Development and Validation of a *Sclerotinia sclerotiorum*-Specific Quantitative PCR Assay to Assess Risk of Sclerotinia Stem Rot of Canola (*Brassica napus*)

By

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Abstract

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is a major disease of canola (*Brassica napus*) commonly managed by the routine application of fungicides. Petal infestation is an important stage of the disease cycle and has been the focus of previously developed Sclerotinia stem rot risk assessment methods. Quantitative PCR (qPCR) analysis can provide a more accurate and rapid assessment of petal infestation level. A hydrolysis probe-based *S. sclerotiorum*-specific qPCR assay was developed that could detect as little as 8.0×10^{-4} ng of *S. sclerotiorum* DNA with a high degree of specificity. Petal infestation level, as determined with this assay at full bloom (40-50% flower), accounted for 60-92% of the variation in Sclerotinia stem rot incidence under field conditions in western Canada. The strength of the relationship, however, varied from year-to-year and over the flowering period. Petal infestation level was influenced by petal age and by the time of day when samples were collected. A diurnal fluctuation in infestation levels was found in young, but not old petals, with significantly higher infestation in afternoon- versus morning-collected samples. Sclerotinia stem rot development also was influenced by environmental conditions, with relative humidity playing a more significant role in disease development than temperature. Inclusion of relative humidity conditions in a forecasting system with petal infestation levels may improve the reliability and accuracy of Sclerotinia stem rot risk assessments. In addition to its potential utility as a risk assessment tool, the *S. sclerotiorum*-specific qPCR assay may also prove useful in studies of the epidemiology of Sclerotinia stem rot.

Preface

This thesis is an original work conducted by Barbara Rosemary Ziesman. Ms. Ziesman conducted all experiments and wrote the first draft of all chapters. The chapters were then examined by Ms. Ziesman's supervisors, Dr. Stephen Strelkov and Dr. Thomas Kelly Turkington. Drs. Strelkov and Turkington provided editorial revisions and suggestions for each chapter, which were then incorporated by Ms. Ziesman.

Chapter 2 of the thesis has been published as: Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2016. A quantitative PCR system for measuring *Sclerotinia sclerotiorum* in canola (*Brassica napus*). Plant Dis. 100:984-990. Ms. Ziesman was responsible for the method development, data collection, analysis, and manuscript composition, while Drs. Strelkov and Turkington developed the initial research concept and assisted with manuscript editing and interpretation of results. Dr. Urmila Basu assisted with method development and contributed to manuscript editing.

Agronomists with Crop Production Services (CPS) (formerly Viterra), Fort Saskatchewan, Alberta, assisted in the selection of fields near Edmonton, Alberta. Fungicide-free check-strips were left and marked by co-operating farmers for all locations near Edmonton. Ms. Ziesman, with the assistance of several University of Alberta summer students, lab personnel, and CPS staff, collected all petal samples from fields located near Edmonton. Dr. Turkington and the staff at the Lacombe Agriculture and Agri-Food Canada research station collected all petal samples from fields near Lacombe, Alberta, and shipped them to Ms. Ziesman. Petal sample collection and disease assessments for fields located in Saskatchewan were conducted by Dr. Bruce Gossen, Ms. Faye Dokken-Bouchard, Dr. Randy Kutcher and Mr. Ken Bassendowski. Petal sample collection and disease assessments for fields located in Manitoba

were conducted by Dr. Khalid Rashid, Mrs. Holly Derksen, and Dr. Debra McClaren. All sample processing and qPCR analysis was conducted by Ms. Ziesman with the support of several summer students and Mrs. Homa Askarian.

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Chapter 1. Introduction and literature review

1.1 General introduction

Canola or oilseed rape (*Brassica napus* L.) is the second most widely grown crop in Canada after wheat, with approximately 8.27 million seeded hectares and an annual average of 15.98 million tonnes of production from 2011 to 2015 (Statistics Canada 2015). Sclerotinia stem rot of canola, caused by the necrotrophic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Willets and Wong 1980), is one of the most economically important diseases of canola. When inoculum levels are high and environmental conditions are conducive to disease development, Sclerotinia stem rot can potentially reduce yields by up to 0.5% for every 1% increase in disease incidence (DI) (del Río et al. 2007). The incidence and severity of Sclerotinia stem rot are sporadic in nature, however, and vary from year-to-year, region-to-region and field-to-field (Morrall and Dueck 1982; Turkington et al. 1988; Turkington et al. 1991a). This variability is in large part due to the influence of environmental conditions on ascospore release and successful host infection by the fungus. Sclerotinia stem rot management options are limited. The movement of ascospores on wind currents over fairly long distances makes crop rotation ineffective as a disease control measure (Morrall and Dueck 1982; Williams and Stelfox 1979; Williams and Stelfox 1980). Tillage to bury the sclerotia (Cook et al. 1975) or decreased seeding rates to reduce the level of lodging (Jurke and Fernando 2008) are other potential management options, but have not had consistent success in the field. In western Canada, Sclerotinia stem rot of canola is managed mainly by the routine application of fungicides during crop flowering.

Fungicide application is typically conducted without any objective indication of disease risk (del Río et al. 2007; Koch et al. 2007; Turkington et al. 2011). As a result, there have been efforts to develop effective Sclerotinia stem rot forecasting systems that can provide growers

with reliable information on which to base their spray decisions. These have included forecasting systems focused on weather-related parameters (Clarkson et al. 2014; Harikrishnan and del Río 2008; McLaren et al. 2004; North Dakota State University 2009; Sharma et al. 2015), field history (Twensgström et al 1998), measures of inoculum pressure (Twensgström et al 1998), or petal infestation levels (Almquist and Wallenhammar 2014; Morrall and Thompson 1991). More recently, there has been an interest in the development of *S. sclerotiorum*-specific polymerase chain reaction (PCR)-based assays to provide estimates of inoculum pressure (Almquist and Wallenhammar, 2015; Parker et al. 2014; Rogers et al. 2009; Yin et al. 2009).

This review will focus on the biology of *S. sclerotiorum* with respect to Sclerotinia stem rot of canola. The life cycle and specific driving factors of Sclerotinia stem rot will be discussed in detail. The main focus will be on the development of forecasting systems that can provide insights and improve Sclerotinia stem rot management.

1.2 Hypotheses and objectives

The specific research objectives of my Ph.D. program were as follows:

1. To develop a hydrolysis probe-based qPCR assay for the sensitive, specific and reliable quantification of *S. sclerotiorum* DNA on canola petals.
2. To examine the potential of using this qPCR assay as the basis of a Sclerotinia stem rot risk assessment tool, by determining the relationship between petal infestation level as determined with qPCR and final Sclerotinia stem rot incidence in the field.
3. To conduct intensive validation in commercial canola fields in western Canada, with the purpose of identifying the impact of environmental conditions (temperature, humidity and precipitation), crop canopy characteristics (density and lodging), timing of petal

sampling (morning vs. afternoon), and petal age on the petal infestation estimates and their relationship to the final incidence of Sclerotinia stem rot in the field.

I hypothesize that:

1. The proposed *S. sclerotiorum*-specific, hydrolysis probe-based qPCR assay will provide specific and reliable quantification of *S. sclerotiorum* DNA levels on field-collected canola petals.
2. Petal infestation levels determined through qPCR analysis will have a strong statistical relationship with Sclerotinia stem rot incidence. The ability to predict disease risk will likely vary between sampling years due to differences in environmental conditions.
3. Petal infestation levels will change over the flowering period and will influence the ability to predict disease incidence risk.
4. Environmental conditions and crop canopy characteristics will influence the development of Sclerotinia stem rot.
5. The levels of petal infestation detected through qPCR analysis will be influenced by the time of day that samples are collected and petal age.
6. The relationship between petal infestation levels and disease incidence, as well as the ability to predict Sclerotinia stem rot risk, will be mostly dependent on the level of petal infestation and environmental conditions.

1.3 History and distribution of *S. sclerotiorum*

Sclerotinia sclerotiorum is a fungus in the Phylum Ascomycota and Family Sclerotiniaceae (Willets and Wong 1980). The pathogen was first described as *Peziza sclerotiorum* in 1837 (Bolton et al. 2006; Purdy 1979), but the name was later changed to

Sclerotinia libertiana when the genus *Sclerotinia* was described (Reviewed in Purdy 1979). The name *S. libertania* was disputed based on the rules of the International Code of Botanical Nomenclature and the proper name of the fungus was subsequently determined to be *S. sclerotiorum* (Bolton et al. 2006). It is a non-specific, necrotrophic pathogen that is known to cause disease in more than 400 plant species from 75 different families (Boland and Hall 1994). The majority of the susceptible hosts belong to the subclass Dycotyledonae, with only 25 potential hosts belonging to the subclass Monocotyledonae (Boland and Hall 1994). In addition to having a broad host range, the fungus is distributed globally. *Sclerotinia sclerotiorum* has been reported from every continent of the world, with the occurrence of the pathogen and associated diseases most common in temperate regions (Adams and Ayers 1979; Purdy 1979; Willets and Wong 1980).

Sclerotinia sclerotiorum is homothallic and reproduces sexually through carpogenic germination, producing apothecia. Asexual reproduction occurs through the myceliogenic germination of sclerotia (Aldrich-Wolfe et al. 2015; Willets and Wong 1980). Outcrossing and recombination result in genetic and phenotypic variation within *S. sclerotiorum* populations (Attanayake et al. 2013; Hemmati et al. 2009). Mycelial incompatibility, identified as a reaction line between paired mycelia in culture, and molecular marker assays indicate a clonal population structure with a high level of intraspecific heterogeneity (Aldrich-Wolfe et al. 2015; Anderson and Kohn 1995; Kohli et al. 1992; Kohli et al. 1995; Kohn et al. 1990; Kohn et al. 1991). Genetic diversity is known to occur between geographic regions, as well as within agricultural fields (Aldrich-Wolfe et al 2015; Anderson and Kohn 1995; Attanayake et al. 2013; Carpenter et al. 1990; Karimi et al. 2011; Kohn et al. 1990 Kohn et al. 1991). Geographically isolated *S. sclerotiorum* populations from China and the United States were genetically differentiated and

found to share no mycelial compatibility groups, and also were differentiated phenotypically based on mycelial pigmentation, growth rate, sclerotia production, oxalic acid levels and sensitivity to some fungicides (benomyl and iprodione) (Attanayake et al 2013). However, these populations could not be distinguished based on their virulence on canola (Attananayake et al. 2013).

Field populations of *S. sclerotiorum* collected from canola fields in Canada were found to contain several clones in a single agricultural field (Kohli et al. 1992; Kohn et al. 1991). There is no evidence of host preference in different isolates, since there was no greater genetic similarity between isolates infecting similar hosts than between isolates from different hosts (Carpenter et al. 1999). In fact, in a study from the United States, a greater amount of genetic diversity was found among populations of the pathogen from the same host crop than from between crops (Aldrich-Wolfe et al. 2015). These results support the non-specific nature and broad host range of *S. sclerotiorum*. An understanding of the level of genetic diversity within fields and between regions is important for the development of management strategies that are effective against all isolates within a pathogen population.

The distribution of *S. sclerotiorum* clones over distances of up to 2000 km has been documented in Canada (Kholi et al. 1992). This is in contrast with findings from New Zealand, where the dispersal of clones in canola fields between different regions was restricted (Carpenter et al. 1999). It was suggested that the scale and longer history of agriculture and canola production in Canada may contribute to the longer dispersal distances between clones (Carpenter et al. 1999). Ascospores carried on the wind can be a means of moving clones between fields, while human activities such as the use of seedlots contaminated with sclerotia could result in dispersal of clones over longer distances (Adams and Ayers 1979; Kohli et al 1992).

1.4 Disease cycle, symptomology and epidemiology

Sclerotinia sclerotiorum spends up to 90% of its lifecycle as sclerotia, which act as the overwintering stage of the fungus (Adam and Ayers 1979). Infection of canola is dependent on the carpogenic germination of sclerotia to produce ascospore-bearing apothecia. When the apothecia are mature, the ascospores will be forcibly released, picked up on wind currents and carried above the crop canopy to initiate infection of susceptible hosts (Abawi and Grogan 1979; Bardin and Huang 2000; Purdy 1979; Willets and Wong 1980). When infection is initiated by ascospores, the fungus must first go through a saprophytic stage in which it infects weak, nonliving or senescent tissue, prior to parasitizing the living host (Hegedus and Rimmer 2005; Jamaux et al. 1995). In Sweden, infestation levels on the lower leaves have been shown to have a stronger relationship to disease incidence than petal infestation levels (Almquist and Wallenhammar 2015). However, research in Canada suggests that assessing the level of petal infestation provides a better indication of disease risk than assessing the infestation levels of leaves and leaf axils (Gugel 1986). As a result, in Canada, petal infection is believed to play a key role in the initiation of the disease cycle and is critical for Sclerotinia stem rot development in canola (Gugel 1986; Jamaux et al. 1995; Turkington 1991a).

As infected canola petals senesce, they fall into the crop canopy and land on leaves or stem branches. They must adhere to these surfaces for 2-3 days for infection of the host plant to occur (Tu 1989). Under conditions of free moisture, the fungal mycelia grow out of infected cast petals and directly infect the living host (Jamaux et al. 1995). Under cool moist conditions, the fungus will invade the stem tissue by producing abundant white mycelium (Purdy 1979; Willets and Wong 1980). The initial symptoms of Sclerotinia stem rot will appear as water-soaked lesions on leaf and stem tissue in contact with the infected cast petals (Abawi and Grogan 1979;

Boland and Hall 1994; Jamaux et al. 1995). The lesions enlarge, with the infected host tissue becoming bleached and often shredded in appearance (Bolton et al. 2006). After several days of mycelial growth, fungal sclerotia will form within the canola stem. Under high humidity, sclerotia may also develop on the outer surface of infected stems (Bolton et al 2006; Purdy et al. 1979; Willets and Wong 1980). The sclerotia are dislodged from the host during harvest, overwintering on or below the soil surface.

Crop phenology and the interaction of crop development with weather events will have an influence on the final incidence of Sclerotinia stem rot in the field. Morrall and Dueck (1982) found that carpogenic germination and apothecium development do not occur until the plant canopy completely shades the soil surface. Canopy closure in crops other than canola can also promote conditions that favour germination of sclerotia. As a result, these researchers suggested that crop seeding date will influence the incidence of Sclerotinia stem rot, through the interaction of the timing of canopy closure and the occurrence of weather events and environmental conditions conducive for carpogenic germination of sclerotia (Morrall and Dueck 1982). Plant density and the degree of lodging within the crop canopy also may influence disease development and account for field-to-field differences in Sclerotinia stem rot incidence. A dense crop canopy can reduce soil surface evaporation and help to maintain cooler soil temperatures, creating conditions that are conducive for carpogenic germination (Abawi and Grogan 1979; Morrall and Dueck 1982). Schwartz and Steadman (1978) reported that the production of apothecia was greater under dense crop canopies than under more open canopies. Lodging within a crop canopy can contribute to an increased incidence of Sclerotinia stem rot, through secondary spread of the disease late in the season via plant-to-plant contact and by the creation of a more conducive microclimate (Huang and Hoes 1980; Jurke and Fernando 2008; Morrall and

Dueck 1982; Tu 1989). The degree of crop lodging and secondary spread of Sclerotinia stem rot within the crop canopy can be influenced by the seeding rate (Jurke and Fernando 2008), row width and plant morphology (Tu 1989).

