a shift in pathogens, and that may have been witnessed in Saskatchewan where ascochyta blight has declined in prominence. Indeed, the last major outbreak was recorded in 2005 (Morrall et al. 2006). Even in a wet year such as 2010, 45 to 100% (provincial mean 84%) of seed samples analyzed at commercial seed testing labs were free of ascochyta blight, which was attributed to the widespread cultivation of ascochyta blight resistant cultivars (Morrall et al. 2011). It appears that as ascochyta blight has declined in importance, stemphylium blight has become more prominent in Saskatchewan (Mwakutuya and Banniza, 2010). This occurred during a period when lentil production has risen to be transformed

from special crop into a major Canadian commodity. Disease surveys in lentil fields and testing of seed samples have reported traces of stemphylium blight in seed and crop samples with low to high levels during 2002 to 2015 (e.g. Chongo et al., 2003; Morrall et al., 2005; Banniza et al., 2009; Miller et al., 2012; Dokken-Bouchard et al., 2016). Other reasons for the increased reports on stemphylium blight could be increased awareness and better detection, or the fact that this disease may have cycles that match the climate cycles observed in the regions where lentil is produced. In Patna India, for example, stemphylium blight was reported as a cyclic disease, showing up at high levels every two to four years (Sinha and Singh, 1993). Similar disease cycles were reported from pear where stemphylium blight (caused by *S. vesicarium*) shifted from 5 to 10% severity in one year to 90% in the subsequent year (Montesinos and Vilardell, 1992). However, the decline in ascochyta blight due to resistant varieties may have given *Stemphylium* spp. a competitive advantage leading to an increase in stemphylium blight on varieties that currently do not have high levels of resistance to this particular disease.

Stemphylium blight on lentil in Saskatchewan was previously reported to be caused by *S. botryosum* (Dokken-Bouchard, 2010; Morrall, 2003), likely based on other reports that indicated this pathogen as the causal agent (Huq and Khan, 2008; Bayaa and Erskine 1998; Bakr and Ahmed, 1992; Chowdhury et al., 1997). A detailed characterization of Canadian isolates from lentil has not been published to date, although the identification of pathogenic species is important since each pathogen may behave differently epidemiologically and may be virulent on different varieties.

The different pathogenic species of *Stemphylium* described from around the world have similar morphology and molecular features, which have made identification difficult (Graf et al., 2016; Kurose et al., 2015) as was observed in the current study of conidia. As concepts around species recognition have evolved over the last decade, the complexity in the identification of fungal species has increased (Taylor et al., 2000). The classical evolutionary species concept comprises morphology, biology and phylogenetic species recognition (Taylor et al., 2000); however, when morphological features overlap or in the absence of sexual states that could be used for the biological species concept based on mating, molecular phylogenetic species recognition should be the approach of choice (Taylor et al., 2000). Unlike morphological characters, DNA is consistent and is not affected by environmental factors as would be the case for morphological characterisation (Kurose et al., 2015).

For consistency in phylogenetic analyses of fungi the ITS region is recommended since it is considered the barcoding gene for fungi (Crous et al., 2014). However, one single locus may only provide a preliminary perspective, and will not necessarily show interspecific differentiation (Leavitt et al., 2015). The use of multiple independent loci under the Genealogical Concordance Phylogenetic Species Recognition proposed by Taylor et al., (2000) has been recommended to increase the accuracy of species differentiation (Leavitt et al., 2015). Most of the *Stemphylium* species recorded as plant pathogens in Japan have been identified by using molecular phylogenetic analysis of four loci (rDNA-ITS, *EF-1a*, *gpd*, and *vmaA-vpsA*) with the addition of morphologic studies (Kurose et al., 2015).

Molecular phylogenetic analyses with the barcoding loci ITS and the conserved gene region *gpd* combined have been consistently used for the study of *Stemphylium* genus. (Camara et al., 2002; Wang et al., 2010). The use of these methods has not related well with morphological methods in very closely related species such as *S. vesicarium* and *S. herbarum* (Camara et al., 2002). Either morphology overlaps or molecular data shows identical DNA sequences. *Stemphylium* morphological traits may have been affected by evolutionary convergence as pointed out by Wang et al. (2010).

Thus to identify, describe and infer phylogenetic relationships of different species of *Stemphylium* it is essential to employ combined analyses of morphological and molecular data (Wang et al., 2010). The observation of overlapping morphology of conidia in this study highlighted that morphologic studies were not a helpful tool to differentiate two closely related *Stemphylium* spp. considering that the isolates in Clusters A and B shared similar conidial features. Future studies may have to include the description of additional structures such as conidiophores and sexual structures such as the ascus and the ascospore.

The statistical methods (NJ, ML and UPGMA) used for the construction of phylogenetic trees provides support of clades, but does not determine the branch representing a separate species. Misrepresentation of *P. tarda* EGS 08-069 as an ex-type species, and identical sequences of ITS and *gpd* for *P. herbarum* EGS 36-138, *S. vesicarium* EGS 37-067 and *S. alfalfa* EGS 36-088 originally identified as separate species by morphology confirm the imprecision of the morphological species concept for fungal species without support by molecular data as pointed out by Crous et al., (2014).

To conclude, in this study yield loss due to stemphylium blight in lentil was not demonstrated, most likely due to the amount of background inoculum that resulted in diseased control treatments that were expected to be free of stemphylium blight. As proposed earlier, future work should focus on reducing, or if possible eliminating, the background inoculum so that uninoculated treatments are disease-free. Only then will it be possible to determine the real effect of stemphylium blight and develop economic thresholds for disease management intervention. This may be achieved through a combination of inoculation and antifungal protection; however, secondary effects of the fungicides on plant development and seed yield in uninoculated treatments would have to be assessed as well (Cooke, 2006).

Results here also suggest the need for studies of epidemics of stemphylium blight under field conditions including the identification of causal species of *Stemphylium / Pleospora* and their respective contributions to an epidemic. Assessment of the effect of the pathogens on different lentil genotypes also warrants further research. Such studies may confirm the importance of early- and mid-flowering infection for potential yield loss.

The species *S. botryosum* as the causal pathogen of stemphylium blight in lentil crop as reported by other authors was confirmed as one of two possible species observed in this study. However, an extensive revision of the genus *Stemphylium* based on detailed morphological description potentially including the sexual state and molecular phylogenetic analyses based on conserved genic regions that can delineate closely related species is required before the causal agent(s) of stemphylium blight in Saskatchewan lentil can be identified.

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Appendix 1: Mass spore production procedure of *Stemphylium* spp. used to produce inoculum for greenhouse and for field experiments

Isolate SB19 was grown in a 90 mm Petri dish with V8-PDA with an average nutrient concentration [V8-PDA(av)] (10 g Potato Dextrose Agar, 10 g agar, 3 g Carbonate calcium CaCO3, 150 ml V8 vegetable juice, 850 ml di-ionized water, and 1 ml of Chloramphenicol solution per 1000 ml of medium to make 1000 ml of medium) and incubated under dark conditions on the bench top at a temperature of approximately 25ºC for 7 to 10 days, at which point more than 80% of the plate was covered with mycelia and spores.

Baking aluminum trays of 33 x 45 x 2 cm were prepare for the second step in the procedure by covering the inside with two layers of cheesecloth that was taped onto the four sides. Then the tray was covered with a second tray as a lid, which was taped to the bottom tray, before being autoclaved at 120 psi for 30 min on the liquid cycle, and being left to cool on the bench top.

In the meantime 700 ml of V8-PDA with high nutrient concentration [V8-PDA(hi)] (175 ml of V8 juice, 3.5 g $CaCO_3$, 10.5 g PDA, 5.11 g agar, 1.75 g $MgSO_4$, 1 ml FeCl3 solution, 560 ml di-ionized water, and 1 ml of chloramphenicol per each 100 ml of medium) was prepared, and poured onto a sterile tray that was then covered again with the second sterile tray serving as a lid, and left overnight to cool. To inoculated trays, two Petri dishes of SB19 were washed with 5 mL of sterile di-ionized water per dish, using a bent glass rod to remove all spores and mycelia. Spores from both plates were combined with 15 ml of di-ionized water to get a volume of 25 mL of spore suspension. The suspension was poured onto the V8-PDA (hi) in the baking trays and spread with a bent glass rod. Trays were wrapped with plastic wrap carefully avoiding touching the medium with the plastic wrap, which was taped to all four sides of the tray. The trays were placed under continuously light on the bench top at room temperature (about 25ºC) for 20 days. After 20 days or until the whole tray was covered with spores and mycelia, trays were unwrapped and placed under dark conditions inside a running biosafety cabinet for two to three days to dry. Once dry, the inside of trays was scrapped with a sterile wire brush to loosen spores that were collected with a sterile filter paper serving as a 'spoon', and using a vacuum with a filter paper. Spores collected on

the same day were mixed and stored together in a glass beaker in an incubator at 35°C for 24 h to remove moisture. They were weighed, and stored in a sterile plastic bottle at room temperature (about 25°C).

Appendix 2: Pre-seeding and post-seeding chemical treatments and agronomic management of the field experiments at the Seed Farm in 2013 and 2014 and Preston Avenue in 2014.

Chemical treatments and agronomic management	2013 Seed Farm	2014 Seed Farm	2014 Preston field
Pre-seeding herbicide	06- May, Edge® a.i. Ethalfluralin 5% (17kg/ha)	No application	No application
Post-seeding herbicide	23-May, Roundup® a.i. glyphosate 48.8% (1L/acre)+ AimEC® a.i. Carfentrazone-ethyl (30mL/acre)	No application	21-May, Roundup® a.i. glyphosate 48.8% (1L/acre)+ AimEC® a.i. Carfentrazone-ethyl (30mL/acre)
Pre harvest chemical	13-August, Reglone® a.i. Diquat ion 240g per L (1L/acre)	12-August, Reglone® a.i. Diquat ion 240g per L (1L/acre)	12-August, Reglone® a.i. Diquat ion 240g per L (1L/acre)
Pesticides chemical	12-July, Orthene® a.i. Acefate 75% (750g/ha)	No application	No application
Seeding date	17-May, 540seeds/plot	13-May, 540seeds/plot	13-May, 540seeds/plot
Irrigation system set up	31-May (14 d.a.s.)	02-June	02-June
Low tunnels set up	11-June (25 d.a.s.)	11-June (29 d.a.s.)	11-June (29 d.a.s.)
Field cleaning (weeding)	once a week	every two weeks	3 weeding during all cycle
Bird net set up	08-August	07-August	08-August
Harvest	20-August	19-August	19-August

d.a.s: days after seeding.

Appendix 3: Temperatures and relative humidity collected from non-covered and covered treatments with green-polyethylene and Novagryl® at the Seed Farm field experiment in 2013, and from non-covered and covered treatments with green-polyethylene at the Seed Farm and Preston Avenue field experiments in 2014.

Site / year	Season	ºC RH (%)	Un-covered			Green-polyethylene			Novagryl®		
			Max	Min	Average	Max	Min	Average	Max	Min	Average
	May	Temp	36.2	12.6	19.7	34.0	16.4	23.1	32.9	16.5	22.5
		HR	100.0	30.3	84.8	100.0	67.7	94.7	100.0	60.9	91.0
Seed Farm 2013	June	Temp	25.2	12.8	16.1	19.0	14.9	17.3	21.3	14.1	17.9
		HR	100.0	40.6	97.2	100.0	100.0	100.0	100.0	97.3	99.8
	July	Temp	24.0	13.1	17.7	26.0	13.3	19.0	24.9	13.3	18.3
		HR	100.0	64.6	85.5	100.0	69.2	92.6	100.0	70.5	93.0
	August	Temp	24.9	9.7	16.8	28.7	11.8	17.6	30.7	11.7	18.5
		HR	100.0	60.3	86.2	100.0	38.3	96.2	100.0	47.3	93.3
	May	Temp	30.4	12.1	20.9	29.8	14.7	21.4	.	.	.
		HR	100.0	51.7	84.0	100.0	75.0	94.7	.	.	.
Seed Farm 2014	June	Temp	30.2	12.0	20.2	31.2	12.8	20.7	.	.	.
		HR	100.0	42.8	82.4	100.0	68.4	92.5	.	.	.
	July	Temp	28.5	12.5	21.3	31.4	13.5	21.1	.	.	.
		HR	100.0	37.7	73.6	100.0	58.7	89.4	.	.	.
	August	Temp	29.8	13.1	20.7	29.3	13.5	20.6	.	.	.
		HR	98.9	41.6	75.6	100.0	68.4	89.7	.	.	.
	May	Temp	29.6	11.7	21.0	37.6	13.9	22.5	.	.	.
		HR	100.0	47.4	82.5	100.0	54.5	88.5	.	.	.
Preston Ave. 2014	June	Temp	28.8	11.5	20.3	40.3	12.1	22.2	.	.	.
		HR	100.0	45.3	82.3	100.0	45.3	86.7	.	.	.
	July	Temp	29.1	12.4	21.2	37.4	12.9	23.2	.	.	.
		HR	100.0	45.0	75.2	100.0	47.4	83.2	.	.	.
	August	Temp	30.4	13.2	20.8	37.0	12.2	22.4	.	.	.
		HR	100.0	43.4	76.1	100.0	53.8	86.2	.	.	.

