DISEASE FORECASTING AND THE ROLE OF POLLINATORS IN THE SPREAD OF BLOSSOM
BLIGHT OF SEED ALFALFA IN SOUTHERN ALBERTA

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DEDICATION

On the edge and in the exactitude

of inexact sciences, I am here, friends,

not knowing how to explain these vocables

that little by little make their way into the sky

and try out their vigorous lives.

We were unsuccessful

burying the ostrich in our head,

or becoming holes in the earth:

"There is nothing more to know, everything is known."

"Trouble us no longer with geometry."

It is certain that an abstract uncertainty

takes leave of each chaos that returns

each time to order,

and how curious, everything

begins with words,

new words that sit alone

at the table, not having been invited,

hateful words that we swallow

and that get into our cupboards,

our beds, our loves,

until they exist: until the beginning

begins again in the verb.

- Pablo Neruda, "I Know Nothing"

(translated by William O'Daly)

ABSTRACT

Blossom blight of seed alfalfa (*Medicago sativa* L.), caused by fungal pathogens *Botrytis cinerea* Pers.:Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary, can contribute to significant yield declines in moist growing seasons. In a greenhouse study that examined the occurrence of pollen infection by *B. cinerea*, pollen infection was observed at very low levels (<1% of pollen infected) in suspension-inoculated treatments. When leafcutter bees (*Megachile rotundata*), hypothesized vectors of *B. cinerea* spores, were collected from seed alfalfa fields, the bees tested positive for *B. cinerea* but their pollen load did not. Frequency of bees carrying *B. cinerea* spores increased over the growing season, with 96-100% of bees from the final collections (~August 20) testing positive for *B. cinerea*. These patterns closely follow patterns of observed airborne *B. cinerea* spore concentrations as detected by a quantitative polymerase chain reaction (qPCR) assay using aerosol samples collected from seed alfalfa fields. A sensitive, species-specific primer and probe for *S. sclerotiorum* was designed for use in the same qPCR assay. Discharge of *S. sclerotiorum* in one field closely followed the discharge of *B. cinerea* spores, with several larger (>200 ascospores per day) discharge days observed beginning in mid-July and continuing to the end of August. In the other two fields surveyed, discharge of *S. sclerotiorum* was concentrated in one or two large events. It is concluded that pollen infection and transmission of blossom blight pathogens by leafcutter bees are not large contributors to the spread of blossom blight in southern Alberta. In addition, using qPCR to quantify airborne inoculum levels will likely be useful in developing a disease forecasting model for blossom blight of seed alfalfa.

Finally, I would like to acknowledge all the efforts of my co-advisors, Dan Johnson and Syama Chatterton. Dan, thanks for the discussions about statistics and for sharing many of your stories with me – they boosted my morale on many occasions. Syama, thank you for your patience and sense of humour. I don't know how you manage to juggle so many people and projects and still have time to edit my attempts at scientific writing over and over. And over. I appreciate it more than you know.

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LIST OF ACRONYMS AND ABBREVIATIONS

°C	degree Celsius
μ	micro
BHQ	black hole quencher
BLAST	basic local alignment search tool
bp	base pairs
BSM	Botrytis semi-selective media
CSGA	Canadian Seed Growers' Association
CSM	confocal scanning microscope
C_t	cycle threshold
CTAB	cetyltrimethyl ammonioum bromide
Cy5	cyanine5
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
f	femto
FAA	formalin alcohol acetic acid
FAM	fluorescein amidite
g	gram
GLM	generalised linear model
h	hours
hpi	hours post-inoculation
HPLC	high performance liquid chromatography
HSD	honest significant difference
IGS	intergenic spacer
ITS	internal transcribed spacer
kV	kilovolt
L	liter
lb(s)	pound(s)
LTP	lipid transfer protein
M	molarity
m	meter
mL	milliliter
NCBI	National Centre for Biotechnology Information
NUV	near ultra-violet
OA	oxalic acid
p	pico
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
qPCR	quantitative (real-time) polymerase chain reaction
RAPD	random amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
RH	relative humidity

s	seconds
SAS	Statistical Analysis System
SCAR	sequence characterized amplification region
SDW	sterile distilled water
SE	standard error of the mean
SEM	scanning electron microscope
SSM	Sclerotinia semi-selective media
VPD	vapour pressure deficit

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Blossom blight of seed alfalfa (*Medicago sativa* L.), caused by the fungal pathogens *Botrytis cinerea* Pers.:Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary, is an economically damaging disease in cool, wet growing seasons on the Canadian Prairies (Gossen et al. 1998; Gossen et al. 1997; Gossen & Platford 1999). In alfalfa, these pathogens establish first on senescent floret petals and grow to colonize the whole raceme. White or grey fluffy mycelia are signs of severe infections of *S. sclerotiorum* and *B. cinerea*, respectively. Infected floret petals may also fall through the canopy onto alfalfa stems, leading to stem rot, which has similar signs as blossom blight. Both of these infection processes may lead to reduced yields by either destroying the stigma or ovaries (blossom blight), or reducing the amount of nutrients reaching the developing seed (stem rot).

Both *B. cinerea* and *S. sclerotiorum* are generalist pathogens, infecting a wide range of field, horticultural, and ornamental crops (Elad et al. 2007; Boland & Hall 1994), and therefore their occurrence in seed alfalfa fields may influence, and may be influenced by, surrounding crops. For instance, Alberta is the largest producer of canola in Canada (StatsCanada 2014), and one of the main pathogens of canola is *S. sclerotiorum* (AAFC 2014). Thus, an outbreak of white mould in a nearby canola field could contribute to *S. sclerotiorum* inoculum in a nearby seed alfalfa field in subsequent years.

Growers of alfalfa seed in southern Alberta have reported decreased yields over the past years, in which healthy seedpods develop but have fewer seeds per pod or fall off the plant before harvest (Chatterton 2013). *Botrytis cinerea* and *S. sclerotiorum* can reduce the number of pods set, the number of seeds set per pod (Huang et al. 2000), and reduce alfalfa pollen viability (Huang et al. 1999, 1997). These two pathogens could be a contributing factor to this reported decline in yields. The purpose of this research project, therefore, is to investigate biological and environmental factors contributing to the spread and development of blossom blight in southern Alberta.

ECONOMY OF SEED ALFALFA

Alfalfa (*Medicago sativa*) grown for seed is a high-value crop whose production is a small but important part of Canadian agricultural exports. In 2009 (the most recent data available), Canadian growers exported $50 million of seed, with Alberta contributing over 35% of the total (AARD 2012a, 2012b; Kosinski 2012). The majority of seed is exported to the United States, but China, The Netherlands, South Korea, and Germany are also major importers of alfalfa seed (Kosinski 2012). Alfalfa seed is used to support one of the largest industries in agriculture – forage production for consumption by livestock – which in Alberta alone had an estimated value at over $1 billion in 2010 (Kosinski 2012).

According to the Canadian Seed Growers' Association (CSGA), total certified seed alfalfa acreage has increased steadily in Canada from 47,456 to 56,319 acres between 2012 and 2014, with nearly 50% of that acreage in Alberta (CSGA 2014). The region near Brooks in Southern Alberta has become well-established for growing seed alfalfa, in part because of the dryer, warmer climate that is ideal for leafcutter bees, and

in part because of an extensive irrigation network, which facilitates more vigorous plant growth (Frank 2003). Currently, growers of seed alfalfa in southern Alberta average at least 400 lbs of seed per acre, with a market value of at least $2.00 per lb (Witdouck 2015; Kosinski 2012). At these rates, seed alfalfa is a highly lucrative crop and growers may earn over twice what they would if they grew wheat on the same land. Growers earn additional income from the sale of leafcutter bees (*Megachile rotundata*), which are intensively managed as pollinators of the crop and also command a high market value at ~$100 per gallon of bee nests (Witdouck 2015; Kosinski 2012). Growers may also contract out their leafcutter bees for pollinating other crops, such as hybrid seed canola (Kosinski 2012).

BIOLOGY OF THE PATHOSYSTEM

The Pathogens: *Botrytis cinerea* and *Sclerotinia sclerotiorum*

Botrytis cinerea, causal agent of grey mould, and *S. sclerotiorum*, causal agent of white mould, are both generalist pathogens, with host ranges numbering in the hundreds (Boland & Hall 1994; Williamson et al. 2007). Both are considered model necrotrophic pathogens, colonizing only dead plant tissue (Agrios 1997). They are both members of the family Sclerotiniaceae (order: Hypocreales), and have a highly similar (>80% homology in amino acid identity) genetic composition (Amselem et al. 2011). *S. sclerotiorum* is considered a more aggressive pathogen of alfalfa than *B. cinerea* (Huang et al. 2000), although there is considerable variation in aggressiveness between isolates of *S. sclerotiorum* (Marciano et al. 1983). The pathogens share many similar life cycle characteristics but have some key differences, as described below.

Biology of *Botrytis cinerea*

Botrytis cinerea has a wide host range of over 200 species including fruit, vegetables, field crops, and flowers, and may cause complete yield losses in some field crops (Williamson et al. 2007; Elad et al. 2007). It is a pathogen most commonly studied for causing blossom blights and post-harvest rots in fruit and flowers including grape (Cadle-Davidson 2008; Coertze & Holz 2002; Nair & Allen 1993), strawberry (Bristow et al. 1986; MacKenzie & Peres 2012; Xu et al. 2000), raspberry (Jarvis 1962a; McNicol et al. 1985), nectarine and plum (Fourie & Holz 1998, 1995), geranium (Bi et al. 1999; Hausbeck & Pennypacker 1991), and rose (de Araujo et al. 2015; Elad 1988).

Signs and symptoms. Also known as grey mould, *B. cinerea* is readily identified by fluffy, grey hyphae growing on dead plant tissue. Often, the hyphae will produce conidia in grape-like clusters, which are slightly lighter in colour than the hyphae and produce a stippling effect. The conidia disperse easily if disturbed by wind or other physical forces. In alfalfa, it is often difficult to see early signs of grey mold, though in more severe infections, racemes of infected florets typically appear matted and clumped (Samac et al. 2015).

Life cycle. B. cinerea is generally considered to reproduce asexually, since it is heterothallic (i.e. each isolate is one mating type) and its sexual, apothecia-producing stage is rarely observed in the natural environment (Elad et al. 2007). In the field, the pathogen survives between seasons in two ways: First, at the end of a growing season, it may produce sclerotia – dense mycelia surrounded by a melanised rind – which allows overwintering in the soil. Sclerotia are formed by a wide range of fungi, and act as long-term survival structures, as they are resistant to chemical and physical degradation (Coley-Smith & Cooke 1971). In the spring, sclerotia may germinate to produce hyphae

and conidia (asexual spores), which are dispersed by wind, rain, or insects and act as the primary inoculum in a crop (Jarvis 1962a; Louis et al. 1996). Second, and more frequently, mycelium overwinters in dead crop residue and continues hyphal growth in the spring to directly infect nearby plants, producing conidia under favourable conditions in as little as 5-10 days (Agrios 1997).

Typically, aggressive growth by *B. cinerea* is favoured by prolonged (>12 h) wetness and temperatures between 18 and 22°C (Broome et al. 1994; Bulger et al. 1987; Hausbeck & Pennypacker 1991), although conidia production may be favoured at temperatures near 15°C (Jarvis 1977). Sudden changes in relative humidity (RH) may stimulate release of conidia (Jarvis 1962a), and production of conidia is stimulated by conidia removal; thus, conidia formation is an ongoing process (Elad et al. 2007). *Botrytis* can repeat this hyphae-conidia cycle many times within a growing season (i.e. it is polycyclic) if growing conditions are favourable.

Infection process. Considered a weak pathogen, *B. cinerea* requires a nutrient source, typically senescent plant tissue, on which to grow prior to infection of other plant parts (Edlich et al. 1989). In alfalfa and other crops, senescent flower petals are a nutrient-rich base for *B. cinerea* to establish (Huang et al. 2000). Upon landing on host tissue, airborne conidia can adhere to the substrate and remain viable for several days (Doss et al. 1993). The conidia require a thin film of water or high relative humidity (>93%) to germinate and actively penetrate host tissue (Cole et al. 1996; Hausbeck & Pennypacker 1991; Coertze et al. 2001; Jarvis 1962b). While dry-deposited conidia may germinate readily on a host, only conidia deposited by suspension produce a mucilaginous sheath that aids in further adhesion and infection (Cole et al. 1996; Doss et

al. 1993; Williamson et al. 1995). Germination may occur as rapidly as 3 hours after landing on susceptible tissue (Elad et al. 2007; Jarvis 1962b).

Once germinated on host tissue, the hyphae may form appressoria, swollen tips of hyphae that facilitate attachment to, and penetration of, the host (Agrios 1997). The hyphae may also release a host of cell wall-degrading enzymes to aid in penetration (Williamson et al. 1995; Choquer et al. 2007). Typically, growth of the hyphae is short (<15 μm) prior to appressoria formation (Fourie & Holz 1995), and conidia deposited in a suspension grew longer germination tubes than those deposited by dusting (Cole et al. 1996). Following the formation of appressoria or protoappressoria, *B. cinerea* breaches the host cuticle by forming an infection peg (Fourie & Holz 1995). Extensive inter- and intracellular colonization of host tissue was observed within 24 h of inoculating ripe plum and nectarine fruits with a suspension of *B. cinerea* conidia (Fourie & Holz 1995).

At the molecular level, infection by *B. cinerea* triggers an oxidative burst response in the host, which is the first step in the host's hypersensitive response and programmed cell death (Williamson et al. 2007). Thus, *B. cinerea* effectively kills host cells prior to colonizing them, a requirement of its necrotrophic lifestyle, which then facilitates rapid colonization of tissue (Govrin & Levine 2000; Van Kan 2005).

Quiescent infections. The ability of *B. cinerea* to survive quiescently in its host for extended periods of time is a well-documented but poorly understood phenomenon. Several studies have documented quiescence of *B. cinerea* conidia, wherein conidia that landed on host tissue survived for days or weeks and germinated only when conditions were conducive to growth (Elad 1988; Barnes & Shaw 2002; Prusky 1996; Prusky et al. 2013). However, *Botrytis* may also germinate and penetrate host tissue, and then become

quiescent at the appressoria stage, or later (Prusky 1996). Such infections are well known in the study of post-harvest grey mould of fruit (Bristow et al. 1986; McNicol et al. 1985; Cadle-Davidson 2008). In alfalfa, *B. cinerea* remained quiescent in the styles of pollinated florets for 5 weeks, and rapidly colonized the tissue once the plant was subjected to high humidity (Huang et al. 2000).

The ability of *B. cinerea* to survive as an endophyte (a microorganism that lives within a plant without causing disease) in its host is an aspect of quiescence that has been studied but not well documented. A handful of studies, however, suggest that *B. cinerea* may survive for long periods of time within its host and only become aggressive once the host is injured or stressed (Sowley et al. 2009; Barnes & Shaw 2003). In both lettuce (*Lactuca sativa*) and primula (*Primula* x *polyantha*), *B. cinerea* was isolated from all parts of the plants that were grown from seeds inoculated with *B. cinerea* conidia, indicating that it had colonized the seedlings and survived in the plant as an endophyte. In primula, *B. cinerea* only became symptomatic at flowering, approximately 3 months after seeding (Barnes & Shaw 2003).

Biology of *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum has a larger reported host range than *B. cinerea*, with over 400 species of gymnosperms and angiosperms acting as hosts (Boland & Hall 1994). It is an economically important pathogen of several crops, including carrot (McDonald et al. 2008; Foster et al. 2011; Parker et al. 2014a), canola (del Río et al. 2007; Bom & Boland 2000; Turkington & Morrall 1993), lettuce (Clarkson et al. 2014), sunflower (Marciano et al. 1983; McCartney & Lacey 1991), and bean (Tu 1988; Olivier et al. 2008).

Signs and symptoms. Often referred to as white mold, *S. sclerotiorum* is identified by the presence of white, cottony mycelium and, eventually, the formation of sclerotia on dead host tissue. Unlike *B. cinerea*, *S. sclerotiorum* does not produce conidia. Like *B. cinerea*, it can rapidly colonize whole racemes of alfalfa, giving a matted and clumped appearance to the racemes (Samac et al. 2015).

Life cycle. S. sclerotiorum is homothallic (i.e. each isolate contains both mating types), and therefore reproduces exclusively sexually. In *S. sclerotiorum*, the sclerotium plays a far greater role than in the life cycle of *B. cinerea*. *S. sclerotiorum* survives almost exclusively as sclerotia in soil or on dead plants, which germinate in the spring under prolonged cool (12-24°C), moist conditions (Agrios 1997; Clarkson et al. 2014). Sclerotia may germinate myceliogenically (i.e. asexually, producing mycelia) or carpogenically (i.e. sexually, producing apothecia), however the most common – and most epidemiologically important – method is carpogenic germination.

When sclerotia germinate carpogenically, stipes emerge from the soil, on the end of which small (<10 mm diameter) mushroom-like structures called apothecia are formed. Apothecia are tan-coloured and bear millions of asci, each containing eight ascospores. The proportion of large to small ascospores within each ascus is used as a main method of distinguishing *Sclerotinia* species from each other (Willetts & Wong 1980). Under changing RH or atmospheric pressure, ascospores are released in a puff, and transported by wind to senescent tissue, thereby acting as the main infectious unit of the pathogen (Bolton et al. 2006). Ascospore release may last for as long as ten days in the field at a rate of 1600 ascospores per hour (Clarkson et al. 2003).

Sclerotia have several temperature and moisture requirements in order to germinate carpogenically. Those isolated from temperate climates must be exposed to cool temperatures (4-10°C) for a period of time before they will produce apothecia; however isolates from different geographic origins may not require this conditioning period (Huang & Kozub 1991; Coley-Smith & Cooke 1971). Sclerotia also require high soil moisture levels (>-100 kPa) in order to produce apothecia (Clarkson et al. 2007; Grogan & Abawi 1974). Thus, apothecia are typically only formed once a crop canopy is closed, which helps retain soil moisture (Kora et al. 2005; Bolton et al. 2006). Due to the environmental conditions required for apothecia production, *S. sclerotiorum* typically only produces inoculum once per year (i.e. it is monocyclic).

While several studies have suggested that ascospores may travel long distances above the canopy and between fields (Parker et al. 2014b, 2014a; Qandah & del Río Mendoza 2011; Williams & Stelfox 1979), others have shown that dispersal of ascospores is spatially aggregated within fields and that inoculum from other fields was uncommon (Qandah & del Rio Mendoza 2012; Boland & Hall 1988).

Infection process. Ascospores are dispersed by wind or water drops and readily infect senescent plant tissues, particularly petals of florets that have been pollinated, as in alfalfa (Jamaux et al. 1995; Huang et al. 2000). Once deposited, ascospores can germinate in as little as two hours (Garg et al. 2010). Under field conditions, ascospores may remain viable for up to 12 days after deposition (Caesar & Pearson 1983). Like *B. cinerea*, *S. sclerotiorum* typically requires a period of saprophytic growth on senesced plant tissue. Under these conditions, *S. sclerotiorum* rapidly formed an infection cushion and penetrated both leaves and stems of canola within three days after inoculation

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(Huang et al. 2008). This same study documented extensive inter- and intracellular hyphal growth within the same period. In some cases, infection of host tissue may occur more rapidly. In sunflower, *S. sclerotiorum* was observed to infect and colonize stems within 12 hours after inoculation (Davar et al. 2012). In most cases, penetration of host tissue is achieved by the formation of appressoria which penetrates directly through the cuticle, and may be facilitated by secreted enzymes (Hegedus & Rimmer 2005). As with *B. cinerea*, a thin film of water is typically required for appressoria formation; without surface water ascospores may directly penetrate the host cuticle or stomata (Grogan & Abawi 1974; Jamaux et al. 1995; Garg et al. 2010).

Temperatures between 20 and 24°C and prolonged moist conditions (RH > 80%) favour ascospore germination (Clarkson et al. 2003; Clarkson et al. 2014; Abawi & Grogan 1974). Survival of ascospores decreases precipitously at temperatures above 25°C, and exposure to UV light or high RH contributes to rapid declines in viability (Clarkson et al. 2003; Caesar & Pearson 1983).

Oxalic acid (OA) has been extensively studied in *S. sclerotiorum* as a necessary pathogenicity factor. While many fungi produce OA, it appears necessary that *S. sclerotiorum* produces large quantities of OA in order to successfully infect host tissue, with aggressiveness in isolates being correlated to OA production levels (Marciano et al. 1989). OA appears to play a number of roles in the establishment of *S. sclerotiorum* on host tissue, including reducing the ambient pH to allow more efficient functioning of cell wall-degrading enzymes (Marciano et al. 1983) and preventing the oxidative burst defense response of plants (Cessna et al. 2000). Mutant strains that do not produce OA are not pathogenic and cannot form sclerotia (Godoy et al. 1990).

The Host: *Medicago sativa*

Alfalfa is a perennial crop, typically grown for use as a hay crop. Alfalfa grown for hay is cut before its flowering stage and the plant matter is used as feed for livestock. Grown for seed, however, alfalfa is allowed to flower and set seed. Seed alfalfa fields are maintained for 4-6 years before seed production declines. Alfalfa requires cross-pollination in order to set seed (Strickler 1999), although its flower structure prevents the possibility of wind pollination. The stamens and style of an alfalfa floret are encased within two keel petals; the keel petals must be physically separated in order to expose the pollen and stigma, a process referred to as "tripping". Thus, alfalfa grown for seed requires the management of insect pollinators, particularly leafcutter bees, that will trip alfalfa florets and cross-pollinate while foraging for other resources (Pitts-Singer & Cane 2011).

Alfalfa grown for seed is particularly susceptible to blossom blight pathogens *B. cinerea* and *S. sclerotiorum* for several reasons. Generally, a field of seed alfalfa is maintained for production for 4-6 years, with the first year of growth yielding very little seed (Frank 2003). Lack of crop rotation or interplanting during this time may contribute to the buildup of certain pathogens and/or inoculum over years. For instance, sclerotia of *S. sclerotiorum* can survive in the soil for up to eight years (Adams & Ayers 1979; Coley-Smith & Cooke 1971), which could lead to a severe epidemic in a year when conditions are conducive. In addition, because of the semi-arid climate of southern Alberta and the high value of seed alfalfa, growers irrigate their fields throughout the season. The additional moisture contributes to the humid environments under the canopy that is conducive to the growth of fungal pathogens (McDonald et al. 2008; Abawi & Grogan 1974; Grogan & Abawi 1974).

Alfalfa's blooming period of several weeks (early July to mid-August), during which it must be pollinated in order to produce seed, provides a large window for blossom blight pathogens to infect the plant. Once a floret has been pollinated, its petals senesce, and the senescent tissue provides a nutrient-rich base for *B. cinerea* and *S. sclerotiorum* spores (Huang et al. 2000; Olivier et al. 2008). Infected petals that remain attached to the raceme may lead to blossom blight – a cottony grey or white mould that grows on the floret and raceme. Infected petals that fall into the canopy may land on stems and lead to stem rot (Samac et al. 2015). The long blooming period provides a potentially continuous supply of nutrients on which the pathogens can feed, grow, and spread. Furthermore, the late harvest of seed alfalfa allows for a long season of pathogen growth and development. Growers harvest seed alfalfa in the early fall (end of September or beginning of October), meaning that even late-season sources of inoculum, particularly *B. cinerea*, or conditions that favour the development of latent infections could contribute to yield losses (Huang et al. 2000).

Management of blossom blight. Integrated pest management strategies to control blossom blight pathogens have been difficult to implement in seed alfalfa systems. Cultural control options such as crop rotation are difficult to employ, given the perennial nature of the crop and the fact that sclerotia, the initial source of inoculum, can survive for several years in the soil (Coley-Smith & Cooke 1971). In some crops, foliar trimming has virtually eradicated white mould caused by *S. sclerotiorum* (McDonald et al. 2008), but this approach in seed alfalfa is unlikely to work due to the spreading growth characteristics of the plant and the fact that significant amounts of seed would be removed with the foliage. Burning has been shown to kill virtually all sclerotia in the top

2 cm of soil and lead to an increase in seed yield of over 40% (Gilbert 1991); however, fire temperature is variable within a field and must reach at least 93 to 107°C in order to kill sclerotia (Hind-Lanoiselet et al. 2005). In addition, seed yields in burned fields may be reduced in subsequent years compared to yields from unburned fields (Frank 2003).

Biological control of blossom blight pathogens, particularly *B. cinerea*, has been studied in various contexts, although few have resulted in commercial field applications. In seed alfalfa, researchers found that application of *Clonostachys rosea* in powder form could significantly reduce infection by *B. cinerea* and increase seed production under field conditions (Li et al. 2004). Similar results using *C. rosea* in a suspension inoculation were reported for grey mould of strawberry under field conditions (Cota et al. 2008). Research on the use of pollinators including the honey (*Apis mellifera*) and bumble bee (*Bombus* spp.) to deliver biocontrol agents to flowers for control of *B. cinerea* in greenhouse and field crops have reported success on small scales (Kapongo et al. 2008; Kovach et al. 2000).