1.5 Biology of *S. sclerotiorum*

1.5.1 Germination and survival of sclerotia

Sclerotia of *S. sclerotiorum* are developed from aggregates of mycelium that start white and turn black as they mature (Willets and Wong 1980). The black color at maturity is due to the presence of melanin, which is thought to play a role in protection against adverse conditions in many fungi (Bell and Wheeler 1986; Bolton et al. 2006; Henson et al. 1999). When a sclerotium is dissected, three distinct regions will be evident: a highly melanized outer rind, a 2-4 cell layer of thin-walled hyaline pseudoparenchymatous cells, and an inner medulla (Willets and Wong 1971). The sclerotia of *S. sclerotiorum* can survive in the soil for approximately 4-5 years (Adams and Ayers 1979), depending on environmental conditions and the soil microbial community (Abawi and Grogan 1979; Adams and Ayers 1979; Niem et al 2013; Zeng et al. 2012).

While soil pH and moderate soil temperatures have not been found to influence the longevity of sclerotium survival, extreme soil temperatures (>35˚C for 3 weeks or more) will have an adverse effect (Adams and Ayers 1979). Flooding of the soil for extended periods of time also has been found to negatively impact the survival of sclerotia and can result in a 100% loss of viability (Adams and Ayers 1979; Niem et al. 2013). More than 30 species of fungi and bacteria are reported to be antagonistic to or are mycoparasites of *Sclerotinia* spp. (Adams and Ayers 1979). The impact of biological activity on the viability of sclerotia was highlighted by Zeng et al. (2012), who found that *Coniothyrium minitans*, *Bacillus subtilis*, *Trichoderma*

harzianum and *Streptomyces lydicus* were able to reduce the survival of *S. sclerotiorum* sclerotia by 29.6-50.0%. The knowledge of which organisms have the most adverse effect on sclerotia is important and may be used to identify candidates for the biological control of *S. sclerotiorum*.

In the spring, the sclerotia can germinate either myceliogenically to produce mycelia or carpogenically to produce sexual apothecia (Bardin and Huang 2001; Purdy 1979; Willets and Wong 1980). The type of germination is determined by environmental conditions. Since carpogenic germination of *S. sclerotiorum* is most important for the initiation of disease in canola, it will be the focus of this discussion. The apothecia are comprised of a stalk (stipe) and a head (disc) (Willets and Wong 1980). The ascospores are produced within asci in the hymenium layer on the upper surface of the head of the apothecium (Willets and Wong 1980).

Soil moisture is very important for carpogenic germination, with even slight moisture tension preventing the formation of apothecia (Abawi and Grogan 1979). At least 10 days of continuous soil moisture are needed for carpogenic germination under field conditions (Abawi and Grogan 1979; Wu and Subbarao 2008), conditions which have been found to occur after canopy closure (Boland and Hall 1987). In contrast, saturated or flooded soils are not optimal for the fungus, due to the lack of oxygen which can reduce the viability of sclerotia and inhibit germination (Adams and Ayers 1979; Morrall 1977; Niem et al. 2013). Carpogenic germination can occur at soil matric potentials of 0 and -0.75 MPa (Boland and Hall 1987; Morrall 1977; Teo and Morrall 1985), although levels between -0.01 and -0.1 MPa are the most optimal (Clarkson et al 2004; Hao et al. 2003; Teo and Morrall 1985; Wu and Subbarao 2008). The need for high soil moisture for carpogenic germination is supported by the findings of Wu and Subbarao (2008), who found that carpogenic germination rates increased as the soil water potential increased from -0.3 to -0.01 MPa. In addition, prolonged dry conditions (>10 days) have been

found to arrest carpogenic germination, but apothecial formation can resume following a period of rewetting (Wu and Subbarao 2008). It has been suggested that carpogenic germination may be possible when sclerotia are exposed to a series of discontinuous, short periods of high soil moisture conditions (Boland and Hall 1987).

Temperature is also important for carpogenic germination of sclerotia. Carpogenic germination has been reported to occur between 5-25 ºC, with reductions in germination observed at temperatures >25 ºC (Clarkson et al. 2004; Hao et al 2003). Other reports suggest that a 5 day period of 30 ºC does not have a significant effect on carpogenic germination (Wu and Subbarao 2008). The temperature during sclerotium formation and the geographic origin of pathogen isolates are known to influence the temperature requirements for carpogenic germination (Huang et al. 1991). Sclerotia from cool climatic regions, such as the Canadian Prairies, germinate more readily at cool temperatures (10ºC) than those produced at warmer temperatures (25-30ºC) (Bardin and Huang 2001). The most commonly reported optimum temperature range for carpogenic germination is between 10 and 20ºC, with the highest germination rate at 15ºC (Abawi and Grogan 1979; Clarkson et al. 2004; Hao et al. 2003; Willets and Wong 1980; Wu and Subbarao 2008). Stipes of the apothecia can form between 5-25ºC, but temperatures > 20ºC or <10ºC can result in abnormal apothecial formation or no formation of apothecia (Willets and Wong 1980). Apothecia and stipe production is stimulated by exposure to light within the UV light spectrum (Thaning and Nilsson 2000). Wavelengths between 276 and 319 nm are required for normal apothecium formation in *S. sclerotiorum*, which are consistent with the requirements for apothecium formation in *S. trifoliorum* (Honda and Vunoki 1977; Thaning and Nilsson 2000).

1.5.2 Ascospore release

Ascospores are borne within asci located in the hymenium layer on the upper surface of the mature, saucer-like apothecium head (Willets and Wong 1980). Release of the ascospores from the apothecia lasts an average of 9 days (Schwartz and Steadman 1978), although it may continue for as long as 20 days (Clarkson et al. 2003). Schwartz and Steadman (1978) reported that as many as 3×10^7 ascospores can be produced per apothecium, but that the average was 2×10^6 ascospores. Clarkson et al. (2003) found a maximum production of 7.6×10^5 ascospores per apothecium over the 20 day fruiting body life span. Ascospores are forcibly released from physiologically mature apothecia in response to slight moisture tension, and are ejected to heights that will introduce the spores to more turbulent above-ground air layers (Abawi and Grogan 1979). After release, the ascospores are carried above crop canopies on air currents, and have been detected at 30.5 to 147.5 cm above the soil surface in barley and canola fields (Williams and Stelfox 1979). The distances travelled by the airborne ascospores can vary, with estimates ranging from >25 m (Suzui and Koayashi 1972) to 100 m (Williams and Stelfox 1979) and up to several kilometers (Brown and Butler 1936). There is evidence that ascospores can also be distributed by pollinating bees, which may contribute to their dispersal over even longer distances (Stelfox et al. 1978).

It has been suggested that light is required for ascospore release (Harthill 1980), but this was disputed by Clarkson et al. (2003), who found that ascospores could be released in darkness under controlled conditions. Indeed, ascospore release during the night has also been reported under field conditions in a dry year, when peak ascospore release was found to occur at 4 am (Qandah et al. 2011). During wet years, however, the peak level of ascospore release occurred around 11 am, suggesting that night release of spores may be in response to adverse

environmental conditions (Qandah et al. 2011). This pattern of diurnal fluctuation, with the highest level of ascospore release occurring mid-day, was consistent with reported patterns of atmospheric ascospore levels and fluctuations in canola petal infestation (Ben-Yephet and Bitton 1985; Harthill 1980; Turkington et al. 1991b). Infestation levels, determined based on visual identification of *S. sclerotiorum* colonies recovered from canola petals on agar, tended to be higher for petals collected in the afternoon versus those collected in the morning (Turkington et al. 1991b). This is consistent with, and likely the result of, the higher numbers of ascospores in the air in the day versus the night (Ben-Yephet and Bitton 1985; Harthill 1980; McCartney and Lacey 1991).

Ascospore release also is influenced by environmental conditions. While it can occur over a wide range of temperatures (4-32ºC), the optimum temperature for ascospore release is around 22ºC (Clarkson et al 2003; Newton and Sequira 1972). However, RH seems to have a greater effect in triggering ascospore release than either temperature or precipitation; few ascospores are detected during periods of low RH (Qandah et al. 2011). Ascospore release can occur at 65-75% RH, but under these conditions there is a decline in apothecium longevity and ascospore release relative to conditions of high humidity (90-95% RH) (Clarkson et al. 2003). Precipitation events may influence ascospore release directly by preventing discharge in the rain or washing ascospores out of the air. Precipitation may also have an indirect effect, by influencing the duration of high RH (Qandah et al. 2011; Turkington et al. 1991b; Turkington et al. 2011).

1.5.3 The infection process

As noted earlier, *S. sclerotiorum* is a necrotrophic fungus that requires weak or senescent tissue as a nutrition source prior to the infection of the living host leaf or stem tissues (Abawi and

Grogan 1975; Cook et al. 1975; Hegedus and Rimmer, 2005). In canola, the infection of both living young and senescing petals by *S. sclerotiorum* has been identified as the first stage of infection by *S. sclerotiorum* (Jamaux et al 1995). Canola flower petals remain attached to the inflorescence for up to 6 days and are most susceptible to infection by ascospores for at least a 2 day period when the flowers are fully expanded and not yet wilted (Penaud 1984 cited in Turkington et al. 1991b). A mucilaginous material has been reported to be discharged with the ascospores, which is thought to aid in spore adhesion to the host surface (Abawi and Grogan 1979). However, no adhesive material was identified on canola petals inoculated with *S. sclerotiorum* ascospores (Jamaux et al. 1995). The penetration of canola petals can occur directly, without the formation of distinguishable appressoria (Jamaux et al. 1995). Ascospores were not observed to germinate and penetrate canola leaves directly, illustrating the importance of petal infection in the development of Sclerotinia stem rot. Recent work by Almquist and Wallenhammar (2015) in Sweden suggests that infection of the lower leaves may be related to final disease incidence, although this has not been investigated under Canadian canola cropping systems.

When infected petals are cast they will fall into the crop canopy and are often caught on canola leaves or stem branches. Fungal mycelium will emerge from the colonized petal tissue and grow towards the leaf tissue (Jamaux et al 1995). The mycelia penetrate the host tissue directly, through the cuticle, via the formation of complex appressoria known as infection cushions (Hegedus and Rimmer 2005; Jamaux et al 1995; Lumsden 1979). Reports of infection through the stomata, without the formation of appressoria or other adhesive mycelial structures, was documented to occur in potato, but has not been observed in canola (Jamaux et al. 1995; Jones 1976). The collapse of host epidermal cells occurs prior to continued fungal growth,

which is both inter- and intracellular (Jamaux et al 1995). The collapse of host cells is likely caused by the production of extracellular lytic enzymes and oxalic acid by *S. sclerotiorum* (Godoy et al. 1990; Hegedus and Rimmer 2005; Jamaux et al 1995; Lumsden 1976; Marciano et al. 1983).

The infection of host tissue by *S. sclerotiorum* is influenced by the temperature, RH and free moisture within the crop canopy (Abawi and Grogan 1975; Clarkson et al. 2014; Koch et al. 2007; Tu 1989; Willets and Wong 1980; Young et al. 2004). Optimal fungal growth and sclerotial development in infected host tissue were found to occur at 15-22ºC (Koch et al. 2007; Willets and Wong 1980). As with requirements for carpogenic germination, the optimal temperature ranges for infection and lesion enlargement may vary slightly between isolates (Willets and Wong 1980). The performance of the fungus at extreme temperatures was reduced, with fungal growth and lesion development lower at temperatures >30º and <7ºC (Abawi and Grogan 1975; Koch et al. 2007; Willets and Wong 1980). On lettuce (*Lactuca sativa*), the rate of symptom development was found to be positively influenced by temperature, with the first symptoms of disease occurring 20-26 days after inoculation at 8-11ºC and 7-9 days after inoculation at 15-25ºC (Young et al. 2004). Periods of free moisture and prolonged leaf wetness are required for infection (Abawi and Grogan 1979; Harikrishnan and del Río 2008; Tu 1989). A free water period within the crop canopy of 42 -78 h was reported as necessary for successful infection and lesion expansion by *S. sclerotiorum* on canola (Tu 1989). In bean (*Phaseolus vulgaris*), 16-24 h of leaf wetness over a 72 h period was required for infection via infested bean blossoms (Abawi and Grogan 1979). If the infected tissue or the tissue surface becomes dry, lesion enlargement will be arrested, but can be reactivated again by conditions of free moisture (Abawi and Grogan 1979; Bardin and Huang 2001; Tu 1989). High moisture conditions also

may influence the adherence of infected petals to the leaf surface; with few petals adhering when leaves are dry (Young and Werner 2012).

Free moisture and leaf wetness are related to high levels of RH which also have been found to be required for successful infection, fungal growth and lesion enlargement (Clarkson et al. 2014; Koch et al. 2007). Although infection of dry bean flowers can occur at RH levels as low as 25% (Harikrishnan and del Río 2006), infection of lettuce occurs more readily under high RH conditions (>70%) (Clarkson et al. 2014). In canola/oilseed rape, a minimum threshold of 80% RH was determined to be required for successful infection by *S. sclerotiorum* (Heran et al. 1999; Koch et al 2007). Relative humidity also may influence the suitability of the environment for Sclerotinia stem rot development, by altering temperature conditions in the canopy (Koch et al. 2007).

1.6 Management of Sclerotinia stem rot in canola

1.6.1 Cultural control

Cultural disease management strategies can be used to avoid infection or reduce levels of Sclerotinia stem rot in canola (Bardin and Huang 2001). However, the wide host range, sporadic occurrence, and potential long distance distribution of ascospores (Brown and Butler 1936; Williams and Stelfox 1979) of *S. sclerotiorum* can reduce the consistency and effectiveness of cultural disease management.

Tillage of the soil has been suggested as a potential tool to manage Sclerotinia stem rot, by burying the sclerotia and preventing their successful germination. Williams and Stelfox (1980) found that tillage of sclerotia to depths of 1.6-15 cm reduced sclerotial germination in years when the tillage treatment was applied. Presumably, the deeper the sclerotia are buried, the

better, since Wu and Subbarao (2008) reported that sclerotia can produce apothecia when buried in the soil down to depths of 4 cm (although Abawi and Grogan (1979) reported that germinated sclerotia rarely produced stipes longer than 3cm). Tillage also may reduce the viability of sclerotia through increased parasitism of the buried sclerotia (Kurle et al. 2001). When considering the use of tillage as a disease management strategy, however, it is also important to consider the benefits that no-till operations have on overall soil health. Moreover, the benefits of tillage for the management of *S. sclerotiorum* have not been conclusively demonstrated. Gracia-Garza (2002) found that fields in no-till production systems, where the crop residue was left on the soil surface, tended to have fewer numbers of apothecia than fields under minimum tillage. This trend was attributed, at least partly, to the effect of soil microbial activity under no-till conditions (Gracia-Garza 2002). Differences in the viability of sclerotia and apothecial production with different tillage systems and different burial depths also have been reported (Gracia-Garza 2002; Kurle et al. 2001; Williams and Stelfox 1980; Wu and Subbarao 2008). Furthermore, the effect of tillage on sclerotial germination will only be evident during the year of the tillage event, since subsequent tillage events may redistribute the sclerotia on or near the soil surface.