Temp: Temperature in Celsius degrees; HR: Relative humidity in percentage; LST: Field Laboratory.

Appendix 4: Results of the assessment of disease severity, yield loss and seed quality in field experiments at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014 (individual analyses).

A4.1. Analysis of the effect of low tunnels compared with non-covered treatments in the field experiments at the Seed Farm in 2013, Seed Farm in 2014, and Preston Avenue in 2014.

A4.1.1. Analysis of variance, linear contrast analysis, and least square estimates of stemphylium blight severity in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2013.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	2	31.2	78.48	<.0001
Inoculation timing	4	36.1	17.71	<.0001
Covering *inoculation timing	8	31.2	3.43	0.0061

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	36.3	156.91	<.0001

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	1081.82	63.0324	6.65	17.16	<.0001
Covering	Novagryl	946.52	55.973	4.5	16.91	<.0001
Covering	Non-covered	562.75	52.5204	3.73	10.71	0.0006

A4.1.2. Analysis of variance, linear contrast analysis, and least square estimates of stemphylium blight severity in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	18.3	136.13	<.0001
Inoculation timing	4	7.24	4.73	0.0347
Covering *inoculation timing	4	7.24	6.38	0.0162

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	18.3	136.13	<.0001

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	2059.4	47.4723	18.3	43.38	<.0001
Covering	Non-covered	1276.1	47.4723	18.3	26.88	<.0001

A4.1.3. Analysis of variance, linear contrast analysis, and least square estimates of stemphylium blight severity in low tunnels and in the non-covered treatments in field experiment at Preston Avenue at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	23.3	63.69	<.0001
Inoculation timing	4	8.86	6.35	0.0107
Covering *inoculation timing	4	8.86	2.91	0.0855

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	23.3	63.69	<.0001

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	1710.58	106.99	3.56	15.99	0.0002
Covering	Non-covered	1032.17	106.99	3.56	9.65	0.0011

A4.1.4. Analysis of variance, linear contrast analysis, and least square estimates of seed yield in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2013.

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Covering	2	10.1	218.54	<.0001
Inoculation timing	4	10.1	1.18	0.3754
Covering *inoculation timing	8	10.1	2.6	0.0783

Contrasts

Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	10.1	436.87	<.0001

Least Square Estimates

Effect	*Covering*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
Covering	Green polyethylene	1357.17	49.3038	10.1	27.53	<.0001
Covering	Novagryl	1389.45	49.3038	10.1	28.18	<.0001
Covering	Non-covered	2635.43	49.3038	10.1	53.45	<.0001

A4.1.5. Analysis of variance, linear contrast analysis, and least square estimates of seed yield in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Covering	1	30	113.08	<.0001
Inoculation timing	4	30	0.57	0.6858

Covering *inoculation timing	4	30	0.7	0.5962

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	30	113.08	<.0001

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	2152.08	121.35	30	17.73	<.0001
Covering	Non-covered	3977.08	121.35	30	32.77	<.0001

A4.1.6. Analysis of variance, linear contrast analysis, and least square estimates of seed yield in low tunnels and in the non-covered treatments in field experiment at the Preston Avenue at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	15.3	3.54	0.0792
Inoculation timing	4	15.3	0.28	0.8858
Covering *inoculation timing	4	15.3	0.41	0.8017

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	15.3	3.54	0.0792

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	7.3716	0.07143	2.92	103.2	<.0001
Covering	Non-covered	7.6604	0.1629	17.7	47.03	<.0001

A4.1.7. Analysis of variance, linear contrast analysis, and least square estimates of thousand seed weight in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2013.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	2	34.7	14.93	<.0001
Inoculation timing	4	35.9	1.87	0.1377
Covering *inoculation timing	8	34.7	0.5	0.8509

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	41.9	9.67	0.0034

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	29.27	0.3035	13.2	96.45	<.0001
Covering	Novagryl	27.37	0.3037	13.6	90.12	<.0001
Covering	Non-covered	29.12	0.1559	5.49	186.73	<.0001

A4.1.8. Analysis of variance, linear contrast analysis, and least square estimates of thousand seed weight in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	27	12.21	0.0017
Inoculation timing	4	27	0.77	0.5526
Covering *inoculation timing	4	27	2.54	0.0631

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	27	12.21	0.0017

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	25.1675	0.5282	7.4	47.65	<.0001
Covering	Non-covered	27.43	0.5282	7.4	51.94	<.0001

A4.1.9. Analysis of variance and linear contrast analysis of thousand seed weight in low tunnels and in the non-covered treatments in field experiment at the Preston Avenue at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	27	3.56	0.0699
Inoculation timing	4	27	2.01	0.121
Covering *inoculation timing	4	27	1.08	0.3871

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	27	3.56	0.0699

A4.1.10. Analysis of variance, linear contrast analysis, and least square estimates of percentage of seed infected with *Stemphylium* spp. in CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2013.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	2	26.9	4.08	0.0284
Inoculation timing	4	24	10.19	<.0001
Covering *inoculation timing	8	24	2.54	0.0369

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	26.9	7.53	0.0107

		Least Square Estimates				
Effect	*Covering*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
Covering	Green polyethylene	2.35	0.3571	26.9	6.58	<.0001
Covering	Novagryl	1.95	0.3571	26.9	5.46	<.0001
Covering	Non-covered	0.95	0.3571	26.9	2.66	0.013

A4.1.11. Analysis of variance, linear contrast analysis, and least square estimates of percentage of seed infected with *Stemphylium* spp. in CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2014.

	Type 3 Tests of Fixed Effects			
Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Covering	1	25.9	5.32	0.0294
Inoculation timing	4	25.9	2.18	0.0993
Covering *inoculation timing	4	25.9	0.76	0.5584

	Contrasts			
Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	25.9	5.32	0.0294

		Least Square Estimates				
Effect	*Covering*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
Covering	Green polyethylene	1.7	0.3082	15	5.52	<.0001
Covering	Non-covered	0.85	0.2021	15	4.21	0.0008

A4.1.12. Analysis of variance, linear contrast analysis, and least square estimates of percentage of seed infected with *Stemphylium* spp. in CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Preston Avenue at Saskatoon in 2014.

	Type 3 Tests of Fixed Effects			
Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Covering	1	15	25.26	0.0002
Inoculation timing	4	5.3	3.87	0.0798
Covering *inoculation timing	4	5.3	4.69	0.0556

Contrasts				
Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	15	25.26	0.0002

Least Square Estimates						
Effect	*Covering*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
Covering	Green polyethylene	2.6	0.4704	12.9	5.53	<.0001
Covering	Non-covered	0.15	0.2424	29	0.62	0.5408

A4.1.13. Analysis of variance and linear contrast analysis of percentage of staining seed in CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2013.

Type 3 Tests of Fixed Effects				
Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Covering	1	30	0.9	0.3499
Inoculation timing	4	30	2.38	0.0738
Covering *inoculation timing	4	30	0.18	0.9484

Contrasts				
Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	30	0.9	0.3499

A4.1.14. Analysis of variance and linear contrast analysis of percentage of staining seed in CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2014.

Type 3 Tests of Fixed Effects				
Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Covering	1	27	1.43	0.2416
Inoculation timing	4	27	2.13	0.1048
Covering *inoculation timing	4	27	1.33	0.2839

A4.1.15. Analysis of variance and linear contrast analysis of percentage of staining seed in CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Preston Avenue at Saskatoon in 2014.

Type 3 Tests of Fixed Effects				
Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Covering	1	15.8	1.68	0.2131
Inoculation timing	4	15.8	5.39	0.0062
Covering *inoculation timing	4	15.8	0.87	0.5059

		Contrasts		
Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	15.8	1.68	0.2131

A4.1.16. Analysis of variance, linear contrast analysis, and least square estimates of seed diameter of CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2013.

Type 3 Tests of Fixed Effects				
Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Covering	2	31.1	49.7	<.0001
Inoculation timing	4	31.2	0.61	0.656
Covering *inoculation timing	8	31.1	0.59	0.782

		Contrasts		
Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	40.3	83.53	<.0001

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	3.978	0.01998	13.1	199.12	<.0001
Covering	Novagryl	3.866	0.0205	16.8	188.54	<.0001
Covering	Non-covered	4.062	0.0108	3.33	376.22	<.0001

A4.1.17. Analysis of variance, linear contrast analysis, and least square estimates of seed diameter of CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	27	21.05	<.0001
Inoculation timing	4	27	0.41	0.7996
Covering *inoculation timing	4	27	1.33	0.2831

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	27	21.05	<.0001

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	3.8385	0.03509	5.05	109.39	<.0001
Covering	Non-covered	3.994	0.03509	5.05	113.82	<.0001

A4.1.18. Analysis of variance, linear contrast analysis, and least square estimates of seed diameter of CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Preston Avenue at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	27	24.28	<.0001
Inoculation timing	4	27	1.21	0.3283

Covering *inoculation timing	4	27	0.54	0.705

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	27	24.28	<.0001

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	3.6625	0.04051	3.8	90.41	<.0001
Covering	Non-covered	3.796	0.04051	3.8	93.71	<.0001

A4.1.19. Analysis of variance, linear contrast analysis, and least square estimates of seed thickness of CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2013.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	2	5.47	26.37	0.0016
Inoculation timing	4	5.47	0.68	0.6325
Covering *inoculation timing	8	5.47	0.64	0.7291

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	5.47	31.02	0.0019

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	2.4259	0.00796	2.68	304.62	<.0001
Covering	Novagryl	2.3818	0.00796	2.68	299.09	<.0001
Covering	Non-covered	2.3583	0.00796	2.68	296.13	<.0001

A4.1.20. Analysis of variance, linear contrast analysis, and least square estimates of seed thickness of CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Seed Farm at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	27	11.79	0.0019
Inoculation timing	4	27	0.09	0.9848
Covering *inoculation timing	4	27	1.55	0.2174

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	27	11.79	0.0019

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	2.0715	0.0288	5.14	71.92	<.0001
Covering	Non-covered	2.1685	0.0288	5.14	75.28	<.0001

A4.1.21. Analysis of variance, linear contrast analysis, and least square estimates of seed thickness of CDC Robin harvested in low tunnels and in the non-covered treatments in field experiment at the Preston Avenue at Saskatoon in 2014.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Covering	1	27	24.45	<.0001
Inoculation timing	4	27	2.03	0.1183
Covering *inoculation timing	4	27	0.36	0.8344

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	27	24.45	<.0001

Least Square Estimates

Effect	Covering	Estimate	Standard Error	DF	t Value	Pr > |t|
Covering	Green polyethylene	1.988	0.02477	4.85	80.25	<.0001
Covering	Non-covered	2.102	0.02477	4.85	84.85	<.0001

A4.2. Analysis of disease development in low tunnels in field experiments at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014.