Research on biocontrol of *S. sclerotiorum* has focused on parasitic bacteria (*Pseudomonas* and *Bacillus* spp.) and other fungi (*Trichoderma* spp. and *Coniothyrium minitans*) that increase decomposition of sclerotia, and has shown some potential for field application (Li et al. 2003; Li et al. 2005; Kim & Knudsen 2008; Fernando et al. 2007; Bardin & Huang 2001). Currently only one biological control agent, Contans[®], a formulation of *C. minitans*, is available for commercial use against *S. sclerotiorum* in Canada. It is applied to the surface of the soil in a suspension and can be incorporated into the soil through cultivation or irrigation (Anonymous 2009b; Butt et al. 2001). However, field trials using Contans[®] have shown inconsistent results in suppressing

sclerotia germination or the final disease intensity (Toussaint 2015; Li et al. 2005), and the product is not used by seed alfalfa growers in southern Alberta.

Breeding for resistance to *B. cinerea* and *S. sclerotiorum* has been relatively unsuccessful, as these pathogens are generalists with no known unique virulence genes (Amselem et al. 2011). As a result, most breeding programs have focused on altering physical (thicker cuticles, upright cultivars) and phenological (flowering date) traits of the host in order to reduce disease intensity (Bolton et al. 2006; Elad et al. 2007).

Fungicides are the most frequently employed methods of disease control for blossom blight, and the most common labels include Headline (active ingredient: pyraclostrobin) and Lance (active ingredient: boscalid) (Bardin & Huang 2001; Frank 2003). These are prophylactic treatments, and, as a result, growers may apply them more frequently than necessary in order to ensure their yields are not compromised by disease. Such heavy reliance on chemical methods of control may be undesirable, however, because species of *Botrytis* and *Sclerotinia* are well documented to rapidly develop resistance to fungicides (Jo et al. 2008; Amiri et al. 2014; Amiri et al. 2013; Leroux et al. 2002). Disease forecasting to alert growers when disease pressure is high is often an integral component to integrated pest management, and can contribute to significant reductions in pesticide use, as discussed below (Bolton et al. 2006).

The Pollinator: *Megachile rotundata*

Leafcutter bees (*Megachile rotundata*) are employed as pollinators in seed alfalfa production because they are much more efficient pollinators of alfalfa florets than honey bees, which results in significantly greater seed yields (Pitts-Singer & Cane 2011; Cane 2002). Only female leafcutters forage for leaves and provisions (pollen and nectar) for

their larvae; in the process, they pollinate alfalfa florets. When a bee lands on the keel petals of a floret, the keel petals separate and release a spring-loaded staminal column (a structure containing both the pistil and stamens). The staminal column strikes the bee, forcibly releasing the pollen and allowing the stigma to come into contact with pollen collected by the bee from previous flowers. Leafcutter bees may travel large distances (>100 m) to collect resources including pollen, nectar, and leaves (Bosch & Kemp 2005; Frank 2003); however in seed production areas, pollination typically occurs within a 4 m radius of where they begin foraging (Amand et al. 2000).

To successfully manage leafcutter bees, growers must provide them with a place to build their nests. These shelters typically take the form of a tent in which boards with drilled holes (~1cm diameter) are placed. The leafcutter bees use these holes to deposit their larvae, provision them with pollen and nectar, and build cells to separate them with harvested leaves. The tents are generally placed 100 m apart from each other, and the bees are active throughout alfalfa's flowering stage (early July to late August).

Leafcutter Bees as Vectors

Insects are well documented vectors of bacterial, viral, and fungal pathogens. Many insects are documented mycophages, receiving most or all of their nutritional requirements from fungi, and the interactions between insects and phytopathogenic fungi may be very complex (Hatcher 1995). Fungus gnats (*Bradysia* spp.) are serious pests of greenhouse crops and are capable of transmitting fungal diseases caused by *Fusarium* spp., *Pythium* spp., and *Verticillium* spp. (Cloyd 2015). Several insects in a variety of plant management systems are capable of transmitting *Botrytis* conidia (Louis et al. 1996; Fermaud & Le Menn 1989, 1992; James et al. 1995). In conifer seedlings, fungus gnats

were found to frequently carry *B. cinerea* conidia (James et al. 1995). In grape, female fruit flies (*Drosophila melanogaster*) could transmit viable *B. cinerea* conidia up to 16 days after foraging on infected grapes (Louis et al. 1996). In addition, the conidia could survive the fruit fly digestive tract and be expelled germinated or ungerminated. In both production systems, insects were implicated as important contributors to the spread of disease.

Like other insects, leafcutter bees could be an important component of the disease cycle of *B. cinerea* and *S. sclerotiorum*. As discussed previously, research into the use of bees to apply biological control agents (fungi and bacteria) has been relatively successful, suggesting that bees vector many types of fungi under natural conditions. One study reported that leafcutter bees transported pollen infected by *Verticillium albo-atrum*. In this study, researchers collected leafcutter bees from alfalfa fields infected by *V. albo-atrum* and examined the bees under scanning electron microscopy (SEM) for spores and pollen infection. While species identification based solely on spore morphology may be inconclusive, the results demonstrated that the bees may act as vectors of fungus between plants (Huang et al. 1986). Another group found that honey bees could transmit ascospores of *S. sclerotiorum* between canola plants (Stelfox et al. 1978). In a greenhouse study of blossom blight of alfalfa using both *B. cinerea* and *S. sclerotiorum*, researchers hypothesized that "the risk of transmission of spores of *S. sclerotiorum* and *B. cinerea* to alfalfa flowers by pollinating insects is high because blossom blight is widespread in seed alfalfa fields in the Canadian prairies," although the role of pollen and pollination was not investigated in this study (Huang et al. 2000).

BIOLOGY OF POLLEN-INFECTING FUNGI

Little research exists on the role of fungus-infected pollen in disease transmission (Ngugi & Scherm 2006). One of the earliest studies of pollen infection used microscopy to examine the infection of *Schizanthus pinnatus* (family: Solanaceae) pollen by *Alternaria* sp. (Nair & Khan 1963). This study identified the relationship between fungus and pollen as a new area of research with potentially significant implications in the disease cycle, however few papers have since been published on the topic. Conversely, a considerable amount of research on virus-infected pollen shows that infected pollen can transmit diseases horizontally (i.e. between plants of the same generation) and vertically (i.e. from parent to offspring) (Agrios 1997; Mink 1993). Virus-infected pollen has been implicated in drastically reduced reproductive abilities in plants such as *Paeonia emodi* (Huang et al. 1983).

While the potential of pollen as a vector for disease has important implications for disease management in certain crops, it is unknown whether this method of transmission is common in the spread of fungal diseases or what affect it may have on plant health and epidemiology. A handful of studies on fungal infection of alfalfa (*Medicago sativa*) or orange (*Citrus sinensis*) pollen by *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Coniothyrium minitans*, *Gliocladium catenulatum*, and *Colletotrichum acutatum* have noted that infection readily occurs *in vitro* (Chang et al. 2003; Huang et al. 1999, 1997; Marques et al. 2013). However, these studies did not examine whether, and to what extent, infection is common under field conditions, nor did they investigate the role of infected pollen in the disease cycle. One study found that leafcutter bees collected from the field carried fungal spores and infected pollen but the study did not quantify the

frequency of this infection (Huang et al. 1986). Another group suggested that fungal-infected pollen could transmit disease in the field; however, researchers did not conclusively distinguish whether fungal spores or fungus-infected pollen was responsible for disease transmission (Stelfox et al. 1978).

Several studies have documented the infection process of fungal spores on flowers. Fungal infection of flowers may occur by hyphae colonizing the base of the flower between the calyx and ovary, as in grape (Viret et al. 2004), or by hyphae mimicking pollen germ tube growth by growing down the style and infecting the ovaries (Ngugi & Scherm 2004). Histopathological studies of sweet pepper flowers have shown that the infection process of *Fusarium lactis* results in colonization of the ovaries and can lead to infected, symptomless seeds (Yang et al. 2010). Conversely, similar work in grape flowers showed no sign of ovary infection (Viret et al. 2004). These studies used concentrated spore suspensions as a source of inoculum, but it remains unknown whether fungal-infected pollen, or even windborne spores, will exhibit similar growth characteristics on host flowers or whether they can reduce fertilization rates and lower seed set.

Jung (1956, as cited in Jarvis, 1977) found that *B. cinerea* could infect all parts of several species of flower, including the style; however, in no case did he observe that *B. cinerea* could penetrate the style as far as the ovary. He hypothesized, therefore, that seed infected by *B. cinerea* was unlikely. However, as discussed previously, *Botrytis*-infected and *Sclerotinia*-infected seed has been found in plants such as primula, lettuce, and alfalfa (Sowley et al. 2009; Barnes & Shaw 2003; Huang et al. 2000).

DISEASE FORECASTING SYSTEMS

Contemporary disease forecasting systems have their origins in systems created in the early part of the 20th Century. Following the Irish potato famine in the mid-19th Century, which demonstrated the devastation of severe plant disease epidemics, the earliest forms of disease forecasting systems were developed in Holland for managing late blight of potato and tomato caused by *Phytophthora infestans* (Miller & O'Brien 1957). Late blight remains one of the most commonly forecasted and economically important diseases worldwide, though disease forecasting systems have been successfully developed to improve yields and reduce pesticide use in many crops (Hardwick 2006; Broome et al. 1994; Foster et al. 2011; Klosterman et al. 2014).

Development of predictive systems requires extensive data on topics including pathogen identification and life cycle, yield loss potential, host biology, and environmental dynamics, and, as a result, fully functional disease forecasting systems develop over long periods of time and in incremental steps (Hardwick 2006). The end goals of these predictive models (i.e. the practice of epidemiology) are twofold: first, a deeper mechanistic understanding of epidemics; second, and following from the first, the control of plant diseases (Madden 2006; Zadoks 2001). For the latter to be successfully implemented in the field, a necessary step is to monitor the presence and development of the pathogen in the field (Madden et al. 2007).

Monitoring the presence of inoculum to develop a predictive model can add sensitivity to more traditional models based solely on environmental variables. In carrot, quantifying the airborne inoculum of *S. sclerotiorum* was a better predictor of final disease intensity than quantifying the number of apothecia in the field (Foster et al.

2011). In strawberry, including inoculum levels of *B. cinerea* increased the predictive power of an infection model based only on environmental variables, though the improvements were not drastic (Xu et al. 2000). On the other hand, quantification of *B. squamosa* conidia in onion greatly improved the reliability of disease monitoring programs of leaf blight (Carisse et al. 2009).

Methods of Inoculum Detection

Several methods of detection are used for quantifying inoculum levels of airborne pathogens. Plate culturing techniques, often using semi-selective media, have been used extensively to detect airborne spores throughout a growing season (Parker et al. 2014b; Edwards & Seddon 2001; Foster et al. 2011), or used to culture susceptible plant tissue collected from the field (Turkington & Morrall 1993; Xu et al. 2000). In greenhouse-grown tobacco, a semi-selective medium was used to collect airborne ascospores of *S. sclerotiorum* to help quantify the spatial and temporal distribution of the pathogen (Gutierrez & Shew 1998). One advantage of this approach is that only viable spores are counted, yielding a measure of the effective inoculum load at a given time. In canola, a plate test for *S. sclerotiorum* is available to growers to help determine whether their crops are at low, moderate, or high risk of *Sclerotinia* infection. The test involves collecting petals of canola at various flowering periods and plating them on agar plates amended with antibiotics and assessing incidence of infection (Turkington & Morrall 1993; Turkington et al. 1991). However, culture-based identification methods face several challenges. First, inoculum counts are delayed by several days because the pathogen must grow to the stage where it can be identified. Second, semi-selective media do not always distinguish species adequately. Consequently, for some pathogens, identification based

on morphology may be difficult and require a high level of expertise. Third, many selective media require expensive reagents and are labour-intensive to make.

An increasingly common method of monitoring airborne pathogens is through the use of spore samplers. The first report of a spore trap described the airborne fungal fauna over one season at Rothamsted, UK (Gregory & Hirst 1957), but this technology has since been used to describe occurrence of plant pathogens (Sreeramulu 1959; Parker et al. 2014b; Hartill 1980; Klosterman et al. 2014; Carisse et al. 2005; Jedryczka et al. 2008; Luo et al. 2007). Broadly, the two main types of air samplers are active and passive (West & Kimber 2015). Active samplers use a vacuum pump to gather and deposit aerosols onto sticky tape or into a collection tube. Many models of active samplers exist, and vary in their efficiencies at trapping small particles, their flow rates (2 to 900 L min^{-1}), or their ability to facilitate processing samples by plate culture, microscopy, or molecular methods (West & Kimber 2015). These samplers are the preferred method of aerosol collection due to their constant and quantifiable rate of air intake, and are discussed in more detail below. Passive samplers allow passive deposition of aerosols onto petroleum-jelly-coated glass slides or agar plates for identification of organisms under microscopy or plate culture. They are typically less expensive, but yield more qualitative results than active samplers.

Two methods have been employed most extensively to collect and quantify spores and pollen with active samplers: The first involves the use of a piece of sticky tape that rotates around a drum at a fixed rate. Aerosols adhere to the tape, and the tape can be sectioned based on time of day (often, hourly) to count spores of interest under the microscope (Inch et al. 2005; Hartill 1980; Jarvis 1962a; Gregory & Hirst 1957; Hirst

1953; Carisse et al. 2005; Qandah & del Río Mendoza 2011; Rodríguez-Rajo et al. 2010).

The second involves molecular methods to identify and quantify species of interest, which is particularly useful when spores of the species of interest may easily be confused with other spores, as in the case of *B. cinerea* and *S. sclerotiorum* (West et al. 2008). With this approach, aerosols are collected in a vial or on a rod in the spore sampler, and the DNA is extracted. Using species-specific primers and quantitative real-time polymerase chain reaction (qPCR) techniques, the amount of inoculum per day can be accurately quantified (Parker et al. 2014b; Carisse et al. 2009; Klosterman et al. 2014; Rogers et al. 2009). Typically, spore samplers used for this latter method collect daily samples in a single vial or rod; thus the method is more useful for investigating seasonal patterns than daily patterns of spore discharge.

Developing a qPCR Assay for Detection of Airborne Fungal Pathogens

Developing a reliable qPCR assay is time-consuming and expensive, though once a protocol is established the quantification of samples may take as little as 5-6 hours – a substantial improvement over plate culturing methods, which require several days of waiting (Parker et al. 2014b). There are many processes involved with developing a qPCR assay: primer and probe selection/design (including specificity and sensitivity testing), qPCR chemistry type (SYBR[®] Green vs TaqMan[®]), developing a standard curve, and finding suitable controls to test for false positives and false negatives.

Assuming a sensitive, species-specific primer set has been designed, there are two main chemistries for qPCR. Both chemistries are based on the detection of fluorescence emitted by fluorophores attached to DNA, which is measured at the end of every PCR

cycle. In SYBR® Green chemistry, a fluorescent dye binds to any double strand of DNA, including any non-specific amplification, such as primer dimers, that may occur in the PCR process. In TaqMan® chemistry, a probe releases a fluorescent dye once it has hybridized to the amplified gene/region (Anonymous 2012). The probe used in TaqMan® assays is also sequence-specific to the target region, effectively acting as an additional level of specificity in the assay. Because probes can be labelled with different fluorophores, TaqMan® chemistry also allows multiplexing of primers to detecting several species in a single reaction by labelling each species-specific probe with a different fluorophore (Anonymous 2012). Target DNA is detected once it has amplified enough for the concomitant increase in fluorescence to pass the detection threshold of the fluorometer. The cycle at which this detection occurs is referred to as the threshold cycle (C_t).

In order for the PCR assay to be quantitative, a standard curve is established. The standard curve is a set of serial dilutions from a known concentration of DNA, and is included in every run of samples for a qPCR assay. The standard curve graph plots the logarithm of the starting mass of DNA against its C_t value. By determining the cycles at which the unknown samples pass the threshold, the initial quantity of template DNA can be calculated by using the regression line of the standard curve. When methods for standard curve development are reported, they typically involve cloning the gene of interest into a plasmid and using dilutions of the plasmid to determine copy number (Carisse et al. 2009; Klosterman et al. 2014; Almquist & Wallenhammar 2014), serially diluting DNA from a known starting concentration (Parker et al. 2014b), or serially diluting spores and extracting DNA (Diguta et al. 2010; Luo et al. 2007). In the first two

cases, further work may be required to relate the template copy number to number of spores.

Increasingly, qPCR assays include the use of an internal control – a plasmid (Parker et al. 2014b; Carisse et al. 2009; Haudenshield & Hartman 2011), non-target fungal species (Diguta et al. 2010), or synthetic oligonucleotide (Haudenshield & Hartman 2011) included in the field samples prior to DNA extraction. Following DNA extraction, the internal control is quantified in the qPCR assay using target-specific primers. This process tests for inhibition in the extraction or amplification process; samples that have no amplification of the internal control are removed from analysis. In addition, the internal control could act as a normalizing agent for environmental samples, although this use has not previously been reported.

Origins of a Predictive Disease Model

Developing a disease forecasting program based on airborne inoculum requires determining the best time(s) of the day/season to monitor the air, and which environmental variables to measure. The times and variables to monitor will vary depending on the species of interest, and only information relevant to *B. cinerea* and *S. sclerotiorum* is discussed here.

When to Monitor: Daily and Seasonal Inoculum Dispersal Patterns

Both *B. cinerea* and *S. sclerotiorum* generally follow a diurnal discharge pattern, though the patterns are not necessarily the same between years or species. For instance, Qandah and del Rio Mendoza (2011) found that in canola fields, *S. sclerotiorum* released most of its ascospores in the late morning (10:00 to 13:00h). However, in dryer years, the

majority of ascospores were released at night (02:00 to 07:00h), and there were fewer ascospores overall. Jarvis (1962), studying *B. cinerea* in raspberry, found that generally the greatest numbers of conidia were released in the late morning and early afternoon (11:00 to 16:00h), however on certain days the greatest numbers of conidia were recorded in the evening (21:00 to 24:00h). Chastagner et al. (1978) found that most *B. cinerea* conidia were released around 12:00h in tomato populations; similarly, Sutton et al. (1978) found greatest numbers of *B. squamosa* conidia between 09:00 and 12:00h in onion fields.

What to Monitor: Environmental Variables

Experiments have shown clear relationships between pathogen development and temperature, relative humidity, soil moisture, and, to a lesser extent, light (Broome et al. 1994; Clarkson et al. 2014; Sun & Yang 2000; Ferraz et al. 1999; Morrall 1977; Wu & Subbarao 2008), however these relationships may be much less obvious when applied to field data.

Temperature. Typically, air or soil temperature is used to determine the peaks and limits of pathogen growth (Clarkson et al. 2004), and will often have interactions with other variables such as hours of moisture or RH (Broome et al. 1994; Carisse et al. 2012). In onion, temperature and hours of leaf wetness were used to develop an empirical model of infection by *B. squamosa* for field applications (Carisse et al. 2012). The model showed that infection of onion leaves was greatest at 20°C for all periods of leaf wetness, and that infection declined rapidly toward 10 and 30°C. In carrot, soil temperature and soil moisture were two factors used to create a risk algorithm for infection by *S. sclerotiorum* (Foster et al. 2011). The greatest risk points were given for soil that had a

daily average of ≤19°C and a daily maximum of 24°C. Similar to the relationship established with *B. squamosa*, the number of risk points dropped as temperature increased.

      Relative humidity. Clarkson et al. (2003), studying ascospore release of *S. sclerotiorum* under lab conditions, found that RH did not affect ascospore release at levels of 65-70% and 90-95%. However, Qandah and del Río Mendoza (2011), studying airborne ascospore densities in fields over several seasons, found that ascospore discharge was associated with a sudden change in RH (a 10-point drop) rather than a constant RH. They also found that days with peak ascospore discharge were preceded by seven consecutive days of >85% RH in the canopy, and their final model included only RH as a predictive factor. Similarly, Jarvis (1962a), studying aerial densities of *B. cinerea* conidia in the field, found the highest concentrations of conidia when RH rose or fell rapidly between 65-85%. Similar findings were reported by Sutton et al. (1978).

      An alternative, but related, measure to RH is vapour pressure deficit (VPD), the difference between the amount of pressure exerted by moisture in the air at a measured temperature and the amount of pressure it would exert in saturated state (i.e. 100% RH). This metric is reported to be more biologically relevant and less temperature dependent, and reduces error compared to RH (Lacy & Pontius 1983). Several studies have found significant correlations between low VPD (comparable to high RH) values and increased infection rates by *Botrytis* spp. under field conditions (Lacy & Pontius 1983; Xu et al. 2000). The average temperature and VPD of the 72 h preceding a large release of conidia by *B. squamosa* in onion described 85% of the variation in conidia numbers recorded the

following day; the model was used to create a sporulation risk index for *B. squamosa* (Lacy & Pontius 1983).

Soil Moisture. For pathogens like *S. sclerotiorum* that have specific temperature and moisture requirements for (carpogenic) germination, measuring the soil matric potential may be useful in predicting apothecia formation (Sun & Yang 2000; Clarkson et al. 2007; Morrall 1977; Kora et al. 2005; Foster et al. 2011). In lettuce, investigators found that soil moisture was a significant variable in predicting apothecia emergence; however, issues associated with measuring soil moisture under variable field microclimates reduced the robustness of this variable (Clarkson et al. 2007). In carrot, soil moisture was positively correlated with apothecia production and ascospore discharge, but not significantly correlated with final disease incidence (Kora et al. 2005). These findings suggest that the presence of apothecia may not be a reliable indicator of final disease intensity because many microclimatic factors influence the germination of apothecia and the release of ascospores (Foster et al. 2011).

Light. Quantity and quality of light is not typically a variable that is included in environmental monitoring for disease forecasting. Some controlled studies in *S. sclerotiorum* have related light intensity to size of apothecia and days to maturity (Sun & Yang 2000); however, others have noted no difference in ascospore release between day and night time (Clarkson et al. 2003). Sporulation by *B. cinerea* is commonly induced by exposure to near-UV (NUV) wavelengths (Tan & Epton 1973), and one approach to reduce *B. cinerea* inoculum in greenhouses is to filter NUV radiation (Elad 1997a). Day/night periodicity of both *B. cinerea* and *S. sclerotiorum* is typically attributed to

other events, such as rapidly changing RH or temperature, rather than changes in light intensity.

Success Stories

Some disease forecasting systems have been highly successful in their ability to dramatically reduce fungicide application by alerting growers only once high risk of infection exists (Broome et al. 1994; Jedryczka et al. 2008). As a result, growers do not needlessly apply fungicides when risk is low, and yields are maintained. In carrot, two models based on canopy closure, soil moisture and temperature, and airborne ascospore concentration were tested for effectiveness at predicting *Sclerotinia* rot. Fungicide use was reduced by up to 80% by adhering to the spray program determined by disease models (Foster et al. 2011). In grape, a model based on wetness duration and temperature was used to predict the risk of *B. cinerea* infection. In some instances, spray programs that followed the model reduced fungicide application by up to 100% (Broome et al. 1994).

On a larger scale, the Canola Council of Canada has developed a Sclerotinia Stem Rot Checklist, available free of charge, to help growers determine the risk of stem rot in their fields (Anonymous 2009a). The risk algorithm uses only environmental variables and includes the number of years since the last canola crop and the incidence of *Sclerotinia* stem rot in that crop, crop density, amount of rain in the previous two weeks, weather forecast (high vs low pressure systems), and a measure of regional risk for apothecia development based on scouting data. In conjunction with the petal test discussed previously, these resources encourage growers to only apply fungicides when risk of disease is high.

While many studies have reported methods to detect and monitor inoculum concentration in aerosols, relatively few of these studies have developed models based on their results. In many cases, further work is required to determine the relationship between inoculum concentration and disease intensity, and to determine threshold concentrations appropriate for alerts. Generally, pathogen inoculum must reach a threshold concentration before it is capable of causing an epidemic with noticeable yield losses. This threshold value may be determined empirically (Carisse et al. 2008; Carisse et al. 2009), and once determined, days are categorized as either "at risk" or "not at risk" for infection by a pathogen (Qandah & del Río Mendoza 2011).

Clearly, threshold values will vary depending on methods used to collect data and, as a result, may need to be developed separately for each research program. In rare cases where thresholds have been established, as in the case of leaf blight of onion caused by *B. squamosa* (Carisse et al. 2005), use of sensitive qPCR techniques to quantify pathogen DNA has increased the reliability of disease forecasting significantly (Carisse et al. 2009). Before including the qPCR techniques, management of *Botrytis* leaf blight was still successful with using microscope counts of conidia, and reduced fungicide use by up to 75% of conventional spray programs (Carisse et al. 2005). When the model included data obtained with the molecular techniques, predictions of days with high disease pressure increased in accuracy, with both an increase in true positives and a decrease in false negatives (Carisse et al. 2009).