Crop rotation, like tillage, is focussed on reducing the amount of inoculum present in the field. Twengström et al. (1998) found that if the previous incidence of Sclerotinia stem rot was high (>31%) in a field, the odds of a heavy infestation increased by a factor of 13. These findings suggest that in-field inoculum pressure (density of sclerotia) can influence disease pressure, indicating that crop rotation can be used as a means to manage the disease. However, crop rotation away from a susceptible host does not ensure a low population of viable sclerotia in the soil (Morrall and Dueck 1982), and a three year rotation away from a susceptible host was

found not to appreciably reduce sclerotia populations in the field (Schwartz and Steadman 1978; Williams and Stelfox 1980). The ineffectiveness of crop rotation as a Sclerotinia stem rot management strategy can be attributed to the dispersal of *S. sclerotiorum* ascospores on wind currents (Brown and Butler 1936; Suzui and Koayashi 1972; Williams and Stelfox 1979), which leads to the infection of host crops by externally produced inoculum (Morrall and Dueck 1982). Moreover, since *S. sclerotiorum* has a wide host range (Boland and Hall 1994), the presence of susceptible weeds and volunteers may help to sustain the levels of inoculum, even in the absence of a canola crop.

Cultural control strategies can be used to alter the crop canopy phenology and the microclimate of the crop canopy. Wider plant spacing between and within rows can reduce disease spread through plant to plant contact (Huang and Hoes 1980; Tu and Zeng 1997). Reductions in seeding rate will influence the microclimate under the crop canopy and the level of lodging, while late seeding will influence the interaction between the crop growth stage and environmental conditions favourable for carpogenic germination and apothecium formation by the fungus (Jurke and Fernando 2008; Morrall and Dueck 1982). The incidence of Sclerotinia stem rot is positively related to seeding rate, which is likely due to higher plant densities and more lodging as seeding rates increase (Jurke and Fernando 2008). This relationship was not, however, consistent across all cultivars and years (Jurke and Fernando 2008). The effectiveness of manipulating crop characteristics to manage Sclerotinia stem rot will likely be dependent on the lodging resistance of the cultivar, and the extent to which the environment is favourable for disease development in a particular year.

Late seeding of canola can be used to reduce Sclerotinia stem rot incidence via manipulation of the overlap between crop canopy closure and the occurrence of environmental

conditions favourable for carpogenic germination of sclerotia and the formation of apothecia (Morrall and Dueck 1982). The effectiveness of this approach will be highest when sclerotia populations are high and early spring conditions are conducive for carpogenic germination (Morrall and Dueck 1982), but will not be equally effective in all years. Other agronomic consequences of late seeding, such as a shorter growth period and the risk of late season frost, should also be kept in mind when considering this as a disease management approach.

1.6.2 Chemical and biological contro1

In Canada, the primary management tool for Sclerotinia stem rot is the routine application of fungicides during flowering of the crop. There are a variety of fungicides registered for the management of this disease in Canada (picoxystrobin, cyprodinil, fludioxonil, azoxystrobin, difenoconazole, boscalid, pyraclostrobin, iprodione, fluxapyroxad, prothioconazole, metconazole, and penthiopyrad), representing six different modes of action (Government of Saskatchewan 2016). All of these fungicides are preventative and are registered for one or two applications during flowering (from 20-50% bloom). While the application of fungicides may provide excellent disease control, infection by *S. sclerotiorum* can vary over time and may not be warranted every year and routine application of fungicides can be economically inefficient for canola producers. Moreover, the frequent application of fungicides can put increased selection pressure on pathogen populations to develop fungicide insensitivity. In Canada, *S. sclerotiorum* populations have been confirmed to have developed resistance to the fungicide benomyl (Benlate), which was previously used to manage diseases of canola and alfalfa caused by *S. sclerotiorum* (Gossen et al. 2001). Application of fungicides when disease risk is low, is economically inefficient for Canadian canola producers, since yield responses are limited and do not cover the cost of chemical and application.

The management of Sclerotinia stem rot with biological control agents also has been explored. Adams and Ayers (1979) reported that more than 30 species of fungi and bacteria that are antagonistic to or are mycoparasites of *Sclerotinia* spp. Many organisms have been investigated for use as possible biological control agents for *S. sclerotiorum*. In Canada, there are three pest control products with biological active ingredients registered for use on canola: Serenade Max (PCP# 28549, Bayer Crop Science), Serenade CPB (PCP# 30143, Bayer Crop Science), and Contans WG (PCP#29066, Bayer Crop Science) (Government of Saskatchewan 2016). Both Serenade Max and Serenade CPB are registered for two applications at 20-50% bloom. These products contain the bacterium *B. subtilis* as the active agent, which inhibits growth of *S. sclerotiorum*. Contans contains the fungus *C. minitans* as the active agent, for the suppression of sclerotial germination. A study conducted by Huang et al. (2000) found that the efficacy of spray applications of the biocontrol agent *C. minitans*, were not as consistent in reducing Sclerotinia stem rot levels as the fungicide benomyl. There is little information available to indicate how widely these biological control products are used in Canada and how their efficacy compares to chemical fungicides.

1.6.3 Host plant resistance

Due to the importance of petal infection in the development of Sclerotinia stem rot of canola, the apetalous trait in oilseed rape was investigated as a mechanism for Sclerotinia stem rot disease avoidance (Young and Werner 2012). Unfortunately, apetalous canola genotypes still developed some disease (Young and Werner 2012), perhaps due to infection of other flower parts such as the stamens (Hoyte 2000; Huang and Kokko 1992). In the apetalous plants, the cast stamens were found to adhere to leaves in numbers equal to those observed for petals in

fully petalled plants, supporting the suggestion that stamens represent an important initial infection route in apetalous canola genotypes (Young and Werner et al. 2012).

Susceptibility to infection by *S. sclerotiorum* has been found to vary between species as well as between accessions within species (Sedun et al. 1989; Uloth et al 2013). Sclerotinia stem rot resistance is quantitative in nature and several quantitative trait loci (QTL) have been identified in the A and C genomes of *B. napus* (Mei et al. 2013; Navabi et al. 2014; Zhao and Meng 2003; Zhao et al. 2006). Of the 19 *B. napus* chromosomes, at least 11 carry QTLs for resistance, which can explain between 5.9 to 39.8% of the phenotypic variation in resistance to *S. sclerotiorum* (Navabi et al. 2014). *Brassica carinata* has been demonstrated to be a promising source of genetic resistance (Navabi et al. 2014; Uloth et al. 2013). Two double haploid lines derived from interspecific crosses between *B. napus* (AACC) × *B. carinata* (BBCC) containing B-genome chromosomes were found to have significantly higher levels of resistance compared with those without B-genome chromosomes (Navabi et al. 2014). While the study was unable to rule out the introgression of resistance from the C genome of *B. carinata*, the results clearly suggested that *B. carinata* may be a useful source of resistance for the improvement of *B. napus* (Navabi et al. 2014).

There are no canola cultivars with complete resistance to Sclerotinia stem rot on the Canadian market. However, there are partially resistant or tolerant cultivars, such as '45S52' from DuPont Pioneer (Pratt 2012). There is no information available publicly on the genetic basis of the tolerance or partial resistance in these cultivars. While they represent another tool for the integrated management of Sclerotinia stem rot, when disease pressure is high these tolerant cultivars may still suffer damage and will benefit from fungicide application (Canola Council of Canada 2014; Pratt 2012).

1.7 Forecasting Sclerotinia stem rot of canola

Given the limited options available for the management of Sclerotinia stem rot of canola, and farmers' reliance on the application of fungicides for effective disease control, there has been considerable interest in the development of forecasting systems for this disease. Over the past three decades, several such systems have been proposed for diseases caused by *S. sclerotiorum* in general (Clarkson et al, 2004; Clarkson et al. 2014; Harikrishnan and del Río 2008; McLaren et al. 2004; Sharma et al 2015), and for Sclerotinia stem rot of canola in particular (Morrall and Thompson 1991; North Dakota State University 2009; Twengström et al. 1998). While some of these systems focus on different aspects of the disease cycle or disease triangle, all aim to reduce the unnecessary application of fungicides and help farmers make informed crop management decisions. Ideally, a risk assessment tool for Sclerotinia stem rot should incorporate information on the level of inoculum present, the susceptible stages of crop development, and a measure of how favourable environmental conditions are for disease development (Bom and Boland 2000a).

1.7.1 Risk assessment checklists

Sclerotinia stem rot checklists take into account factors such as the number of years since the last oilseed crop, disease incidence in the last host crop, crop density, the level of rainfall, weather forecasts and a measure of inoculum potential (Twengström et al. 1998). Risk points are assigned to levels within each risk factor, and the risk points are totalled and compared with a threshold to determine whether or not a fungicide application should be considered. When a checklist developed by Twengström et al. (1998) was validated under Swedish conditions, a threshold value of 40 risk points resulted in the application of a fungicide to 75% of fields where it was required, and to 16% of fields where it was not required. Checklists provide quick, field-

specific, and qualitative assessments of disease risk that incorporate most factors known to influence Sclerotinia stem rot of canola. However, these tools may lack an accurate or practical measure of inoculum potential. In the checklist developed by Twengström et al. (1998), inoculum potential was estimated by counting the number of apothecia developing from 100 sclerotia in the field. Not only is this a time-consuming process that requires some mycological expertise, it also does not take into account the contribution of inoculum disseminated from other fields. Moreover, since Sclerotinia stem rot has been reported in fields in the absence of apothecia (Gugel 1986), scouting for these structures may not always provide an accurate indication of risk. Only a small number of apothecia are required to cause a localized epidemic and little quantitative information is available to determine the number of apothecia that are associated with economically significant disease levels (Bom and Boland 2000a).

1.7.2 Weather-based forecasting systems

Weather-based risk assessment systems have been developed for a number of diseases caused by *S. sclerotiorum*. A model for infection of lettuce by this pathogen was developed by Clarkson et al. (2014), which incorporated environmental conditions by including measures of RH and temperature. However, Clarkson et al. (2014) found that a measurement of ascospore density also was needed to make accurate predictions of disease risk. Another recent model, aimed at predicting the risk of Sclerotinia rot of Indian mustard (*Brassica juncea*), also incorporated various weather-based parameters including temperature, RH, hours of sunshine, and soil moisture (Sharma et al. 2015). In bean, a logistical regression model, which takes in account the total rainfall in June, minimum temperatures in July, and the number of rain days in August (all measured during the first half of each month) was found to have good accuracy in predicting white mold incidence in North Dakota when white mold incidence level is high

(Harikrishnan and del Río 2008). However, the described model was found to overestimate white mold incidence in years when incidence was low (<20%).

In canola, weather-based maps have been generated to indicate the regional risk of Sclerotinia stem rot. Risk maps based on environmental conditions were developed for use in the Prairies of western Canada (McLaren et al. 2004), but are no longer produced or made available to producers. In North Dakota, risk maps for Sclerotinia stem rot are produced approximately every 3 days over the canola flowering period (North Dakota State University 2009). Forecasts based on weather maps indicate the risk in an area due to weather conditions that are favorable for disease development, but apply only to fields that are flowering at the time that the map is produced (McLaren et al. 2004). The major limitation of this type of forecast is that the maps indicate risk on a regional versus a field scale; they do not take into account the conditions or microclimate in specific fields. Moreover, weather-based systems do not incorporate any information regarding inoculum levels into their forecasts.

1.7.4 Forecasts based on inoculum pressure

Assessments of inoculum density provide an indication of the amount of pathogen propagules available to initiate disease. Many previously developed risk assessment tools have included some measure of inoculum pressure (Clarkson et al. 2014; Sharma et al. 2015; Twengström 1998). In the case of diseases caused by *S. sclerotiorum*, ascospore density has been found to be particularly important in forecasting risk (Clarkson et al. 2014; Harikrishnan and del Río 2006; Heran et al. 1999). In canola, lesion size increased with increasing ascospore concentration, up to approximately 80 ascospores per leaf, in experiments where leaves were inoculated with petals infected with known concentrations of ascospores (Heran et al. 1999).

Inoculum pressure can be measured in various ways, including by counting apothecia, measuring the levels of airborne ascospores, and by determining the level of petal infestation. As noted earlier, assessments of apothecia, as used in the risk assessment checklist developed by Twengström (1998), account only for the inoculum present in a field and are very labour intensive (Bom and Boland 2000a). The monitoring of airborne ascospores over time can be useful in determining when fungicide application would be most effective, and may be accomplished with spore traps (Almquist and Wallenhammar 2015; Parker et al. 2014; Rogers et al 2009). However, an assessment of petal infestation may be most useful for obtaining field-specific forecasts of disease risk, particularly since there is no guarantee that airborne inoculum will be deposited on a crop.

Petal infestation can be measured in a number of ways. Traditionally, petal infestation was monitored by plating field-collected canola petals on potato dextrose agar (PDA) and determining the percentage of petals infested with *S. sclerotiorum*, through the visual identification of fungal colonies (Morrall and Thompson 1991; see Section 1.7.5) or via qPCR analysis (see Section 1.7.6). Immunological assays also have been developed, which include a serological test for the detection of *S. sclerotiorum* on young canola petals (Jamaux and Spires 1994). The relationship between petal infestation estimates derived from immunodetection of the pathogen versus plating of petals on PDA was examined and found to be influenced by the viability and clumping of ascospores (Lefol and Morrall 1996; McLaren et al. 2004). Evaluation of three polyclonal antibody-based immunoassays for the detection of *S. sclerotiorum* on canola petals revealed a poor correlation with the level of petal infestation as determined by plating on semi-selective growth media (Bom and Boland 2000b).

1.7.5 Agar plate test

Petal infestation is known to be a critical stage in the Sclerotinia stem rot disease cycle on canola (Gugel 1986; Jamaux 1995; Koch et al. 2007; Morrall and Dueck 1982). Given this fact, estimates of petal infestation may provide accurate and field-specific indications of disease risk. Flower infestation levels have been shown to predict disease incidence levels with a higher sensitivity than algorithms that take into account crop rotation history, the incidence of disease in the last host crop and plant density, whether or not environmental conditions are also considered (Makowski et al. 2005). Assessment of petal infestation provides a quantitative estimate of disease risk, and may be more informative than counting apothecia, since disease has been reported in fields in the absence of apothecia (Gugel 1986). In addition, petal infestation accounts for inoculum produced internally within and externally to specific fields (Bom and Boland 2000a).