A4.2.1. Analysis of variance and least square estimates of area under the disease progress curve (AUDPC) of lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2013 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	11.2	29.37	<.0001

Least Square Estimates

Effect	Inoculation timing (stage)	Estimate	Standard Error	DF	t Value	Pr > |t|
stage	Early-flower	1229.88	67.3707	6.09	18.26	<.0001
stage	Mid-flower	900.19	70.9594	6.47	12.69	<.0001
stage	Control	750.25	51.4009	5.05	14.6	<.0001
stage	Podding	912.69	87.8595	6.57	10.39	<.0001
stage	Seedling	1277.88	36.1973	5.04	35.3	<.0001

A4.2.2. Analysis of variance and linear contrast analysis of area under the disease progress curve (AUDPC) of lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Inoculation timing (stage)	3	1	5.50	0.3014

Contrasts

Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Inoculated *vs* un-inoculated	1	2.3	10.78	0.0676

A4.2.3. Analysis of variance, linear contrast analysis and least square estimates of area under the disease progress curve (AUDPC) of lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Preston Avenue at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Inoculation timing (stage)	4	5.65	25.22	0.0009

Contrasts

Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	15	69.25	<.0001

Least Square Estimates

Effect	*Inoculation timing (stage)*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
stage	Early-flower	1543.69	35.7836	4.86	43.14	<.0001

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stage	Mid-flower	1848.75	145.12	3.43	12.74	0.0005
stage	Control	1250.5	42.065	13.4	29.73	<.0001
stage	Podding	1927.41	61.8865	15	31.14	<.0001
stage	Seedling	1982.56	150.97	3.27	13.13	0.0006

Figure A4.2.1. Area under the disease progress curve (AUDPC) of lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2013 and 2014, and at Preston Avenue in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages. Different letters within the same color column are significantly different. Separation of means by Fisher's LSD ($P < 0.05$).
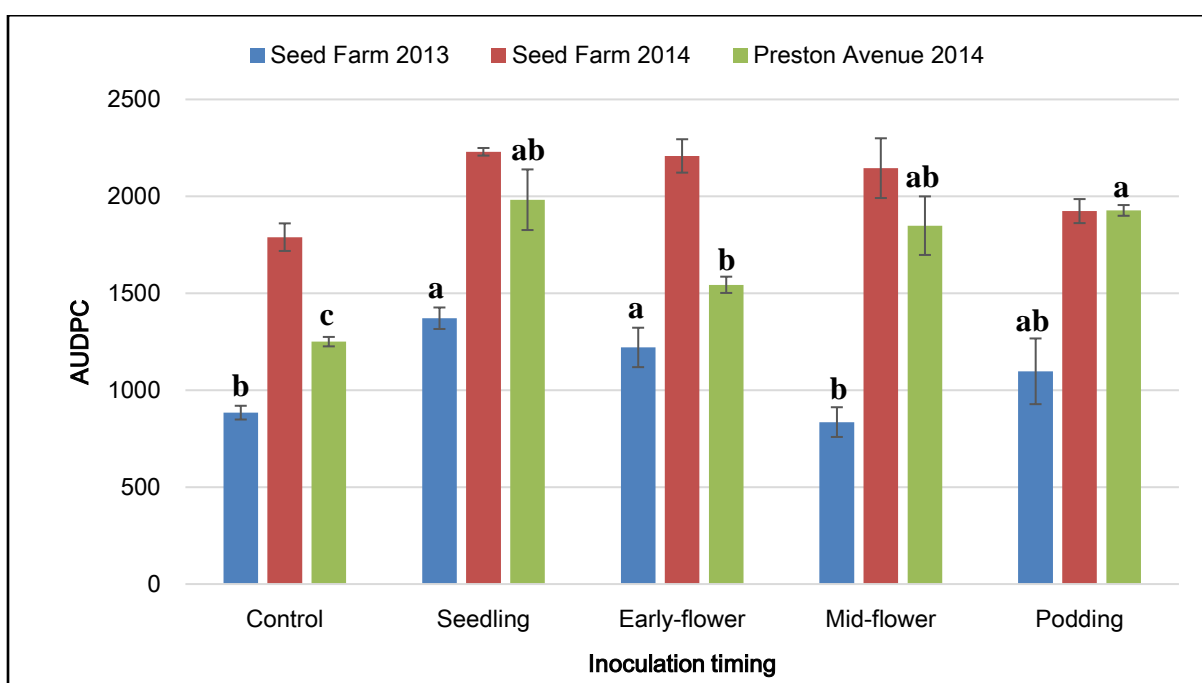
A4.3. Analysis of lentil seed yields harvested from low tunnels in field experiments at the Seed Farm in 2013and 2014, and at Preston Avenue in 2014.

 A4.3.1. Analysis of variance and linear contrast analysis of seed yield of lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in

2013 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	3.41	13.91	0.02020

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	3.41	7.67	0.0602

A4.3.2. Analysis of variance and linear contrast analysis of seed yield of lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	15	0.56	0.6951

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	15	0.96	0.3437

A4.3.3. Analysis of variance and linear contrast analysis of seed yield of lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Preston Avenue at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	1	0.94	0.6394

Contrasts				
Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	10	1.53	0.2424

Figure A4.3.1. Seed yields of lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2013 and 2014 and Preston Avenue in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages. Different letters within the same color column are significantly different. Separation of means by Fisher's LSD ($P < 0.05$).
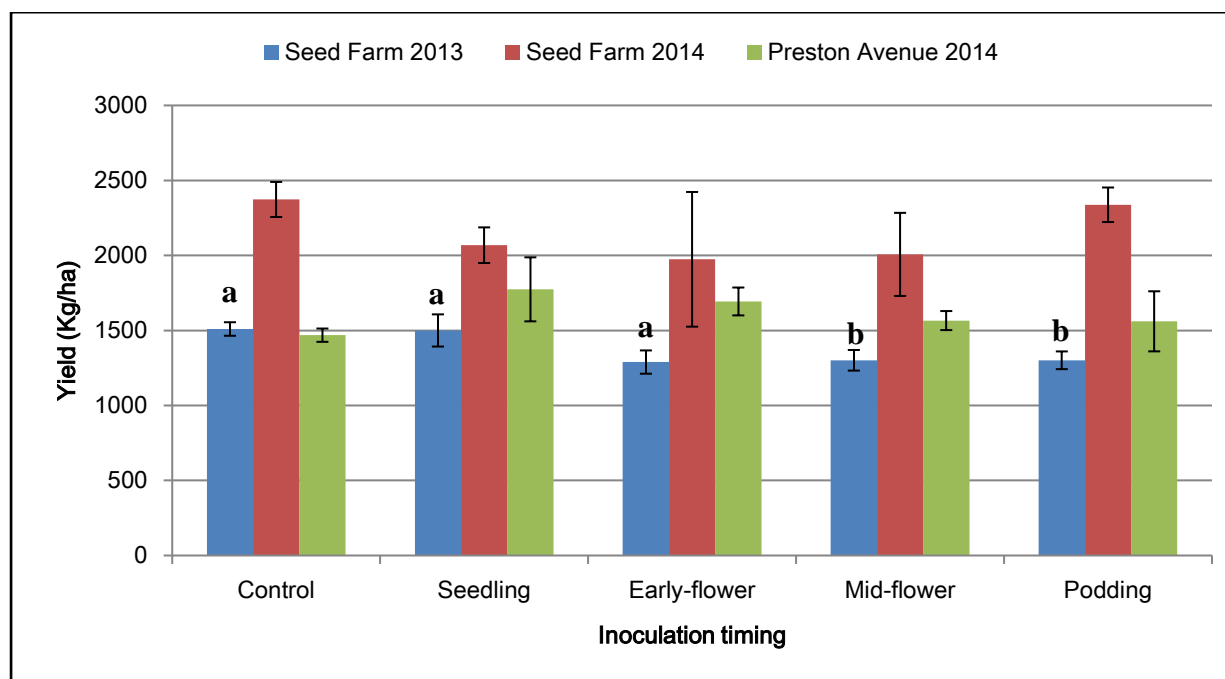
A4.4. Analysis of thousand seed weight of seed harvested from low tunnels in field experiments at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014.

A4.4.1. Analysis of variance and linear contrast analysis of thousand seed weight (TSW) harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at

the Seed Farm at Saskatoon in 2013 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	30	1.35	0.2735

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	30	1.72	0.2000

A4.4.2. Analysis of variance and linear contrast analysis of thousand seed weight (TSW) harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	15	2.25	0.1128

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	15	8.01	0.0127

A4.4.3. Analysis of variance and linear contrast analysis of thousand seed weight (TSW) harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Preston Avenue at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F

Inoculation timing (stage)	4	15	1.30	0.3157

	Contrasts			
Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	15	1.03	0.3266

Figure A4.4.1. Thousand seed weight (TSW) harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at Saskatoon at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10³ conidia mL^{-1} at four different growth stages.
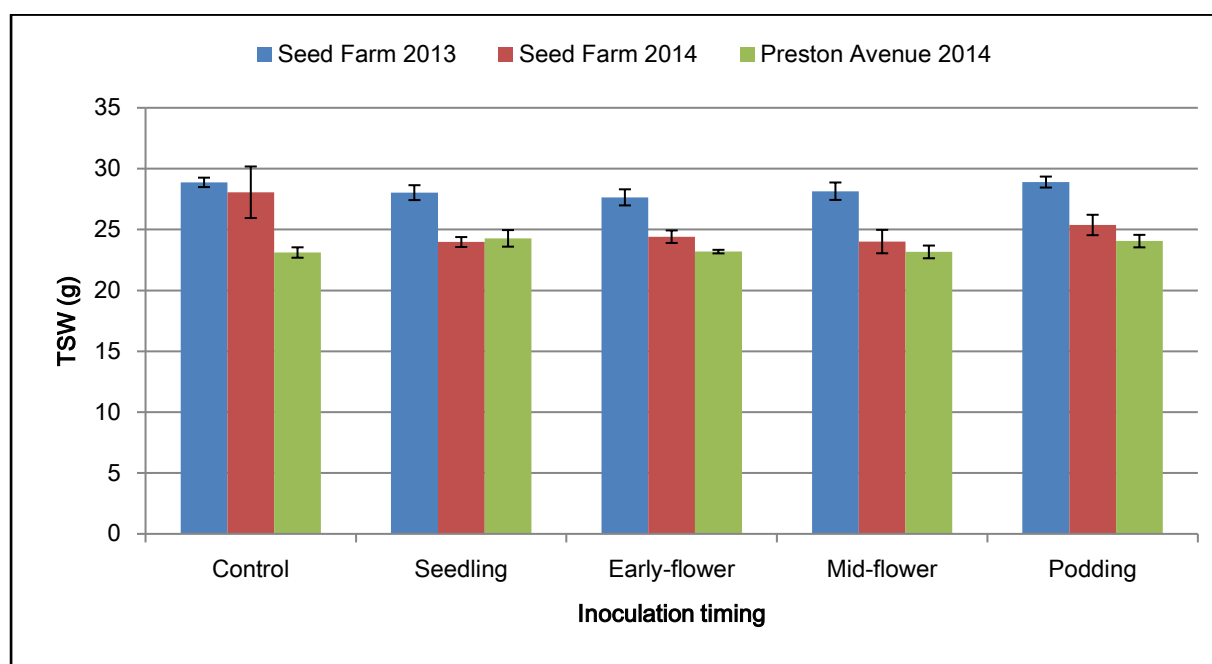
A4.5. Analysis of percentage of seed infected with *Stemphylium* spp. in seeds harvested from low tunnels in the field experiments at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014.