On a larger scale, the Institute of Plant Genetics PAS and DuPont Poland have implemented a nation-wide program using volumetric spore samplers to monitor levels of airborne inoculum of *Leptosphaeria maculans* and *L. biglobosa*, causal agents of

blackleg in canola (Jedryczka et al. 2008). Ten spore samplers throughout the country collect daily data on spore concentrations and days are categorized as low, moderate, or high risk for <10, <20, and ≥20 spores m^{-3}, respectively. Alerts are disseminated to growers via websites, text messages, and email. At the same time, researchers are collecting meteorological data in order to determine the effects of weather variables on the dispersal of blackleg spores.

CONCLUSION

Seed alfalfa is an important industry in southern Alberta and its product is the base for a much larger forage alfalfa industry. In moist growing seasons, blossom blight has been reported to be a major constraint on seed production across the Canadian Prairies. It is known that blossom blight pathogens can infect alfalfa pollen and that leafcutter bees may carry fungal spores or fungal-infected pollen. It remains unknown whether, and to what extent, blossom blight pathogens are able to colonize pollen under field conditions and whether, and to what extent, leafcutter bees are acting as vectors of these pathogens. Furthermore, neither the intensity of blossom blight nor the seasonal patterns of blossom blight pathogens in seed alfalfa fields of southern Alberta have been studied. Quantifying the patterns of spore dispersal and their relationships to disease intensity can provide the basis for a disease forecasting model aimed at reducing unnecessary fungicide use.

RESEARCH OBJECTIVES

This thesis has three main objectives:

1. Describe the role of pollen infection and pollinators in the spread of blossom blight pathogens in seed alfalfa in southern Alberta.

2. Develop the tools and methodology to detect and quantify DNA of blossom blight pathogens *B. cinerea* and *S. sclerotiorum*.

3. Describe the seasonal occurrence of blossom blight pathogens in seed alfalfa fields of southern Alberta using classical disease surveys and spore sampling methods.

CHAPTER 2: DETERMINING THE ROLE OF POLLEN AND POLLINATORS IN THE DEVELOPMENT OF BLOSSOM BLIGHT OF ALFALFA CAUSED BY *BOTRYTIS CINEREA*

ABSTRACT

The fungal pathogen *Botrytis cinerea* Pers.: Fr. is well documented to infect flowers of many species of plants, including alfalfa (*Medicago sativa*). In southern Alberta – the largest seed alfalfa production region in Canada – blossom blight, caused in part by *B. cinerea*, can be damaging to yields in cool, wet growing seasons. Previous studies have suggested that *B. cinerea* uses pollen, a nutrient-rich substrate, to begin infection, and that the fungal-infected pollen may be vectored by alfalfa leafcutter bees (*Megachile rotundata*). A longitudinal greenhouse study was performed to investigate the role of pollen in the infection process of alfalfa florets by *B. cinerea*. The effects of pollination (pollinated vs unpollinated) and inoculation method (dry vs suspension) on floret infection, pollen number, pollen infection, and pollen viability were tested. Alfalfa plants were inoculated, maintained at 100% RH for 24 hours, and then restored to ambient RH. Florets were harvested at 0, 12, 24, 48, and 94 hours after inoculation. In florets that were dry inoculated, those that were pollinated had significantly greater levels of infection (84%) than those that were not pollinated (38%, p<0.001), however no pollen infection was observed in dry inoculated samples. In contrast, 90% of suspension inoculated florets were infected and a small (<1%) but significant (p<0.0001) level of infected pollen was observed in these samples only. Leafcutter bees were also collected from seed alfalfa fields in 2013 and 2014 and plated on selective media to estimate the prevalence of *B. cinerea* on pollen. Pollen removed from bees collected in the field exhibited no growth of *B. cinerea*; however, the bees that were directly plated exhibited

an increased load of *B. cinerea* as the growing season progressed. These data suggest that, under typical field conditions, alfalfa pollen is not likely to be a significant factor contributing to the establishment and spread of *B. cinerea*.

INTRODUCTION

The fungal pathogen *Botrytis cinerea* Pers.: Fr. is well documented to infect flowers of a broad range of plant species, causing diseases such as blossom blight, soft rot, and blossom-end rot, as well as numerous post-harvest diseases (Elad et al. 2007). Flowers are particularly susceptible parts of plants because they contain nutrient-rich resources that provide ideal growth conditions for various fungi, including *B. cinerea*. In particular, pollen and nectar contain many proteins, sugars, minerals, and amino acids that stimulate conidia germination and may enhance growth rates of *B. cinerea* (Ngugi & Scherm 2006; Huang et al. 1999; Chou & Preece 1968). Petals, thin-walled and lacking a waxy cuticle, are also easily infected by pathogenic fungi, particularly if they have senesced (Ngugi & Scherm 2006; Huang et al. 2000; Olivier et al. 2008). Infection of flowers by *B. cinerea* has been described in many plants including strawberry (Bristow et al. 1986), raspberry (McNicol et al. 1985), grape (Viret et al. 2004), geranium (Sirjusingh & Sutton 1996), and rose (Elad 1988).

Previous histopathological studies on flower-infecting fungi have documented the growth of fungi down the style towards the ovaries, resulting in infected seed (Yang et al. 2010; Shinners & Olson 1996; McNicol et al. 1985). In a study of the etiology of blossom blight of seed alfalfa (*Medicago sativa* L.) (Huang et al. 2000), *B. cinerea* could infect developing seeds, and researchers hypothesized a similar mechanism of infection as

above. This hypothesis was supported by the observation that *B. cinerea* grows more rapidly in the presence of alfalfa pollen (Chou & Preece 1968; Huang et al. 1999).

Less well documented, however, is the potential for *B. cinerea* to be transmitted vertically – that is, from parent to offspring – via seed and to exist as an asymptomatic endophyte in the offspring. *B. cinerea* infection of flax seed (*Linum usitatissimum*) (Harold et al. 1997), primula seed (*Primula* x *polyantha*) (Barnes & Shaw 2003), lettuce seed (*Lactuca sativa*) (Sowley et al. 2009), and alfalfa seed (Huang et al. 2000) results in poor seed germination or increased seedling death. Surviving seedlings may give rise to plants harbouring *B. cinerea* as a symptomless endophyte, which could become pathogenic only once its host is stressed or environmental conditions are conducive for disease development (Barnes & Shaw 2003; Sowley et al. 2009).

Southern Alberta is Canada's largest producer of seed alfalfa, a highly lucrative crop that involves the management of leafcutter bees (*Megachile rotundata*) for pollination (Kosinski 2012; CSGA 2014; Pitts-Singer & Cane 2011). As in other flowering plants, alfalfa grown for seed is susceptible to blossom blight caused, in part, by *B. cinerea*, which has been identified as a major constraint in seed production on the Canadian Prairies in moist growing seasons (Gossen et al. 1998; Gossen et al. 1997). Following floret pollination, petals senesce, and provide a nutrient-rich base for *B. cinerea* establishment (Huang et al. 2000; Olivier et al. 2008). Under warm (18 to 22°C), humid (>93% RH) conditions, *B. cinerea* can rapidly colonize a whole raceme (Broome et al. 1994; Sirjusingh & Sutton 1996). While a severe blossom blight epidemic would destroy alfalfa florets entirely, mild to moderate epidemics could result in conditions

suitable for infection of pollen, ovaries, and seed. Infection of these tissues under field conditions has not been documented.

Insect vectors, particularly leafcutter bees, could be contributing to the spread of *B. cinerea* in fields. Insects transmit many economically important phytopathogenic fungi over a range of production systems (Hatcher 1995). Fungus gnat larvae transmit *Pythium aphanidermatum* in greenhouse cucumber production (Jarvis et al. 1993), and adults transmit *B. cinerea* in conifer seedling production (James et al. 1995). Fruit fly (*Drosophila melanogaster*) is capable of transporting viable *B. cinerea* conidia for many days after foraging on infected grape (Louis et al. 1996). Bee pollinators and other insects are known to carry fungal spore loads of both beneficial and pathogenic fungi, including *Verticillium albo-atrum* (Huang et al. 1986), *Sclerotinia sclerotiorum* (Stelfox et al. 1978), *Trichoderma harzianum* (Kovach et al. 2000), and *Chlonostachys rosea* (Kapongo et al. 2008). Consequently, insects have also been implicated as vectors of infected pollen (Huang et al. 1986; Stelfox et al. 1978). It remains unclear, however, whether and under what conditions bee pollinators are significant contributors to disease spread in field contexts, whether they disperse fungal spores or fungal-infected pollen, and whether these methods of dispersal increase the likelihood of seed infection.

The objectives of this study, therefore, were to: i) describe the infection process of *B. cinerea* on alfalfa florets under greenhouse conditions, ii) describe the frequency of infected pollen under these conditions, iii) determine whether *B. cinerea* could infect the ovaries under these conditions, and iv) evaluate potential for pollinators to vector *B. cinerea* conidia under field conditions.

MATERIALS AND METHODS

Plant and Fungal Material. Alfalfa (cultivar: AC BlueJ) plants were grown from seed in Cornell mix (Boodley & Sheldrake Jr. 1977) in one-gallon pots. Greenhouse conditions were: 16 h/8 h and 22°C/18°C (day/night), and plants received 600 mL of water a day by drip irrigation.

Colonies of *B. cinerea* isolated from geranium were grown in the dark on potato dextrose agar (PDA) for 5-7 days and then transferred to an incubation chamber and grown under a 12 h dark, 12 h near-UV cycle at 22°C to induce sporulation (16-21 days). For the second repetition of the experiment, *B. cinerea* colonies were reisolated from infected florets in the first experiment, and these isolates were used for inoculations. Plants were cut back to ~5 cm 2-3 times prior to the experiment to stimulate stem and flower production. The plants were 12 and 14 months old for the first and second repetitions of the study, respectively.

Experimental design. Treatments tested the effects of pollination (untripped vs tripped) and inoculation method (dry vs suspension) in a 2x2 factorial design, with a control for each treatment, for a total of 8 treatments. The treatments were: i) not tripped, ii) tripped, iii) not tripped and sprayed with H_2O, iv) tripped and sprayed with H_2O, v) not tripped and dry inoculated, vi) tripped and dry inoculated, vii) not tripped and suspension inoculated, and viii) tripped and suspension inoculated. An individual alfalfa plant served as the experimental unit, and seven unpollinated racemes per plant were marked to receive the treatment assigned to that plant. At each sampling time (0, 24, 48, and 96 hours post inoculation (hpi)), a single raceme was removed from each plant and the florets were subdivided into vials for processing. An additional sample at 12 hpi was

taken for confocal and electron microscopy only. A final raceme was harvested 6 weeks after inoculation to test for seed yield and infection. Each treatment was replicated three times, and plants were placed on a greenhouse bench in a complete randomized block design. The study was performed twice.

Inoculation Methods. For treatments in which plants were both pollinated and inoculated, the florets of the racemes were tripped prior to inoculation to expose the pollen to the conidia. A metal dissecting tool was used to pollinate florets; it was loaded with pollen by tripping florets from one unmarked raceme prior to tripping the florets receiving treatment. For dry inoculated plants, a Petri dish of sporulating *B. cinerea* was held inverted over the raceme and the dish was tapped to release the spores. To determine the number of spores released while tapping, the lid of a sterile Petri dish was held below the raceme while tapping the conidia. Two lids were used for each plant (=7 racemes per plant), and the number of spores on the lids were estimated by viewing the Petri dishes under a light microscope (Olympus CK40, 20x magnification), and counting three randomly selected fields of view. The average of the top and bottom Petri dish counts was used to determine the number of conidia per mm^2 per inoculation event. This method determined that between 0.7 to 10.7 conidia per mm^2 were deposited per raceme inoculation.

For plants receiving inoculation with a suspension, Petri dishes of sporulating *B. cinerea* were flooded with water, scraped with a scalpel, and transferred to a Waring 2-Speed Blender (Cole-Parmer). The solution was mixed on high for 60 s, strained through cheesecloth, and the concentration of spores was adjusted to 6×10^5 spores ml^{-1} using a hemacytometer. Two drops of Silwet L-77 (Momentive Performance Materials Inc.) were

added to the spore suspension, and an atomizer was used to apply the suspension until the raceme glistened.

To maintain 100% relative humidity conditions, a clear plastic bag was placed over each treated raceme and twist-tied to the stem immediately after the raceme received treatment. Plastic bags were removed after 24 h and plants were maintained under normal greenhouse conditions, described previously.

Floret and Pollen Infection. At each sampling time, five florets were surface sterilized with 0.05% NaOCl for 60 s, rinsed in distilled water three times, and plated on Botrytis semi-selective media (BSM) (Edwards & Seddon 2001). Plates were incubated for 7 days in the dark, and were rated positive for *B. cinerea* if both i) the plate colour changed from pink to brown, and ii) spreading hyphae (>2 cm) and/or conidia were present.

To harvest the pollen from the florets, three florets were placed in a 1.5 mL vial, 1 mL of 1 x phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and adjusted to pH 7.4 with HCl) was added to the vials, and the vials were vortexed for 30 s. The florets were removed with forceps and the vials were placed in a centrifuge at 13,000 rpm for three minutes. After centrifugation, the supernatant was removed, 100 μL PBS added to the vial, and the pellet resuspended. 10 μL of the suspension were counted on a hemacytometer to estimate number of pollen grains per floret. In addition, the number of fungal-infected pollen grains out of one-hundred randomly counted pollen grains was recorded. Both counts were performed twice for each sample.

Pollen Viability. To determine the proportion of viable pollen at each sampling period, florets were placed in a 2 mL vial and 1 mL of Carnoy's fixative (6 ethanol: 3 chloroform: 1 glacial acetic acid, v:v:v) was added. Samples were set for at least two hours but no more than twenty-four hours. Pollen was then isolated in the same manner as above, except that after centrifugation, approximately 900 µL of supernatant was removed, leaving approximately 100 µL in the vial. The pellet was resuspended and 10 µL of the suspension was added to a glass slide. Two to three drops of differential stain for pollen viability (10 mL 95% EtOH, 1 mL malachite green (1% w:v in 95% EtOH), 54.5 mL distilled H_2O, 25 mL glycerol, 5 mL acid fuchsin (1% w:v in H_2O), 0.5 mL Orange G (1% w:v in H_2O), and 4 mL glacial acetic acid) (Peterson et al. 2010) were added and the slide was heated over a flame for 20 s. The slides were then viewed using a light microscope (Olympus CX41) under 10x objective magnification, and 100 pollen grains were counted and assessed for viability. Pollen stained red or orange was considered viable, and pollen stained blue was considered not viable.

Scanning Electron Microscopy. At each sampling time, three florets from each treatment were fixed in 1 mL of FAA (10 EtOH (95%) : 1 glacial acetic acid : 5 formalin : 4 deionized H_2O) until processing for scanning electron microscopy (SEM). Samples were rinsed twice with distilled water, dehydrated through a graded series of ethanol washes (30, 50, 70, 85, 95, and three changes of 100% EtOH for 15 minutes each), and critical point dried in CO_2. Samples were then mounted on aluminum platforms, sputter coated with gold, and viewed on a scanning electron microscope (Hitachi S-3400N) at 5.0 kV at 400x magnification.

Confocal Scanning Microscopy. As with SEM, at each sampling time, three florets from each treatment were placed in 1 mL of FAA until processing for confocal scanning microscopy (CSM). Processing the samples was similar to SEM preparation, with two alterations: first, after the 85% wash, samples were stained with a 0.005% aniline blue in 95% EtOH (w:v) solution for 25 minutes and the dehydration was completed as per SEM; second, after the three 100% EtOH washes, samples were cleared using methyl salicylate (Sigma-Aldrich). Samples were viewed with 455 and 633 nm lasers on an Olympus Fluoview FV1000 laser microscope under 10x and 20x magnification.

Data collection of SEM and CSM samples. Data were collected for the 96 hpi collection of inoculated treatments only. For each sample, two florets were dissected and one calyx, one keel petal, one banner petal, and two staminal columns (stamens + style) were examined. Each tissue sample was scanned for conidia. If conidia were found, the following data were recorded: an estimated number of conidia, whether the conidia had germinated, an estimate of the length of hyphal growth, whether the hyphae had infected pollen, and an estimate of the proportion of pollen infected.

Seed Counts and Viability. Seed pods were harvested six weeks after inoculating plants. Pods were surface sterilized as described previously, split open to expose seeds, and the seeds were plated on BSM and examined for infection, as described previously.

Collection and processing of leafcutter bees from seed alfalfa fields. To investigate whether leafcutter bees may transmit *B. cinerea* spores and/or infected pollen, leafcutter bees were collected from three seed alfalfa fields in southern Alberta throughout the 2013 and 2014 growing seasons. In each season, fields were surveyed on

three dates and on each date ten bees from ten nests were collected in Falcon tubes, though numbers of bees collected depended on weather conditions. Female bees with a large pollen load returning from the field were preferentially collected, and, once caught, were transported in a cooler and stored at -20°C until processing.

To process the samples, 5 mL of 1 x PBS were added to the tubes and the tubes were vortexed for 60s. The concentration of pollen in the suspension was estimated by counting two sides on a hemacytometer, and the suspension was adjusted to 1×10^4 pollen grains mL^{-1}. From each sample, one thousand pollen grains were plated on each of three BSM plates. Following pollen removal, the bees were removed from the vial, patted dry, and plated on BSM. Plates were observed after 7 days for evidence of *B. cinerea* growth.

Statistical analyses. Data from both repetitions of the experiment were tested for homogeneity of variance and were combined for final analysis. Control plants showed trace levels of infection by *B. cinerea* not statistically different from zero, and were therefore excluded from all analysis, with the exception of the preplanned contrasts described below. Analysis was performed using the GLM procedure in SAS (SAS Institute Inc.), testing for treatment, time, and interaction effects. Means were separated by Tukey's HSD. There were not enough pods and seeds produced at the end of six weeks for analysis of seed yields, so these data were not analysed.

Preplanned orthogonal contrasts were used to test the effects of i) pollination and ii) inoculation on both pollen viability and pollen number. The first contrast tested all pollinated treatments (including controls) against all unpollinated treatments. The second contrast tested all inoculated treatments against all controls. Because there were

significant time effects on pollen viability, these contrasts were repeated for each sampling time.

RESULTS

Floret and pollen infection. There was a significant effect of time ($p<0.05$) on floret infection only, so these data were separated by time (Table 2.1). Overall, florets collected at 0 hpi had lower infection levels than those collected at 24, 48, and 96 hpi. There were also significant effects of both inoculation method and pollination. Within the dry inoculated samples, florets that were not tripped had significantly ($p<0.0001$) lower levels of infection (48%) than florets that were tripped (80%). Within the wet inoculated samples, however, there was no significant effect of tripping on infection (87% and 93% for untripped and tripped treatments, respectively).

Pollen infection occurred at levels significantly greater than zero ($p<0.001$), but was still rare (<1% pollen infection) and occurred in wet inoculated treatments only (Table 2.2). Under light microscopy, infection was typically observed on small clusters of pollen grains and less frequently on single pollen grains (Figure 2.1).

Pollen counts and viability. There was a large variation in the number of pollen grains per floret (ranging from 200 to 7,500, data not shown) within and between plants, and therefore no significant effects of treatment or time were found on pollen number ($p>0.05$). Neither pollination nor inoculation method significantly affected pollen viability ($p=0.5$). However, when contrasts were run including the controls, all treatments exhibited a similar decrease of about twelve percentage points in pollen viability from 0 hpi to 96 hpi ($p<0.05$, Table 2.3).

Histopathology. By 96 hpi, conidia were found germinated with hyphae growing on all parts of the floret observed, including the calyx, banner and keel petals, anthers, filament, style, and stamen (Table 2.4; Figures 2.2, 2.3, 2.5-2.7). The majority of conidia (germinated or ungerminated) and hyphal growth were observed on the keel and banner petals in regions of rougher texture, particularly in suspension inoculated treatments (Figures 2.2, 2.3, 2.8). Collapsed cells, indicative of lesions, were observed frequently on keel and banner petals (Figure 2.4). Hyphae were also seen growing directly on the style, on top of the ovaries (Figure 2.7). Appressoria were observed infrequently across all treatments. While inoculations had deposited conidia on all flower parts, by 96 hpi not all conidia had germinated, and this was true for all treatments (Figure 2.3).

In some instances, *B. cinerea* colonized the anthers (Figures 2.5, 2.6), in which case a large proportion of infected pollen was observed. The percentage of pollen infection was greater when estimated by confocal and electron microscopy (1.5 to 2.5% of pollen infected, data not shown) than when estimated by light microscopy (<1%). No pollen infection of dry inoculated treatments was observed.

Leafcutter bees. Pollen collected from leafcutter bees collected from the field and plated on semi-selective media showed very low levels of pollen infection (<0.001%, data not shown). The bees, however, had a much higher proportion of *B. cinerea* growth, particularly those collected at the end of the season, from which *B. cinerea* was detected almost 100% of the time (Table 2.5).

DISCUSSION

The goal of this project was to investigate whether, and to what extent, *Botrytis cinerea* can infect alfalfa pollen under conditions that are likely to be experienced in fields of southern Alberta. The region is semi-arid in climate, and, consequently, fields in this area typically experience warm, dry growing seasons. Most seed alfalfa fields, however, are irrigated throughout the growing season, and this added moisture may contribute to conditions conducive for *B. cinerea* growth (Broome et al. 1994).

The greenhouse experiment tested the effects of pollination (tripped vs untripped) and inoculation method (dry vs suspension) on the infection process of alfalfa florets by *B. cinerea* following 24 h of high humidity. Florets collected at 0 hpi had significantly lower infection levels than those collected subsequently, which suggests that the conidia may have been rinsed off during the surface sterilization procedure prior to full attachment to the tissue. Other studies have shown that adhesion is a passive process and is enhanced by moisture or humidity (Doss et al. 1993; Elad et al. 2007). Both living and dead conidia of *B. cinerea* adhered to a tomato cuticle following dry inoculations, similar to the dry inoculation procedure reported here. The levels of conidia adhesion, however, were greater if the conidia were subsequently hydrated by exposing the tomato tissue to 100% RH (Doss et al. 1993). Thus, the greater levels of floret infection detected at 24, 48, and 96 hpi in this study may be a result of conidia hydration resulting from exposure to 100% RH conditions applied immediately after floret sampling at 0 hpi.

Within dry inoculated florets, there was a significantly greater proportion of infection on tripped than on untripped treatments. Conidia counts from Petri dishes did not reveal any obvious differences between numbers of conidia deposited between the

dry inoculated treatments, and, consequently, these results could be explained by either the presence of pollen (Chou & Preece 1968) or of senescing petals on tripped treatments (Huang et al. 2000). Because no infected pollen was observed in dry inoculated treatments, the greater infection levels of pollinated florets support the hypothesis that the senescing petals are more readily infected by *B. cinerea*. This conclusion supports findings by Bristow et al. (1986), who detected no significant increase in virulence of strawberry flowers by *B. cinerea* in the presence of pollen under field conditions.

Within the suspension inoculated samples there was no statistically significant difference in the proportion of florets infected between treatments that had been tripped and those that had not been tripped. That the overall infection levels were high (87 and 93%) in both treatments suggests that this inoculum delivery method is highly effective at initiating disease. These results are to be expected, given that the high concentration of the suspension (10^5 conidia mL^{-1}) likely resulted in each floret receiving 2-3 orders of magnitude more conidia than dry inoculated florets. Although there was no significant effect of tripping on floret infection, it was observed that, when plated, *B. cinerea* grew more rapidly from those florets that were pollinated than those that were not (data not shown). This observation suggests that conidia deposited on tripped florets had already germinated and colonized the tissue to a greater extent than conidia deposited on untripped florets, which is consistent with the hypothesis that senescing petals are more readily colonized than non-senescing petals (Li et al. 2004; Elad 1997b).

The differences between dry and suspension inoculations of *B. cinerea* conidia on infection processes have been the subject of several studies (Cole et al. 1996; Williamson et al. 1995). Similar to those studies, results presented here show that dry inoculated

conidia grew shorter germ tubes than those that were inoculated by suspension, and neither inoculation method resulted in appressoria formation. The presence of water can greatly facilitate tissue colonization by *B. cinerea* (Nair & Allen 1993), which likely explains why extensive growth was observed on treatments inoculated with a suspension (Figure 1 D). Williamson et al. (1995), controlling for condensation at high RH levels, found high colonization of rose petals by maintaining high humidity (>94%) for 48h. Sowley et al. (2009), investigating quiescence of *B. cinerea* in lettuce, used a similar method for inoculating lettuce seeds as the dry inoculation method presented here: a Petri dish with a sporulating *B. cinerea* colony was inverted over lettuce seeds, the bottom of the Petri dish was tapped to dislodge the conidia, and the inoculated seeds were covered in a plastic bag for 24 h. This method resulted in infected seeds and, subsequently, in asymptomatic plants that hosted *B. cinerea* as an endophyte. These results demonstrate that under humid conditions, dry inoculations can result in *B. cinerea* colonization and delayed disease development.