As noted above, the traditional agar plate test involves culturing field-collected canola petals onto Petri dishes filled with PDA. The plates are incubated for 3-5 days, following which the percentage of petals infected by *S. sclerotiorum* is assessed visually by colony morphology (Morrall and Thompson 1991). This general protocol is the basis of the petal test kits that are available commercially in Canada (Discovery Seed Labs Ltd. Saskatoon, Saskatchewan). Disease risk assessments were determined by examining the relationship between petal infestation level and final disease incidence (DI) in the field (Turkington et al. 1991a). At early bloom, the agar plate test was found to have an overall success rate of 73%, with predictions of low disease risk being the most accurate (Turkington et al. 1991a). Petal infestation levels were found to vary over the growing season, and the accuracy of forecasts could be improved by repeated assessments over the flowering period (Turkington and Morrall 1993). Petal

infestations levels determined with the agar plate test were found to be related to DI in most cases, with the strength of the relationship influenced by environmental conditions, canopy density, flowering stage and time of day of petal collection (Gugel 1986; Turkington et al. 1991a; Turkington et al. 1991b; Turkington and Morrall 1993). As such, the influence of weather and canopy density need to be taken into consideration when making assessments of Sclerotinia stem rot risk based on petal infestation (Turkington and Morrall 1993).

The agar plate test has the advantage, relative to some of the other methods described thus far, of providing a field-specific, quantitative assessment of inoculum available to initiate infection over the period in which fungicide application decisions are being made. When environmental conditions are favorable for Sclerotinia stem rot development, petal infestation levels alone can provide a reliable assessment of disease risk, but can be improved by including a measure of soil moisture (Bom and Boland 2000a). Even with the advantages of this risk assessment system, the uptake of the commercially available petal test kit by agronomists and farmers has been limited. This is likely due to the 3-5 day incubation period required for fungal growth, which represents a significant delay in a short fungicide application window (McLaren et al. 2004). In addition, the assay is labour intensive and the accuracy of predictions and petal infestation estimates is reliant on the correct identification of *S. sclerotinia* colonies, which may be difficult for non-expert personnel. Some of the weaknesses associated with the agar plate test could be mitigated by the development of a *S. sclerotiorum*-specific quantitative PCR (qPCR) assay to measure petal infestation.

1.7.6 Quantitative PCR

As with conventional PCR-based assays, qPCR assays utilize template-specific oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), a heat-stable DNA polymerase,

and the manipulation of temperature to create the conditions for DNA amplification (Kubista et al. 2006). However, qPCR assays also require the inclusion of a fluorescent reporter, such as an intercalating agent (e.g., SYBR green) or fluorogenic probe (e.g., hydrolysis probe) (Gachon et al. 2004; Kubista et al. 2006). During the qPCR assay, as DNA amplification occurs, fluorescence is produced by the reporter molecule and detected in real-time to quantify the level of DNA in the original sample (Kubista et al. 2006; Mirmajlessi et al. 2015). Measurements obtained via qPCR analysis are sensitive, robust and highly reproducible, making qPCR a useful tool across many disciplines (Smith and Osborn 2008).

Many qPCR assays have been developed to quantify plant pathogens in or on various substrates, including soil (Ceccherini et al. 2013; Etebu and Osborn 2010; Migliorini et al. 2015; Rennie et al. 2011), seeds and tubers (Duressa et al. 2012; Rennie et al. 2011), and host plant tissue (Ceccherini et al. 2013; Migliorini et al. 2015; Wieczorek et al. 2014). Quantitative PCR assays also have been used to predict disease risk by measuring inoculum levels (Bithell et al. 2012; Wieczorek et al. 2014). Once DNA extraction from the substrate is complete, qPCR analysis can be completed in 20-120 min, depending on the specific type of qPCR and thermocycler used (Gachon et al. 2004). The time required for the quantification of canola petal infestation with a S. *sclerotiorum*-specific qPCR assay would be significantly less than the time need for the agar plate test (McLaren et al. 2004). Another advantage of a qPCR-based assay, if properly validated, is that it carries a smaller risk of identifying false positives, as can occur by the misidentification of fungal colonies when relying on visual identification.

If it is to serve as an alternative to agar plate testing, a qPCR-based assay for estimating petal infestation level must be able to measure *S. sclerotiorum* DNA with a high level of sensitivity, specificity and reliability. The design of primers specific for *S. sclerotiorum* DNA is

complicated by genetic diversity in the pathogen and by its similarity to other organisms, particularly *Botrytis cinerea*. A comparison of the *S. sclerotiorum* and *B. cinerea* genomes indicated that approximately 83% of the total amino acid identity is shared between these two organisms (Amselem et al. 2011). This suggests that *S. sclerotiorum*-specific primers must be designed carefully and tested extensively against *B. cinerea* and other common microorganisms and the host. Several *S. sclerotiorum*-specific PCR and qPCR assays have been reported (Almquist and Wallenhammar 2015; Freeman et al. 2002; Rogers et al. 2009; Parker et al. 2014; Yin et al. 2009). Freeman et al. (2002) developed a non-quantitative, touchdown PCR assay for the detection of *S. sclerotiorum* DNA on oilseed rape petals and air samples. This assay targeted a 278 bp region of ribosomal DNA and was specific to the genus *Sclerotinia*. The primer set could not differentiate between *S. sclerotiorum* and other *Sclerotinia* spp., including *S. minor*, *S. trifoliorum* and *S. glacialis*. The threshold for reliable detection of *S. sclerotiorum* DNA on canola petals was 50 ascospores per petal, with the best sensitivity obtained if the petals were not incubated after inoculation. This assay was not quantitative, and if it were to be employed for assessing the risk of Sclerotinia stem rot, biologically meaningful thresholds would need to be determined.

To measure (as opposed to just detect) *S. sclerotiorum* DNA, a qPCR assay is required. Parker et al. (2014) developed an intercalating dye-based qPCR assay for the quantification of *S. sclerotiorum* DNA in air samples, as part of a model for forecasting Sclerotinia rot of carrots (*Daucus carota*). The assay targeted a 122 bp region of mitochondrial small subunit (mt SSU) rRNA and was highly sensitive, with the ability to detect as few as 0.5 ascospores of *S. sclerotiorum*. However, while it did not amplify DNA of *B. cinerea* or nine other non-target fungi, the assay amplified the DNA of only 93% of *S. sclerotiorum* isolates tested. It also was

unable to differentiate *S. sclerotiorum* from *S. minor* or *Monilinia vaccinia-corymbosi*. The inability to reliably detect the DNA of all isolates of *S. sclerotiorum* is a significant drawback of this particular assay, since it increases the chances of false negatives. Rogers et al. (2009) also developed an intercalating dye-based qPCR assay to quantify *S. sclerotiorum* DNA in air samples, using a primer set that amplified a 125 bp region of the mt SSU rRNA intron and open reading frame 1 (ORF1). The limit of detection of this assay was 5.0×10^{-5} ng of *S. sclerotiorum* DNA, and it was highly specific for *S. sclerotiorum* DNA even in a high background of *B. cinerea* DNA. Yin et al. (2009) also developed a qPCR assay that was based on the amplification of a 252 bp fragment of DNA (from a 1.2 kb region amplified by the microsatellite marker M13; Ma et al. 2003) and intercalating dye technology (Ma et al. 2003). This assay was designed for the quantification of *S. sclerotiorum* DNA on field-collected canola petals under Chinese conditions, and had a limit of detection of 5.0×10^{-4} ng *S. sclerotiorum* DNA.

A disadvantage of intercalating dye detection systems is that these dyes will release a fluorescent signal when bound to any double-stranded DNA molecule, including primers and non-target DNA (Ririe et al. 1997). Thus, this type of qPCR assay can result in the amplification of non-target, double-stranded DNA, which must be differentiated from the target through a melting curve analysis (Smith and Osborn 2008). Almquist and Wallenhammar (2015) developed the first *S. sclerotiorum*-specific, hydrolysis probe-based qPCR assay, using the primers reported by Freeman et al. (2002). The assay was specific for *Sclerotinia* spp., but had a greater sensitivity than the touch-down PCR assay of Freeman et al. (2002), detecting as few as 5 copies of the DNA fragment (Almquist and Wallenhammar 2015). The assay of Almquist and Wallenhammar (2015) was evaluated on various substrates, including air samples, canola petals

and canola leaves, and has the potential for use as the basis of a risk assessment system in Sweden, where it was developed.

Given the high level of genetic variation in *S. sclerotiorum* populations (Aldrich-Wolfe et al. 2015; Anderson and Kohn 1995; Carpenter et al. 1999; Karimi et al. 2011; Kohli et al. 1992; Kohn et al 1991; Kohn et al. 1990), a hydrolysis probe-based qPCR assay for determining petal infestation levels in Canadian canola cropping systems should be specific to isolates from Canada. Furthermore, any new system should also include an internal amplification control (IAC). An IAC is a non-target DNA sequence added in known quantities to the sample, and which can be amplified with other primers to control for and identify false negatives (Hoorfar et al. 2004). A false negative occurs when a negative PCR result is obtained despite the presence of the target DNA, and may result from factors such as a faulty thermocycler, poor DNA polymerase activity, or the presence of PCR inhibitors in the sample (Hoorfar et al 2004; Paterson 2007).

1.8 Conclusion

The overarching goal of this thesis was to develop tools that will contribute to the sustainable management of Sclerotinia stem rot in canola cropping systems, enabling farmers to make more informed fungicide spray decisions. This was accomplished through the development and validation of a hydrolysis probe-based qPCR assay for the quantification of *S. sclerotiorum* DNA in canola petals, and an assessment of the relationship between petal infestation, as determined by qPCR, and final DI in the field. The impact of environmental factors on this relationship also was examined. Ultimately, the tools and analysis presented in the subsequent chapters will add to understanding of the biology and epidemiology of Sclerotinia stem rot of canola.

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Chapter 2. A quantitative PCR system for measuring *Sclerotinia sclerotiorum* in canola (*Brassica napus*)[1]

2.1 Introduction

Sclerotinia stem rot, caused by the ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, is an economically important disease of canola (*Brassica napus* L.) (Purdy 1979; Willets and Wong 1980). Yield losses can be as high as 50%, with a yield reduction of 0.5% estimated for every 1% increase in disease incidence (del Río et al. 2007). The fungus overwinters as sclerotia, which can germinate carpogenically in the spring to produce apothecia (Abawi and Grogan 1979; Bardin and Hwang 2001; Willets and Wong 1980). Mature apothecia will forcibly release wind-borne ascospores (Abawi and Grogan 1979). The ascospores come into contact with canola petals onto which they adhere, germinate and penetrate the petal tissue to initiate infection (Jamaux et al. 1995; Purdy 1979). Petal infestation has been shown to be an important stage in the Sclerotinia stem rot disease cycle (Jamaux et al. 1995; Morrall and Dueck 1982). After senescence, the infected petals fall into the crop canopy and the fungus grows from these petals, infecting the leaf and stem tissues. The first visible symptoms of disease consist of browning on and around the cast petals. As the fungal infection spreads to the stems, the characteristic symptoms of Sclerotinia stem rot appear, including bleached whitish-grey lesions where the infected host stem tissues are very brittle, shredding and shattering when dry (Bolton et al. 2006; Jamaux et al. 1995; Young and Werner 2012). Once the infection is well-established, hard black survival structures known as sclerotia are produced by the fungus inside the stem. The sclerotia can be dislodged during harvest and serve as inoculum in subsequent years.

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The development of Sclerotinia stem rot of canola is influenced by environmental conditions, in particular temperature and moisture. For carpogenic germination of the sclerotia to occur, continuous soil moisture, near saturation, for about 10 days and moderate temperatures are required (Abawi and Grogan 1979; Schwartz and Steadman 1978; Wu and Subbarao 2008). Ascospore release has been found to be highest at 20-25°C, with the ascospores being able to survive for up to 2 weeks in the environment (Abawi and Grogan 1979; Clarkson et al. 2003). Infection of stem and leaf tissue by *S. sclerotiorum* is favored in the presence of free water and moderate temperatures (15-25°C), while at extreme temperatures (<4°C and >30°C) fungal growth is restricted (Bolton et al. 2006; Willets and Wong 1980).

The wide host range of *S. sclerotiorum*, along with the influence of temperature and moisture conditions on apothecial development and subsequent plant infection, have limited the effectiveness of cultural stem rot management practices. Crop rotations that include four years away from a susceptible host were found to be ineffective for Sclerotinia stem rot management, as a consequence of the survival of viable sclerotia in the soil and the introduction of ascospores from external sources (Morrall and Dueck 1982). Sclerotinia stem rot tolerant canola cultivars have become available recently in Canada, but when the risk of disease is high even these cultivars can suffer damage and require fungicide application (Canola Council of Canada 2014; Pratt 2012). As a consequence, the application of fungicides is the primary management tool for Sclerotinia stem rot of canola. To be effective, fungicides need to be applied during the key stage for infection, i.e., at flowering and before the appearance of symptoms in the crop. Therefore, the application of fungicide often must be made without any objective indication of disease risk (del Río et al. 2007; Koch et al. 2007; Turkington et al. 2011).

In an effort to predict the likelihood of Sclerotinia stem rot development in a given canola crop, a petal test was developed whereby field-collected petals were plated onto potato dextrose agar (PDA) and incubated for 3 to 5 days (Morrall and Thomson 1991). The cultured petals were then examined for growth and the resulting colonies were identified. The proportion of petals that yielded colonies of *S. sclerotiorum* was taken as the percent petal infestation. Risk assessments were provided based on the statistical relationship between petal infestation and final Sclerotinia stem rot development in the field, and were calculated based on percent petal infestation (Gugel and Morrall 1986; Turkington et al. 1991a). The percent petal infestation was used to identify the Sclerotinia stem rot risk level as low, moderate, or high based on the relationship between this parameter and disease incidence (Turkington and Morrall 1993; Turkington et al. 1991a). The 3 to 5 day incubation period that is required for accurate estimates of petal infestation represents a potential disadvantage of this system, since timely spray decisions need to be made during a fairly narrow window of crop development (McLaren et al. 2004; Turkington et al. 1991a). A molecular approach, such as quantitative polymerase chain reaction (qPCR)-based analysis, may represent an alternative method to determine petal infestation levels without the time delay associated with incubation of the petals. Moreover, PCR-based methods can lower the risk of human error associated with the misidentification of the fungal cultures growing out of the infested petals.