A4.5.1. Analysis of variance, linear contrast analysis and least square estimates of percentage of seed infected by *Stemphylium* spp. in seeds harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2013 and

inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	15.5	6.18	0.0035

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	21.1	21.49	0.0001

Least Square Estimates

Effect	Inoculation timing (stage)	Estimate	Standard Error	DF	t Value	Pr > |t|
stage	Early-flower	2.35	0.4072	17.2	5.77	<.0001
stage	Mid-flower	1.95	0.4072	17.2	4.79	0.0002
stage	Control	5	1	6	5	0.0025
stage	Podding	1.75	0.3953	6	4.43	0.0044
stage	Seedling	0.5	0.27	6	1.85	0.1135

A4.5.2. Analysis of variance and linear contrast analysis of percentage of seed infected by *Stemphylium* spp. in seeds harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	15	1.80	0.1809

Contrasts

Label	Num DF	Den DF	F Value	Pr > F

| covered *vs* non-covered | 1 | 15 | 3.79 | 0.0706 |

A4.5.3. Analysis of variance, linear contrast analysis and least square estimates of percentage of seed infected by *Stemphylium* spp. in seeds harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Preston Avenue at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	4.86	9.44	0.0161

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	4.62	3.23	0.1373

Least Square Estimates

Effect	Inoculation timing (stage)	Estimate	Standard Error	DF	t Value	Pr > |t|
stage	Early-flower	2.25	1.0597	2.68	2.12	0.1346
stage	Mid-flower	5.5	1.2153	2.75	4.53	0.0242
stage	Control	1.25	0.7892	2.42	1.58	0.2324
stage	Podding	0	0.001953	1	0	1
stage	Seedling	4	1.2492	2.77	3.2	0.0551

Figure A4.5.1. Percentage of seed infected by *Stemphylium* spp. in seeds harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at Saskatoon at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014 and inoculated with *Stemphylium*

botryosum field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages. Different letters within the same color column are significantly different. Separation by LSD ($P < 0.05$).
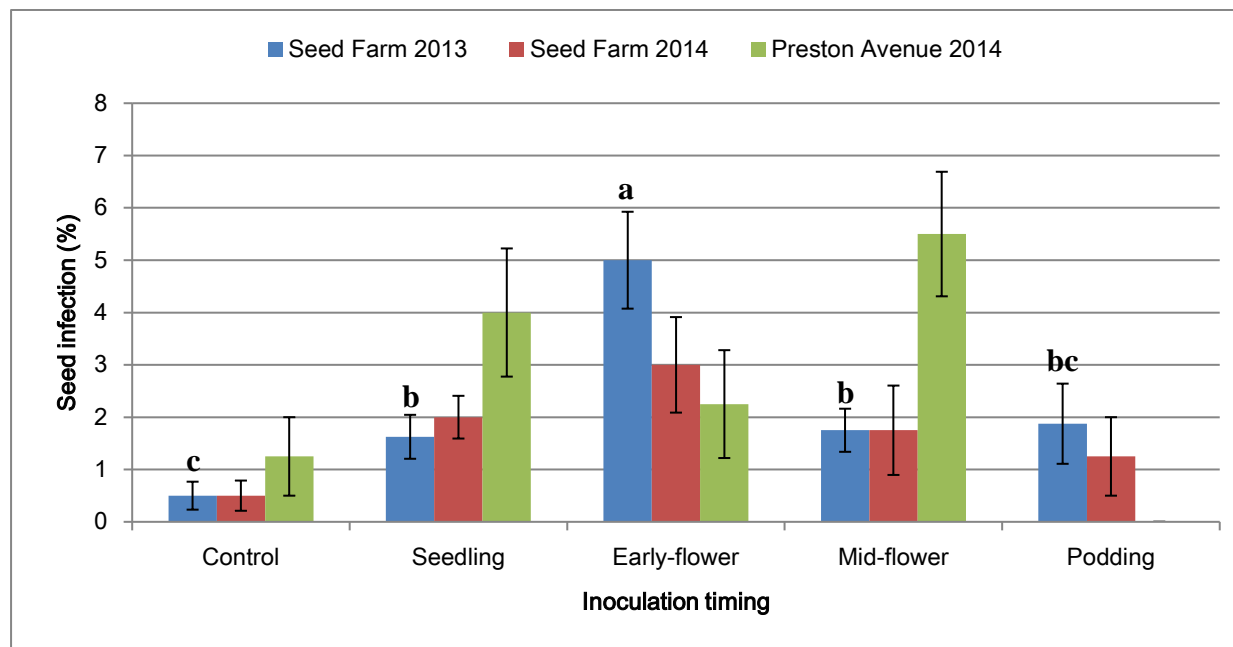
A4.6. Analysis of percentage of seed staining of seeds harvested from low tunnels in field experiments at the Seed Farm in 2013 and 2014, and Preston Avenue in 2014.

A4.6.1.Analysis of variance and linear contrast analysis of percentage of staining seed in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2013 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects				
Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Inoculation timing (stage)	4	3.76	1.01	0.4995

Contrasts				
Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	6.91	3.29	0.1132

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A6.2. Analysis of variance and linear contrast analysis of percentage of staining seed in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Inoculation timing (stage)	4	12	1.13	0.3885

Contrasts

Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	12	2.47	0.1418

A6.3. Analysis of variance, linear contrast analysis and least square estimates of the percentage of staining seed in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Preston Avenue at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Inoculation timing (stage)	4	15	4.14	0.0186

Contrasts

Label	*Num DF*	*Den DF*	*F Value*	*Pr > F*
covered *vs* non-covered	1	15	7.77	0.0138

Least Square Estimates

Effect	*Inoculation timing (stage)*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
stage	Early-flower	13.5	1.9257	15	7.01	<.0001

stage	Mid-flower	16.5	1.9257	15	8.57	<.0001
stage	Control	6.5	1.9257	15	3.38	0.0042
stage	Podding	8.75	1.9257	15	4.54	0.0004
stage	Seedling	11.25	1.9257	15	5.84	<.0001

Figure A6.1. Percentage of staining seed in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at Saskatoon at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1×10^3 conidia mL^{-1} at four different growth stages. Different letters within the same color column are significantly different. Separation of means by LSD ($P < 0.05$).
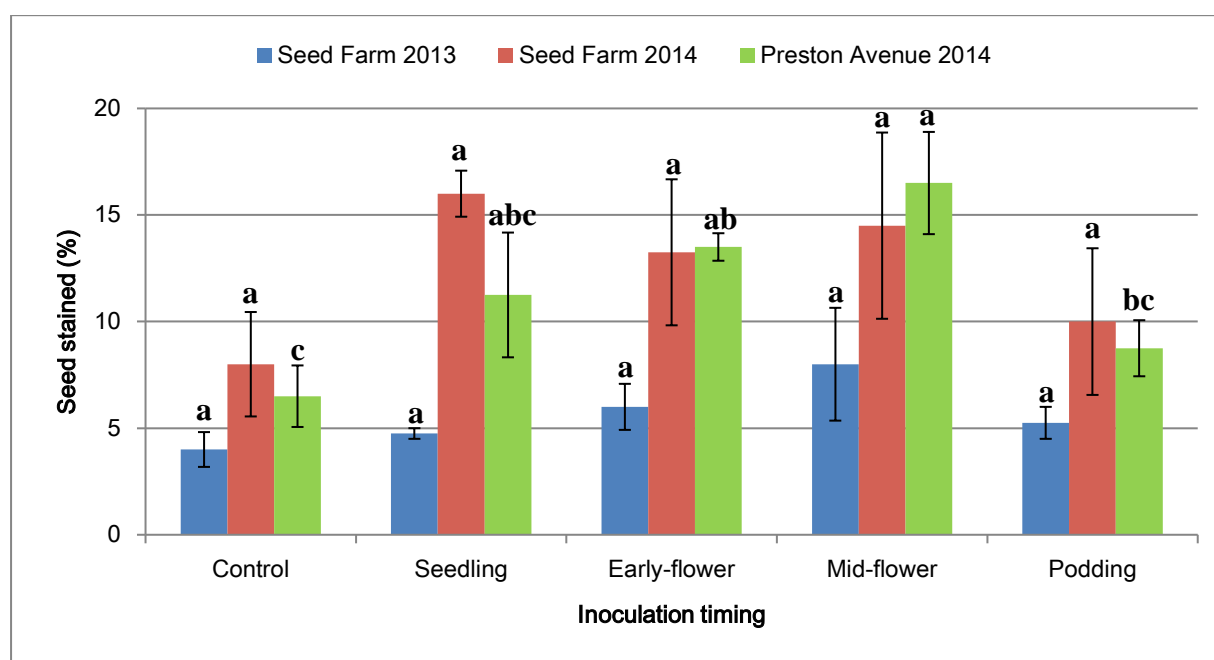
A4.7. Analysis of seed diameter in seeds harvested from low tunnels in field experiments at the Seed Farm in 2013, Seed Farm in 2014, and Preston Avenue in 2014.

A4.7.1. Analysis of variance and linear contrast analysis of seed diameter in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed

Farm at Saskatoon in 2013 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	27	0.89	0.4858

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	27	0.12	0.7371

A4.7.2. Analysis of variance and linear contrast analysis of seed diameter in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	12	1.27	0.3337

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	12	3.41	0.0894

A4.7.3. Analysis of variance and linear contrast analysis of seed diameter in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Preston Avenue at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	12	0.55	0.7012

Contrasts				
Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	12	1.01	0.3340

Figure A4.7.1. Assessment of seed diameter in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at Saskatoon at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

A4.8. Analysis of of seed thickness in seeds harvested from low tunnels in field experiments at the Seed Farm in 2013, Seed Farm in 2014, and Preston Avenue in 2014.

A4.8.1. Analysis of variance and linear contrast analysis of seed thickness in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed

Farm at Saskatoon in 2013 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	2.82	0.98	0.5334

Contrasts

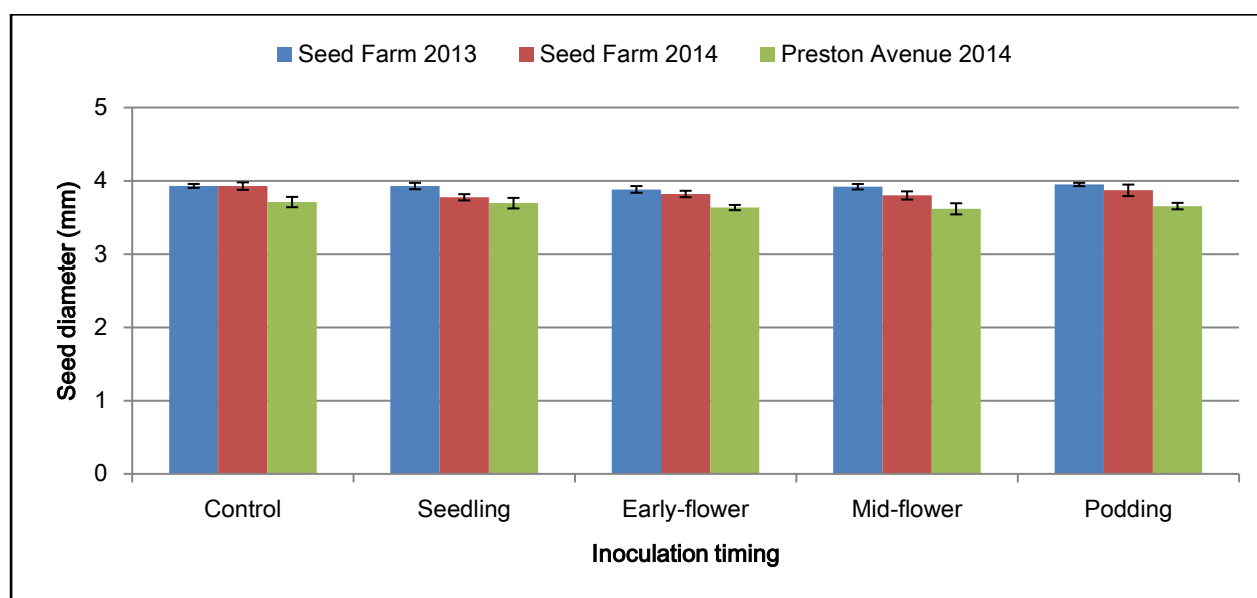| Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	2.82	2.07	0.2508

A4.8.2. Analysis of variance and linear contrast analysis of seed thickness in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Seed Farm at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	12	1.21	0.3576

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	12	3.09	0.1040

A4.8.3. Analysis of variance and linear contrast analysis of seed thickness in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at the Preston Avenue at Saskatoon in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1 x 10^3 conidia mL^{-1} at four different growth stages.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Inoculation timing (stage)	4	12	0.55	0.7010

Contrasts				
Label	Num DF	Den DF	F Value	Pr > F
covered *vs* non-covered	1	12	0.26	0.6218