Previous work in alfalfa has documented the possibility of *B. cinerea* to act as a quiescent pathogen (Huang et al. 2000). Quiescence is typically defined as "the period from host penetration to activation of fungal colonization and symptom appearance" (Prusky et al. 2013), with the main difference from *latency* being that in quiescence, the pathogen halts growth for an extended period of time. In a greenhouse experiment (Huang et al. 2000), alfalfa florets were pollinated with a toothpick dipped in a *B. cinerea* conidia suspension. Plants were maintained under greenhouse conditions and five weeks later, the pods were subjected to 100% RH for several days. Grey mould developed following the high humidity treatment. In the present study, *B. cinerea* exhibited

extensive growth on alfalfa florets after exposure to 24 h of 100% RH. Infection levels of florets remained steady after a return to ambient conditions, and obvious signs of increased disease (grey mould) were not observed.

Many ungerminated conidia were observed attached to all floret parts when examined under CSM and SEM. In strawberry, Bristow et al. (1984) found that *B. cinerea* conidia were present on anthers, filaments, and sepals six days after inoculation, and, when these parts were placed in a humidity chamber, *B. cinerea* could still colonize the tissue. While not a quiescent infection, these findings indicate that a large release of conidia on one day may still become infectious several days later when conditions are conducive. Therefore, even if hot or dry conditions halt colonization after initial infection, the quiescent infection may continue to grow incrementally whenever favourable conditions are present.

CSM and SEM revealed that *B. cinerea* hyphae grew more extensively on the anthers and stamens than other parts of the florets. This finding supports many others that pollen acts as a readily available nutrient source (Huang et al. 1999; Chou & Preece 1968; Fourie & Holz 1998). However, it was not obvious that *B. cinerea* grew preferentially toward pollen; in some cases it did, in others, *B. cinerea* hyphae grew directly beside the pollen but no interactions were observed. Thus, as discussed above, pollen alone unlikely explains increased virulence on pollinated treatments (Bristow et al. 1986). Presence of *B. cinerea* did not affect pollen viability, but viability did decrease over time for all treatments (Table 2.3). This declining trend is likely the result of the florets ageing over the course of the experiment, or due to the high humidity treatment, which has been documented to reduce pollen viability (Hanson 1961).

Ultrastructural results from the confocal and electron microscope showed that *B. cinerea* primarily infects the banner and keel petals, and to a lesser extent the style and anthers (Table 2.4). Viret et al. (2004) observed *B. cinerea* germination on all parts of grape flowers following 24 h of 100% RH. When the style was inoculated, few germ tubes were observed and there was no indication that hyphae grew towards the ovaries. These results agree with our findings and those of Jung (1956, as cited in Jarvis 1977) , who found only very limited growth of *B. cinerea* down the style of all florets from 61 plant species that were tested, including alfalfa.

The pollen collected from the leafcutter bees showed very low levels of fungal infection, as indicated by plating on semi-selective media. However, when the bees were plated following pollen removal, *B. cinerea* was detected at much greater levels (Table 5). This finding indicates that leafcutter bees do carry *B. cinerea* conidia, but that the conidia may be trapped in regions of the body that make them difficult to remove. The number of infected bees increased in the last collection date (August 19-21) in both seasons, which closely follows the trends of *B. cinerea* incidence observed on plants in the alfalfa fields from which the bees were collected (Chatterton & Reich 2014; Reich & Chatterton 2015). By this time, however, few florets remain on the plants, and the majority of pollination has been completed (Frank 2003).

Given the low levels of pollen infection by *B. cinerea* detected in this study, and because *B. cinerea* inoculum is spread primarily by air, it is unlikely that leafcutter bees contribute significantly to the spread of blossom blight – particularly under conditions that are likely to be encountered in the field. In addition, under the tested conditions, it is unlikely that *B. cinerea* infection will lead to seed infection. Indeed, in surveys of seed

alfalfa fields, very little *B. cinerea* infection was observed on seed, and these levels dropped further after harvest (Chatterton & Reich 2014; Reich & Chatterton 2015). As previous literature indicates, and these data support, risk of infection is likely to increase as spore load and precipitation increase (Xu et al. 2000; Bulger et al. 1987; de Araujo et al. 2015; Huang et al. 2000; Sirjusingh & Sutton 1996). Conidia may remain viable for long periods on host tissue and germinate only when conditions are conducive, and the length of this viability period in the field should be determined experimentally. A long viability period could mean that a single large discharge of conidia early in the season would put a crop at high risk of seed losses for the remainder of the season. Methods of describing seasonal discharge of conidia in seed alfalfa fields of southern Alberta are currently being developed.

Table 2.1: The effect of inoculation method and pollination on the infection of alfalfa florets by *B. cinerea* over 96 hours. Data are the mean (SE) of six replicates over two trials, with five florets plated per treatment per harvest time per trial.

Treatment[a]	% Floret Infection								Overall[b]
	0 hpi		24 hpi		48 hpi		96 hpi		
DNT	30.0	(14.4)	60.0	(18.2)	43.3	(8.0)	56.7	(13.7)	47.5 a
DT	63.3	(11.5)	90.0	(6.8)	83.3	(10.0)	83.3	(6.1)	80.0 b
SNT	76.7	(15.8)	90.0	(9.5)	96.7	(3.3)	83.3	(13.1)	86.7 b
ST	80.0	(13.1)	100.0	(6.1)	100.0	(13.1)	93.3	(4.2)	93.3 b
Overall[b]	62.5 a		85.5 b		80.8 ab		79.2 ab		

[a] DNT – dry inoculated, not tripped; DT = dry inoculated, tripped; SNT = suspension inoculated, not tripped; ST = suspension inoculated, tripped

[b] Means followed by different letters are significantly different at p = 0.05.

Table 2.2: Table 2: The proportion of alfalfa pollen infected by *B. cinerea* over 96 hours and four collection times. Data are the means (SE) of 100 pollen grains counted under light microscopy at four collection times and six replicates.

Treatment[a]	% Infected[b]	
DNT	0.013	(0.25)
DT	0.083	(0.24)
SNT	0.861*	(0.25)
ST	0.875*	(0.24)

[a] DNT – dry inoculated, not tripped; DT = dry inoculated, tripped; SNT = suspension inoculated, not tripped; ST = suspension inoculated, tripped

[b] Means followed by * are significantly different from zero at p<0.001.

Table 2.3: The effect of inoculation method of *B. cinerea* and pollination on the viability of alfalfa pollen over 96 hours as indicated by a differential stain. Orthogonal contrasts were run for each collection period and are the mean (SE) of six replicates, with 100 pollen grains counted per harvest time under a light microscope. Inoculated vs Not Inoculated tests all inoculated florets (dry and suspension) against all controls; Tripped vs Not Tripped tests all tripped florets (treatments and controls) against all not tripped florets.

Contrast	% Viable Pollen			
	0hpi	24 hpi	48 hpi	96 hpi
Inoculated	75.8 a	74.9 a	67.7 a	68.2 a
Not Inoculated	73.6 a	74.0 a	63.7 a	60.2 a
Tripped	74.5 a	74.0 a	64.6 a	61.7 a
Not Tripped	75.0 a	74.9 a	66.9 a	66.7 a

[a] Means within each set of contrasts followed by the same letter in the same column are not significantly different at p = 0.05.

Table 2.4: Percentage of alfalfa floret parts on which conidia were observed at 96 hpi. Data are pooled results of samples viewed under confocal and electron microscopy, and include both germinated and non-germinated conidia (n=12 for calyces, banners, and keels; n=24 for pistils/stamens). Statistical analyses were not performed on these data.

Treatment[a]	% With Conidia Present			
	Calyx	Banner	Keel	Pistil/Stamen
DNT	12.5	55.6	28.6	5.6
DT	11.1	18.2	22.2	22.7
SNT	40.0	80.0	44.4	45.0
ST	54.5	100.0	55.6	47.6

[a] DNT – dry inoculated, not tripped; DT = dry inoculated, tripped; SNT = suspension inoculated, not tripped; ST = suspension inoculated, tripped.

Table 2.5: Average percentage of bees carrying *B. cinerea* conidia over two years of collections from four commercial seed alfalfa fields. Data are the mean (SE) of about five bees sampled from ten nests in each field for each collection period. Following pollen removal, bees were plated on *Botrytis* semi-selective media and examined for *Botrytis* growth 7 days after plating.

| Collection Date | 2013 | | | 2014 | | |
	Field 1	Field 2	Field 4	Field 1	Field 2	Field 21
July 15 - 23	.[a]	.	.	19.4 (9.0)	17.5 (8.8)	0.0 (0.0)
Jul 29 - Aug 5	36.0 (11.9)	4.0 (2.7)	8.0 (4.4)	5.0 (3.3)	65.0 (11.6)	12.5 (6.7)
Aug 19 - 21	100.0 (0.0)	.	100.0 (0.0)	.	96.0 (2.7)	.

[a] No bees collected for the date specified.

52
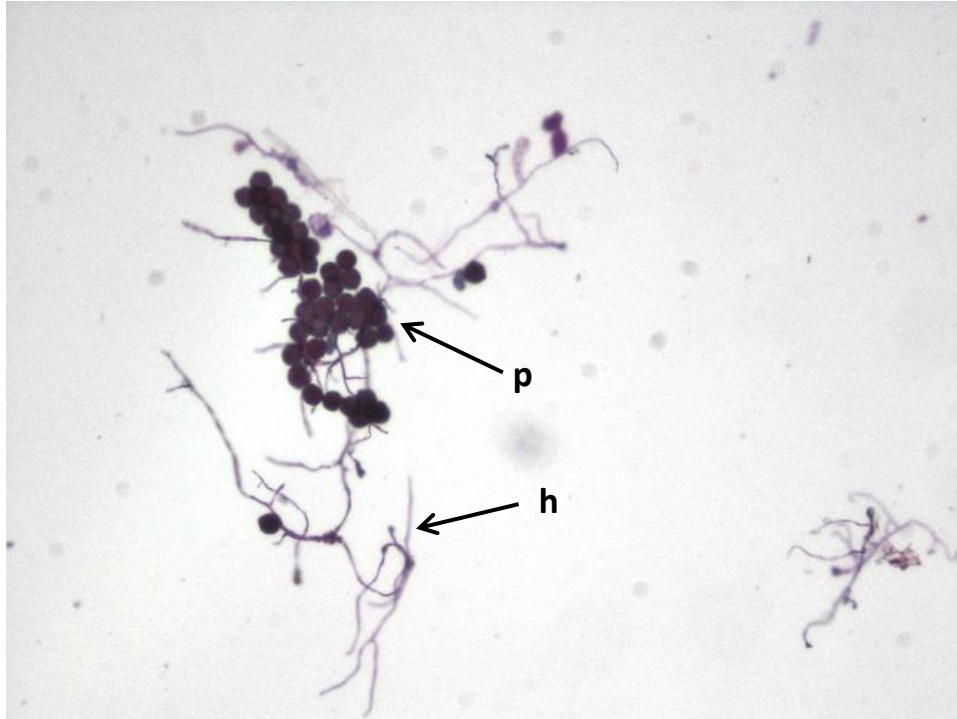
Figure 2.1: Pollen infection by *B. cinerea* as observed by light microscopy 48 hpi (treatment: not tripped, suspension inoculated); p = alfalfa pollen, h = *B. cinerea* hyphae.
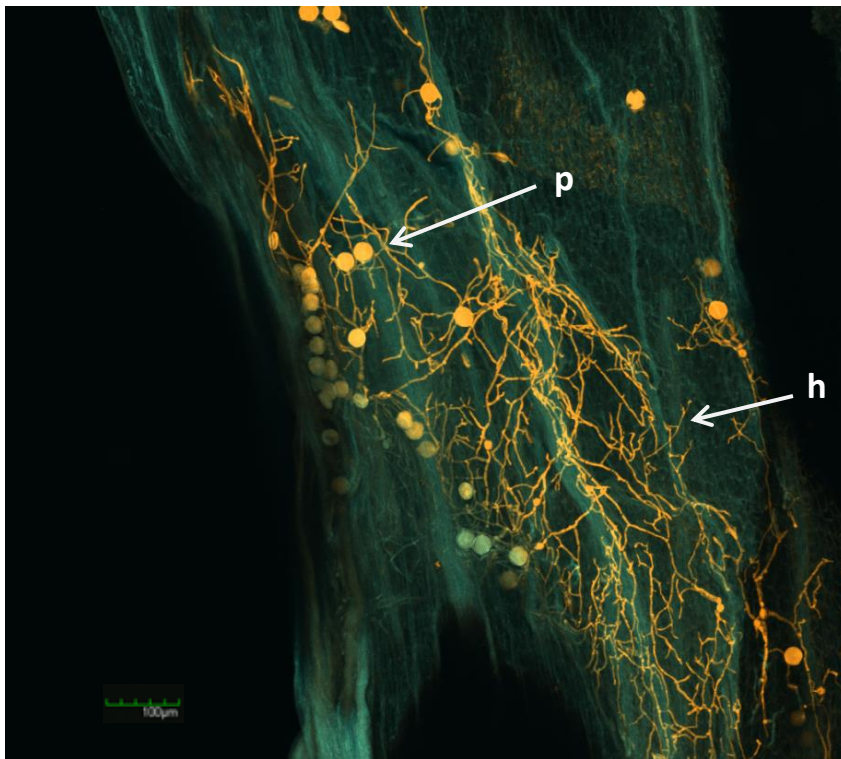
Figure 2.2: A severely infected banner petal as observed by CLM 48 hpi (treatment: tripped, suspension inoculated); p = alfalfa pollen, h = *B. cinerea* hyphae.
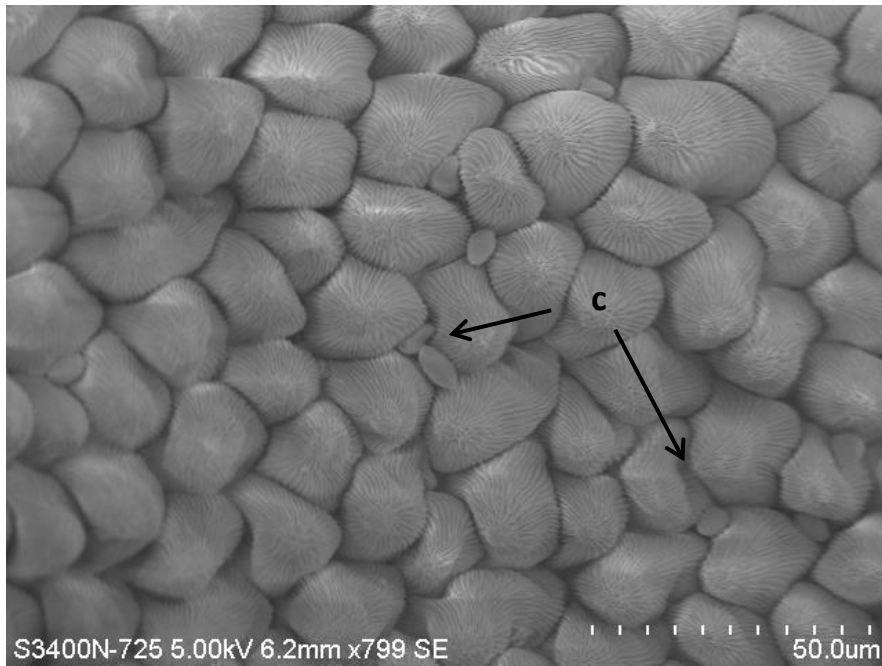
Figure 2.3: Conidia of *B. cinerea* are lodged in the grooves of the banner petal but none have germinated, as observed by SEM 96 hpi (treatment: not tripped, dry inoculated); c = *B. cinerea* conidia.
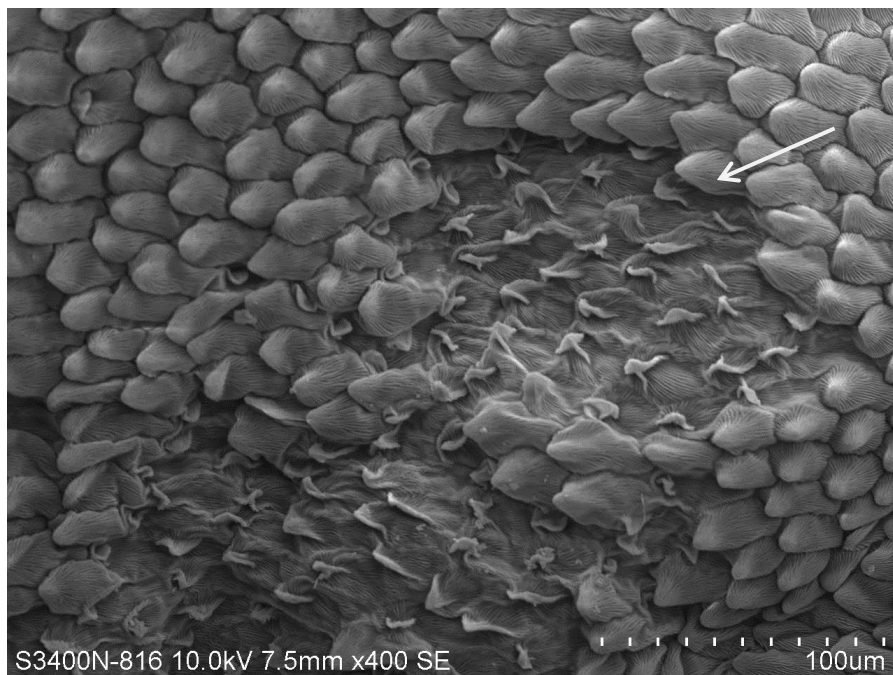
Figure 2.4: Collapsed cell walls on the banner petal associated with *B. cinerea* infection and observed by SEM 96 hpi (treatment: not tripped, dry inoculated); arrow indicates boundary of collapsed cells.
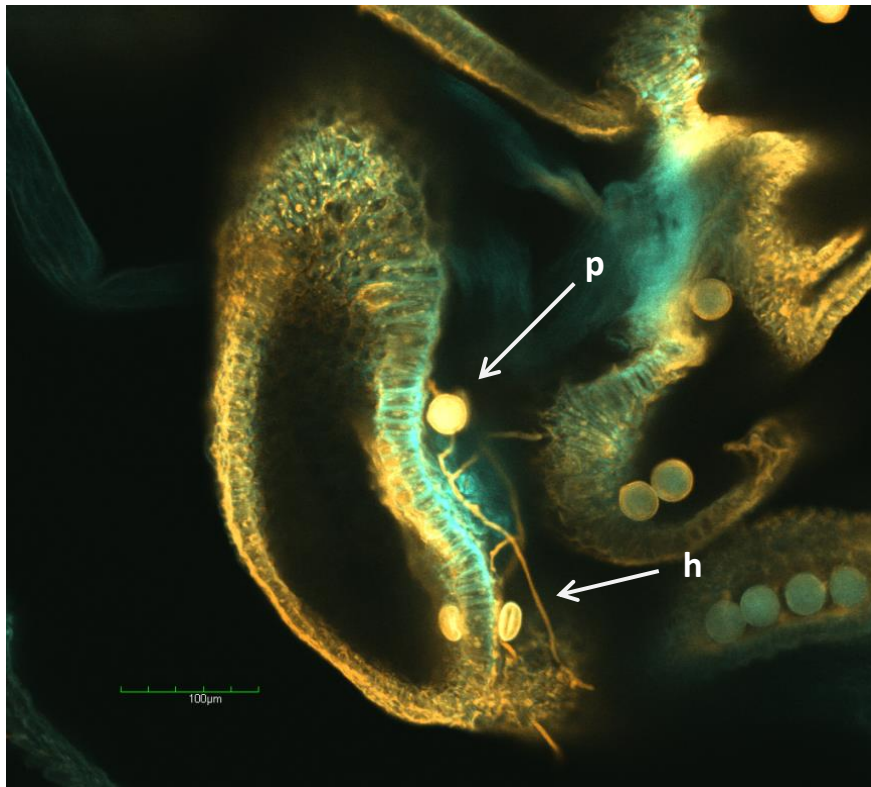
Figure 2.5: Hyphal growth of *B. cinerea* on alfalfa anthers as observed by CLM 96 hpi (treatment: tripped, suspension inoculated); p = alfalfa pollen, h = *B. cinerea* hyphae.
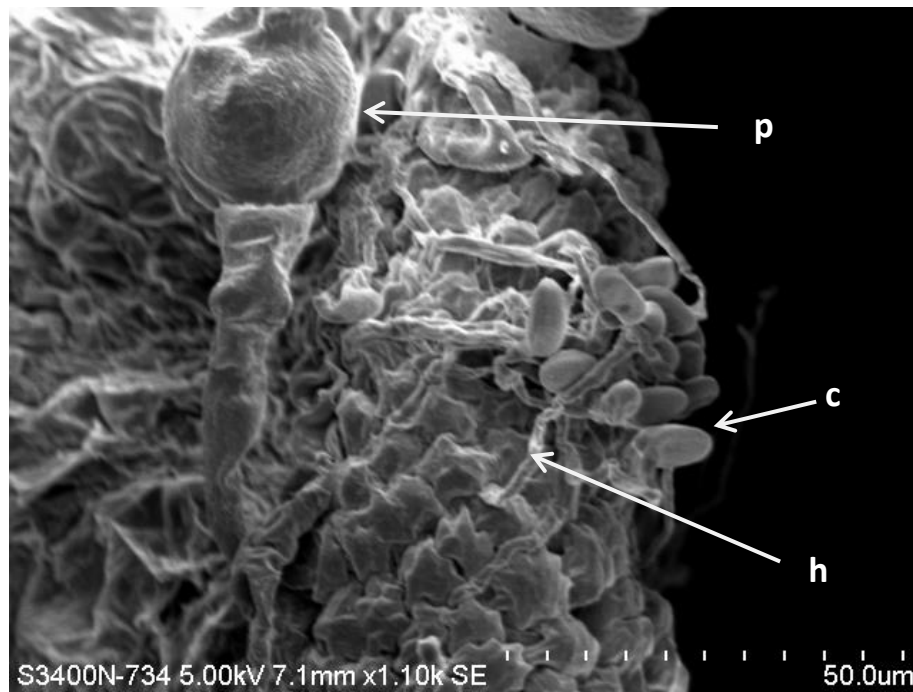
Figure 2.6: Conidia of *B. cinerea* germinate on the anthers of an alfalfa floret, 96 hpi (treatment: tripped, dry inoculated), as observed by SEM; p = alfalfa pollen, c = *B. cinerea* conidia, h = *B. cinerea* hyphae.

Figure 2.7: Hyphae of *B. cinerea* germinates directly on the style, directly over top of the ovaries 96 hpi (treatment: tripped, suspension inoculated), as observed by CSM; o = alfalfa ovary, h = *B. cinerea* hyphae.
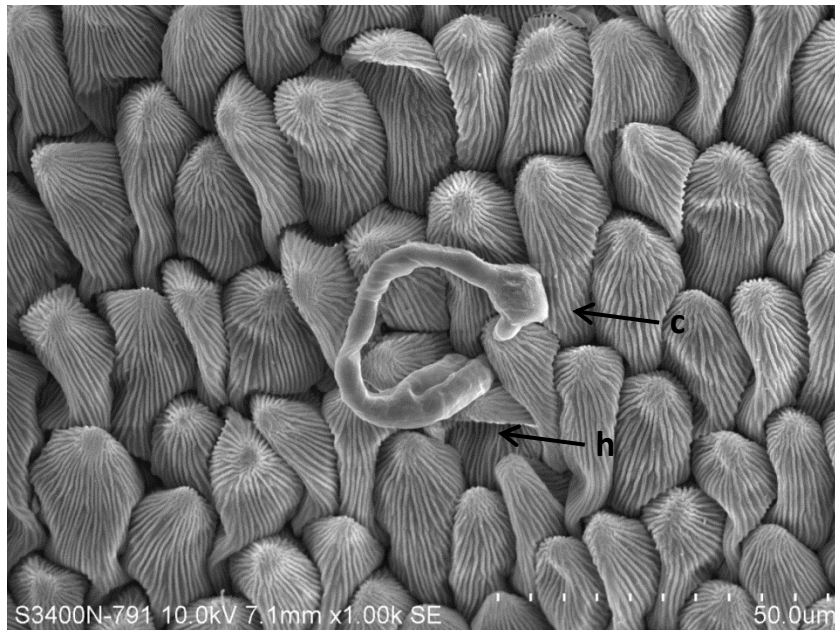
Figure 2.8: A conidium of *B. cinerea* penetrates the banner petal directly, 96 hpi (treatment: not tripped, suspension inoculated), as observed by SEM; c = conidium of *B. cinerea*, h = hypha of *B. cinerea*.