In recent years, many qPCR-based methodologies have been developed to detect and quantify plant pathogens including *S. sclerotiorum* (Almquist and Wallenhammar 2015; Freeman et al. 2002; Parker et al. 2014; Rogers et al. 2008; Yin et al. 2009). Freeman et al. (2002) established a touchdown PCR assay to quantify *S. sclerotiorum* DNA in airborne ascospores of the pathogen and inoculated canola petals. The primers SSFWD and SSREV amplified a 278-bp

fragment of ribosomal DNA and were found to be specific to *S. sclerotiorum*, even in the presence of a 40-fold excess of *Botrytis cinerea* DNA. However, this primer set was unable to distinguish between the DNA of *S. sclerotiorum* and that of close relatives such as *S. minor* and *S. trifoliorum* (Freeman et al. 2002). Nonetheless, Almquist and Wallenhammar (2015) were able to use the primers of Freeman et al. (2002) to develop a hydrolysis probe-based qPCR assay for detection of *Sclerotinia* spp. DNA in air samples and infected leaves and petals of oilseed rape (*B. napus*) in central Sweden. Rogers et al. (2008) described an intercalating dye-based qPCR assay to quantify airborne ascospores of *S. sclerotiorum* around oilseed rape crops near Rothamsted, United Kingdom, using primers designed to amplify a region of the mitochondrial small subunit rRNA and open reading frame 1 (ORF1) of the fungus. This assay could detect *S. sclerotiorum* DNA with a high level of sensitivity and specificity, even in the presence of DNA from the closely related fungus *B. cinerea*, except at 5×10^{-5} ng of DNA where the sensitivity of the quantification of *S. sclerotiorum* DNA was reduced (Rogers et al. 2008). Parker et al. (2014) developed another intercalating dye-based qPCR assay for the quantification of airborne ascospores of *S. sclerotiorum*, as part of a model for forecasting Sclerotinia rot of carrot (*Daucus carota* ssp. *sativus*). The assay targeted the group I intron of the mitochondrial small subunit (MtSSu) rRNA, and could detect 93% of the *S. sclerotiorum* isolates tested, although the quantification cycle (C_q) values varied for different isolates. While this assay may be useful for the detection of airborne ascospores of *S. sclerotiorum* on a regional scale, 7% of the isolates tested could not be detected, indicating the potential for false negatives. The variation in C_q values observed for different isolates also is a cause for concern, since it is indicative of differences in the ability to quantify *S. sclerotiorum* isolates. This likely would result in inconsistencies in the quantification of the same amount of inoculum, depending upon which

isolate is tested, particularly if the standard curve is based on another isolate. Moreover, on carrots there is no initial petal infestation stage, with infection first occurring on senesced leaves in a closed canopy, and the additional risk of postharvest infection (Kora et al. 2005).

Most relevant to the current study, Yin et al. (2009) developed an intercalating dye-based qPCR assay for the quantification of *S. sclerotiorum* DNA on canola petals in China. The primers (SsF and SsR) targeted a region amplified by the microsatellite primer M13 (Ma et al. 2003). The authors reported that this assay was very specific to *S. sclerotiorum* and could quantify as little as 5.0×10^{-4} ng DNA of the fungus. The specificity of the primers SsF/SsR, however, was not validated by testing with DNA of pure cultures of other *Sclerotinia* species such as *S. minor* or *S. trifoliorum.* Analysis of infected canola petals resulted in quantifications ranging from 2.5×10^{-2} ng to 1.1×10^{-1} ng *S. sclerotiorum* DNA per mg canola petal tissue. This assay may have value as the basis for a risk assessment tool in canola production systems in Canada. Nevertheless, because of evidence of a high level of genetic variation in the *S. sclerotiorum* population (Carpenter et al. 1999; Sirjusingh and Kohn 2001), specificity testing would be needed to ensure that Canadian isolates of the fungus can be detected with no false positives. In addition, it would be important to ensure that there is no difference in the detection and quantification of different *S. sclerotiorum* isolates.

The objective of the current study was to develop a *S. sclerotiorum*-specific qPCR assay for the quantification of Canadian isolates of *S. sclerotiorum* on canola petals using a hydrolysis probe. Development of this assay was focused on petals rather than airborne inoculum because of the importance of petal infestation in the disease cycle of Sclerotinia stem rot of canola. In addition, estimates of petal infestation may provide a better field-specific indication of disease risk, whilst detection of airborne inoculum would be best for regional risk assessments. The

qPCR assay described in this study also was compared with the qPCR assay developed by Yin et al. (2009), to evaluate the potential utility of each method for quantifying *S. sclerotiorum* DNA on canola petals.

2.2 Materials and methods

2.2.1 DNA isolation from fungal mycelium

Pure fungal cultures of *S. sclerotiorum* and the other fungal species used for specificity testing (Table 2.1) were grown on Difco potato dextrose broth (Dickinson and Company, Sparks, MD, USA) that was amended with 25 ppm ampicillin (Life Technologies, Carlsbad, CA, USA) and streptomycin (Sigma Chemical Company, St, Louis, MO, USA) in 200 ml Erlenmeyer flasks. The cultures were grown at room temperature (approximately 20-24^{o}C) under natural light provided by a north-facing window with gentle agitation for approximately 7 days, until a large mass of mycelium had formed. The supernatant was decanted and the mycelium washed with sterile water, frozen at -80ºC, and lyophilized in a freeze-drier. Approximately 20 mg of lyophilized tissue was homogenized to a powder in a 1.5 ml microcentrifuge tube with a hand-held plastic pestle, and the DNA was isolated with a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) as per the manufacturer's instructions, but with the addition of a phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) purification step. Briefly, following the protein precipitation step, three phenol/chloroform/isoamyl alcohol extractions were performed. In each extraction, 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) was added and the solution was mixed and centrifuged. The DNA was quantified with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the 260 nm/280 nm and 260 nm/230 nm absorbance ratios were calculated as an indication of DNA quality prior to qPCR analysis.

2.2.2 DNA isolation from field-collected canola petals

To validate the qPCR assay, canola petals were collected from 10 commercial fields in the Edmonton, AB, region in 2012. These 10 fields were farmed by three different farmers and were known to have a history of Sclerotinia stem rot. The fields were sampled once at 20-30% bloom and again at 40-50% bloom, as determined by visual assessment (Harper and Berkenkamp 1975). These levels of flowering correspond to growth stages 62-63 and 64-65, respectively, on the BBCH scale (Lancashire et al. 1991; Weber and Bleiholder 1990). Sampling dates varied slightly in each field depending on crop stage, but in general, sampling at 20-30% bloom was carried out in the first week of July, followed by sampling at 40-50% bloom 3-4 days later. The incidence of Sclerotinia stem rot also was assessed at the end of the growing season for future reference. Petals were collected at each of five sampling sites within each field. The sampling sites were located 100 m from the edge of each field and were situated 50 m apart along a fungicide-free check-strip. At each sampling site, the top 10-20 cm of the inflorescences from each of 20 plants were collected at random, placed in a plastic bag, and stored in a cooler on ice for transport back to the laboratory. A minimum of 80 flowers were selected randomly from the sampled inflorescences and stored in an ultra-low temperature freezer (-80°C) until further processing.

DNA was isolated according to the protocol of Liang et al. (2013) from a randomly chosen subsample of 20 petals from each sampling site. Petal samples were homogenized with a TissueLyser II (Qiagen, Toronto, ON, Canada) and a single 5-mm stainless steel bead in a 2-ml locking tube. The TissueLyser II adapters and petal samples were frozen at -80°C prior to homogenization to prevent damage to the DNA. Prior to elution, the DNA pellets were dried in a Vacufuge Plus (Eppendorf, Mississauga, ON, Canada) for 7 min at 45°C. The extracted DNA

was eluted with 30 µl of nuclease free water (Life Technologies, Carlsbad, CA, USA) and quantified with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) as above.

2.2.3 Development of *S. sclerotiorum*-specific primers

Forward and reverse primers and a hydrolysis probe were designed using Primer 3 Express (Applied Biosystems, Carlsbad, CA, USA) based on the 468-bp sequence of a single-copy gene (SS1G_00263) encoding the hypothetical secreted protein ssv263 (Liang et al. 2013) from *S. sclerotiorum* (GenBank accession no. XM_001598127). This gene is highly specific to *S. sclerotiorum* and orthologous to a protein-encoding gene BC1G_00896 (GenBank accession no. XM_001560818) from *B. cinerea* (Liang et al. 2013; Shah et al. 2009). The forward (SSBZF) and reverse (SSBZR) primers were designed to amplify a 70-bp product in a region of SS1G_00263 that exhibits the greatest difference with the *B. cinerea* orthologue. The hydrolysis probe (SSBZP) was labeled with a non-fluorescent quencher-mini groove binder (NFQ-MGB) on the 3' end, and with the reporter dye FAM (6-carboxyfluorescein) on the 5' end (Table 2.2). The amplified region shares 71% similarity with the orthologous gene in *B. cinerea*, but includes 20 mismatches of base pairs, 17 of which are covered by the primer and probe sequences. The probe and primer sequences were used to query the GenBank databases using the Basic Local Alignment Search Tool (BLAST) in order to identify any similar sequences from other organisms that could lead to false positives.

2.2.4 Development of the exogenous positive internal control

To identify false negatives resulting from failed DNA extraction or inhibition of the PCR, an exogenous internal control was included in the analysis of all samples. The *ToxB* gene from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat (Lamari and Strelkov 2010), was

selected as the exogenous internal control since it is unlikely to be found in canola petals. The pSilent1 plasmid (Nakayashiki et al. 2005) containing a 432-bp fragment of *ToxB* from *P. tritici-repentis* (Aboukhaddour et al. 2012) was used as the template for the design of the primers and hydrolysis probe. The *ToxB*-specific primers (ToxBF and ToxBR) and a probe (ToxBP) were designed using Primer 3 Express (Applied Biosystems, Carlsbad, CA, USA) (Table 2.2). The *ToxB*-specific hydrolysis probe was labelled with NFQ-MGB on the 3' end and with the reporter dye VIC on the 5' end.

For use as an internal control, every canola petal sample was spiked with 2×10^6 plasmids containing the *ToxB* gene prior to DNA isolation. Each sample was analyzed in two separate singleplex qPCR reactions, one with the *ToxB* primers and probe set and another with the *S. sclerotiorum*-specific primers and probe. To identify outliers and potential error, the standard deviation of the C_q values obtained for the internal control in a set of samples was calculated. If the standard deviation was close to or < 1.0 for a set of samples, the variation in the internal control and thus the risk of false negatives as a result of failed DNA isolation or PCR inhibition were regarded as low. Any samples that were outside of this range were discarded and DNA was isolated again.

2.2.5 qPCR analysis

The terminology used to describe the qPCR analysis are as suggested in the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al. 2009). All qPCR analyses were conducted in a ViiA7 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using the Universal FastStart Master (Rox) mix (Roche, Indianapolis, IN, USA) in a MicroAmp® Fast Optical 96-well reaction plate (Applied Biosystems, Carlsbad, CA, USA), which was sealed with MicroAmp optical adhesive film

(Applied Biosystems). Each qPCR was conducted in a total volume of 10 µl, including 0.1 µl of each forward and reverse primer (50 µM SSBZF and 50 µM SSBZR, respectively), 0.03 µl of the hydrolysis probe (100 µM SSBZP), 5 µl of the 2× master mix (Rox), 0.77 µl of molecular grade water (Life Technologies), and 4 µl of the template DNA or negative control. For the quantification of the internal control, the reaction mixture was as above except that 0.025 µl of the 100 µM ToxBP hydrolysis probe and 0.775 µl of molecular grade water (Life Technologies) were added and the primers ToxBF/ToxBR were substituted for SSBZF/SSBZR. The reaction conditions included a hot start at 95ºC for 10 min, followed by 40 cycles of 95ºC for 10 sec and 60ºC for 30 sec as per the manufacturer's instructions for the master mix. Each of the primer/probe sets was analyzed separately and reactions were not multiplexed. Each sample was analyzed in triplicate for quantification of the SS1G_00263 target, while samples were analyzed in duplicate for assays of the internal control. If the standard deviation of the mean C_q obtained for a set of triplicates of a given sample was > 0.5, the sample was reanalyzed, and if an outlier was present, it was removed. Similarly, if the standard deviation of the mean C_q obtained for duplicates of the *ToxB* internal control was > 0.5, the sample was re-extracted.

2.2.6 Specificity testing

The specificity of the SS1G_00263 primer and probe set was confirmed by separately testing 100 ng of DNA from 13 fungal species and the canola host as templates in the qPCR analysis (Table 2.1). In addition, amplification of a total of eight isolates of *B. cinerea* and seven isolates of *S. sclerotiorum* was compared. No amplification of any product, or a C_q value > 2 cycles below that of the smallest standard, were considered to be indicative of no detection. The reproducibility of the standard curve was assessed by calculating the standard deviation at each point, for 4 replications included in separate 96-well plates. Plasmids containing the *ToxB* insert

were analyzed with the SS1G_00263-specific primer set to ensure that SSBZF/SSBZR did not amplify the internal control.

2.2.7 Sequencing of PCR products

Amplicons obtained with the SSBZF/SSBZR primer set were sequenced to confirm the identity of the product. Conventional PCR was used to increase the amount of amplicon obtained from pure mycelial DNA of six *S. sclerotiorum* isolates (UAMH 6321, UAMH 4514, UAMH 9192, SSA-11, SSB-11, SSD-11) for sequencing purposes. The PCR analysis was carried out in a 25 µl reaction volume, which consisted of 15.4 µl molecular grade water (Life Technologies), 2 µl of 10× PCR buffer (no magnesium chloride) (Invitrogen by Life Technologies, Carlsbad, CA, USA), 1 µl 2.5mM each dNTP, 1 µl 50 mM $MgCl_2$, 0.2 µl of each 50 mM SSBZF and SSBZR, 0.2 µl Platinum Taq DNA Polymerase (Intvitrogen), and 5 µl (100 ng) template DNA. The reaction conditions were 94ºC for 3 min, followed by 40 cycles of 94ºC for 30 sec, 60ºC for 20 sec and 72ºC for 5 min, and ended with an incubation at 72ºC for 5 min followed by a constant 4ºC. Polymerase chain reaction products were resolved on a 2% agarose gel run at 90V for 50 min. The amplicon band was extracted using a Qiaquick Gel Extraction Kit (Qiagen) and sent to the Molecular Biology Service Unit, University of Alberta, Edmonton, AB, for sequencing. Sequencing was conducted on a 3730 Genetic Analyzer (Applied Biosystems) with Sanger cycle sequencing using fluorescently labelled dye terminators and BigDye Terminator v1.1 chemistry.

2.2.8 Sensitivity testing

To test the limit of detection (LOD) of the assay, standard curves were generated with 1:10 serial dilutions of purified *S. sclerotiorum* DNA. All standard curves tested consisted of five serial dilutions ranging from 8.0 ng to 8.0×10^{-4} ng or from 5.0 ng to 5.0×10^{-4} ng. Each

standard was included in triplicate in the same 96-well plate as the samples being analyzed. The LOD was regarded as the lowest quantity of DNA that could be detected with confidence in all three of the triplicates. The efficiency and coefficient of determination (R^2) of the standard curves were determined for each replication. The reproducibility of the standard curve was assessed by calculating the standard deviation at each point, for 4 replications included in separate 96-well plates. A standard deviation of < 0.5 for the average Cq values of the 4 replications was considered to indicate that the assay was reliable.