Figure 4.8.1. Assessment of seed thickness in seed harvested from lentil cultivar CDC Robin grown in green polyethylene low tunnels in field experiments at Saskatoon at the Seed Farm in 2013 and 2014, and at Preston Avenue in 2014 and inoculated with *Stemphylium botryosum* field isolate SB19 at 1×10^3 conidia mL^{-1} at four different growth stages.
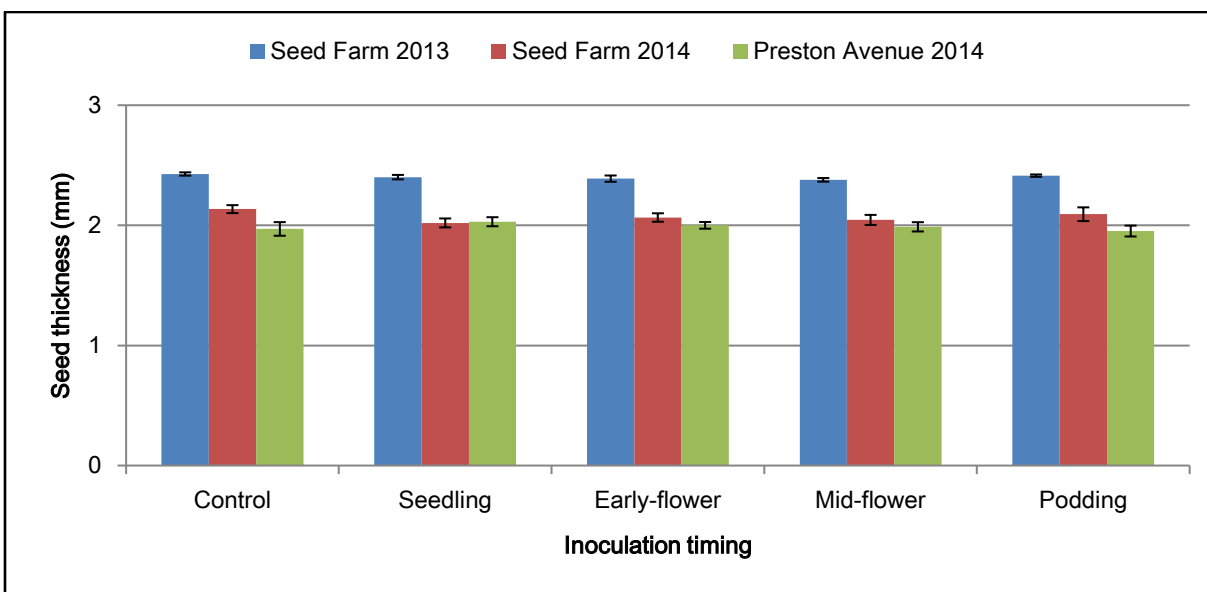
Appendix 5: Phylogenetic trees of the 23 isolates of *Stemphylium* spp., and an *Alternaria* sp. sequenced in this study, and sequences of ex-type/type of five *Stemphylium* species and one isolate of *Alternaria alternata* retrieved from GenBank based on ITS and *gpd* gene regions and constructed by using the Neighbor-Joining method. Bootstrap values ≥ 50% are shown above or below branches. The evolutionary analyses were conducted in MEGA6. a) ITS phylogenetic tree, b) *gpd* phylogenetic tree.

a) **ITS**

Appendix 5: Continued.   **b)** *gpd*
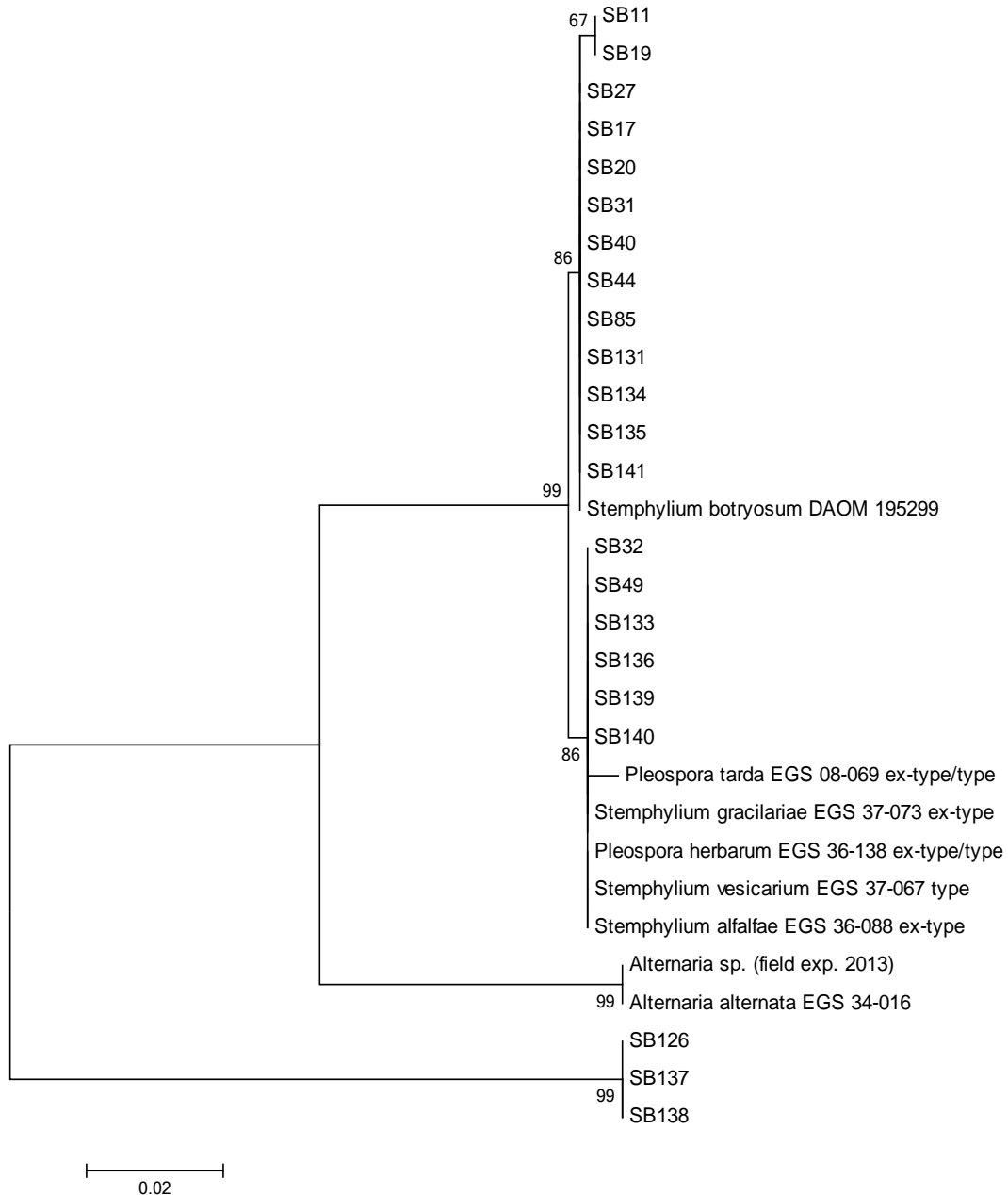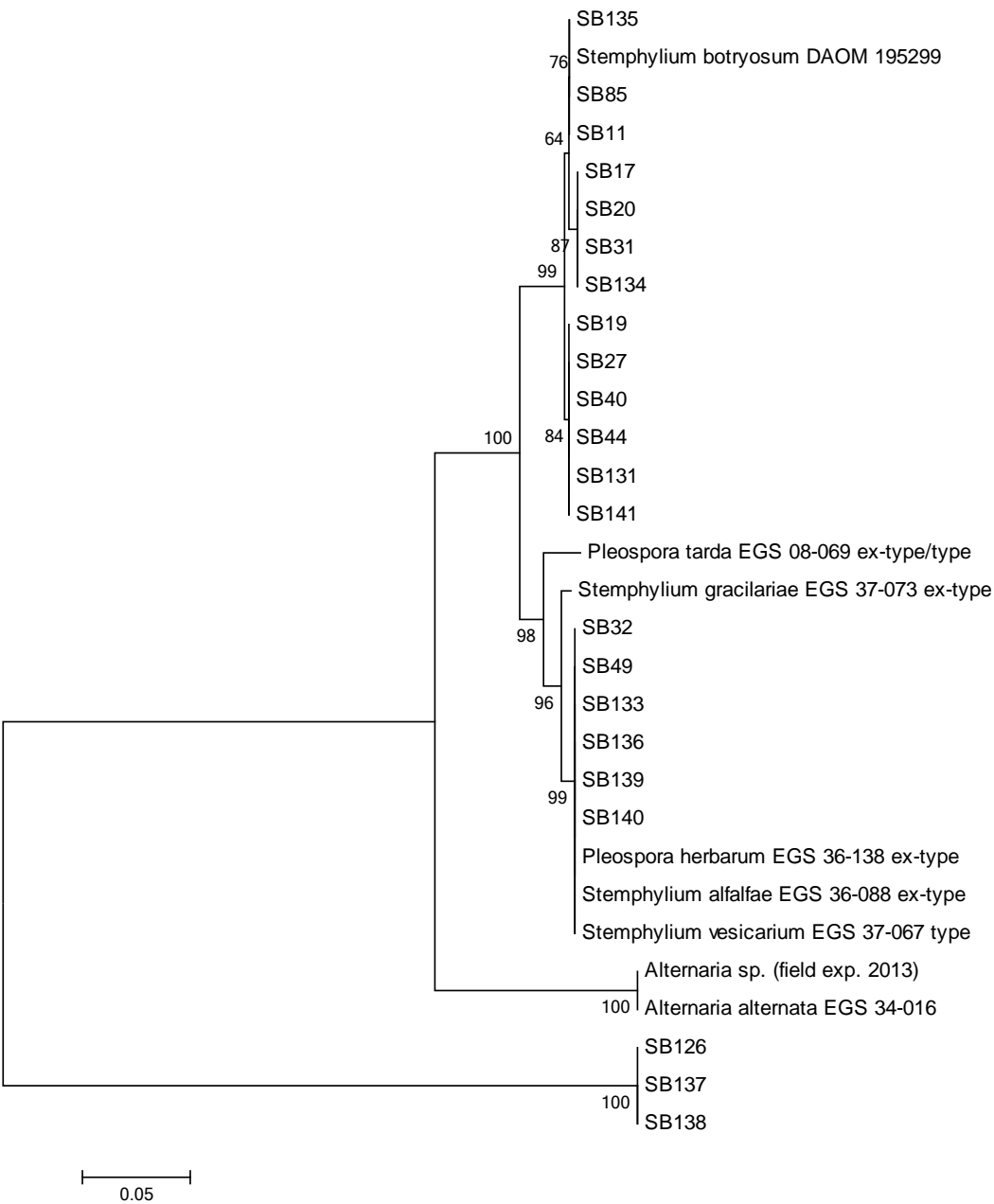
Appendix 6: Phylogenetic trees of the 23 isolates of *Stemphylium* spp., and an *Alternaria* sp. sequenced in this study, and sequences of ex-type/type of five *Stemphylium* species and one isolate of *Alternaria alternata* retrieved from GenBank based on ITS and *gpd* gene regions and constructed by using the Unweighted Pair Group Method with Arithmetic Mean method. Bootstrap values ≥ 50% are shown above or below branches. The evolutionary analyses were conducted in MEGA6. a) ITS phylogenetic tree, b) *gpd* phylogenetic tree.

a) ITS

Appendix 7: Phylogenetic trees of the 23 isolates of *Stemphylium* spp., and an *Alternaria* sp. sequenced in this study, and sequences of ex-type/type of five *Stemphylium* species and one isolate of *Alternaria alternata* retrieved from GenBank based on ITS and *gpd* gene regions and constructed by using the Maximum Likelihood method. Bootstrap values ≥ 50% are shown above or below branches. The evolutionary analyses were conducted in MEGA6. a) ITS phylogenetic tree, b) *gpd* phylogenetic tree.

a) ITS

b) *gpd*

- SB135
- Stemphylium botryosum DAOM 195299
- SB85
- SB11
- SB17
- SB20
- SB31
- SB134
- SB19
- SB27
- SB40
- SB44
- SB131
- SB141
- Pleospora tarda EGS 08-069 ex-type/type
- Stemphylium gracilariae EGS 37-073 ex-type
- SB32
- SB49
- SB133
- SB136
- SB139
- SB140
- Pleospora herbarum EGS 36-138 ex-type
- Stemphylium alfalfae EGS 36-088 ex-type
- Stemphylium vesicarium EGS 37-067 type
- Alternaria sp. (field exp. 2013)
- Alternaria alternata EGS 34-016
- SB126
- SB137
- SB138