CHAPTER 3: DEVELOPMENT OF A MULTIPLEX TAQMAN qPCR ASSAY TO DETECT AND QUANTIFY *BOTRYTIS CINEREA* AND *SCLEROTINIA SCLEROTIORUM*

ABSTRACT

Fungal pathogens *Botrytis cinerea* Pers.:Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary have a highly homologous genetic code, making it difficult to distinguish them from each other using molecular techniques. The goal of this research was to develop a specific, sensitive primer set for *S. sclerotiorum* that could be used for quantitative real-time PCR (qPCR) on field aerosol samples. Nine primer and probe sets were designed that targeted the intergenic spacer (IGS) region of the ribosomal DNA and genes of several hypothetical proteins. Primers passing initial screening were tested against 34 fungal species and field isolates of *S. sclerotiorum* and *B. cinerea*. All primers targeting hypothetical proteins amplified both *S. sclerotiorum* and *S. trifoliorum*. The primer/probe set, SsIGS_5, targeting the IGS region was highly specific to *S. sclerotiorum*, but also faintly amplified *Penicillium aurantiogriseum* and *Leptosphaeria maculans*. Sensitivity tests were run with this primer set, which was able to reliably detect 0.05 pg target DNA. SsIGS_5 was then multiplexed with a previously published primer set for *B. cinerea* in a TaqMan assay; both retained high sensitivity and were able to amplify as little as 0.05 pg of target DNA.

INTRODUCTION

The generalist fungal pathogens *Botrytis cinerea* Pers.:Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary each have an identified host range numbering in the hundreds of species (Boland & Hall 1994; Williamson et al. 2007). They disperse primarily by

airborne ascospores and conidia, respectively, which, when deposited on senescent plant tissue, germinate and begin colonization. Though *B. cinerea* can form sclerotia – hard, melanised structures resistant to physical and chemical degradation – it typically survives between seasons as hyphae in plant debris (Agrios 1997). Under moist conditions, the hyphae colonize senescent tissue, form conidia, and the conidia are dispersed by wind, rain, or insects (Jarvis 1977, 1962a). This hyphae-conidia cycle may repeat itself whenever conditions are conducive for growth. Conversely, *S. sclerotiorum* survives primarily through formation of sclerotia, which remain in the soil until conditions are suitable for carpogenic germination (Adams & Ayers 1979; Coley-Smith & Cooke 1971). Under prolonged moist soil conditions, the sclerotia germinate to form small mushroom-like structures called apothecia. Apothecia release millions of ascospores over the course of days, and the ascospores are dispersed primarily by wind. Because sclerotia typically require a period of cold conditioning prior to carpogenic germination (Huang & Kozub 1991), this sclerotia-ascospore cycle only occurs once a season. These, and other, airborne pathogens have been the subject of several pilot disease forecasting programs that use vacuum pump spore samplers to monitor aerosols for quantifying inoculum concentrations (Carisse et al. 2005; Rodríguez-Rajo et al. 2010; Parker et al. 2014b).

In contexts where both pathogens are present on the same crop – as in the case of seed alfalfa (*Medicago sativa*), tobacco (*Nicotiana tabacum*), or lentil (Huang et al. 2000; Hartill 1980; Huang & Erickson 2005; Boland & Hall 1994) – differentiating between the two is an important first step for disease forecasting and management. Traditionally, plate culturing techniques are used to differentiate the two fungi (Turkington et al. 1991; Edwards & Seddon 2001; Parker et al. 2014b); however this can be labour intensive and

time consuming, as cultures typically require at least 3-5 days of incubation before identification can be made.

For applications involving aerosol monitoring, using microscopy to identify spores caught on sticky tape or glass slides based on morphology is a rapid method of identification and quantification (Jedryczka et al. 2008; Qandah & del Río Mendoza 2011; Carisse et al. 2005). However, because of the high morphological similarity between ascospores and conidia of *Sclerotinia* and *Botrytis* species, using spore count methods that rely on microscopic observations may require a high degree of specialization to accurately distinguish them (Rogers et al. 2009). Molecular techniques may be more reliable at differentiating between closely related species. An increasing body of literature has demonstrated that quantitative (real-time) polymerase chain reaction (qPCR), when coupled with aerosol sampling, provides reliable identification and quantification of fungal airborne inoculum (Carisse et al. 2009; Parker et al. 2014b; Luo et al. 2007; Rogers et al. 2009).

At the molecular level, *S. sclerotiorum* and *B. cinerea* share a high amino acid identity (83%) in their genetic code (Amselem et al. 2011), which creates difficulties in designing molecular tools that reliably differentiate between the two. A recent comparative genomics paper found no virulence genes that were unique to either species that could be used for differentiating these two organisms, supporting previous pathogenicity and genomics work (Amselem et al. 2011). Several primer sets for both species have been reported that target a range of regions, including the intergenic spacer (IGS), internal transcribed sequence (ITS) of nuclear ribosomal DNA (rDNA), mitochondrial small subunit (mtSSU), calmodulin gene, aspartyl protease gene, β-tubulin

gene, or using RAPD and SCAR markers (Parker et al. 2014b; Rogers et al. 2009; Suarez et al. 2005; Kim & Knudsen 2008; Freeman et al. 2002; Abd-Elmagid et al. 2013; Yin et al. 2009). The primer set for *B. cinerea*, developed by Suarez et al. (2005) and targeting the IGS region, has been shown to be specific (amplifying only *B. cinerea* DNA) and sensitive (able to detect low quantities of *B. cinerea* DNA) (Saito et al. 2013), and our preliminary trials agreed with these findings. Reliable primer sets for *S. sclerotiorum*, however, have been more difficult to develop and many are not species-specific (Freeman et al. 2002; Young et al. 2007).

Parker et al. (2014) determined that previously published primers (Yin et al. 2009; Rogers et al. 2009) for *S. sclerotiorum* failed initial screening tests, and thus developed their own for use in a SYBR Green qPCR assay. However, when our lab tested this new primer set in a SYBR Green assay, we found that it amplifed *B. cinerea* DNA at relatively low C_t values. For disease forecasting purposes in seed alfalfa, this primer set was inadequate at differentiating between the two species at the potentially low quantities that might be expected in field samples. The difficulty in finding a primer set for *S. sclerotiorum* may suggest that isolates of this species vary between regions or crop type (Clarkson et al. 2013; Petrofeza & Nasser 2012), and, as a result, new primers may be required for each application.

The primary objective of this research, therefore, was to design a specific and sensitive primer set for *S. sclerotiorum* that could be used in a qPCR assay for aerosol environmental samples. The second objective was to design a multiplex TaqMan assay for simultaneous quantification of *S. sclerotiorum* and *B. cinerea*.

MATERIALS AND METHODS

Identifying candidate genes: A previous proteomics study on the secretome of *B. cinerea* found thirteen hypothetical proteins with no known function (Shah et al. 2009), and subsequent work reported that at least one of these hypothetical proteins has a homologue in *S. sclerotiorum* (Liang et al. 2013). Therefore, all of these hypothetical proteins were investigated as potential targets. The IGS region between the 28S and 18S genes of the rRNA was also targeted. This region is known to evolve more rapidly than the ITS regions in fungi, and has been used previously to distinguish closely-related species in the family Sclerotiniaceae (Suarez et al. 2005).

Primer and probe design. Sequences for the IGS region and for four of the hypothetical proteins (chosen randomly, accession numbers: XM_001585379, XM_001598127, XM_001596079, and XM_001598892) were obtained from NCBI. Accession numbers were entered into AlleleID 7.7 (Apticraft Systems Pvt. Ltd.) and were aligned with any homologous sequences found in a BLASTn search. TaqMan primers and probes were designed using the default settings of AlleleID 7.7. The resulting primers, probes, and amplicons were entered into BLASTn of the GenBank database to search for any significant alignment with other organisms.

Fungal cultures, DNA extractions, and species confirmation. Fungal isolates of *S. sclerotiorum* and *B. cinerea* were collected from seed alfalfa fields in southern Alberta in the 2014 growing season. Alfalfa florets and pods were harvested, surface sterilized, and plated on *Sclerotinia* semi-selective media (Gutierrez & Shew 1998). Plates were incubated at room temperature for 5-7 days, and *S. sclerotiorum* colonies were identified by a colour change of the medium from purple to yellow and by characteristic white,

fluffy mycelium and subsequent sclerotia formation. Twenty-two more fungal isolates were obtained from greenhouse-grown alfalfa, field pea, and the Lethbridge Research Centre (Agriculture and Agri-Food Canada) isolate collection.

For all isolates, colonies were transferred to PDA and incubated at room temperature until hyphae filled the plate. One hundred milligrams of mycelium were harvested and lyophilized prior to DNA extraction using a DNeasy Plant DNA extraction kit (Qiagen), following manufacturer's instructions. DNA was quantified with a Qubit (Life Technologies) fluorometer. All isolates were sequenced using primers targeting the ITS region using standard PCR protocols and universal primers (White et al. 1990); additionally, the β-tubulin gene was sequenced for the *Botrytis* spp., *B. cinerea*, and *S. sclerotiorum* isolates using standard PCR protocols and universal primers (Glass & Donaldson 1995). PCR products were sequenced using Genome Quebec sequencing services, and sequences were compared to the GenBank database using BLASTn to determine species.

PCR conditions. All primers (salt purified) and probes (HPLC purified, Cy5 labelled and quenched with BHQ-2) were ordered from Eurofins MWG Operon LLC. For endpoint PCR, each 20 μL reaction contained 10 μL HotStarTaq Plus Master Mix (Qiagen), 500 nM of each forward and reverse primer, 20 ng of template DNA, and 6 μL nuclease-free water. PCR conditions were as follows: 95°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 10 min. Reactions were performed in an Eppendorf Mastercycler Pro S thermocycler. Following amplification, the product was stained with EZ-Vision DNA Dye (Amresco Inc., USA) and visualized on a 2.0% (w/v) agarose gel following electrophoresis.

For qPCR, each 25 µL reaction contained 12.5 µL Environmental Master Mix 2.0 (Life Technologies), 300 nM of each forward and reverse primers, 200 nM probe, 20 ng DNA, and 8.5µL nuclease-free water. Reactions were performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following cycle conditions: 95°C for 10 min followed by 40 cycles of 60°C for 1 min and 95°C for 15 s.

Specificity and sensitivity tests. Endpoint PCR using eight samples (four *S. sclerotiorum* isolates, three *B. cinerea* isolates, and a plasmid containing a lipid transfer protein from wheat (TaLTP3, Foroud 2011), was used for preliminary screening of primers. The plasmid served as an internal control for environmental samples, as described below. Primer sets passing the initial screening were used in advanced specificity tests using 9 field isolates of *S. sclerotiorum*, 8 field isolates of *B. cinerea* and *Botrytis* sp., and 19 other common or closely related species on both end-point PCR and TaqMan qPCR (Table 3.1). For specificity TaqMan assays, samples were run in duplicate and the average C_t value was calculated if both replicates amplified before 40 cycles.

For sensitivity tests, DNA from *S. sclerotiorum* isolate LRC 2421, obtained from the Lethbridge Research Centre collection, was obtained from plate culture as described previously. The standard curve contained 10^6 to 10^0 fg of target DNA in ten-fold serial dilutions, and each point was run in technical triplicates. PCR conditions were the same as those described previously. Efficiency and R^2 values were calculated in Bio-Rad CFX Manager 3.1.

Multiplexing. Once the above screening process was completed, the most promising primer and probe set (SsIGS_5) was optimized for multiplexing with a previously published primer and probe set for *B. cinerea* (Suarez et al. 2005). To create

the multiplex standard curves, equal concentrations of *S. sclerotiorum* and *B. cinerea* DNA were mixed and serially diluted in ten-fold dilutions from 10^6 to 10^0 fg. Each 25 µL multiplex reaction contained 12.5 µL 2x Environmental Master Mix (Life Technologies), 500 nM of each SsIGS_5 primer, 300 nM of each Bc3 primer, and 200 nM of each probe. On the same plate, singleplex standard curves were run for *S. sclerotiorum* and *B. cinerea* to compare reaction efficiencies. Thermocycler conditions were the same as described previously for qPCR, and samples were analyzed in technical triplicates.

Testing for inhibition from environmental samples. Aerosol samples collected from seed alfalfa fields in southern Alberta in 2014 were tested with the SsIGS_5 primer and probe set. Aerosol samples were collected daily by a Burkard Multi-Vial Cyclone Sampler (Burkard Manufacturing Co. Ltd.) and deposited in 1.5 mL tubes. DNA was extracted from the tube following the protocol of Parker et al. (2014b). To test for possible PCR inhibitors in environmental samples, four samples that showed no amplification were spiked with 5.0 pg of *S. sclerotiorum* DNA and the C_t values were compared with the C_t values of reactions containing 5.0 pg of pure *S. sclerotiorum* DNA.

RESULTS

Identifying candidate genes; primer and probe design. When entered into a BLASTn search, nine of the thirteen hypothetical proteins identified by Shah et al. (2009) in *B. cinerea* had low homology (all <85%) to genes only in *S. sclerotiorum*. Accession numbers for four of the *S. sclerotiorum* genes were chosen randomly and used for primer design (Table S1), including the possible virulence factor targeted by Liang et al. (2013). Alignment of *B. cinerea* and *S. sclerotiorum* IGS sequences resulted in several candidate

primer sets based on the default scoring algorithm of AlleleID 7.7, five of which were chosen for further screening (Table S1).

Primer and probe specificity. All nine primer sets amplified a region of predicted size (Table S1). Of the nine primer sets designed, all of those targeting hypothetical proteins and two of those targeting the IGS region passed preliminary specificity tests and were used for advanced specificity tests (data not shown). Of the six primer sets remaining, all showed high specificity towards *S. sclerotiorum* (Table S2). All primer sets targeting hypothetical proteins amplified all the *S. sclerotiorum* isolates and the *S. trifoliorum* isolate, but no other fungi. Primer set SsIGS_2 did not amplify *S. trifoliorum* and *S. sclerotiorum* isolate 14.2.13.1 but did faintly amplify several other species, most notably *Leptosphaeria maculans*, *Penicillium aurantiogriseum*, and *Rhizoctonia solani*. Primer set SsIGS_5 was similar to SsIGS_2, except it amplified both *S. trifoliorum* and the *S. sclerotiorum* isolate 14.2.13.1.

Probes were tested for primer sets SsP_1, SsP_4, and SsIGS_5, but not for primer sets SsP_2 and SsP_3 because the latter two exhibited relatively weak amplification of *S. sclerotiorum* DNA on end-point PCR. The probe for SsP_1 did not hybridize during amplification, so only the remaining two primer and probe sets (SsP_4 and SsIGS_5) were used for specificity and sensitivity analyses. Results from these TaqMan assays were similar to results using endpoint PCR. For SsP_4, fluorescence was detected for all *S. sclerotiorum* isolates at approximately 25 cycles and the *S. trifoliorum* isolate at approximately 33 cycles; for SsIGS_5, fluorescence was detected for all *S. sclerotiorum* isolates at approximately 23 cycles as well as for one *Botrytis* sp. isolate, *L. maculans*, *P. aurantiogriseum*, and *R. solani*, at C_t values greater than 36 (Table 1). There was no

amplification or fluorescence detected for *S. trifoliorum* DNA when SsIGS_5 was used in the TaqMan assay. Under the reaction conditions described, SsP_4 was less sensitive than SsIGS_5, with amplification occurring on average 2-3 cycles later than SsIGS_5. To confirm amplicon size, products of the TaqMan qPCR assay were visualized on an agarose gel following electrophoresis, as described previously. SsP_4 primers amplified a region of 194 bp and SsIGS_5 primers amplified a region of 79 bp.

Sensitivity. Sensitivity tests were run with only the SsIGS_5 primer set (Table 2) for two reasons: first, it exhibited higher specificity than SsP_4 within the *Sclerotinia* species (Table 1); and second, it had lower C_t values for the *S. sclerotiorum* isolates than SsP_4 at an annealing temperature of 60 °C (Table 1). From ten-fold serial dilutions, a linear response was observed from 5 ng to 0.05 pg of *S. sclerotiorum* DNA with SsIGS_5 (Figure 3.1). SsIGS_5 also amplified 5 fg of target DNA at approximately 38+ cycles, but this point was excluded from the standard curve due to consistently higher standard deviations (0.7 to 1.2 C_t values) between the technical triplicates. R^2 and efficiency of the standard curves were consistently >0.99 and >85%, respectively.

Multiplexing. Both SsIGS_2 and Bc3 primer sets showed similar sensitivity when multiplexed as when run singly (Figure 2), and consistently detected 0.05 pg of target DNA at approximately 35 cycles of amplification. The standard curve equations from multiplexed reactions were: $y = -3.5471 \times \log(x) + 42.273$ for SsIGS_5 and $y = -3.5016 \times \log(x) + 40.765$ for Bc3, where *x* is the mass of DNA and *y* is the C_t value. R^2 values and efficiencies of the standard curves for both primer and probe sets were consistently >0.99 and >85%, respectively.

Testing for inhibition from environmental samples. The C_t value of 5.0 pg of pure *S. sclerotiorum* DNA was 29, and the C_t values of the environmental samples spiked with the same quantity of DNA was 28-29 (Table 3). Thus, no significant PCR inhibition was observed.

DISCUSSION

Fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* have a host range that includes hundreds of field and horticultural plant crops. In rare instances these pathogens have the same host, as in seed alfalfa in which they both cause blossom blight (Huang et al. 2000). Inoculum of these pathogens is primarily airborne, allowing the possibility of disease forecasting based on monitoring aerial spore concentrations and environmental variables (West et al. 2008; Carisse et al. 2005; Carisse et al. 2009). Due to their similarity in spore morphology, the relative inoculum levels of each pathogen may be difficult to quantify using visual counts alone. Plate culturing techniques can be time consuming and do not provide data fast enough to make important disease management decisions. Thus, the development of quantitative molecular techniques to identify these pathogens provides a fast, reliable alternative to more traditional methods.

The aim of this study was to design a species-specific primer set for *S. sclerotiorum* for use in a quantitative PCR assay. We designed nine *S. sclerotiorum* primers that targeted the IGS region and several hypothetical proteins; six of these primers showed high specificity, and two were used in a TaqMan assay with fluorescently-labelled probes. The primers targeting the IGS region (SsIGS_5) were highly specific within the genus *Sclerotinia*, giving clear negative results for both *S.*

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borealis and *S. trifoliorum* and clear positives for *S. sclerotiorum* in a TaqMan assay. However, they also faintly amplified several other species, including *L. maculans*, *P. aurantiogriseum*, and *R. solani*. On an agarose gel, amplified DNA appeared as weak bands, and on TaqMan qPCR these appeared at C_t values above 36. These C_t values were above the limit of quantification, and may be considered negative. Like *S. sclerotiorum*, *L. maculans* (causal agent of blackleg in canola) is an airborne pathogen, and may be found in southern Alberta where large hectares of canola are routinely grown (Gugel & Petrie 1992). *P. aurantiogriseum* is primarily documented as a disease of pear and may not be present in Alberta (Shim et al. 2002), while *R. solani* is a common soilborne fungus that does not commonly disperse via air (Papavizas et al. 1975), and so these latter two species are unlikely to be found in aerosol samples.

The four primers targeting hypothetical proteins had similar specificity to each other, all showing clear positives for the *S. sclerotiorum* and *S. trifoliorum* isolates. One of these primers (SsP_4) was used in a TaqMan assay and showed amplification of *S. trifoliorum* at a C_t value of 33, but no other nonspecific amplification was observed. *S. trifoliorum* is the causal agent of crown and stem rot of alfalfa, and ascospores of this pathogen are typically released during the fall (Samac et al. 2015). It has not been reported as a major pathogen of seed alfalfa in Alberta, although the signs and symptoms are similar to those caused by *S. sclerotiorum* which could make differentiating the two without the use of molecular tools problematic (Frank 2003; Samac et al. 2015). Interestingly, when the *B. cinerea* primers and probe were tested against these same 36 isolates, we found amplification of *L. maculans*, *P. aurantiogriseum*, and *R. solani* at

similar C_t values as for testing with the SsIGS_2 primers (data not shown). These species were not tested in the original design of the Bc3 primers (Suarez et al. 2005).

Under the preliminary screening conditions, SsIGS_5 was determined to be the best primer set because it was the most sensitive and had the greatest intra-genus specificity. As a result, it was subsequently optimized for use, and specificity, sensitivity, and multiplex assays were repeated under the new optimized conditions. Final master mix concentrations were: 12.5 μL Environmental Master Mix 2.0, 500nM of each forward and reverse primers, and 200 nM of probe, with the same qPCR cycle conditions as stated previously. Under these conditions, the limit of quantification was 0.05 pg target DNA (Figure 3.1). However, C_t values for some of the other species tested also decreased, and, as a result, a cut-off value for positive reactions was set at 36 cycles.

When primers for *S. sclerotiorum* and *B. cinerea* were run in multiplex, both primers showed similarly high sensitivity as when run singly, consistently amplifying 0.05 pg DNA of each target organism. In addition, no non-specific amplification was observed when the DNA of one species was run in reactions containing both primer sets. When a known quantity of *S. sclerotiorum* DNA was spiked into environmental samples that had no *S. sclerotiorum* DNA, no PCR inhibition was observed. Efficiencies remained high, at >85% for all reactions.

Many of the previously designed primers for *S. sclerotiorum* have been reported to perform inconsistently when tested by others (Parker et al. 2014b; Qin et al. 2011; Young et al. 2007; Yin et al. 2009). When our lab tested the most recently published primers (Parker et al. 2014b), we found that *B. cinerea* was still amplified at relatively low C_t values. In the case of blossom blight of seed alfalfa, clearly differentiating

between *B. cinerea* and *S. sclerotiorum* at very low concentrations is important to determine what the most prevalent and aggressive pathogen is. The primer and probe set presented here is highly specific to *S. sclerotiorum* isolates from the seed alfalfa fields surveyed, and no amplification from any *B. cinerea* isolate was detected in a TaqMan assay. In addition, the primer and probe set reported here is highly sensitive. At a limit of quantification of 0.05 pg, and a genome size of 38 Mb (Amselem et al. 2011), approximately 2.4 copies of *S. sclerotiorum* genomic DNA (corresponding to 1.2 ascospores) could be quantified when added as a template in the qPCR assay. This sensitivity is comparable to, or better than, other reports which have determined sensitivities of 5 pg (Yin et al. 2009), 0.5 pg (Rogers et al. 2009), or 0.05 pg (Qin et al. 2011) of template DNA in qPCR assays.

In summary, the PCR assays developed were both specific and sensitive to *S. sclerotiorum* using TaqMan chemistry. As a result, the primers were able to detect and quantify airborne ascospores from environmental samples, which can be used to follow the seasonal discharge patterns of inoculum in seed alfalfa fields.

Table 3.1: C_t values (mean of two duplicates) of primer sets SsIGS_5 and SsP_4 when tested using Cy5-labelled probes in a TaqMan qPCR assay. Results for SsIGS_5 are those reported following primer and probe optimization.

Species	Isolate	Host	Primer Set[a]	
			SsIGS_5	SsP_4
Alternaria alternata	34	alfalfa	-	-
Alternaria alternata	14.2.3.6	alfalfa	-	-
Alternaria sp.	13.2.20.10	alfalfa	-	-
Alternaria tenuissima	G-05	pea	-	-
Aspergillus ochraceus	32	alfalfa	-	-
Botrytis cinerea	13.4.4.8	alfalfa	-	-
Botrytis cinerea	#36	alfalfa	-	-
Botrytis cinerea	J1	alfalfa	-	-
Botrytis cinerea	SA3	alfalfa	-	-
Botrytis cinerea	SA6	alfalfa	-	-
Botrytis sp.	14.1.10.10	alfalfa	38.61	-
Botrytis sp.	14.1.6.3	alfalfa	.	-
Botrytis sp.	14.2.23.5	alfalfa	-	-
Chaetomium globosum	H-01	pea	-	-
Cladosporium cladosporioides	33	alfalfa	-	-
Clonostachys rosea	C-04	pea	-	-
Fusarium avenaceum	R1201	pea	.	-
Fusarium redolens	BF-50	pea	-	-
Fusarium solani	BF-68	pea	-	-
Leptosphaeria maculans	LRC 2468	canola	38.19	-
Penicillium aurantiogriseum	LRC 2450	bean	36.06	-
Penicillium tricolor	A-06	pea	-	-
Rhizoctonia solani	LRC 2115	canola	37.52	-
Sclerotinia borealis	LRC 2035	turf grass	-	-
Sclerotinia sclerotiorum	14.1.23.8	alfalfa	24.92	27.55
Sclerotinia sclerotiorum	14.2.11.3	alfalfa	24.61	25.73
Sclerotinia sclerotiorum	14.2.13.1	alfalfa	22.91	25.28
Sclerotinia sclerotiorum	14.2.7.7	alfalfa	22.61	25.25
Sclerotinia sclerotiorum	14.2.9.2	alfalfa	23.37	25.62
Sclerotinia sclerotiorum	14.3.1.10	alfalfa	23.44	25.16
Sclerotinia sclerotiorum	14.3.3.2	alfalfa	23.08	24.32
Sclerotinia sclerotiorum	14.3.4.8	alfalfa	23.57	25.56
Sclerotinia sclerotiorum	14.3.5.9	alfalfa	23.31	25.99
Sclerotinia trifoliorum	LRC 2100	white clover	-	33.13
Trichocladium asperum	D-03	pea	-	-
Trichoderma paraviridescens	C-01	pea	-	-

[a] '-' denotes no fluorescence observed in 40 cycles.