To determine if inclusion of the internal control and the presence of *B. napus* DNA reduced the LOD for *S. sclerotiorum*, fungal DNA was quantified in the presence or absence of the *ToxB* internal control and *B. napus* DNA. A 1:5 dilution series ranging from 4.0×10^{1} ng to 5.0×10^{-4} ng *S. sclerotiorum* DNA was spiked with 2 µl *B. napus* DNA and 2×10^{5} copies of the plasmid containing the internal control. The *B. napus* DNA represented a 1:5 dilution of total genomic DNA extracted from a set of 20 canola petals that were not infected with *S. sclerotiorum*. The mean C_q and standard deviation of the mean for each point in the dilution series were compared between the spiked and non-spiked samples.

2.2.9 Evaluation of canola petals

Total genomic DNA isolated from samples consisting of 20 canola petals each was diluted by a factor of 1:5 with nuclease free water (Life Technologies) prior to qPCR analysis with the SS1G_00263- and *ToxB*-specific primers and probes. DNA was analyzed from samples collected at five sampling sites from each of 10 commercial canola fields at each of two sampling dates as described above. Results are expressed on a per petal basis by first accounting for the dilution, then by dividing the estimate by 20. The qPCR estimates were averaged over each field on each sampling date and the standard deviation of the mean was calculated for each

field. Any sample from which no *S. sclerotiorum* DNA could be amplified, or which was below the lowest standard outside the range of the standard curve, was recorded as 0 ng *S. sclerotiorum* DNA per canola petal.

2.2.10 Assessment of the SsF and SsR primer set

Isolates of *S. sclerotiorum* collected from canola fields in central Alberta were subjected to qPCR analysis with the primers SsF (5' AGTCGAGGGACGGGTACTAA 3') and SsR (5' CTTGTCCTCATTGCCGTTT 3') developed by Yin et al. (2009). The primers were evaluated using Dynamite qPCR Mastermix (Molecular Biology Service Unit, University of Alberta, Edmonton, Canada) instead of the SYBR Premix Ex *Taq* (TaKaRa Biotechnology Co. Ltd., Dalian, China) used by Yin et al. (2009), but both of these master mixes rely on the intercalating dye SYBR Green as the basis of detection. The reaction conditions consisted of an initial heat denaturation at 95ºC for 2 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 60 sec, as recommended by the manufacturer. These differ slightly from the conditions used by Yin et al. (2009), but are optimized for the Dynamite qPCR Mastermix utilized in the current analysis. Quantitative PCR assays were conducted in a Step One Plus Real-Time PCR System (Applied Biosystems) in a 10 µl total reaction volume, containing 5 µl Dynamite qPCR Mastermix, 0.8 µl molecular grade water, 0.1µl of each of 50 mM SsF and SsR, and 4 µl template DNA solution. After the reaction was complete, a melting point analysis was conducted to confirm the presence of a single amplification product that had a melting temperature (T_M) consistent with the predicted T_M of 84ºC. The specificity of the SsF/SsR primer set also was evaluated as described above for the other primer and probe sets.

2.3 Results

2.3.1 Amplicon generated with SSBZF/SSBZR

The amplicons generated from six isolates of *S. sclerotiorum* with the SSBZF/SSBZR primer set targeting the gene SS1G_00263 were sequenced and confirmed to be of the expected 70-bp size. Moreover, the sequences were identical to each other, and a query of the GenBank database using BLASTN revealed 100% identity with accession number XM_001598127.1 from *S. sclerotiorum* (e-value = 3e-31).

2.3.2 Specificity testing

While the SS1G_00263-specific primers consistently amplified a 70-bp product from DNA of each of the *S. sclerotiorum* isolates evaluated, they did not generate an amplicon from any of the other 13 species tested, including the closely related *B. cinerea, S. trifoliorum*, or *S. minor* (Table 2.3). Similarly, no amplicon was obtained from DNA of the host canola plant. Specificity testing with the *ToxB*-specific primers did not yield an amplicon from *S. sclerotiorum* or any of the non-target organisms tested.

2.3.3 Sensitivity testing

The lowest reliable LOD was 8.0×10^{-4} ng *S. sclerotiorum* DNA. Although the assay could detect quantities of *S. sclerotiorum* DNA as small as 5.0×10^{-4} ng, such detection could not be accomplished with a standard deviation of the mean $C_q < 0.5$. As such, the lowest point of the standard curve was set to 8.0×10^{-4} ng DNA. To compare replicates of the standard curve, the C_q values were averaged for a set of comparisons and the standard deviation of the mean for each point on the curve was calculated. An average standard deviation of 0.19 was obtained for the mean C_q over all five points on the curve, among four replicates of the standard curve in separate

96-well plates; none of the points had a standard deviation > 0.5 (Fig. 2.1). For the four replicates of the standard curve generated in separate 96-well plates, the PCR efficiencies ranged from 88.90 to 90.25 and the R^2 values ranged from 0.9928 to 0.9999.

Curves generated with the SS1G_00263-specific primers in the presence or absence of the internal control and *B. napus* DNA were compared to identify any potential reduction in the quantification of *S. sclerotiorum* DNA. The C_q values were averaged for each dilution point in the two dilution series and the standard deviation of the C_q values for the replicates was calculated. Each point on the standard curves was compared individually and no single point had a standard deviation of the mean $C_q > 0.54$ (Fig 2.2).

2.3.4 Quantification of *S. sclerotiorum* in canola petals

Total genomic DNA extracted from 20 petals from each of five sampling sites in each of 10 commercial canola fields on two different sampling dates (100 DNA samples in total) was used as a template for qPCR analysis. The petal infestation estimates for individual sampling sites ranged from 0 ng to 3.3×10^{-1} ng *S. sclerotiorum* DNA per petal. The amount of *S. sclerotiorum* DNA in each of the 5 sampling sites per field was averaged to give a single petal infestation estimate for each field on each sampling date (Fig. 2.3). On the first sampling date, when the canola was at 20-30% bloom, average infestation in the 10 fields ranged from a mean (± standard deviation) of 6.0×10^{-3} ng ± 7.0×10^{-3} ng to 3.4×10^{-2} ng ± 2.8×10^{-2} ng *S. sclerotiorum* DNA per petal (C_q values = 30.5-36.1). On the second sampling date, at 40-50% bloom, average infestation in the 10 fields ranged from a mean (± standard deviation) of 1.0×10^{-3} ng ± 1.0×10^{-3} ng to 8.0×10^{-2} ng ± 1.4×10^{-1} ng *S. sclerotiorum* DNA per petal (C_q values = 28.9-36.1).

In addition to the analysis with the SS1G_00263-specific primer and probe set, all samples were analyzed with the *ToxB* (internal control)-specific primer and probe set in separate, singleplex qPCR assays. The mean and standard deviation of the mean C_q value for duplicates was calculated for early bloom and late bloom sample sets to get an estimate of the amount of variation. At 20-30% bloom, the overall mean C_q (± standard deviation) was 29.6 ± 1.08, and at 40-50% bloom, the overall mean C_q (± standard deviation) was 27.6 ± 0.70.

2.3.5 Assessment of the SsF and SsR primers

The primers SsF and SsR (Yin et al. 2009) were evaluated to determine their suitability for quantifying *S. sclerotiorum* DNA in canola petals under Canadian conditions. The primers amplified a product of approximately 225-bp from all isolates of *S. sclerotiorum*, and did not amplify DNA of *S. minor*, *Aspergillus niger*, *Cladosporium* sp., *Mucor* sp., *Alternaria alternata*, or *Leptosphaeria maculans* (Table 2.3). However, the primers were found to amplify products from DNA of *S. trifoliorum,* as well as from all isolates tested of *B. cinerea* and *B. fuckeliana, Rhizopus* sp.*, Trichoderma* sp.*, Rhizoctonia solani, Penicillium* sp., and *Fusarium graminearum*. The amplicons obtained from *B. cinerea, S. trifoliorum* and *Trichoderma* sp. all had temperature peaks between 84 and 85ºC, which was within the range expected for the product from *S. sclerotiorum.* For the products amplified from DNA of *B. fuckeliana, Rhizopus* sp., *R. solani, Penicillium* sp., and *F. graminearum*, the melting curve analysis revealed the presence of multiple temperature peaks associated with non-specific amplification. The sensitivity of the SsF/SsR primer set was similar to that of the SSBZF/SSBFR primers, with a consistent LOD of 8.0×10^{-4} ng DNA.

2.4 Discussion

This study focussed on the development of a qPCR-based assay for the quantification of *S. sclerotiorum* DNA in canola petals, to enable rapid and accurate estimates of infestation levels. The assay targets a 70-bp region of a single-copy gene encoding the hypothetical secreted protein ssv263 (Liang et al. 2013). While the target region of the gene shares 71% similarity with an orthologue in *B. cinerea*, specificity testing indicated that there was no amplification of DNA from any of six *B. cinerea* isolates tested. The qPCR assay also did not amplify DNA from any of the 13 other species evaluated in this study, including *S. minor, S. trifoliorum* and the host plant, *B. napus*. These results indicate that the assay is highly specific for *S. sclerotiorum*, and can be used to estimate pathogen biomass in canola petals.

The sensitivity tests revealed a consistent LOD of 8.0×10^{-4} ng *S. sclerotiorum* DNA. Rogers et al. (2008) reported that an ascospore of *S. sclerotiorum* is equivalent to about 3.5×10^{-4} ng of DNA, suggesting a LOD of 2.3 ascospores per petal for the current assay. When adjusted to account for the dilution of DNA from canola petals during extraction and analysis, the LOD would correspond to 1.5×10^{-3} ng *S. sclerotiorum* DNA or 4.3 ascospores per petal. An issue with the use of ascospores as the basis for a standard curve to quantify petal infestation, however, is that this approach will not reflect increases in pathogen biomass associated with spore germination. Ascospores have been shown to begin germination as early as 3 hours after release from the apothecium under favorable environmental conditions (Willets and Wong 1980). Therefore, it is likely that any assay examining the amount of *S. sclerotiorum* DNA on canola petals is measuring DNA from the ascospores as well as from hyphae developing from germinated spores. As such, quantification of *S. sclerotiorum* on petals is more meaningful and accurate when expressed as the total amount of pathogen DNA. The specificity of the assay was

not affected by the presence of *B. napus* DNA or of an internal control in the samples. These are important considerations if the assay is to be used to measure infestation of petals collected in the field.

Yin et al. (2009) developed a qPCR assay to measure *S. sclerotiorum* DNA in canola petals using intercalating dye (SYBR Green) detection technology that detected between 2.52×10^{-2} ng to 1.11×10^{-1} ng pathogen DNA per mg of canola petal tissue. Evaluation of the primers developed by Yin et al. (2009) on DNA extracted from Canadian isolates of *S. sclerotiorum* and other fungal species, using a different master mix, but the same dye detection technology, indicated sensitivity similar to that of the assay described in this report. However, the specificity of the primers seemed considerably lower and amplicons were obtained from various non-target species including *S. trifoliorum, B. cinerea, F. graminearum, Rhizopus* sp., and *Penicillium* sp. In their original report, Yin et al. (2009) found no amplification of DNA other than from *S. sclerotiorum*, but only evaluated one isolate of *B. cinerea* (*B. fuckeliana*) and did not test *S. minor, S. trifoliorum,* or *Rhizopus* sp. They did test *R. solani, Penicillium* sp. and *F. graminearum*, but did not detect any product from the isolates evaluated. This lower specificity represents a challenge for application of the earlier protocol (Yin et al. 2009) in the analysis of field samples, and hence for its use as the basis for a Sclerotinia stem rot risk assessment system. While it may be possible to identify non-specific amplification products based on a melting curve analysis, this would complicate the procedure and introduce the possibility of additional errors. Moreover, the quantification of field samples containing a range of different fungal species in addition to *S. sclerotiorum* may be difficult. The similar sensitivity, but increased specificity of the assay developed in this study with the primers SSBZF/SSBZR and probe SSBZP may reflect the use of a hydrolysis probe instead of an intercalating dye. An

intercalating dye, such as SYBR green, binds to any double-stranded DNA and releases a fluorescent signal (Ririe et al. 1997). This potentially can result in fluorescent amplification of non-target double-stranded DNA that must be further differentiated through melting curve analysis (Smith and Osborn 2008). The use of a hydrolysis probe ensures that a fluorescent signal is produced only when there is an exact match between the probe and the target (Smith and Osborn 2008). This increased level of specificity reduces the likelihood of false positives when analysing diverse field samples, without compromising sensitivity. As a result, the newly developed qPCR assay may represent a more reliable method to quantify *S. sclerotiorum* DNA in canola petals.

When the hydrolysis probe-based qPCR assay was used to quantify *S. sclerotiorum* DNA in field-collected canola petals, considerable variation was observed in the amount of petal infestation in different fields and at different crop stages. Environmental conditions in 2012 were conducive to stem rot development in the sampled fields. In five of 10 fields, infestation levels were highest at 40-50% bloom, while in the remaining five fields, the infestation levels were highest at 20-30% bloom. This is consistent with the findings of Turkington and Morrall (1993), who reported changes in petal infestation from early to late bloom and at different locations when culturing petals on growth medium. Similarly, using qPCR analysis, Almquist and Wallenhammer (2015) also observed differences in *S. sclerotiorum* incidence at different stages of flowering. The evaluation of petals by qPCR analysis over the flowering period could provide an indication of when inoculum levels are highest, and thus when the crop may benefit most from fungicide application. The variation in the amount of *S. sclerotiorum* DNA between locations may reflect differences in inoculum level or in the timing of ascospore release, crop seeding date and growth stage, canopy density, or microclimatic conditions. It also indicates the

importance of assessing petal infestation and risk potential for a particular field as opposed to an assessment of risk based on regional conditions. The inclusion of an internal control helped to identify outliers resulting from possible human error or other artifacts, such as inhibition of the PCR, thereby improving the accuracy of qPCR-based estimates of petal infestation.

The qPCR assay described in this study may serve as the basis for a risk assessment system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem rot of canola. It can quantify the level of petal infestation, a key stage in the Sclerotinia stem rot disease cycle, thereby providing a measure of disease risk when timely fungicide application decisions need to be made. To further investigate the possibility of developing a forecasting system based on this technology, the relationship between the petal infestation estimates and final Sclerotinia stem rot incidence must be determined. The strength of the relationship between petal infestation as assessed through qPCR analysis and final disease incidence can be investigated with the use of regression analysis. It is also important to emphasize that a forecasting system based on qPCR quantification of petal infestation should be linked to environmental conditions, as well as cropping history, seeding date and crop canopy conditions, which may influence Sclerotinia stem rot development and the need to spray a fungicide. Validation as a predictive tool under field conditions is the focus of a follow-up study.

2.5 Tables

Table 2.1. List of species used to test the specificity of primers and hydrolysis probes developed for the detection and quantification of *Sclerotinia sclerotiorum* and a *ToxB* internal control.