80
87
85
70
82
54
94
98
100
100

0.1
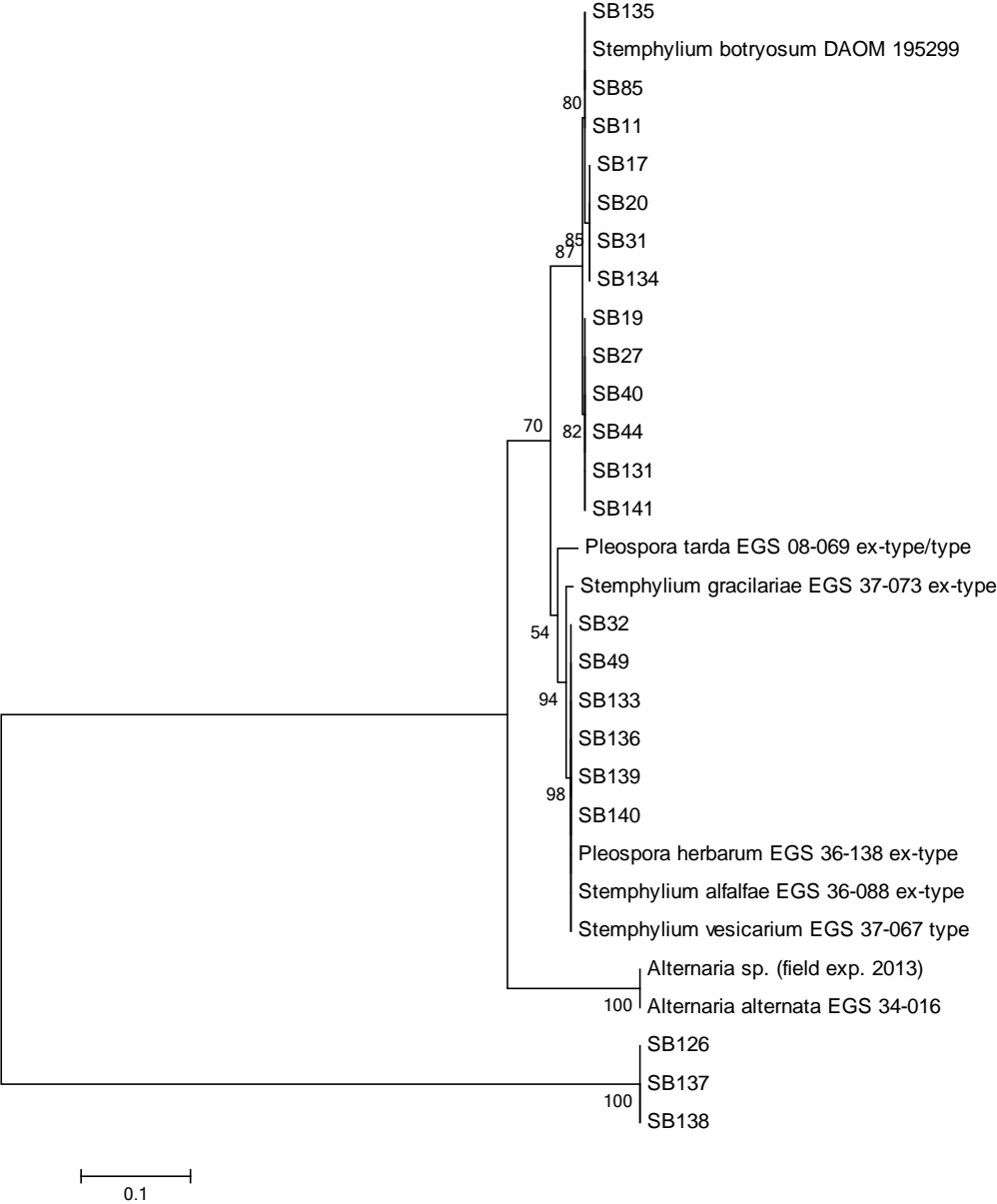
Appendix 8: Phylogenetic trees of the 23 isolates of *Stemphylium* spp., and an *Alternaria* sp. sequenced in this study, and sequences of ex-type/type of five *Stemphylium* species and one isolate of *Alternaria alternata* retrieved from GenBank based on the sequences of the ITS and *gpd* concatenated gene regions and constructed by using the Neighbor-Joining method. Bootstrap values ≥ 50% are shown above or below branches. The evolutionary analyses were conducted in MEGA6.
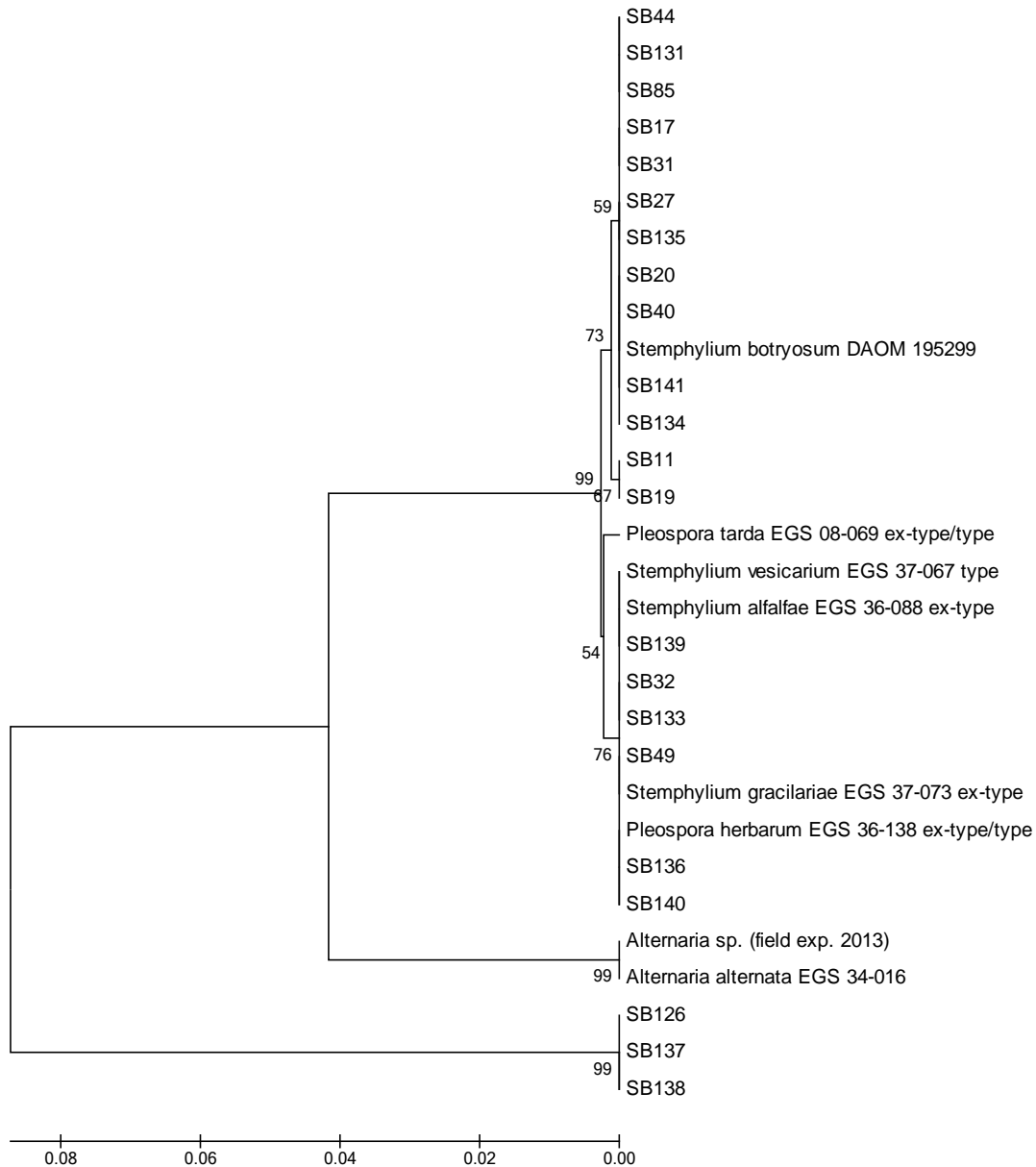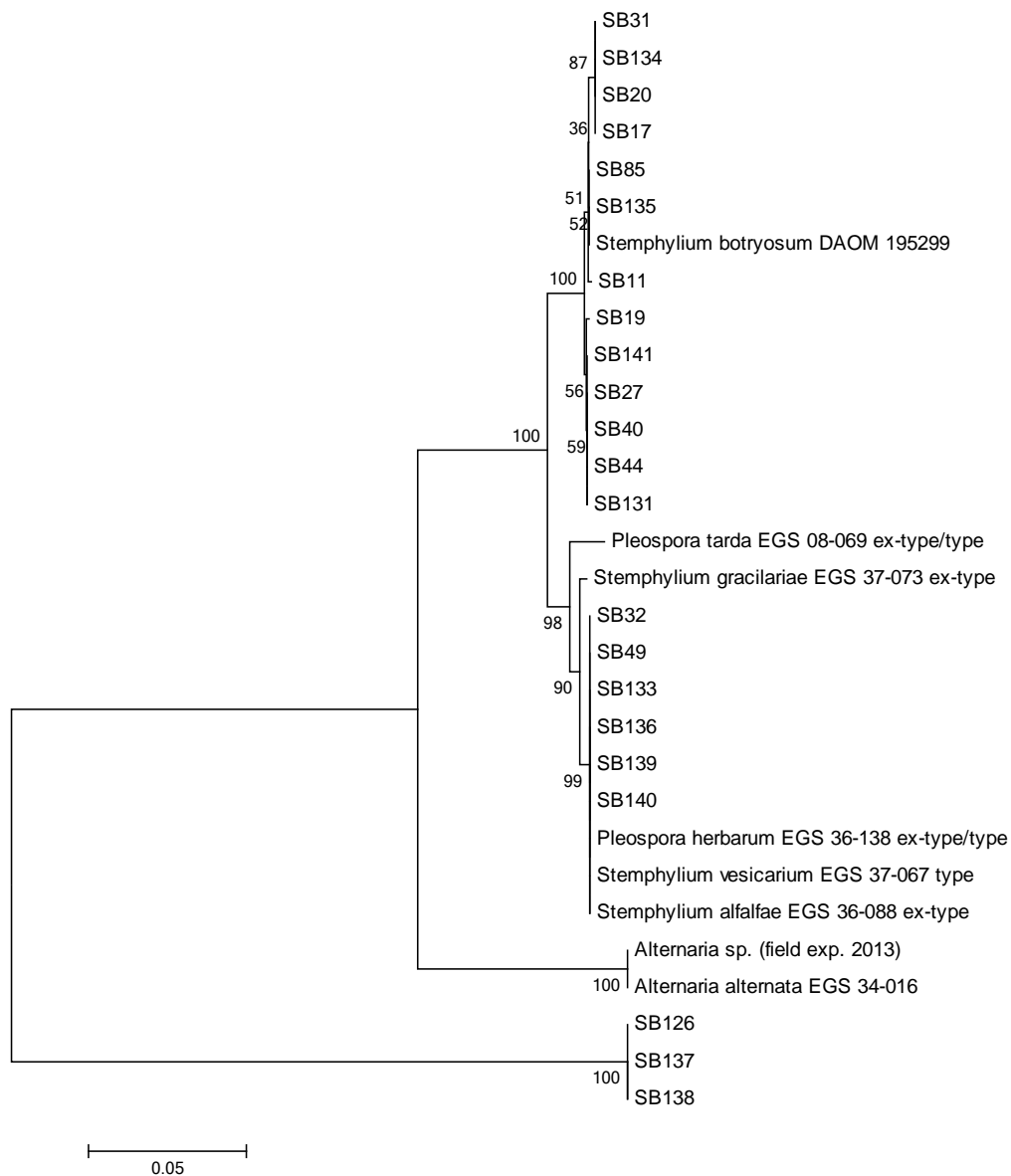
Appendix 9: Phylogenetic trees of the 23 isolates of *Stemphylium* spp., and an *Alternaria* sp. sequenced in this study, and sequences of ex-type/type of five *Stemphylium* species and one isolate of *Alternaria alternata* retrieved from GenBank based on the sequences of the ITS and *gpd* concatenated gene regions and constructed by using the Unweighted Pair Group Method with Arithmetic Mean method. Bootstrap values ≥ 50% are shown above or below branches. The evolutionary analyses were conducted in MEGA6.