Table 3.2: Description of final primer and probe set for *S. sclerotiorum*.

Primer/Probe	Sequence (5' - 3')	Target	Amplicon Size
SsIGS_5F	CCTACATTCTACTTGATCTAGTA		
SsIGS_5R	GTTGGTAGTTGTGGGTTA	IGS spacer	79
SsIGS_5P	ACTTCCAAGTGAATACCAGWGATAA		
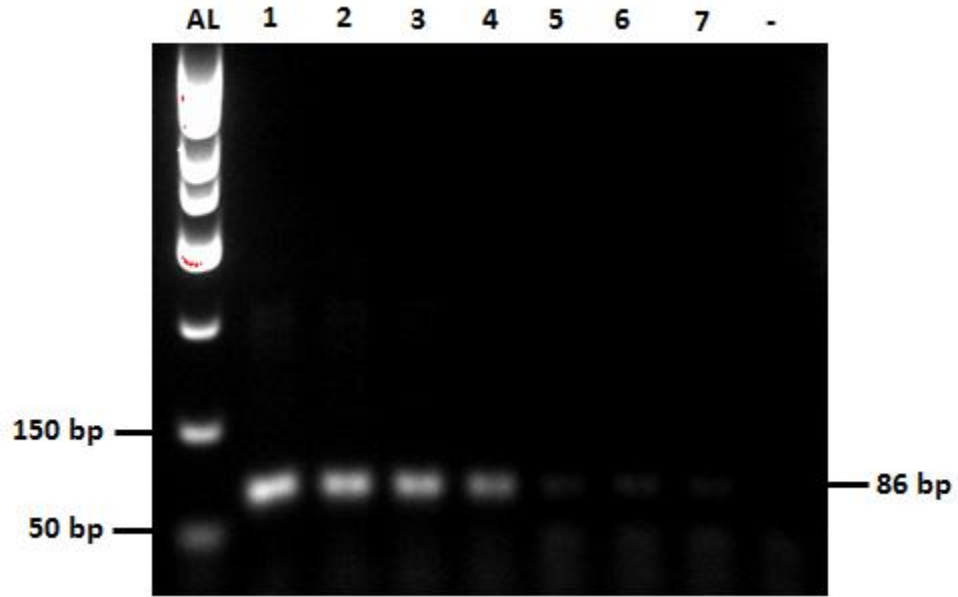
Figure 3.1: Ten-fold serial dilutions of *S. sclerotiorum* DNA amplified by SsIGS_5 on endpoint PCR (35 cycles) and visualized on a 2% agarose gel. Lane 1 contains 7.6 ng of target DNA, which was serially diluted to 7.6 fg in Lane 7.
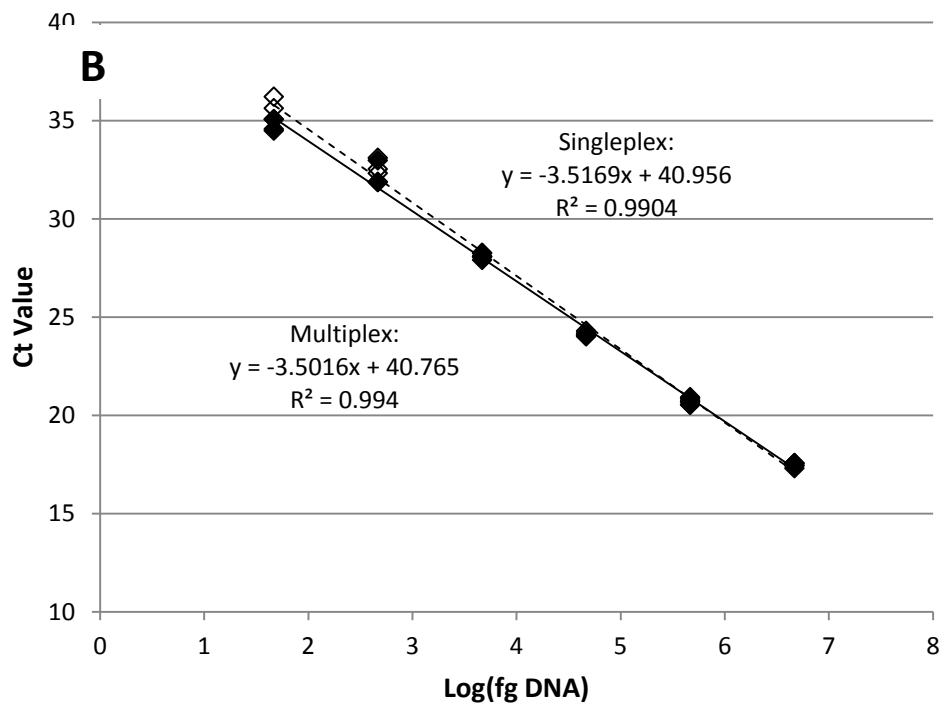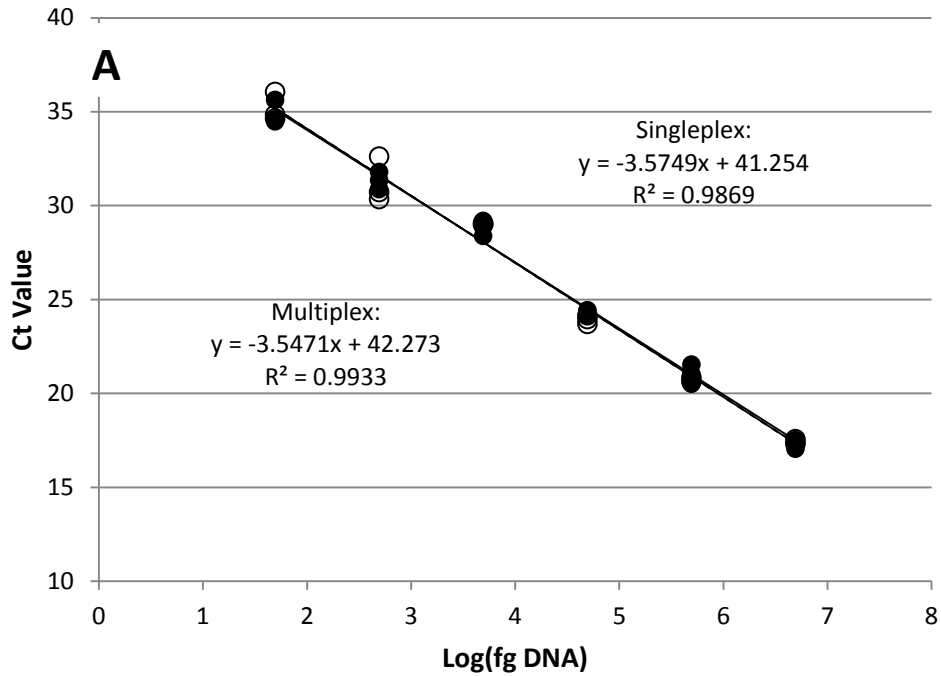
Figure 3.2: Standard curves of (A) *S. sclerotiorum*-specific primer set SsIGS_5 and (B) *B. cinerea*-specific primer set Bc3 when run singly (dashed lines, white shapes) and when multiplexed (solid lines, black shapes) with each other. The assay was based on optimized primer concentrations for SsIGS_5 (see Discussion). Both primer sets retained high r^2 values and sensitivities, detecting 0.05 pg DNA and when multiplexed.

Table 3.3: Mean C_t values of 5.0 pg *S. sclerotiorum* DNA when run directly from extraction (Control) and when diluted in DNA from environmental samples (1-181, 183, 184, 195).

Background DNA	C_t
Control	29.01
1-181	28.06
1-183	28.35
1-184	28.57
1-195	29.87

Table S3.1: Summary of the newly designed primers and probes for specificity tests.

Species	Region Targeted	Primer/Probe[a]	Sequence (5' - 3')	Amplicon Size (bp)
S. sclerotiorum	IGS	SsIGS_1F	AGTGGAATGTGAAATTATCTAA	169
		SsIGS_1R	CATCCTTACCTTYGGAAC	
		SsIGS_1P	CCGCCATCCTGTCCTCGAAT	
S. sclerotiorum	IGS	SsIGS_2F	GTGAGGACCCTAAAGCTA	157
		SsIGS_2R	AGAGCACATACCTACCTA	
		SsIGS_2P	CCAACCTTCAACTGCAAATATGAGC	
S. sclerotiorum	IGS	SsIGS_3F	GTGAGGTATATCTATATGTACTAA	96
		SsIGS_3R	CTCCTAACAGGCTATTTG	
		SsIGS_3P	AGAAACCTGACCCTGACCCTAA	
S. sclerotiorum	IGS	SsIGS_4F	CCTGATTTGAACCCTAAC	289
		SsIGS_4R	CATCCTTACCTTTGGAAC	
		SsIGS_4P	CCGCCATCCTGTCCTCGAAT	
S. sclerotiorum	IGS	SsIGS_5F	CCTACATTCTACTTGATCTAGTA	79
		SsIGS_5R	GTTGGTAGTTGTGGGTTA	
		SsIGS_5P	ACTTCCAAGTGAATACCAGWGATAA	
S. sclerotiorum	Predicted protein	SsP_1F	AGTGGATTTGATGTTAAGC	119
		SsP_1R	GGAGATATCGATGATAGTTTG	
		SsP_1P	TATTATCTGGTCCTTCAACAAGAGCA	
S. sclerotiorum	Hypothetical virulence gene[b]	SsP_2F	AGCTCGACTCAAATCTAC	171
		SsP_2R	CCAAGTTCCATCCTCAAA	
		SsP_2P	ACCTCGTCAACTGCTTCAACAA	
S. sclerotiorum	Predicted protein	SsP_3F	GTCGGAGTACAATACGATA	70
		SsP_3R	TCACGGTGTAAGGATAAC	
		SsP_3P	TGATAACCTCCTACAATTACGGCG	
S. sclerotiorum	Predicted protein	SsP_4F	TCCTGTATCTCAGAAATGC	194
		SsP_4R	CCATCAATCCATTCACATC	
		SsP_4P	CTACTGGTCTCTGCGAATGCC	

[a] F denotes forward primer, R reverse primer, and P probe; all probes were labelled with a cyanine 5 fluorophore [Cy5] on the 5' end and quenched with a Black Hole Quencher [BHQ-2] on the 3' end.

[b] Identified by Liang et al. (2013).

Table S3.2: Specificity tests of primers designed to target the IGS region (SsIGS_2) or hypothetical proteins (SsP_1-4) of *S. sclerotiorum* run on end-point PCR.

Species	Isolate	SsIGS_ 2	SsIGS_ 5	SsP_ 1	SsP_ 2	SsP_ 3	SsP_ 4
		Primer Sets[a]					
Alternaria alternata	34	-	-	-	-	-	-
Alternaria sp.	13.2.20.10	-	-	-	-	-	-
Alternaria tenuissima	G-05	-	-	-	-	-	-
Aspergillus ochraceus	32	-	-	-	-	-	-
Botrytis cinerea	13.4.4.8	o	o	o	-	-	-
Botrytis cinerea	2421	-	-	-	-	-	-
Botrytis cinerea	J1	-	-	-	-	-	-
Botrytis cinerea	SA3	-	-	-	-	-	-
Botrytis cinerea	SA6	-	-	-	-	-	-
Botrytis sp.	14.1.10.10	o	o	o	-	-	-
Botrytis sp.	14.1.6.3	o	o	o	-	-	-
Botrytis sp.	14.2.23.5	o	o	o	-	-	-
Chaetomium globosum	H-01	-	-	-	-	-	-
Cladosporium cladosporioides	33	-	-	-	-	-	-
Clonostachys rosea	C-04	-	-	-	-	-	-
Fusarium avenaceum	R1201	-	-	-	-	-	-
Fusarium redolens	BF-50	-	-	-	-	-	-
Fusarium solani	BF-68	-	-	-	-	-	-
Leptosphaeria maculans	LRC 2468	o	o	o	-	-	-
Penicillium aurantiogriseum	LRC 2450	o	o	o	-	-	-
Penicillium tricolor	A-06	-	-	-	-	-	-
Rhizoctonia solani	LRC 2115	o	o	o	-	-	-
Sclerotinia borealis	LRC 2035	-	-	-	-	-	-
Sclerotinia sclerotiorum	14.1.23.8	+	+	+	+	+	+
Sclerotinia sclerotiorum	14.2.11.3	+	+	+	+	+	+
Sclerotinia sclerotiorum	14.2.13.1	-	+	+	+	+	+
Sclerotinia sclerotiorum	14.2.7.7	+	+	+	+	+	+
Sclerotinia sclerotiorum	14.2.9.2	+	+	+	+	o	+
Sclerotinia sclerotiorum	14.3.1.10	+	+	+	+	+	+
Sclerotinia sclerotiorum	14.3.3.2	+	+	+	+	+	+
Sclerotinia sclerotiorum	14.3.4.8	+	+	+	+	+	+
Sclerotinia sclerotiorum	14.3.5.9	+	+	+	+	+	+
Sclerotinia trifoliorum	LRC 2100	-	+	+	+	+	+
Trichocladium asperum	D-03	-	-	-	-	-	-
Trichoderma paraviridescens	C-01	-	-	-	-	-	-
TaLTP3 plasmid		-	-	-	-	-	-

[a] '-' denotes no amplification, 'o' denotes a faint amplification, and '+' denotes a strong amplification.

CHAPTER 4: DISEASE AND INOCULUM SURVEYS FOR THE DEVELOPMENT OF A DISEASE FORECASTING SYSTEM OF BLOSSOM BLIGHT PATHOGENS IN SEED ALFALFA OF SOUTHERN ALBERTA

ABSTRACT

Blossom blight of seed alfalfa (*Medicago sativa* L.), caused by fungal pathogens *Botrytis cinerea* Pers.:Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary, is a potentially devastating disease on the Canadian Prairies in moist growing seasons. Because these pathogens are dispersed primarily by wind, monitoring the seasonal airborne concentration of spores is a potentially useful approach to developing a disease forecasting model – particularly if coupled with molecular detection and quantification methods. Nineteen seed alfalfa fields in southern Alberta were surveyed throughout the growing seasons of 2013 and 2014. All fields were surveyed for blossom blight incidence and severity, and plant samples were collected and plated on semi-selective media for blossom blight pathogens at each collection time. Trace levels of blossom blight symptoms were found in both years; however, plated samples indicated that *B. cinerea* increased over the growing season, while *S. sclerotiorum* decreased. In 2014 in three of the fields, Burkard 7-day volumetric spore samplers collected daily aerosol samples, and weather stations recorded environmental variables. Conidia and ascospores collected per day were quantified with a quantitative real-time polymerase chain reaction (qPCR) assay. Spore quantification indicated that both *B. cinerea* and *S. sclerotiorum* remained low in July and increased in August. Both species had multiple days with high spore discharge, with seasonal maxima of 21,137 and 2,265 conidia and ascospores, respectively. Results suggest that spore discharge is correlated with precipitation events

or relative humidity. The development of a disease forecasting model for blossom blight pathogens is in progress.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) grown for seed is a lucrative, specialty crop, whose production in Canada is concentrated in southern Alberta (CSGA 2014). Blossom blight, caused by fungal pathogens *Botrytis cinerea* Pers.:Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary, have been associated with severe losses in alfalfa seed across the Canadian Prairies in moist seasons (Gossen et al. 1998; Gossen et al. 1997; Gossen & Platford 1999; Huang et al. 2000). These pathogens produce conidia and ascospores, respectively, which are transported by wind and act as the main infectious units. Spores land on senescent petal tissue, which provides a rich nutrient source for pathogen establishment, and readily spread to infect whole racemes, reducing seed production (Huang et al. 2000; Olivier et al. 2008).

Several production practices may contribute to the development of blossom blight. First, because the majority of seed alfalfa fields in southern Alberta are irrigated, the additional moisture may contribute to the prolonged humid microclimates favoured by blossom blight pathogens (Broome et al. 1994; Clarkson et al. 2014). Second, alfalfa grown for seed is cultivated as a perennial, and is consequently particularly susceptible to pathogens that can survive between seasons and compound inoculum over years (Hind-Lanoiselet et al. 2005). Sclerotia of *S. sclerotiorum* are able to remain viable in soil for several years (Adams & Ayers 1979; Coley-Smith & Cooke 1971), meaning that

inoculum may build up over years and cause a severe epidemic under prolonged cool, humid conditions (Huang et al. 2000; Samac et al. 2015). Third, alfalfa has a long flowering period, lasting from the beginning of July until mid-August, during which it is most susceptible to infection by blossom blight pathogens, particularly the polycyclic *B. cinerea* (Frank 2003; Samac et al. 2015).

Disease forecasting plays an important role in the management of diseases in field and horticultural crops by alerting growers when disease pressure is high. Growers can therefore avoid unnecessary applications of fungicides (i.e. when the pathogen is not present or conditions are not favourable for pathogen growth), and reduce fungicide use while maintaining yields. Disease forecasting models based on environmental variables have led to reductions in fungicide application by up to 80 and 100% in carrot and grape, respectively, when growers apply fungicides according to the model rather than the standard spray regime (Foster et al. 2011; Broome et al. 1994).

A growing body of research has shown that the coupling of aerial monitoring and molecular techniques can result in rapid and reliable quantification of airborne plant pathogens (Parker et al. 2014b; Carisse et al. 2009; Klosterman et al. 2014; Rogers et al. 2009), the benefits of which have been recently reviewed by West et al. (2008), West and Kimber (2015) and Peccia and Hernandez (2006). Due to the sensitivity and specificity of molecular techniques such as quantitative (real-time) polymerase chain reaction (qPCR), significant advances in understanding daily and seasonal spore release patterns have been made (Luo et al. 2007; Almquist & Wallenhammar 2014; Lang-Yona et al. 2012). In conjunction with environmental and disease intensity monitoring, these data can be used to develop a disease forecasting model whose purpose is to reduce fungicide applications

by timing applications when disease risk is high (Rodríguez-Rajo et al. 2010; Xu et al. 2000; Carisse et al. 2005). Monitoring airborne inoculum levels has been shown to increase the reliability of disease forecasting systems based solely on environmental variables (Xu et al. 2000; Carisse et al. 2009).

Currently, detection methods for *S. sclerotiorum* and *B. cinerea* are based on visual signs of disease (grey or white, fluffy mycelia on blossoms or stems) (Samac et al. 2015; Gossen et al. 1997; Gossen & Platford 1999), or on using semi-selective media to capture airborne spores or to plate florets for quantifying infection levels (Turkington & Morrall 1993; Parker et al. 2014b). The latter approach, however, requires waiting several days for the fungi to grow to an identifiable stage and a certain level of expertise in fungal identification. This delay in data collection does not allow growers to take preventative action if high disease risk is predicted. In a comparison of the plate culture and qPCR methods for detecting airborne ascospores of *S. sclerotiorum* in carrot, researchers found that both approaches quantified ascospores with equal accuracy, but that quantification by qPCR was more rapid (Parker et al. 2014b).

A disease forecasting model for blossom blight in seed alfalfa has not yet been developed, although this pathosystem is a good candidate for such a model. Seed production in southern Alberta is concentrated within a relatively small region, meaning that, broadly, environmental conditions across fields would be similar and that a disease forecasting model could be implemented for the whole region. Because the infectious units of blossom blight pathogens are primarily airborne, their life cycle facilitates monitoring inoculum levels by air sampling (West et al. 2008). Spore discharge patterns of *S. sclerotiorum* and *B. cinerea* have not been studied in this region, and because the

population dynamics are unknown, these pathogens are managed primarily by fungicides (Bardin & Huang 2001; Frank 2003). A robust disease forecasting system would reliably predict when disease pressure is high and alert growers to take preventative action only when necessary.

The goals of this project, therefore, were to i) survey commercial seed alfalfa fields in southern Alberta to determine the intensity of blossom blight, and ii) quantify seasonal changes of airborne inoculum of *S. sclerotiorum* and *B. cinerea* over the growing season to provide the foundation for a disease forecasting model.

MATERIALS AND METHODS

Field surveys and sample collection. Nineteen commercial seed alfalfa fields were surveyed every two weeks during blossoming and pod development (July to August) in 2013-2014. The fields were located in southern Alberta, near the towns of Enchant, Rosemary, and Brooks. For each field, ten sites were sampled in a U-shape pattern and were at least 20 m apart from each other and from the field's edge. At each site, twenty stems (~1 m row) of alfalfa were assessed for severity and intensity of blossom blight and stem rot. For blossom blight, severity was rated on a scale of 0 to 4 (0 = no florets infected; 1 = 1-25% florets infected; 2 = 26-50% florets infected; 3 = 51-75% florets infected; 4 = 76-100% florets infected), and incidence was recorded as the number of stems out of twenty that had blossom blight symptoms. For stem rot, severity was rated on a scale of 0 to 5 (0 = no infection; 1 = <1% of stem infected; 2 = 1-10% of stem infected; 3 = 11-25% of stem infected; 4 = 26-50% of stem infected; 5 = >50% of stem

infected) and incidence was rated as the number of stems out of twenty showing stem rot symptoms. From each site, racemes were harvested (three each of florets and/or pods depending on development of the plants) for processing in the lab. Racemes that had recently been pollinated and showed some petal senescence were preferentially chosen, because they were more likely to be infected by blossom blight pathogens (Huang et al. 2000). A final seed sample of approximately 500 mL volume was collected from growers once the crop had been harvested.

Processing floret, pod, and seed samples. From samples collected in the field, ten florets and/or pods from each site were surface sterilized (60 s in 0.5% NaClO plus 2-3 drops of Tween 20), triple rinsed with double distilled water, and dried on paper towel. Five florets and/or pods were plated on each of *Sclerotinia* semi-selective medium (SSM) (Edwards & Seddon 2001) and *Botrytis* semi-selective medium (BSM) (Gutierrez & Shew 1998). For the last field collection in late August, seeds were extracted from surface sterilized pods, surface sterilized using the previous method, and ten seeds were plated on each of SSM and BSM. Seeds obtained after harvest were processed in the same manner, except with the sterilization period extended to 120 s. Twenty seeds were plated on each of five plates of SSM and BSM.

Identification of pathogens from plate culture. Plates were incubated at room temperature in the dark and samples were rated for pathogen incidence at 5 and 7 days. Samples on SSM were positive for *S. sclerotiorum* if the medium changed from purple to yellow, and if white, fluffy mycelia and/or sclerotia were present. Colour change was not a sufficient criterion because *B. cinerea* also produces oxalic acid, the chemical responsible for the colour change, but does not produce white mycelia or sclerotia.

Samples on BSM were positive for *B. cinerea* if the medium changed from pink to brown and also either i) had extensive hyphal growth (> 1.5 cm after 7 days) that was morphologically consistent with the pathogen or ii) formed conidia. When culture morphology was ambiguous, the use of a stereomicroscope revealed formation of microsclerotia on the plant tissue. These criteria were necessary because species of other common airborne fungi turned the medium from pink to brown.

Collection of airborne ascospores and conidia. In 2014, three fields with known histories of blossom blight symptoms were chosen for air sampling. The fields were located near Enchant, Brooks, and Rosemary, and were chosen so that sampling would represent the main seed growing regions in southern Alberta. One Hirst-type, 7-day multi-vial cyclone sampler (Burkard Manufacturing Co. Ltd.) was placed in each of three fields, with the air intake valve 1.0 m above the ground, and approximately 0.2 m above the top of the alfalfa canopy at maturity. Each sampler was powered by a 12 V battery, which was attached to a solar panel to maintain charge throughout the sampling period. The samplers intake air at a rate of 16.5 L min^{-1}, or approximately 1 m^3 hr^{-1}. Due to differences of maximum spore dispersal times between species and between seasons (Qandah & del Río Mendoza 2011; Jarvis 1962a), samplers ran continuously throughout the day (00:05 to 23:55 h) for the growing season (mid-June to early September) and vials were collected and replaced each week. Collected vials were stored at -20ºC until DNA was extracted.

DNA extraction of aerosols. DNA extraction from frozen samples followed the protocol described by Parker et al. (2014). Briefly, 100 µL CTAB buffer (100 mL 1 M Tris HCl pH 8.0, 280 mL 5 M NaCl, 40 mL 0.5 M EDTA, 20 g CTAB, and ddH$_2$O to 1

L) were added to each tube and the tubes were vortexed for 10 min. The tubes were subjected to three freeze-thaw (liquid $N_2 - 60°C$) cycles for two minutes each, with the final thaw period extended to 30 min. The tubes were then spiked with 10^6 copies of the internal exogenous control TaLTP3 plasmid, followed by DNA extraction using a PowerSoil DNA extraction kit (MoBio Laboratories Inc.).