Species	Isolate	Origin	Source	Supplier
Sclerotinia sclerotiorum[ab]	UAMH 6321	Devon, AB	Potted *Garzania* sp.	University of Alberta Microfungus Collection and Herbarium (UAMCH)
S. sclerotiorum[b]	UAMH 4514	Central Alberta	Rapeseed	UAMCH
S. sclerotiorum[b]	UAMH 9192	Maryland, USA	Bean stem	UAMCH
S. sclerotiorum[b]	SSA-11	Edmonton, AB	Canola petals	B. Ziesman, University of Alberta (U of A)
S. sclerotiorum[b]	SSB-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. sclerotiorum	SSC-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. sclerotiorum[b]	SSD-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. minor	CBS 207.25	Unknown	Unknown	Centraalbureau Voor Schimmelcultures (CBS), Royal Netherlands Academy of Arts and Sciences, Uppsalalaan, NL
S. trifoliorum	CBS 122377	Netherlands	Unknown	CBS
Botrytis cinerea	DR12-5	Unknown	Potato tuber tissue	R.J. Howard, Alberta Agriculture and Rural Development (AARD), Brooks, AB
B. cinerea	414JV	Unknown	Alfalfa	R.J. Howard, AARD
B. cinerea	DAOM 192631	Winnipeg, Manitoba	*Fragaria chiloensis*	Canadian Collection of Fungal Cultures (CCFC), Ottawa, Ontario

B. cinerea	DAOM 189076	Charlottetown, PEI	Potato tubers	CCFC
B. cinerea	DAOM 166439	Beaverlodge, AB	*Festuca rubra*	CCFC
B. cinerea	CGC5	PEI	Winter wheat	Canadian Grain Commission, Winnipeg, MB
Botryotinia fuckeliana[c]	UAMH 16	Unknown	Bean	UAMCH
B. fuckeliana	UAMH 1784	Beaverlodge, AB	Indoor air exchange strip from *Apis mellifera* equipment cleaning warehouse	UAMCH
Rhizoctonia solani	N/A	Unknown	Unknown	J.P. Tewari, U of A
Rhizopus sp.	N/A	Unknown	Unknown	J.P. Tewari, U of A
Trichoderma sp.	N/A	Unknown	Soil	J.P. Tewari, U of A
Penicillium sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, University of Alberta (U of A)
Aspergillus niger	N/A	Unknown	Unknown	J.P. Tewari, U of A
Cladosporium sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, U of A
Mucor sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, U of A
Alternaria alternata	N/A	Unknown	Unknown	J.P. Tewari, U of A
Fusarium graminearum	G-1	Unknown	Unknown	A. Tekauz, Agriculture and Agri-Food Canada, Winnipeg, MB
Leptosphaeria maculans	N/A	Unknown	Unknown	J.P. Tewari, U of A
Brassica napus	N/A	Edmonton, AB	N/A	B. Ziesman, U of A

[a]*S. sclerotiorum* UAMH 6321 was originally designated as *S. minor* in the UAMCH, but later identified as *S. sclerotiorum*.
[b]Amplicon obtained from the isolate sequenced to confirm identity of the PCR product.
[c]Teleomorph of *B. cinerea*; species names are given as provided by the original supplier.

Table 2.2. Primers and hydrolysis probes for detection and quantification of *Sclerotinia sclerotiorum* and a *ToxB* internal control. The primer set SSBZF/SSBZR amplifies a 70-bp fragment of the gene SSIG_00263 in *S. sclerotiorum* and was used in conjunction with SSBZP in a hydrolysis probe-based assay to quantify *S. sclerotiorum* DNA. The primer set ToxBF/ToxBR amplifies a 70-bp fragment of the *ToxB* gene from *Pyrenophora tritici-repentis* and was used in conjunction with ToxBP to quantify the exogenous internal control in a hydrolysis probe-based assay.

Primer/probe name	**Sequence**	**Size (bp)**
SSBZF	5'-GCTCCAGCAGCCATGGAA-3'	18
SSBZR	5'-TGTTGAAGCAGTTGACGAGGTAGT-3'	24
SSBZP	5'-CAGCGCCTCAAGC-3'	13
ToxBF	5'-CCATGCTACTTGCTGTGGCTAT-3'	22
ToxBR	5'-CGCAGTTGGCCGAAACA-3'	17
ToxBP	5'-CTCCCTGCTGCCCC-3'	13

Table 2.3. Results of specificity testing with the SsF/SsR primer set (Yin et al. 2009) and SSBZF/SSBZR primer set. The quantification cycle (Cq) value indicates the cycle number at which fluorescence from amplification of a product exceeds background fluorescence.

Fungal species	**C_q value (SsF/SsR)**	**C_q value (SSBZF/SSBZR)**
Sclerotinia trifoliorum	27.673	ND[a]
Sclerotinia minor	ND	ND
Botryotinia fuckeliana UAMH 1784	33.219	ND
B. fuckeliana UAMH 16	35.017	ND
Botrytis cinerea DR12-5	34.954	ND
B. cinerea DAOM 192631	34.099	ND
B. cinerea DAOM 166439	35.017	ND
Rhizopus sp.	34.285	ND
Trichoderma sp.	30.617	ND
Rhizoctonia sp.	35.330	ND
Penicillium sp.	31.550	ND
Aspergillus niger	ND	ND
Fusarium graminearum	30.037	ND
Cladosporium sp.	ND	ND
Mucor sp.	ND	ND
Alternaria alternata	ND	ND
Leptosphaeria maculans	ND	ND
8.0×10^{-4} ng of *S. sclerotiorum* DNA	33.068	33.662

[a]ND, no detection; indicates that there was no amplification of the fungal DNA or that the C_q value was at least 3 cycles greater than the lowest standard (8.0×10^{-4} ng of *S. sclerotiorum* DNA).

2.6 Figures

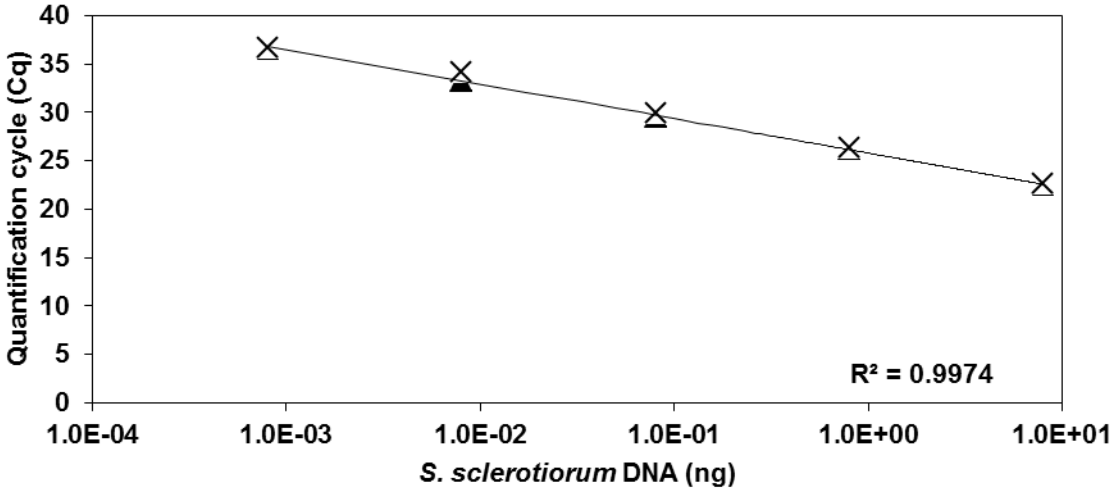

Fig. 2.1. Standard curves obtained with DNA extracted from *Sclerotinia sclerotiorum* and subjected to qPCR analysis with the primer set SSBZF/SSBZR and hydrolysis probe SSBZP. Four replicates of the standard curve were run in separate 96-well plates. The standards were prepared from a serial dilution of 8.0 ng to 8.0×10^{-4} ng *S. sclerotiorum* DNA. The four replications are denoted by different symbols, which may not be clearly visible because the curves overlap. The PCR efficiency (E) of the four standard curves ranged from 88.9 to 90.25 and the R^2 values for the individual standard curves ranged from 0.9928 to 0.9999 (average: 0.996).

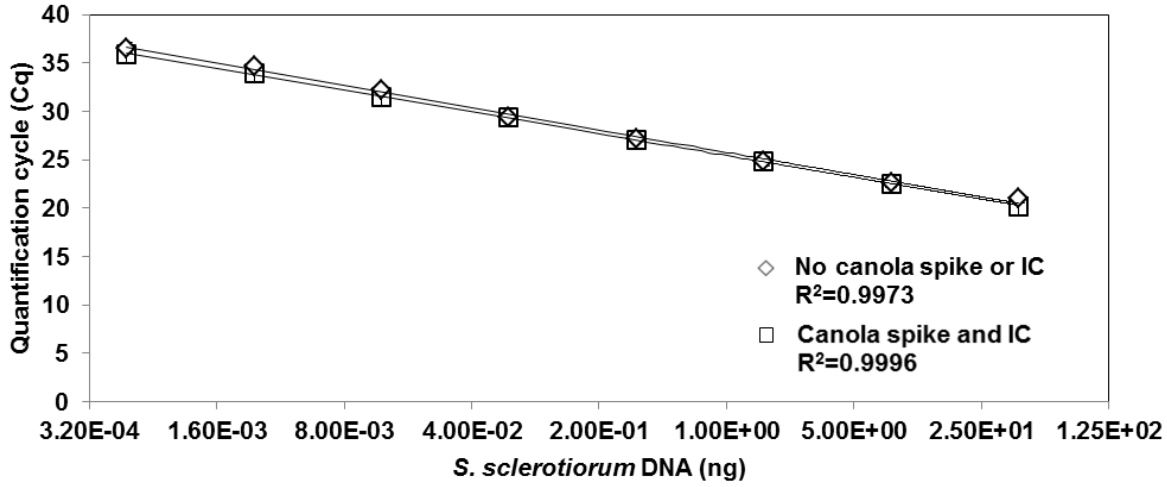

Fig. 2.2. Quantification of *Sclerotinia sclerotiorum* DNA in the presence or absence of canola (*Brassica napus*) DNA and a *ToxB* internal control. The fungal DNA was serially diluted by a factor of 1:5 from 4.0×10^{1} ng to 5.0×10^{-4} ng, and analyzed by qPCR with the primer set SSBZF/SSBZR and hydrolysis probe SSBZP. Samples were analyzed in the absence (diamonds) or presence of a *ToxB* internal control (IC) and *Brassica napus* DNA ('Canola spike') (squares). The standard deviation of the mean C_q for any point along the curve was not > 0.54, suggesting that the presence of host DNA or the internal control did not affect the quantification of *S. sclerotiorum* DNA. The standard deviation at each point of the standard curve is reflected by the error bars.

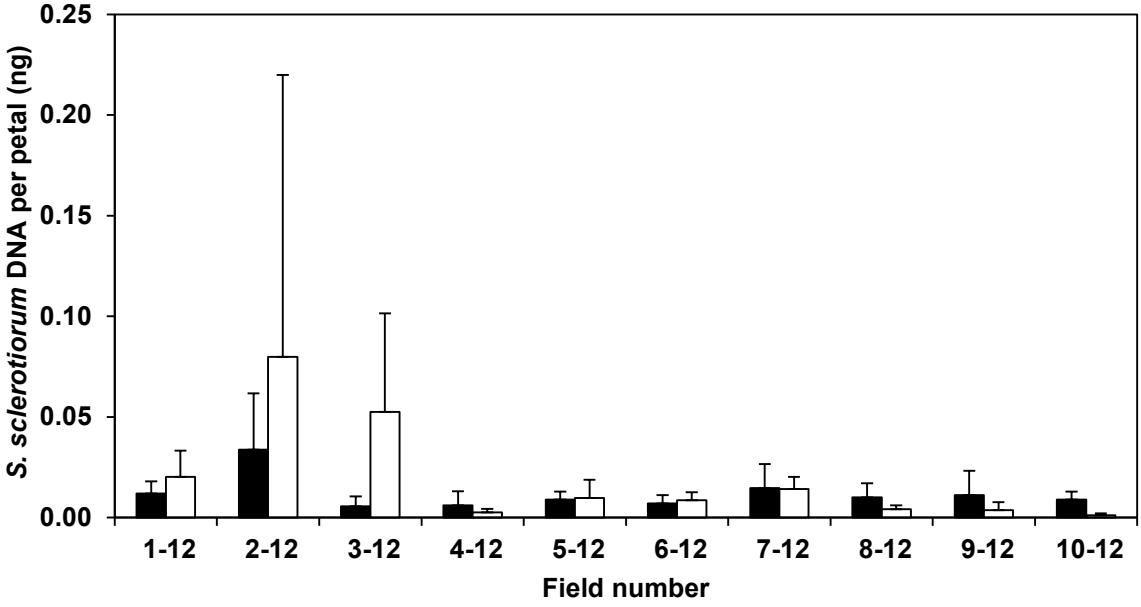

Fig. 2.3. The quantification of *Sclerotinia sclerotiorum* DNA on canola petals collected from commercial fields in the Edmonton, AB, region, as determined by qPCR analysis with the primer set SSBZF/SSBZR and hydrolysis probe SSBZP. Five sites per field in each of 10 fields were sampled at 20-30% bloom (black bars) and 40-50% bloom (white bars). The error bars represent the standard deviation of the mean for each field and reflect the amount of variation across the five sampling sites within each field.

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Chapter 3. Evaluation of PCR-based quantification of *Sclerotinia sclerotiorum* infestation levels as a tool to predict the risk of Sclerotinia stem rot of canola (*Brassica napus*) in western Canada

3.1 Introduction

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most economically important diseases of canola (*Brassica napus* L.), causing yield losses as high as 50% (del Río et al. 2007). Ascospores produced through carpogenic germination of the overwintering sclerotia serve as the primary inoculum and initiate disease development (Abawi and Grogan 1979; Bardin and Hwang 2001; Purdy 1979; Willets and Wong 1980). Airborne ascospores land on canola petals, germinate and penetrate the petal tissue. The petals senesce and fall into the crop canopy, at which point the fungus grows out of the cast petals and infects the living stem and leaf tissues (Jamaux et al. 1995; Purdy 1979). Infection of the petals has been demonstrated to be an important stage in the Sclerotinia stem rot life cycle (Jamaux et al. 1995; Morrall and Dueck 1982). Successful infection of canola is heavily dependent on the environmental conditions, particularly temperature and precipitation, to promote both the carpogenic germination of sclerotia (Abawi and Grogan 1979; Schwartz and Steadman 1978; Wu and Subbarao 2008) and the growth of the fungus (Bolton et al. 2006; Willets and Wong 1980).

In the Prairie region of western Canada, Sclerotinia stem rot is managed primarily by the routine application of fungicides. However, as a result of the influence of environmental conditions on the success of infection, Sclerotinia stem rot is not present at high levels every year. As a result, the application of fungicides without any indication of disease risk can result in fungicides being applied when Sclerotinia stem rot risk is low and fungicides are not required to prevent yield loss. To improve the sustainability and accuracy of fungicide application, a

timely and reliable risk assessment tool is required to assist producers in making informed Sclerotinia stem rot management decisions.