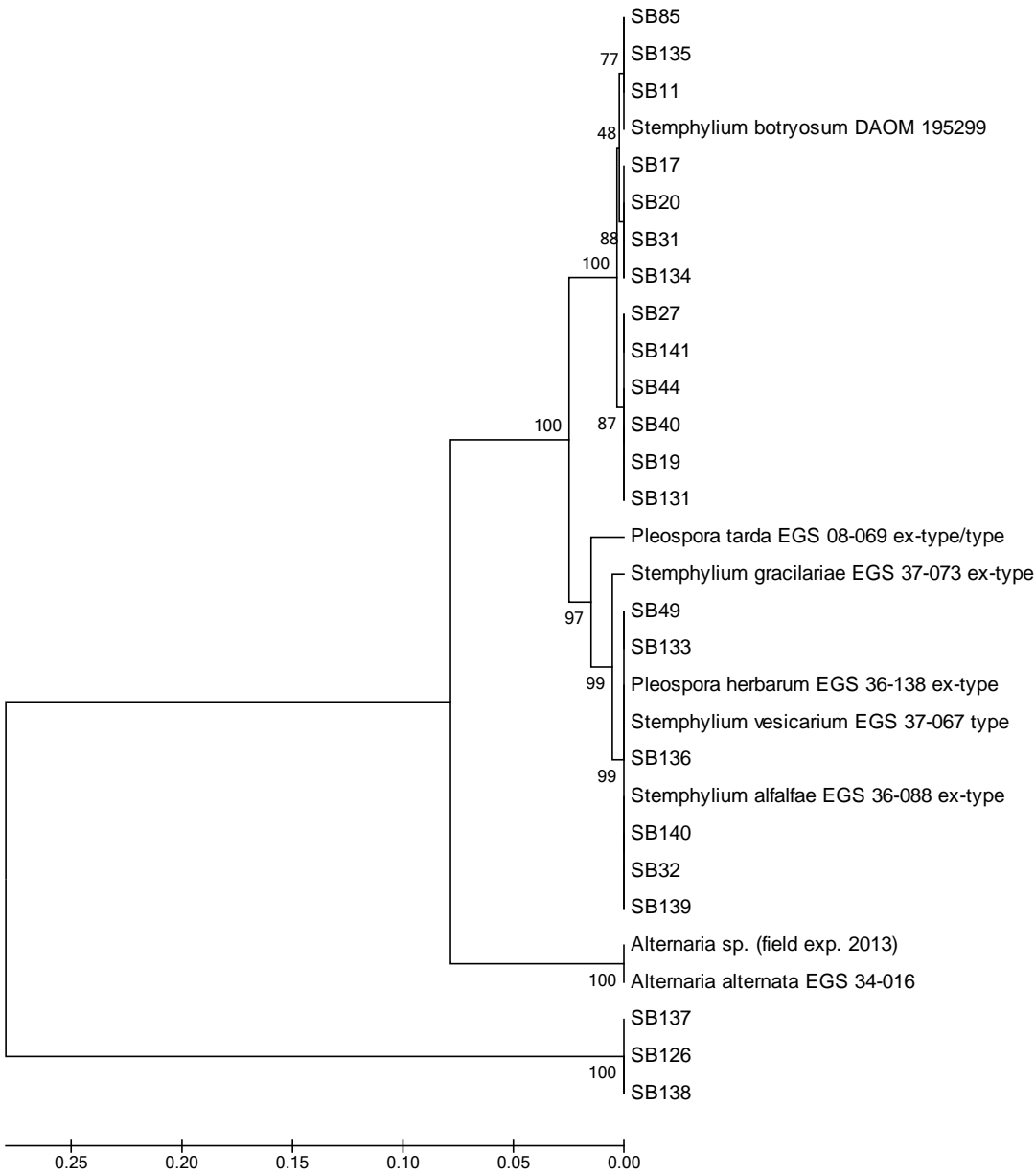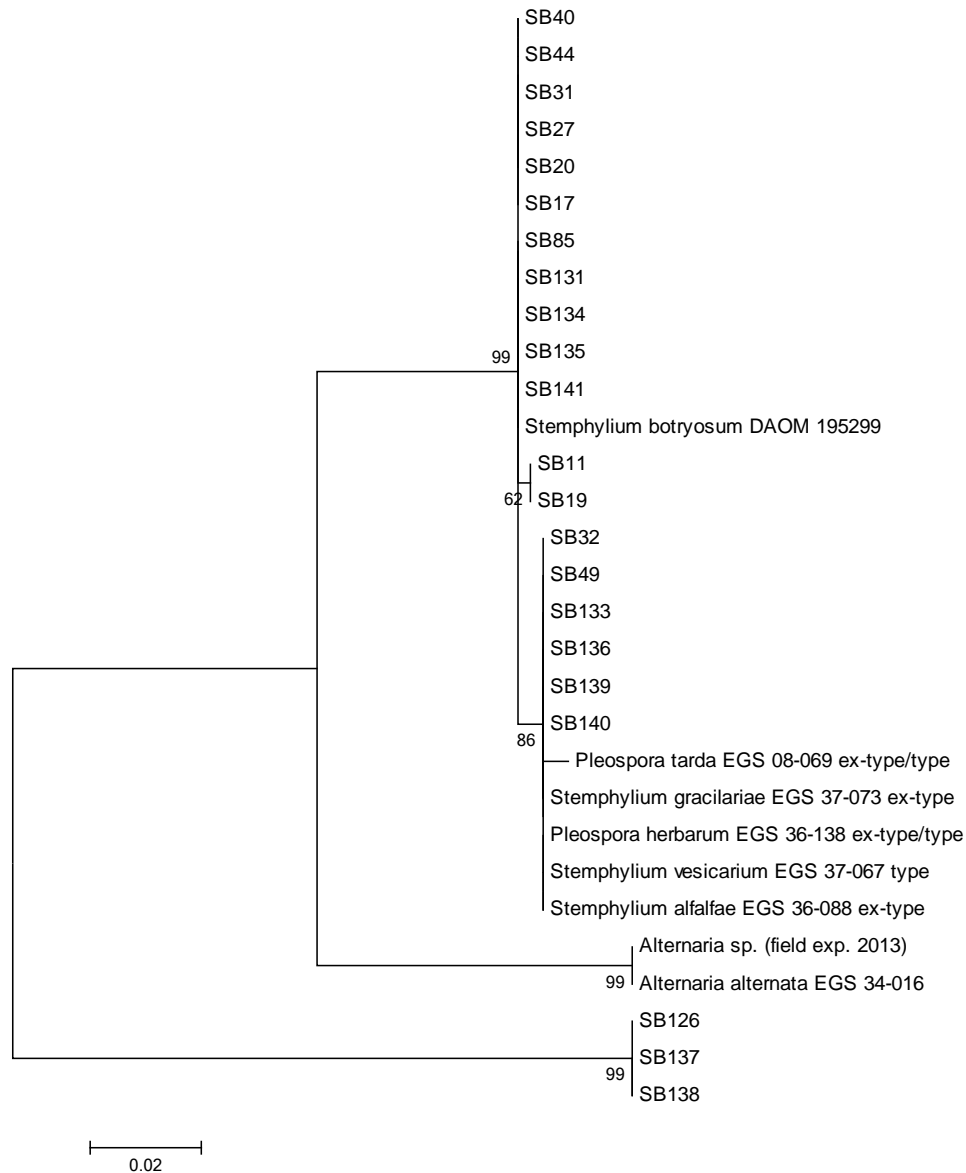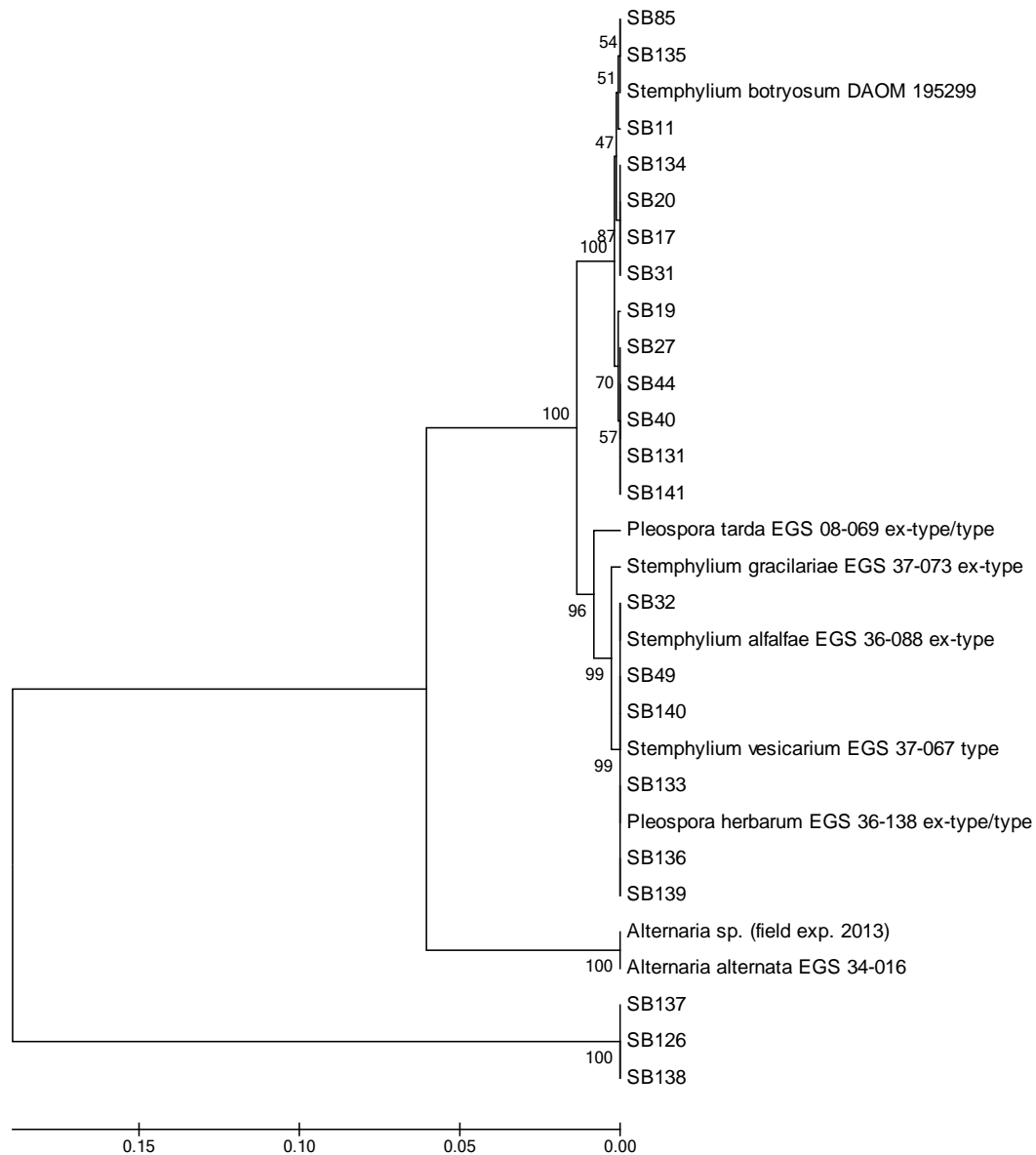
Appendix 10: Phylogenetic trees of the 23 isolates of *Stemphylium* spp., and an *Alternaria* sp. sequenced in this study, and sequences of ex-type/type of five *Stemphylium* species and one isolate of *Alternaria alternata* retrieved from GenBank based on the sequences of the ITS and *gpd* concatenated gene regions and constructed by using the Maximum Likelihood method. Bootstrap values ≥ 50% are shown above or below branches. The evolutionary analyses were conducted in MEGA6.
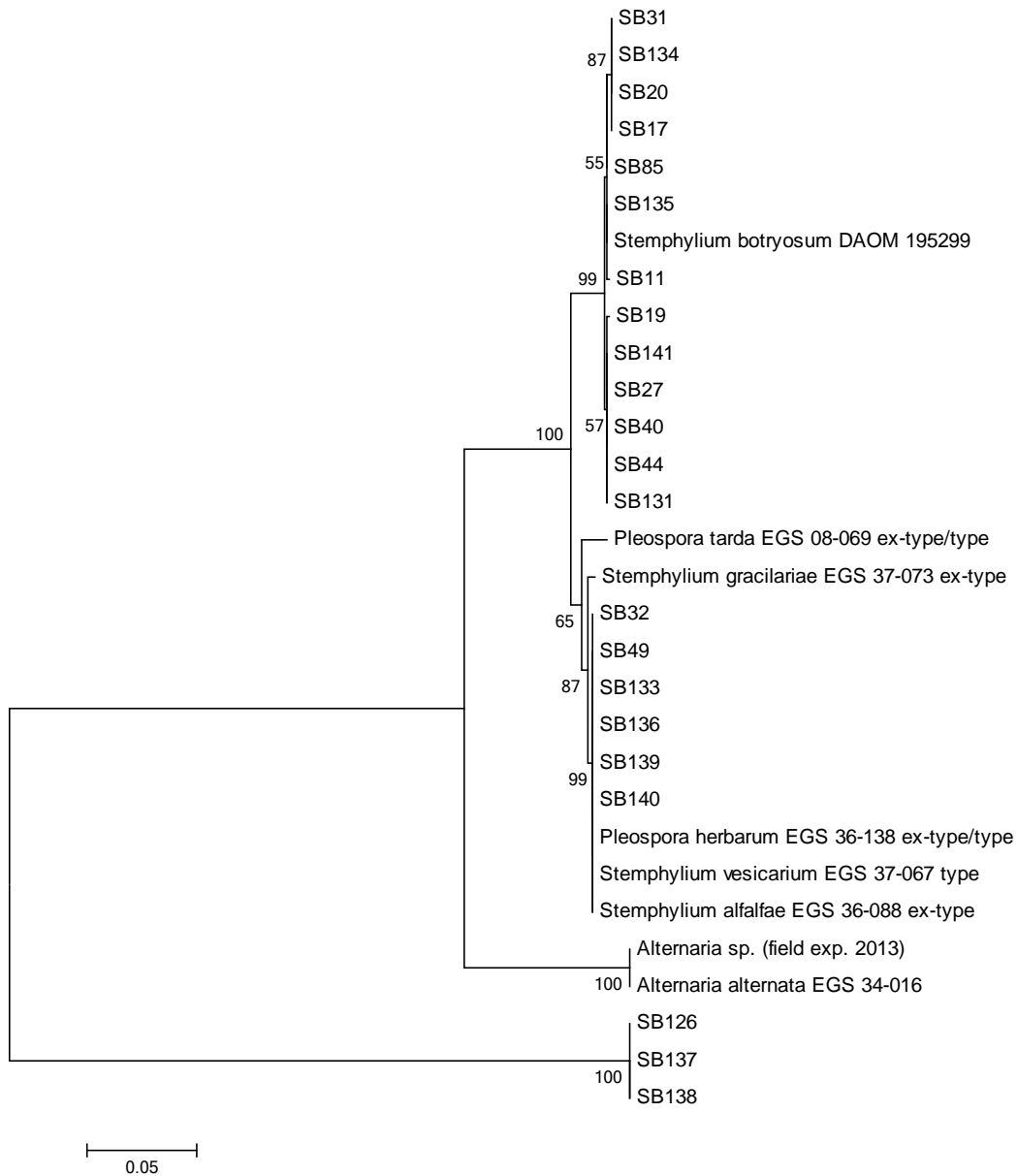
Appendix 11: Percent identity matrix of the concatenated DNA sequences of the ITS and *gpd* of the 23 isolates of *Stemphylium* spp., and *Alternaria* sp. sequenced in this study and sequences of five strains of *Stemphylium* ex-type/type species and one strain of *Alternaria alternata* retrieved from GenBank created with multiple sequence alignment by Clustal2.1 Omega (EMBL-EBI, www.ebi.ac.uk).

	Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	SB126	100	100	100	58.3	58	59.2	59.2	59.2	59.2	59.2	59.2	58.9	58.9	58.9	58.92
2	SB137	100	100	100	58.3	58	59.2	59.2	59.2	59.2	59.2	59.2	58.9	58.9	58.9	58.92
3	SB138	100	100	100	58.3	58	59.2	59.2	59.2	59.2	59.2	59.2	58.9	58.9	58.9	58.92
4	*Alternaria* sp.	58.3	58.3	58.3	100	100	86.1	86.1	86.1	86.1	86.1	86	86.2	86.2	86.2	86.22
5	*A. alternata* EGS 34-016	58	58	58	100	100	86.3	86.3	86.3	86.3	86.3	86.1	86.4	86.4	86.4	86.36
6	SB27	59.2	59.2	59.2	86.1	86.3	100	100	100	100	100	99.7	97.1	97.1	97.1	97.05
7	SB40	59.2	59.2	59.2	86.1	86.3	100	100	100	100	100	99.7	97.1	97.1	97.1	97.05
8	SB44	59.2	59.2	59.2	86.1	86.3	100	100	100	100	100	99.7	97.1	97.1	97.1	97.05
9	SB131	59.2	59.2	59.2	86.1	86.3	100	100	100	100	100	99.7	97.1	97.1	97.1	97.05
10	SB141	59.2	59.2	59.2	86.1	86.3	100	100	100	100	100	99.7	97.1	97.1	97.1	97.05
11	SB19	59.2	59.2	59.2	86	86.1	99.7	99.7	99.7	99.7	99.7	100	96.8	96.8	96.8	96.76
12	SB17	58.9	58.9	58.9	86.2	86.4	97.1	97.1	97.1	97.1	97.1	96.8	100	100	100	100
13	SB20	58.9	58.9	58.9	86.2	86.4	97.1	97.1	97.1	97.1	97.1	96.8	100	100	100	100
14	SB31	58.9	58.9	58.9	86.2	86.4	97.1	97.1	97.1	97.1	97.1	96.8	100	100	100	100
15	SB134	58.9	58.9	58.9	86.2	86.4	97.1	97.1	97.1	97.1	97.1	96.8	100	100	100	100
16	SB11	58.9	58.9	58.9	85.9	86	97	97	97	97	97	97.3	99.5	99.5	99.5	99.53
17	SB85	58.9	58.9	58.9	86	86.2	97.2	97.2	97.2	97.2	97.2	97	99.8	99.8	99.8	99.81
18	SB135	58.9	58.9	58.9	86	86.2	97.2	97.2	97.2	97.2	97.2	97	99.8	99.8	99.8	99.81
19	DAOM195299	58.9	58.9	58.9	86	86.2	97.2	97.2	97.2	97.2	97.2	97	99.8	99.8	99.8	99.81
20	*P. tarda* EGS 08-069	59.1	59.1	59.1	86	86	94.3	94.3	94.3	94.3	94.3	94.1	95.3	95.3	95.3	95.34
21	Sgracilariae	59	59	59	86.8	87	95.4	95.4	95.4	95.4	95.4	95.2	96.6	96.6	96.6	96.6
22	*P. herbarum* EGS 36-138	58.7	58.7	58.7	86.7	86.8	95	95	95	95	95	94.9	96.1	96.1	96.1	96.13
23	SB32	58.7	58.7	58.7	86.8	86.9	95.1	95.1	95.1	95.1	95.1	94.9	96.2	96.2	96.2	96.23
24	SB49	58.7	58.7	58.7	86.8	86.9	95.1	95.1	95.1	95.1	95.1	94.9	96.2	96.2	96.2	96.23
25	SB133	58.7	58.7	58.7	86.8	86.9	95.1	95.1	95.1	95.1	95.1	94.9	96.2	96.2	96.2	96.23
26	SB136	58.8	58.8	58.8	86.8	86.9	95.1	95.1	95.1	95.1	95.1	94.9	96.2	96.2	96.2	96.22
27	SB139	58.7	58.7	58.7	86.8	86.9	95.1	95.1	95.1	95.1	95.1	94.9	96.2	96.2	96.2	96.23
28	SB140	58.7	58.7	58.7	86.8	86.9	95.1	95.1	95.1	95.1	95.1	94.9	96.2	96.2	96.2	96.23
29	*S. vesicarium* EGS 37-067	58.7	58.7	58.7	86.8	86.9	95.1	95.1	95.1	95.1	95.1	94.9	96.2	96.2	96.2	96.23
30	*S. alfalfa* EGS 36-088	58.7	58.7	58.7	86.8	86.9	95.1	95.1	95.1	95.1	95.1	94.9	96.2	96.2	96.2	96.23

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Appendix 11: continued.

	Isolates	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	SB126	58.9	58.9	58.9	58.9	59.1	59	58.7	58.7	58.7	58.7	58.8	58.7	58.7	58.7	58.74
2	SB137	58.9	58.9	58.9	58.9	59.1	59	58.7	58.7	58.7	58.7	58.8	58.7	58.7	58.7	58.74
3	SB138	58.9	58.9	58.9	58.9	59.1	59	58.7	58.7	58.7	58.7	58.8	58.7	58.7	58.7	58.74
4	*Alternaria* sp.	85.9	86	86	86	86	86.8	86.7	86.8	86.8	86.8	86.8	86.8	86.8	86.8	86.79
5	*A. alternata*	86	86.2	86.2	86.2	86	87	86.8	86.9	86.9	86.9	86.9	86.9	86.9	86.9	86.91
6	SB27	97	97.2	97.2	97.2	94.3	95.4	95	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.13
7	SB40	97	97.2	97.2	97.2	94.3	95.4	95	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.13
8	SB44	97	97.2	97.2	97.2	94.3	95.4	95	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.13
9	SB131	97	97.2	97.2	97.2	94.3	95.4	95	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.13
10	SB141	97	97.2	97.2	97.2	94.3	95.4	95	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.13
11	SB19	97.3	97	97	97	94.1	95.2	94.9	94.9	94.9	94.9	94.9	94.9	94.9	94.9	94.94
12	SB17	99.5	99.8	99.8	99.8	95.3	96.6	96.1	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.23
13	SB20	99.5	99.8	99.8	99.8	95.3	96.6	96.1	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.23
14	SB31	99.5	99.8	99.8	99.8	95.3	96.6	96.1	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.23
15	SB134	99.5	99.8	99.8	99.8	95.3	96.6	96.1	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.23
16	SB11	100	99.7	99.7	99.7	95.3	96.6	96.1	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.23
17	SB85	99.7	100	100	100	95.5	96.8	96.3	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.42
18	SB135	99.7	100	100	100	95.5	96.8	96.3	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.42
19	DAOM195299	99.7	100	100	100	95.5	96.8	96.3	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.42
20	*P. tarda* EGS 08-069	95.3	95.5	95.5	95.5	100	97.8	98.1	98.1	98.1	98.1	98.1	98.1	98.1	98.1	98.07
21	Sgracilariae	96.6	96.8	96.8	96.8	97.8	100	99.2	99.3	99.3	99.3	99.3	99.3	99.3	99.3	99.34
22	*P. herbarum* EGS 36-138	96.1	96.3	96.3	96.3	98.1	99.2	100	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.91
23	SB32	96.2	96.4	96.4	96.4	98.1	99.3	99.9	100	100	100	100	100	100	100	100
24	SB49	96.2	96.4	96.4	96.4	98.1	99.3	99.9	100	100	100	100	100	100	100	100
25	SB133	96.2	96.4	96.4	96.4	98.1	99.3	99.9	100	100	100	100	100	100	100	100
26	SB136	96.2	96.4	96.4	96.4	98.1	99.3	99.9	100	100	100	100	100	100	100	100
27	SB139	96.2	96.4	96.4	96.4	98.1	99.3	99.9	100	100	100	100	100	100	100	100
28	SB140	96.2	96.4	96.4	96.4	98.1	99.3	99.9	100	100	100	100	100	100	100	100
29	*S. vesicarium* EGS 37-067	96.2	96.4	96.4	96.4	98.1	99.3	99.9	100	100	100	100	100	100	100	100
30	*S. alfalfa* EGS 36-088	96.2	96.4	96.4	96.4	98.1	99.3	99.9	100	100	100	100	100	100	100	100