Internal control plasmid. A plasmid containing a lipid transfer protein from wheat (*Triticum aestivum*) was obtained from Dr. Nora Foroud (Agriculture and Agri-Food Canada, Lethbridge) for use as an internal control. The plasmid, TaLTP3, was chosen because DNA from wheat is unlikely to be found in the aerosol samples and primers targeting this gene would not amplify fungal DNA. 10^6 copies of the TaLTP3 plasmid were added to each sample prior to extraction to account for extraction efficiencies, false negatives, and to act as a normalizing agent. Because the variation in extraction efficiency may be relatively high (Wagner et al. 2015; Hospodsky et al. 2010), all samples were normalized to the internal control prior to statistical analysis. To normalize, the mean number of plasmid copies detected by qPCR was determined for each set of DNA extractions. The detected plasmid copy number for each sample was used to determine the normalizing factor for that sample by dividing the detected copy number by the mean copy number for the set of extractions. The number of spores detected in that sample was then multiplied by the normalizing factor.

Multiplex qPCR. To enable quantification of both target and internal control DNA in the same reaction, TaqMan chemistry was used for quantification. Reactions to quantify *S. sclerotiorum* and *B. cinerea* DNA were run separately, and each species-specific reaction was multiplexed with primers/probe to detect the internal control

plasmid (Table 1). Each 25 μL multiplex reaction contained 12.5 μL 2x Environmental Master Mix (Life Technologies), either 500 nM of each SsIGS_2 primer pair or 300 nM of each Bc3 and TaLTP3 primer pairs, 200 nM of each probe, and 4 μL of DNA. Reactions were performed in technical triplicates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following cycle conditions: 95°C for 10 min followed by 40 cycles of 60°C for 1 min and 95°C for 15 s. All primers (salt purified) and probes (HPLC purified) were ordered from Eurofins MWG Operon LLC.

Conidia and ascospore production for standard curves. To produce conidia, *B. cinerea* (isolate LRC 2421) was grown on 2% (w/v) malt extract agar for 7 days in the dark at room temperature. Cultures were then exposed to near-UV light (12h/12h night/day) until conidia developed, approximately 10 days. Conidia were harvested by flooding the plates with sterile distilled water (SDW) and filtering through four layers of cheesecloth. The suspension was adjusted to 3.2×10^6 conidia mL^{-1} using a hemocytometer. To produce ascospores of *S. sclerotiorum*, the method described by Huang and Kozub (1989) was followed, with some modification. Briefly, *S. sclerotiorum* (isolate LRC 2148) sclerotia were conditioned at 10°C for 10 weeks, transferred to Petri dishes containing sterilized Cornell mix, and the Petri dishes were flooded with SDW. The cultures were incubated at room temperature under constant light for 2-3 weeks; water levels were maintained at the flooded state during this period by adding SDW to the Petri dishes every 3-4 days. After ~2 weeks, ascospores were harvested by lifting the lid slightly off the Petri dish, which created a puff of ascospores from the apothecia that would attach to the lid. This harvesting procedure was repeated over a number of days in order to obtain enough ascospores for standard curve assays. To collect the ascospores,

the lid was flooded with SDW and the ascospore concentration was adjusted to 3.6 x 10^6 ascospores mL^{-1} using a hemocytometer.

Creating standard curves. To create the standard curves, spores of each pathogen were serially diluted in 10 ng/uL sheared salmon sperm DNA (Invitrogen, Life Technologies) to simulate matrix effects (Carisse et al. 2009) and DNA was extracted from the dilutions. This approach quantifies aerosol samples more accurately than extracting DNA first and serially diluting the DNA (An et al. 2006; Diguta et al. 2010; Luo et al. 2007). The serial dilutions were ten-fold from 10^5 to 10^1 spores mL^{-1}, with additional two-fold dilutions at the 10^3 and 10^2 concentrations. One hundred microliters of each suspension were pipetted into 1.5 mL centrifuge tubes, and DNA extractions were performed as described previously. Thus, the extractions were from 3.2 x 10^4, 3.2 x 10^3, 3.2 x 10^2, 1.6 x 10^2, 32, 16, and 3.2 conidia for *B. cinerea*, and 3.6 x 10^4, 3.6 x 10^3, 3.6 x 10^2, 1.8 x 10^2, 36, 18, and 3.6 ascospores for *S. sclerotiorum*. The extractions were repeated three times for each conidia or ascospore concentration and, following extraction, were mixed to create the DNA stock for the standard curve. This DNA was run in technical triplicates and included with the field samples in each 96-well reaction plate to produce a standard curve specific for each run.

Collection of weather data. WatchDog Mini (model 2450) weather stations (Spectrum Technologies Inc.) were mounted on PVC pipe and placed at 0.8 m above ground level in each of the fields containing spore samplers. A rain gauge was placed 1.0 m above ground level. Temperature, rainfall, and relative humidity were recorded hourly, and these were used to calculate the daily maximum, minimum, and mean for each day throughout the growing season.

Aerosol spore quantification and data quality control. Median C_t values of each triplicate qPCR sample were used for calculating spore number. Spore numbers are reported as the total number of conidia or ascospores detected in a single day, and were not normalized to spores hr^{-1} or spores m^{-3}. Samples were excluded from analysis if their wells had no or very low (<1,000 copies) amplification of the internal control plasmid. Any sample that had a detected quantity of spores <32 or 36 – the limits of detection for *B. cinerea* and *S. sclerotiorum*, respectively (Chapter 3, this thesis) – were considered zero.

Statistical analysis. Means and standard errors for the number of plasmid copies detected per DNA extraction were calculated using the UNIVARIATE procedure in SAS (SAS Institute Inc.).

RESULTS

Field surveys and floret, pod, and seed plating. In both 2013 and 2014, alfalfa began blossoming by the beginning of July, with the first pods forming by mid-July. By the middle of August, very few florets remained, with most of the racemes at the pod stage. Field surveys revealed only trace signs of blossom blight and stem rot incidence and severity (data not shown). Samples that were plated, however, showed higher levels of infection (Table 4.2). In general, for both years, *S. sclerotiorum* incidence was greatest (11%) in the first sampling periods and decreased towards the last sampling period (4 – 7%); conversely, *B. cinerea* incidence was lowest in the first sampling periods (65% and 5% in 2013 and 2014, respectively) and increased towards the last sampling period (99% and 10% in 2013 and 2014, respectively). The fields containing the spore samplers

exhibited similar trends for both pathogens, and overall disease incidence was greater than the average in these fields (Table 4.3).

qPCR standard curves and sample inclusion/exclusion. DNA extractions were more efficient at extracting DNA from *B. cinerea* conidia than *S. sclerotiorum* ascospores, as indicated by the difference of 4-5 C_t values between conidia/ascospores of the same concentration (Figures 4.1 and 4.2). Because quantification of DNA was generally unreliable for points below DNA extracted from 32 and 36 conidia and ascospores (equivalent to DNA of ~1 spore per reaction), respectively, these were considered to be the limits of detection. Efficiencies ranged from 92 to 103% for *B. cinerea* and from 99 to 120% for *S. sclerotiorum* (Figures 4.1 and 4.2); r^2 values were >0.96 for all standard curves.

Ten sets of DNA extractions were performed over the sampling period. For each sample, the TaLTP3 plasmid was expected to amplify 10^4 copies, and, as predicted, the copy number clustered around a value of 10^4 copies for each set of extractions (Table 4.4). These results indicated that overall, the extraction efficiency was equal across extraction sets, although efficiency was more variable between individual samples. Based on the exclusion criteria, twenty data points were excluded from analysis as a result of poor extractions or sampler malfunctioning.

Seasonal conidia discharge and environmental conditions. Seasonal discharge patterns and weather (precipitation and RH) data for each of the three fields surveyed in 2014 are presented in Figures 4.3-4.5. Overall, 2014 was wetter than average, with a number of significant rainfall events in June and again in August. In general, most conidia and ascospores were released in August, at the pod development stage. In

Enchant, *B. cinerea* was detected on July 17 (initial pod formation stage) and quantified at 278 conidia per day. Several spikes of conidia (500 to 1,000 conidia per day) were detected over the next two weeks. August had several days when daily conidia collections were >1,000 per day, with the greatest number (21,137 conidia per day) collected on August 18. *S. sclerotiorum* ascospore discharge followed a similar pattern as *B. cinerea*, with a daily maximum discharge of 8,639 ascospores detected on August 18, and several smaller (200 to 700 ascospores per day) discharges from July 15 until the end of the season.

In Brooks, no data were collected prior to July 23 due to technical difficulties with the spore sampler, but similar patterns were observed to those in Enchant. Spores of both species had a noticeable discharge on July 24 (835 and 216 conidia and ascospores per day, respectively), and conidia of *B. cinerea* were quantified at high levels (>1,000 conidia per day) on several occasions throughout August. By contrast, *S. sclerotiorum* had virtually no release in August, except on August 4, when 1,283 ascospores per day were detected. One last major discharge of ascospores was detected at the end of the sampling season, on September 2.

In Rosemary, a severe hailstorm damaged most of the crop on August 8. Prior to the hailstorm, several peaks of *B. cinerea* conidia (200 to 600 conidia per day) were observed. *B. cinerea* conidia were quantified again at >2,000 conidia per day in the last week of August. On August 10, two days after the hailstorm, the only substantial discharge of *S. sclerotiorum* ascospores was detected at 2,265 ascospores per day.

Enchant received less intense rainfall than Brooks and Rosemary throughout the growing season, with a maximum rain event of 21 mm on July 23 (Figure 4.3), whereas

Brooks and Rosemary experienced several rainfall events over 50 mm throughout July and August (Figures 4.4 and 4.5). The timing of precipitation was similar between all sites and occurred mid- to late July and sporadically through August. Nevertheless, it appears that conidia dispersal may be correlated with precipitation events 1-2 weeks prior to their release. In Rosemary, for instance, larger dispersal days from July 27 to August 7 and August 26 to 28 were preceded by rain events from July 21 to July 26 and on August 15, respectively. Similar trends were seen in both Enchant and Brooks. Obvious trends were not apparent in the data for *S. sclerotiorum* ascospore levels, and more formal statistical analyses will be required to determine significant associations between spore number and environmental conditions from multiple years of study.

DISCUSSION

This study was designed to provide a basis for a disease forecasting system for blossom blight pathogens *B. cinerea* and *S. sclerotiorum* in seed alfalfa fields of southern Alberta. Because minimal signs of disease were observed in field surveys over the 2013 and 2014 season, correlating inoculum and/or environmental variables with disease intensity was not possible. These results may be confounded by the fact that the growers whose fields were surveyed applied fungicides according to their own schedule throughout the season. If the fungicides were effective, the disease intensity observed in the field may not accurately reflect the true disease pressure. Thus, future studies should attempt to record growers' fungicide use and to survey fields where fungicides are not routinely used. Samples collected at each surveying period and plated on semi-selective

media showed a greater frequency of pathogen inoculum, which will be more useful in developing a disease model (Xu et al. 2000).

In general, over both the 2013 and 2014 seasons, presence of *B. cinerea* on plant parts increased throughout the growing season, while presence of *S. sclerotiorum* declined (Table 4.2). These patterns suggest that *B. cinerea* is more prevalent during the pod stage, which corresponds to conidia levels measured by the spore samplers and qPCR assay (Figures 4.3 to 4.5). In 2013, *B. cinerea* was detected at very high levels on the BSM assay, approaching 100% of pods plated by the middle of August (Table 4.2). These findings are a result of errors in species identification that were based solely on a colour change of BSM in the first year. Many common fungal species were found to cause a colour change from pink to brown, including *Cladosporium* spp., *Alternaria* spp., *Penicillium* spp., *Chaetomium* spp., and *Plectosphaerela* spp. Following this discovery, the criteria for positive *B. cinerea* identifications were made more stringent for the 2014 collection season, as described above. Even with these new criteria, several of the presumptive *B. cinerea* isolates were sequenced with ITS and β-tubulin universal primers (Glass & Donaldson 1995; White et al. 1990), and, when the sequences were entered into a BLASTn search for homologous sequences in GenBank, the results indicated that the isolates were a *Botrytis* species, but not *B. cinerea.* The species identity of these isolates is currently being determined by PCR using species-specific primers for a range of common *Botrytis* species. These identification issues highlight certain drawbacks associated with relying on plate cultures and morphology for identifying and quantifying fungal species.

According to plated florets and pods results, *S. sclerotiorum* was more prevalent during the flower stage and early pod stage (Table 4.3), but according to aerosol samples analyzed by qPCR, the majority of *S. sclerotiorum* inoculum was detected towards the end of the flowering stage and well into the pod stage. In field surveys, signs of *S. sclerotiorum* were only evident at these latter stages (surveys completed in August), although the overall incidence of blossom blight was low. These results seem contradictory, as a greater amount of inoculum should translate to greater levels of tissue infection, particularly as evidenced by samples plated on SSM. One explanation could be related to contradictory findings on the dispersal patterns of *S. sclerotiorum*. Some studies have shown that the dispersal of ascospores in canola and carrot crops is spatially aggregated within a field, in part because dense canopy reduces the ability of ascospores to escape (Qandah & del Rio Mendoza 2012; Boland & Hall 1988). Under these conditions, one would expect to collect few ascospores from air samples, but still find localized disease in a field. Other studies, however, have shown that ascospores are dispersed long distances by wind (Williams & Stelfox 1979; Parker et al. 2014a). Under these conditions, one would expect to find varying concentrations of ascospores in air samples, correlating with disease intensity across the field. Thus, infected florets and pods from the beginning of the season (Table 4.2 and 4.3) may reflect inoculum produced within the field and unable to reach above the canopy, as suggested by the low numbers of ascospores collected in the spore samplers (Figures 4.3 to 4.5). Conversely, the lower floret and pod infection in August could be indicative of less inoculum produced within the field. The greater ascospore counts in August may reflect inoculum from other fields, arriving at time when alfalfa racemes are in the pod stage and therefore less vulnerable to

infection. The fact that a large discharge of ascospores (2,265 ascospores per day) was found in Rosemary two days after a hailstorm destroyed most of the foliage in that field may lend support to the hypothesis that the dense foliage prevents ascospores from escaping the canopy.

The data collected here are similar to other surveys for blossom blight of seed alfalfa conducted in Manitoba and Saskatchewan. In particular, Gossen and Platford (1999) found that in Saskatchewan, incidence of *B. cinerea* increased from early to late bloom; in Manitoba, however, *B. cinerea* incidence peaked at mid-bloom and was very low at late bloom. For *S. sclerotiorum*, the patterns in both Saskatchewan and Manitoba followed the patterns detected here, namely, a gradual decrease in incidence from early to late bloom.

The qPCR assays for *B. cinerea* and *S. sclerotiorum* were specific and sensitive (Chapter 3), and could detect DNA extracted from less than 40 spores. However, the greater C_t values detected in the *S. sclerotiorum* standard curve (Figure 4.1) could suggest that ascospores are more difficult to extract DNA from than *B. cinerea* conidia, whose standard curve amplified at about 4-5 cycles earlier. While the results suggest that both species followed similar discharge patterns, there are some noticeable dates where one was detected in excess, while the other was not detected at all (i.e. August 10 and 26 in Rosemary, Figure 4.5).

Results for quantifying the presence of *B.* cinerea and *S. sclerotiorum* spores are comparable to other aerobiological studies. For *B. cinerea*, previous work in strawberry (Xu et al. 2000) and raspberry (Jarvis 1962a) found maximum daily conidia discharge from 10^3 and 10^4, respectively, which is comparable to our findings of a daily maximum

of 2×10^4 conidia. In raspberry, the daily number of conidia collected remained close to zero for most of July, but increased steadily throughout August, with massive discharge events on August 11 and 18 (Jarvis 1962a). Similar trends were observed in the three alfalfa fields surveyed, which had low conidia numbers until the end of July, followed by several large discharge events throughout August. These findings are consistent with the knowledge that *B. cinerea* is a polycyclic pathogen, releasing conidia whenever conditions are conducive. As the season progresses, it is expected that the discharges of conidia become greater as inoculum levels increase exponentially.

For *S. sclerotiorum*, previous work in canola found maximum daily ascospore discharge in the 10^3 range, with significant variation between years dependent on precipitation levels (Qandah & del Río Mendoza 2011). In one year, the maximum discharge occurred on June 24, at the beginning of the canola flowering, and remained low over the rest of the season. In another year, however, ascospore discharge began at the end of June and continued at high (10^2 to 10^3) levels throughout the flowering period until the end of July. As *S. sclerotiorum* is only capable of producing inoculum once per season, this seemingly continuous presence of ascospores may suggest that heterogeneity in microclimates results in a staggered maturation of apothecia and ascospore release. Similar discharge patterns were observed for ascospore levels in canola fields in England (Rogers et al. 2009). In the present study, Enchant had constant ascospore discharge throughout July and August (Figure 4.3). Rosemary, however appeared to follow the more typical pattern of a monocyclic pathogen, with one large spike detected on August 11 (Figure 4.5).

Comparisons to inoculum threshold values determined in other studies are difficult to make, due to differences in sampling methodology and inoculum quantification. Typically inoculum in other studies is reported as spores m^{-3} or spores m^{-3} day^{-1}. In onion, threshold values for conidia of *B. squamosa* were determined at 10 and 15 conidia m^{-3} (Carisse et al. 2005). Because researchers only sampled the equivalent of 2.5 m^3 per day, this would translate to threshold values of 25 and 37.5 conidia per day, the units reported here. Studies of ascospore discharge in carrot and canola provide a much broader range of threshold values. In carrot, researchers sampled 3 m^3 each day and determined thresholds of 2 and 9.5 ascospores m^{-3}, or roughly 6 and 28.5 ascospores caught each day (Parker et al. 2014b). In canola, investigators arbitrarily decided on a threshold value of 20 ascospores m^{-3} day^{-1} to separate high discharge days from low discharge days (Qandah & del Río Mendoza 2011). They sampled approximately 14.5 m^3 each day, which means their total daily catch at that threshold value was 290 ascospores.

In light of the studies described above, the spore dispersal patterns described in the present study (Figures 4.3 to 4.5) indicate that spore dispersal patterns may vary greatly between crop type and season. The variation may be due, in part, to unique microclimates created under each crop canopy. This conclusion highlights the need for studies like this one to be performed for each crop type and climate.

Cursory examination of two weather variables suggests significant correlations exist between precipitation events and spore discharge. In other studies, models for these pathogens have included temperature and leaf wetness (Broome et al. 1994; Xu et al. 2000), dew point (Rodríguez-Rajo et al. 2010), relative humidity (Qandah & del Río Mendoza 2011; Rodríguez-Rajo et al. 2010), or vapour pressure deficit (Rodríguez-Rajo

et al. 2010), but more rigorous statistical analyses will be required to determine the strength of these relationships in the alfalfa-blossom blight system. Such analyses are beyond the scope of this thesis, but more in-depth analyses are planned for future projects when data from multiple years has been collected.

Given these preliminary results, a model describing the occurrence of blossom blight pathogens based on environmental data will be possible. The challenge with such a model, however, is determining the threshold values – that is, the concentration of spores at which appropriate preventative action should be taken. Empirically determining these thresholds is a procedure that has been done relatively rarely (Carisse et al. 2005), but is an important step to complete before such a model becomes useful to growers.

Table 4.1: List of primers and probes used in qPCR assays.

Target	Reference	Primer/Probe	Sequence (5' - 3')	Amplicon Size
S. sclerotiorum	This thesis	SsIGS_5F	CCTACATTCTACTTGATCTAGTA	79
		SsIGS_5R	GTTGGTAGTTGTGGGTTA	
		SsIGS_5P	[Cy5]ACTTCCAAGTGAATACCAGWGATAA[BHQ2]	
B. cinerea	Suarez et al. 2005	Bc3F	GCTGTAATTTCAATGTGCAGAATCC	95
		Bc3R	GGAGCAACAATTAATCGCATTTC	
		Bc3P	[FAM]TCACCTTGCAATGAGTGG[BHQ1]	
TaLTP3 (plasmid)	Foroud 2011	TaLTP3-178F	GCAGGTGGACTCCAAGCTC	143
		TaLTP3-320R	GGCACCTGCACGCTATCT	
		TaLTP3-232P	[HEX]CTCGATCAGCAAGGAGTGCT[BHQ1]	

Table 4.2: Mean incidence (percent) of blossom blight pathogens on floret, pod, and seed samples from nineteen seed alfalfa fields in southern Alberta in 2014. Data are the means of samples collected from ten sites from 19 fields at each collection time. Samples were surface sterilized and plated on semi-selective media for pathogen detection and identification. Data from Chatterton and Reich (2014) and Reich and Chatterton (2015).

Date	Florets		Pods		Seeds	
	Bc[a]	Ss[b]	Bc	Ss	Bc	Ss
2013						
Jun 24 – Jul 5	65.3	10.7	-[c]	-	-	-
Jul 15 – Jul 18	66.5	4.4	76.7	10.7	-	-
Jul 29 – Jul 31	87.9	5.6	95.1	18.6	-	-
Aug12 – Aug21	-	-	99.1	6.8	13.5	0.0
2014						
Jul 3 – Jul 5	4.5	11.8	-[c]	-	-	-
Jul 21 – Jul 24	3.8	12.0	1.6	11.6	-	-
Aug 4 – Aug 6	9.7	7.8	6.4	8.5	-	-
Aug18 – Aug20	-	-	5.6	4.0	0.4	1.1

[a]Bc = *Botrytis cinerea*; [b]Ss = *Sclerotinia sclerotiorum*; [c] no plant samples were collected for the period specified.

Table 4.3: Mean incidence (percent) of blossom blight pathogens on floret, pod, and seed samples from three fields containing the spore samplers in the 2014 field season. Data are the means of samples collected from ten sites at each collection time. Samples were surface sterilized and plated on semi-selective media for detection and identification.

Collection Date	Floret				Pod				Seed			
	Bc[a]		Ss[b]		Bc		Ss		Bc		Ss	
Field 1												
Jul 4	4.0	(2.7)	24.0	(6.5)	-[c]	-	-	-	-	-	-	-
Jul 24	4.0	(2.7)	8.0	(4.4)	2.0	(2.0)	36.0	(7.2)	-	-	-	-
Aug 4	2.0	(2.0)	8.0	(6.1)	6.0	(3.1)	12.0	(4.4)	-	-	-	-
Aug 20	-	-	-	-	22.0	(5.5)	10.0	(5.4)	0.0	(0.0)	1.0	(1.0)
Oct 10	-	-	-	-	-	-	-	-	0.0	(0.0)	0.0	(0.0)
Field 2												
Jul 3	0.0	(0.0)	2.0	(2.0)	-	-	-	-	-	-	-	-
Jul 22	14.0	(7.9)	30.0	(6.8)	0.0	(0.0)	24.0	(5.8)	-	-	-	-
Aug 4	10.0	(4.5)	10.0	(6.1)	4.0	(4.0)	4.0	(2.7)	-	-	-	-
Aug 19	-	-	-	-	4.0	(2.7)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
Oct 10	-	-	-	-	-	-	-	-	0.0	(0.0)	1.0	(1.0)
Field 21												
Jul 3	18.0	(4.7)	22.0	(4.7)	-	-	-	-	-	-	-	-
Jul 22	6.0	(3.1)	34.0	(8.5)	0.0	(0.0)	34.0	(7.9)	-	-	-	-
Aug 5	26.0	(6.0)	4.0	(2.7)	6.0	(3.1)	26.0	(6.7)	-	-	-	-
Aug 19	-	-	-	-	16.0	(5.0)	16.0	(4.0)	0.0	(0.0)	17.0	(8.8)
Oct 10	-	-	-	-	-	-	-	-	0.0	(0.0)	0.0	(0.0)

[a]Bc = *Botrytis cinerea*; [b]Ss = *Sclerotinia sclerotiorum*; [c] no plant samples were collected for the period specified.

Table 4.4: Mean (SE) TaLTP3 copy number ($\times 10^4$) detected by qPCR from the ten DNA extractions performed during the 2014 season. There were 12-24 samples per extraction, and plasmid copy number for each sample was calculated twice: once when multiplexed with *B. cinerea* primers, and once when multiplexed with *S. sclerotiorum* primers. For each multiplex, samples were run in technical triplicates, and copy number was calculated from the plasmid standard curves run on the same plate.

Extraction	Bc[a]		Ss[b]	
1	4.53	(0.69)	5.57	(0.84)
2[c]	0.50	(0.11)	0.51	(0.12)
3	2.13	(0.36)	2.02	(0.36)
4	2.95	(0.24)	2.95	(0.39)
5[d]	0.49	(0.27)	0.40	(0.31)
6	2.28	(0.27)	2.82	(0.32)
7	2.14	(0.22)	2.51	(0.24)
8	1.82	(0.25)	2.22	(0.25)
9	2.31	(0.30)	2.78	(0.37)
10	2.46	(0.16)	3.46	(0.35)

[a] Bc = *B. cinerea*; [b] Ss = *S. sclerotiorum*; [c] Several samples from this set of extractions were excluded from analysis due to poor amplification of the TaLTP3 plasmid; these samples are the cause of the low average of copy numbers in this set of extractions; [d] This whole set of extractions was excluded from analysis due to poor amplification of the TaLTP3 plasmid.
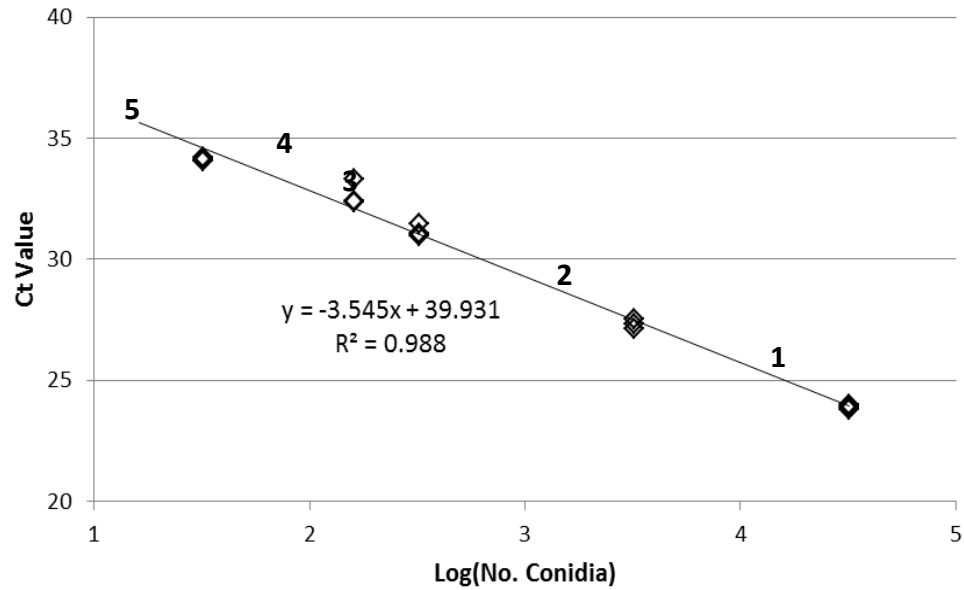
Figure 4.1: Typical standard curve of *B. cinerea* conidia. Conidia were serially diluted prior to DNA extraction, and the DNA from three replicates were pooled for each point on the standard curve. Points are representative of DNA extracted from (1) 3.2×10^4, (2) 3.2×10^3, (3) 3.2×10^2, (4) 1.6×10^2, and (5) 32 conidia. Efficiency = 102%.
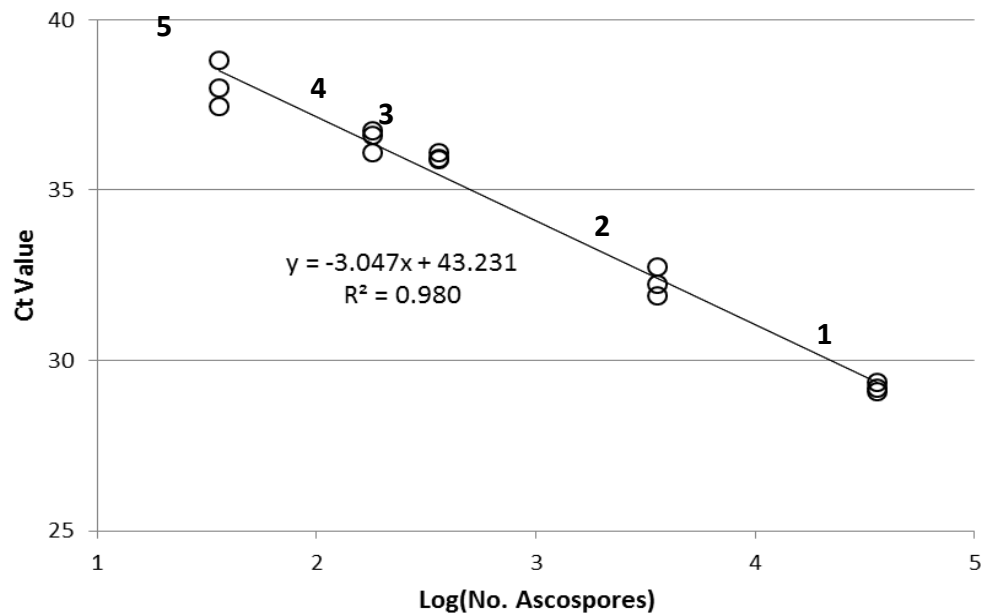
Figure 4.2: Typical standard curve of *S. sclerotiorum* ascospores. Ascospores were serially diluted prior to DNA extraction, and the DNA from three replicates were pooled for each point on the standard curve. Points are representative of DNA extracted from (1) 3.6×10^4, (2) 3.6×10^3, (3) 3.6×10^2, (4) 1.8×10^2, and (5) 36 ascospores. Efficiency = 113%.
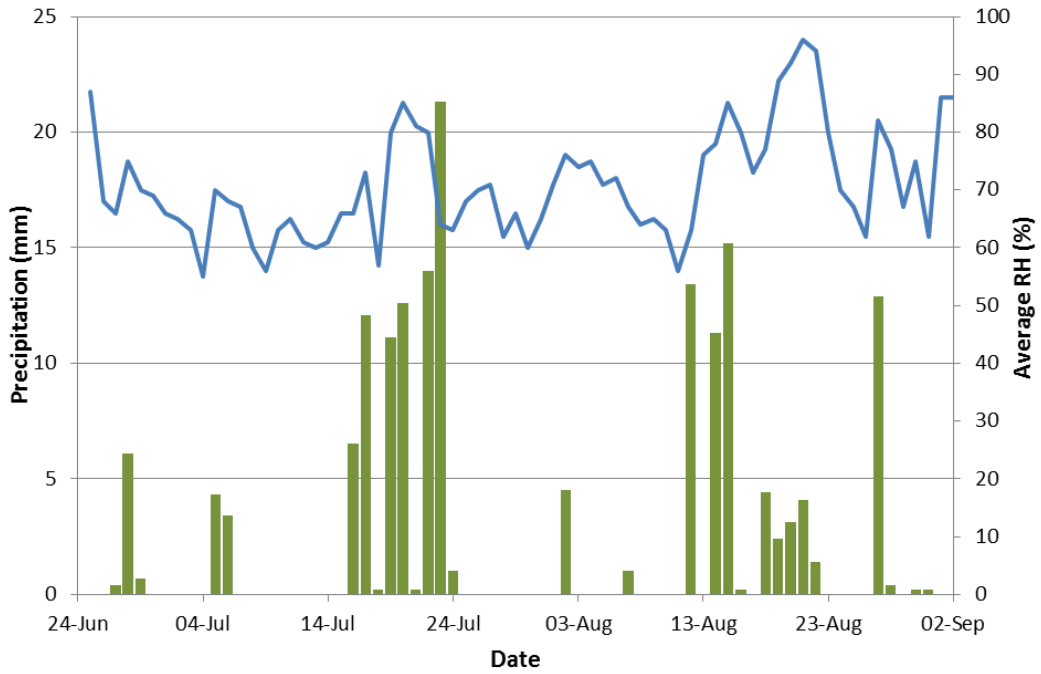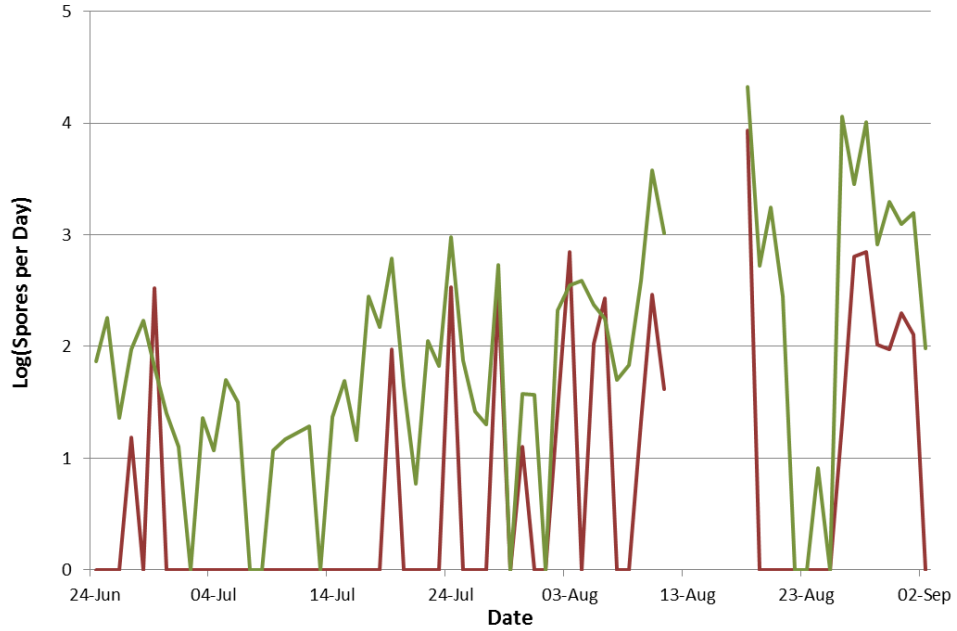
Figure 4.3: Seasonal discharge of *B. cinerea* conidia (green line) and *S. sclerotiorum* (red line) ascospores (top) and select environmental variables, precipitation (bars) and RH (line, bottom) in Field 1, near Enchant, AB.

Figure 4.4: Data for Field 2, near Brooks, AB. Refer to caption below Figure 1.3 for details.
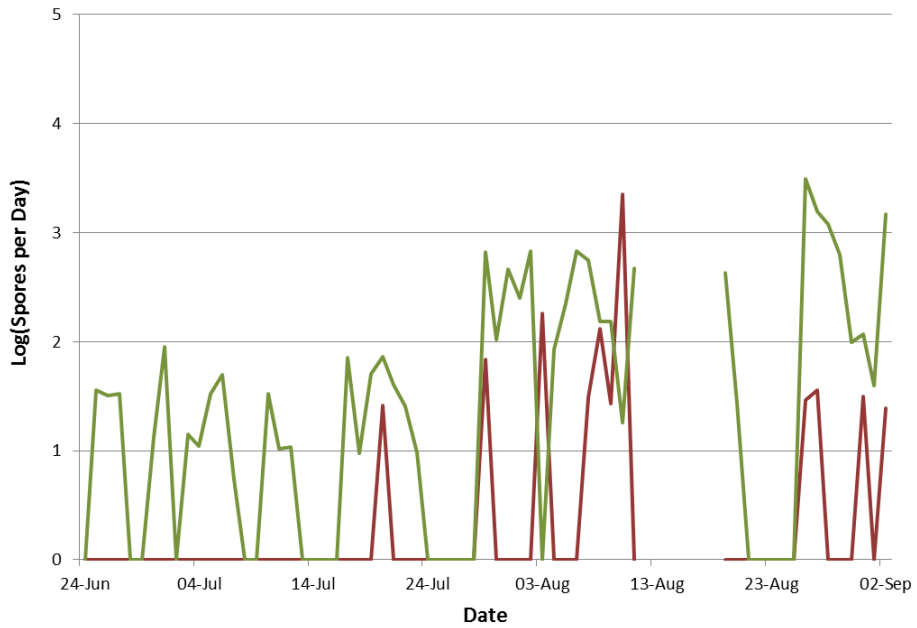
Figure 4.5: Data for Field 21, near Rosemary, AB. Refer to caption below Figure 1.3 for details.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

This thesis investigated the biological and environmental factors contributing to the spread of blossom blight pathogens in seed alfalfa fields of southern Alberta. While spores of *B. cinerea* and *S. sclerotiorum* are generally considered to be dispersed primarily by wind, pollinato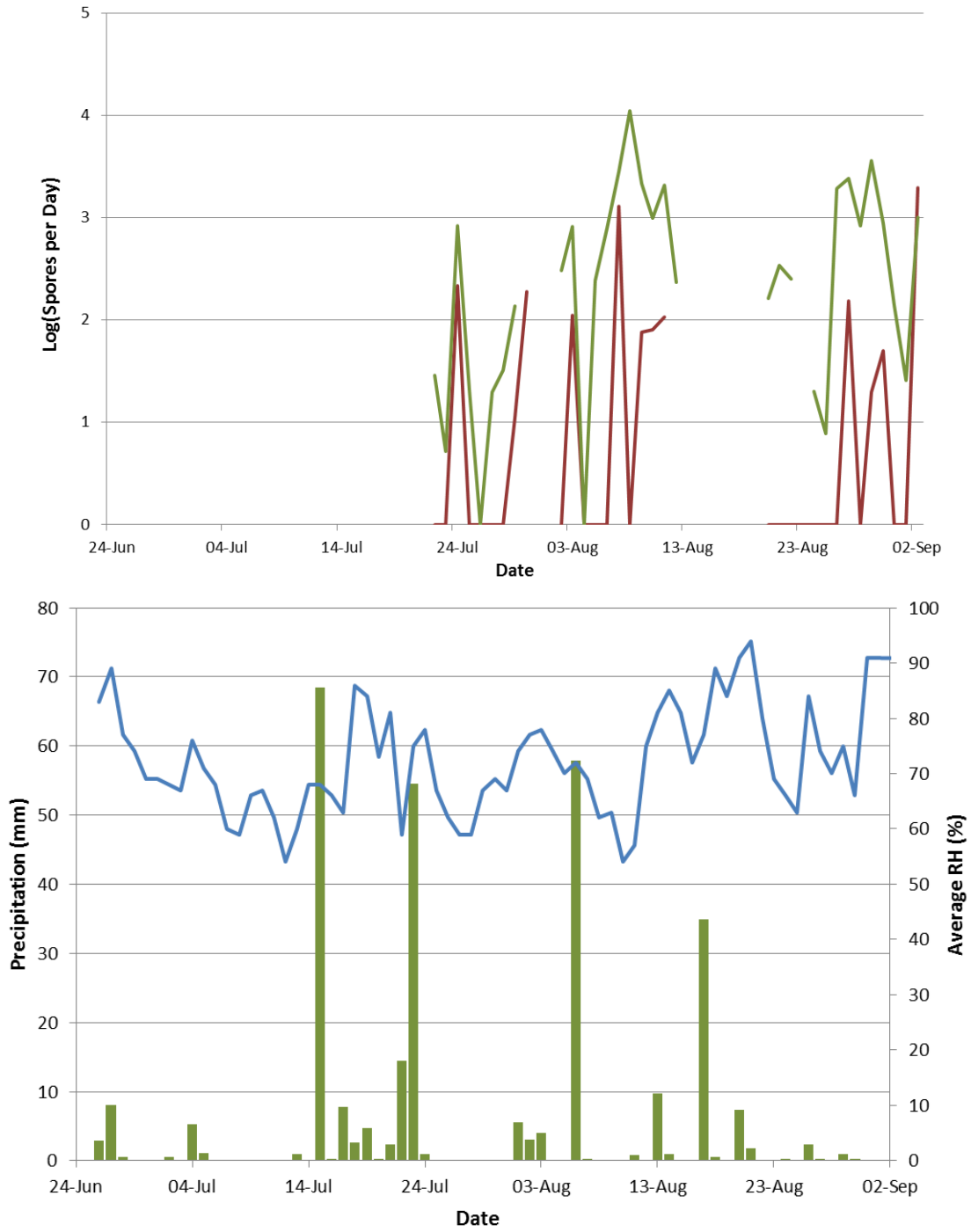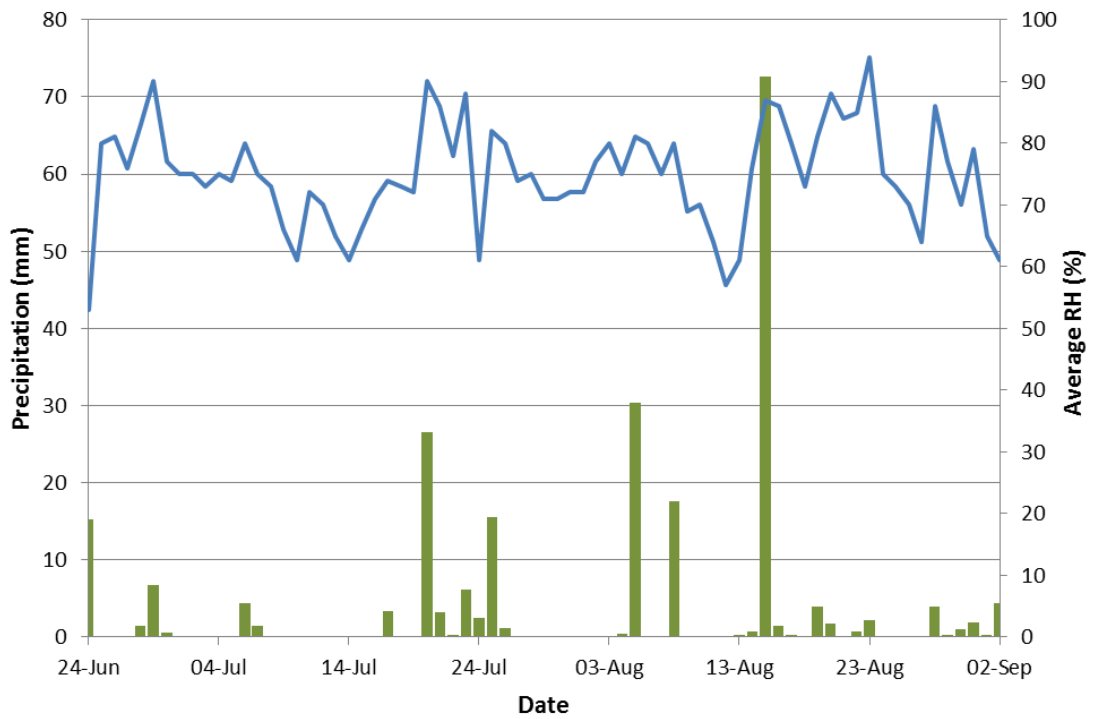rs and other insects have also been implicated in the transmission of these pathogens (Stelfox et al. 1978; Huang et al. 2000; James et al. 1995; Louis et al. 1996). In the case of alfalfa grown for seed, leafcutter bees have been hypothesized to act as vectors by transporting either fungal spores, or infected pollen (Huang et al. 2000; Huang et al. 1999, 1997).

Results from the greenhouse study presented in this thesis suggest that infection of alfalfa pollen grains by *B. cinerea* was unlikely to occur under field conditions. Pollen infection was observed at low levels (<1%) only under suspension-inoculated treatments. The suspension contained 10^5 conidia mL^{-1}, and, consequently, florets were inoculated with a much greater number of conidia than they are likely to encounter under field conditions (Jarvis 1962a; Xu et al. 2000). Indeed, results from spore quantification of aerosols collected from seed alfalfa fields showed that the greatest conidia discharge was estimated at 2.1 x 10^4 conidia in a single day, averaging 8.8 x 10^2 conidia m^{-3} h^{-1} – a notably smaller amount of inoculum reaching its host. As a result, pollen infection in the field may only be likely on days that have both high airborne conidia concentrations and precipitation/irrigation.

A large proportion of leafcutter bees collected at the end of the 2013 and 2014 growing seasons were positive for *B. cinerea* when plated on BSM. These results are

consistent with field surveys of blossom blight during the same years (Chatterton &
Reich 2014; Reich & Chatterton 2015) and with the concentrations of airborne conidia
measured in 2014 (this thesis). Because leafcutter bees typically like to stay warm and
dry (Frank 2003), and *B. cinerea* germinates only under high (>93%) RH or extended wet
periods (Bulger et al. 1987; Broome et al. 1994; Jarvis 1962b), it may be uncommon for
B. cinerea to infect pollen attached to a bee. Rather, the data suggest that airborne conidia
more commonly attach directly to the bodies of leafcutter bees, as has been observed for
some fungi in previous ultrastructural studies of leafcutter bees (Huang et al. 1986). That
few conidia were isolated when pollen was removed from leafcutter bees indicates that
conidia are not readily removed when a bee pollinates a flower. By the time conidia
concentrations were high in mid-August – with nearly 100% of leafcutter bees testing
positive for *B. cinerea* and multiple large discharges of conidia detected – the alfalfa is
well into the pod development stage. Florets pollinated at this time do not mature in time
for seed development and harvest, and so, even if infected, would likely have negligible
effects on disease intensity or yield (Frank 2003).

A greenhouse study on the effect of *S. sclerotiorum* and *B. cinerea* on alfalfa seed
production showed that a small inoculation of spores on the alfalfa style resulted in
significantly fewer pods set and fewer seeds per pod than a non-inoculated control
(Huang et al. 2000). No seeds were infected, but >90% of styles were infected, and
infections became severe if the pods were subjected to high humidity conditions 2 or 5
weeks after inoculation. These results indicate that exposure to relatively low levels of
inoculum at a susceptible stage can significantly reduce seed production, and that
quiescent infections can result in severe disease intensity if under conducive conditions

later in the season. Both findings support the usefulness of monitoring the airborne inoculum loads of these pathogens to detect when risk of infection is high.

The reliability of quantifying fungal spores in aerosol samples is directly related to the specificity and sensitivity of the molecular tools employed. Many primers previously developed for *S. sclerotiorum* were reported to be not species-specific, either producing non-specific amplification, or not amplifying the species of interest (Parker et al. 2014b; Rogers et al. 2009; Freeman et al. 2002). Other primers were not sensitive enough for quantifying DNA from a small number of ascospores (Rogers et al. 2009; Parker et al. 2014b). The primer and probe set developed in this thesis was specific to all *S. sclerotiorum* isolates collected in seed alfalfa fields of southern Alberta. It amplified all *S. sclerotiorum* isolates but no *B. cinerea* isolates or the closely related *S. trifoliorum*. The addition of the probe increased the specificity of the assay, and enabled multiplexing with primers for an internal control plasmid or *B. cinerea*. While not as specific as primers targeting hypothetical proteins, the primers targeting the IGS region were much more sensitive, which was an important criterion for detecting low levels of ascospores from environmental samples.

When multiplexed with a previously published primer and probe set for *B. cinerea* (Suarez et al. 2005), both primers exhibited similar efficiencies and sensitivities for their target DNA, both consistently amplifying 0.05 pg DNA – the equivalent of roughly 2.4 copies of genomic DNA, or 1.2 ascospores (Amselem et al. 2011). However, following DNA extraction of serially diluted conidia and ascospores, amplification of *S. sclerotiorum* ascospores was consistently 4-5 cycles later than amplification of the same

number of *B. cinerea* conidia. These results suggest that ascospores of *S. sclerotiorum* may be more resistant to DNA extraction than conidia of *B. cinerea*.

The standard curves developed for spore quantification were based on DNA extractions of serially diluted spores. This method has been shown to more accurately reflect the true value of organisms in the air (An et al. 2006). According to this study (An et al. 2006), atomizing the spore suspensions and collecting them under a vacuum prior to enumeration and DNA extraction would have increased the accuracy of the quantification method presented in this thesis. As reported, our results likely underestimate the true concentration of blossom blight pathogen inoculum in the environment.

Application of the *S. sclerotiorum* and *B. cinerea* qPCR assay to aerosol samples collected in seed alfalfa fields in the 2014 growing season revealed considerable variation in spore concentrations between days. In the three fields surveyed, the first ascospore and conidia discharges were detected by the middle of July. For *B. cinerea*, conidia were detected at increasing concentrations until the end of sampling at the beginning of September. This finding is consistent with the polycyclic life cycle of *B. cinerea*, which predicts that inoculum dispersal will increase exponentially over time as the pathogen undergoes multiple life cycles. Conversely, high concentrations of ascospores of *S. sclerotiorum* were detected with less frequency (1-2 times) in two of the fields surveyed, but with a much greater frequency (~8 times) in the third field surveyed. For a monocyclic pathogen such as *S. sclerotiorum*, one would expect few spore release days, as observed in the first two fields. The more frequent spore releases observed in the third field could be indicative of physiological differences between *S. sclerotiorum* isolates,

microclimatic factors below the canopy, or the presence of airborne ascospores transported from nearby crops.

This thesis shows that aerosol sampling combined with qPCR for detection of blossom blight pathogens could be used to develop a disease-forecasting system for seed alfalfa. Many studies have documented similar conclusions for crops including canola (Rogers et al. 2009), spinach (Klosterman et al. 2014), onion (Carisse et al. 2009), and carrot (Parker et al. 2014b), however, relatively few studies have determined, either experimentally or empirically, threshold inoculum concentration levels, above which pathogens will cause economic losses. It is critical to establish this relationship for a disease forecasting model to be reliable and useful to growers. One of the constraints with the current research project – and several other published studies – is that the commercial seed alfalfa fields that were surveyed were treated with fungicides according to the growers' schedules. The fungicides may explain, in part, why only trace signs of blossom blight were observed in field surveys. Thus, future research should focus on establishing the relationship between spore concentration and disease progress over the growing season on untreated seed alfalfa crops.

In addition, while other studies have implicated insects as important vectors of fungal disease, few have quantified the relative contribution of insects to pathogen dispersal under field conditions. The data presented in this thesis suggest that, although leafcutter bees vector spores of *B. cinerea* and *S. sclerotiorum*, they contribute little to the overall disease pressure within a field – contrary to previous hypotheses (Huang et al. 2000). Thus, disease management measures should be focused on improving host resistance, reducing initial inoculum sources, slowing the spread and development of the

pathogens, and disease forecasting. Possibly, leafcutter bees could be employed as vectors of biocontrol agents against blossom blight pathogens.

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