In Canada, several risk assessment tools have been developed for Sclerotinia stem rot of canola, including weather-based risk maps, risk point tables and an agar plate-based petal test. Weather-based risk maps take into account soil moisture, daily precipitation and air temperature to provide regional assessments of Sclerotinia stem rot risk (McLaren et al. 2004). The weather risk maps do not take into account the amount of inoculum available, but rather focus on indicating whether or not the environment is favorable for disease development. Risk point tables essentially are qualitative assessments of risk based on a variety of factors that influence the Sclerotinia stem rot disease cycle (Twengström et al. 1998). Risk point tables assign weight to factors such as crop rotation history, the amount or frequency of precipitation events, the presence or absence of apothecia in the field, and the incidence of Sclerotinia stem rot in the last susceptible crop, all of which are known to influence disease development. Although the number of apothecia represents a quantitative estimate of inoculum, disease has been found in fields in the absence of apothecia (Gugel 1986), indicating that this may not be a reliable predictor of the amount of inoculum available to initiate infection. In contrast, the agar plate test developed by Morrall and Thompson (1991) provides a quantitative assessment of risk. This method involves plating field-collected canola petals onto potato dextrose agar (PDA), incubating for 3-5 days and then calculating the percentage of petals infested with *S. sclerotiorum* based on a visual assessment. Percent petal infestation has been shown to be related to Sclerotinia stem rot incidence in canola (Gugel 1986; Turkington and Morrall 1993; Turkington et al. 1991a). However, the delay associated with the incubation period needed for fungal growth, as well as the need to correctly identify colonies of *S. sclerotiorum* emerging

from plated petals has led to limited uptake of this risk assessment tool by canola producers and agronomists in Canada.

Quantitative polymerase chain reaction (qPCR)-based methodologies can be used to measure the amount of *S. sclerotiorum* DNA on infected canola petals. Quantitative PCR analysis can provide sensitive, robust and highly reproducible quantification without the time delay associated with the traditional agar plate test (Smith and Osborn 2008). Several qPCR-based protocols have been reported for the quantification of *S. sclerotiorum* DNA on canola petals, in air, or on other substrates (Almquist and Wallenhammar 2015; Parker et al. 2014; Rogers et al. 2008; Yin et al. 2009). Most recently, a hydrolysis probe-based qPCR assay was described to measure *S. sclerotiorum* DNA in canola petals (Chapter 2). This qPCR assay was found to be highly specific to *S. sclerotiorum* and did not amplify DNA of a variety of closely related fungi. The assay was shown to accurately quantify the amount of *S. sclerotiorum* DNA in field-collected canola petals down to 8.0×10^{-4} ng of *S. sclerotiorum* DNA. Nevertheless, the relationship between the levels of petal infestation as measured by qPCR analysis and the eventual incidence of Sclerotinia stem rot in the sampled crops was not established. An evaluation of this relationship is needed if qPCR analysis of petal infestation is to serve as the basis of a Sclerotinia stem rot risk assessment system.

The primary aim of this study was to establish the relationship between levels of petal infestation, as measured at 10-20% and 40-50% bloom by qPCR analysis, and final Sclerotinia stem rot incidence at harvest, to ascertain whether the qPCR-based assay described in Chapter 2 could serve as a Sclerotinia stem rot risk assessment tool. A secondary objective was to determine whether or not the results obtained by qPCR analysis are correlated with petal infestation estimates based on the agar plate test of Morrall and Thompson (1991).

3.2 Materials and methods

3.2.1. Field selection and sample collection

Two experiments (Exp. 1 and Exp. 2) were conducted to evaluate the relationship between petal infestation level and final Sclerotinia stem rot disease severity. Experiment 1 was conducted over two years (2011 and 2012) by monitoring petal infestation and subsequent disease incidence in fields located in Alberta, Manitoba and Saskatchewan. In 2011, a total of 35 fields were sampled, with 14 fields located in Alberta [near Edmonton (9) and Lacombe (5)], 10 fields located in Manitoba [near Brandon (4), Carman (3) and Morden (3)], and 11 fields located in Saskatchewan [near Melfort (7) and Saskatoon (4)]. In 2012, a total of 34 fields were sampled, with 14 fields located in Alberta (near Edmonton (10) and Lacombe (4)), 10 fields located in Manitoba [near Brandon (5), Carman (3) and Morden (2)], and 10 fields located in Saskatchewan [near Melfort (5) and Saskatoon (5)]. The fields were not the same in each year of the study. With the exception of the fields near Edmonton, the fields in Exp. 1 were selected at random and no information was available as to whether or not fungicides were applied for Sclerotinia stem rot control during the growing season. In the fields near Edmonton, all sampling sites were located in fungicide-free check-strips, and also were included in Experiment 2 (see below). Five sampling sites were selected from each field, and were spaced approximately 50 m apart and at least 75 m from the field edge. Sampling sites were marked with either a flag, Global Positioning System (GPS) coordinates, or identified based on specific field characteristics.

Canola petals were collected at early flowering (10-20% bloom) and full flowering (40-50% bloom). Throughout this paper the first sampling date will be referred to as early bloom and the second sampling date will be referred to as full bloom. In 2012, for the fields near

Edmonton, Alberta, the first sampling date was delayed until 20-30% bloom. Samples were collected between 11:00 am and late afternoon. Fields in the Edmonton area were sampled before 11:00 am in 2012 due to high temperatures during the flowering period. No sampling occurred during or immediately after a heavy rain. At each sampling site, the top 20-30 cm of 20 randomly selected inflorescences were collected and placed into clean plastic bags, which were labelled with the field and site number and kept on ice in a cooler during transport back to the laboratory. In the laboratory, the samples were kept at 4˚C prior to processing, which was carried out within 24 h of sample collection. From each sampling site, approximately 100 randomly selected and fully open flowers and some loose petals were placed in a small plastic bag with forceps and stored at -80˚C for DNA isolation. The forceps used to select flowers and petals were dipped in 70% ethanol and flamed between individual sampling sites to avoid contamination. Sampling sites were revisited at the end of the growing season, but prior to swathing to assess the crops for Sclerotinia stem rot incidence. Twenty plants were randomly selected at each sampling site to give a total of 100 plants assessed per field. Sclerotinia stem rot disease incidence (DI) was recorded as the percentage of plants assessed per field that had any symptoms of the disease.

Experiment 2 was conducted in commercial canola fields near Edmonton, Alberta, in 2011, 2012 and 2013. The fields in 2011 and 2012 were the same fields included in Exp.1. Samples from these fields were collected from check-strips that had not been treated with fungicides. The experiment was focussed on regional validation of the qPCR assay and examining the relationship between the qPCR assay and the traditional petal test methods of determining petal infestation levels. For these analyses, nine fields were included in 2011, 10 fields in 2012, and 11 fields in 2013. These fields were farmed by a group of three growers, and

were selected because they had a history of Sclerotinia stem rot. Other than one field that was sampled in both 2011 and 2013, different fields were included in each year of the study. There were five sampling sites per field arranged 50-100 m apart in a linear fashion. The sampling sites were within a fungicide-free check-strip in each field, at least 50 m from the field edge. The fields selected were known to be at high risk for infection by *S. sclerotiorum* based on the occurrence of high levels of Sclerotinia stem rot in previous years. The seeding date and canola variety was recorded when known and were used to identify outliers in the data set. Sampling occurred twice during the flowering period, at 10-20% flower (early bloom) and 40-50% flower (full bloom) and followed the sampling protocol described above. The early bloom sampling occurred at 20-30% flower in 2012, as described above. Sampling sites were revisited to determine disease incidence at the end of the growing season, but prior to swathing as stated above.

In contrast to Exp. 1, DI for Exp.2 was defined as the percentage of plants assessed with symptoms of Sclerotinia stem rot having a severity rating of ≥ 2 on a five point rating scale, where: 0 = no symptoms, 1 = infection of pods only, 2 = lesion located on the main stem or the stem of a branch (es) with the potential of affecting up to 25% of seed formation and seed filling on the plant, 3 = lesion located on the main stem or stems of a number of branches with the potential of affecting up to 50% of seed formation and seed filling on the plant, 4 = lesion located on the main stem or stems of a number of branches with the potential of affecting up to 75% of seed formation and seed filling on the plant, and 5 = a main stem lesion with the potential of affecting seed formation and wilting of the entire plant (Kutcher and Wolf 2006). The adjusted DI values only excluded upper plant infections that would have limited impact on final yields and would be the result of late season infestations. Statistical analysis for Exp. 2 indicated

that the adjusted DI values improved the R^2 values slightly, but did not change the significance of the regression models. As a result, the calculations of DI in Exp. 1 were not adjusted as these were calculated by collaborators of this study and the raw data was not available for all locations. For Edmonton fields included in both Exp. 1 and Exp. 2, the DI values were calculated both ways.

3.2.2 Agar plate test

Petals collected in Exp. 2 were plated onto 9 cm diameter Petri dishes containing sterile potato dextrose agar (Dickinson and Company, Sparks, MD, USA), amended with 25 ppm each of ampicillin (Life Technologies, Carlsbad, CA, USA) and streptomycin (Sigma Chemical Company, St. Louis, MO, USA), as described by Turkington et al. (1988). Forty petals for each sampling site were randomly selected from the bottom of the sampling bag and from fully opened flowers, with no more than one petal from each flower. Four petals were plated with equal spacing in each Petri dish and incubated for 4-5 days in darkness at room temperature (approximately 20-24˚C). The percentage of petals in each field infested with *S. sclerotiorum* was determined through visual assessment and was calculated as the proportion of infested petals per field (out of 200).

3.2.3 DNA extraction and qPCR analysis

Total genomic DNA was extracted from 20 randomly selected petals per sampling site, following the protocol of Liang et al. (2013). Prior to DNA extraction, the petals were homogenized with a TissueLyser II (Qiagen, Toronto, ON, Canada) and a single 5 mm stainless steel bead as described in Chapter 2. To control for false negatives, each sample was spiked with 2×10^6 pSilent1 plasmids (Nakayaahiki et al. 2005) containing the *ToxB* gene from *Pyrenophora tritici-repentis* (Aboukhaddour et al. 2012), which served as an exogenous internal control

(Chapter 2). All qPCR analysis was conducted in a ViiA7 Real-Time PCR System (Life Technologies, Carlsbad, CA). The risk of a false negative due to failed DNA isolation or PCR inhibition was determined by first analyzing each sample in a qPCR assay with the *ToxB*-specific primers ToxBF (5'CCATGCTACTTGCTGTGGCTAT3') and ToxBR (5'CGCAGTTGGCCGAAACA 3'), and the hydrolysis probe ToxBP (3'CTCCCTGCTGCCCC3'), as described in Chapter 2. Variation in the internal control and the risk of a false negative were considered to be low when the standard deviation of the average threshold cycle (C_q) value for a set of samples was near or <1. For both experiments, a set of samples was defined to be all samples from all fields at a single location at each sampling date.

The amount of *S. sclerotiorum* DNA was measured by qPCR analysis with the *S. sclerotiorum*-specific primers SSBZF (5'GCTCCAGCAGCCATG GAA3') and SSBZR (5'TGTTGAAGCAGTTGACGAGG TAGT3'), and the hydrolysis probe SSBZP (5'CAGCGCCTCAAGC3'), as described in Chapter 2. A standard curve was generated with 1:10 dilutions of purified *S. sclerotiorum* DNA, enabling quantification of sample DNA in a range from 8.0 ng to 8.0×10^{-4} ng. Any sample for which no *S. sclerotiorum* DNA could be detected, or which was below the range of the standard curve, was recorded as zero. Results are expressed on a per petal basis by first accounting for the dilution, then by dividing the estimate by 20. The qPCR estimates were averaged for each field on each sampling date, with the standard deviation of the mean calculated for each field.

3.2.4 Statistical analysis

For both Exp. 1 and Exp. 2, the relationship between qPCR estimates of petal infestation and final Sclerotinia stem rot levels was determined with quadratic regression using Proc Reg in the Statistical Analysis System (SAS) version 9.3 (SAS Institute, Cary, NC, USA). Assumptions

of regression were tested and no transformations were required. Scatter plots indicated a non-linear regression. This was confirmed with lower Akaike information criterion (AIC) and Bayesian information criterion (BIC) values for quadratic regression models over simple linear regression models indicating a better fit. The AIC/BIC values were calculated for both linear and quadratic models using proc mixed and the Residual maximum likelihood (REML) method. Regression analysis to associate field average petal infestation levels as determined with qPCR analysis was conducted separately for each sampling date and year of study. Analysis was conducted separately for each year of the study due to known differences in environmental conditions. The year-to-year variation in the strength of the statistical relationship was used to determine whether or not qPCR-based estimates of petal infestation could be used to indicate Sclerotinia stem rot risk without the consideration of other factors. Regression models were considered significant when the slopes of the independent variables and the overall models were significant at $p = 0.05$.

In Exp. 1, the data were further sorted in three different ways to account for differences in the location: separated by province, by average annual precipitation, and by the average annual regional precipitation from April to August (summer precipitation). The separation of the data set was based on average 10 year (1981-2010) regional precipitation values from the nearest Environment Canada weather station (Environment Canada 2015).

The data were separated into three main groups based on average annual precipitation: > 500 mm (Carman and Morden), between 400 -500 mm (Brandon, Lacombe and Edmonton), and < 400 mm (Melfort and Saskatoon). Average summer precipitation was averaged for the months of April through August and was used to separate the data into two main groups: > 300 mm

(Morden, Edmonton, Lacombe, Brandon and Carman) and < 300 mm (Melfort and Saskatoon) of total precipitation.

In Exp. 2, the relationship between Sclerotinia stem rot DI and percent petal infestation (PPI), assessed with the plate test, at early bloom and full bloom was determined with simple linear regression using Proc Reg in SAS. Regression analysis with non-transformed DI did not meet the assumptions of regression. DI values were Arcsine transformed as discussed by Turkington and Morrall (1993), and the resulting residuals met more closely the assumptions of regression. Thus, arcsine transformed DI (TDI) was used for all regressions with PPI values. Simple linear regression models were confirmed to have lower AIC and BIC values than the quadratic regression models, indicating a better fit for the linear models. Regression analysis was conducted separately for each year of the study. Regression models were considered significant when the slopes of the independent variables and the overall models were significant at $p = 0.05$.

Correlation analysis with Proc Reg in SAS was used to determine the relationship between the amount of *S. sclerotiorum* DNA per canola petal, as determined by qPCR analysis, and the PPI values from Exp. 2. In all three years, there was a slight deviation from normality for at least one variable. As a result, Spearman Rank Correlation was used.

3.3 Results

3.3.1 Experiment 1

Sclerotinia stem rot incidence (DI) and the qPCR estimates of petal infestation for individual fields were variable over the sampling years and between locations (Table 3.1). In both 2011 and 2012, DI ranged from 0 to 92% among the different fields, but mean DI for all

fields, presented as mean DI ± standard deviation, was higher in 2012 (27.2% ± 29.5%) than in 2011 (11.9% ± 17.7%). The mean amount of *S. sclerotiorum* DNA per petal measured by qPCR analysis for all fields, presented as mean ± standard deviation, was higher at full bloom in both 2011 (1.3×10^{-2} ng ± 1.8×10^{-3} ng) and 2012 (6.8×10^{-2} ng ± 1.8×10^{-1} ng). There was variation in the amount of *S. sclerotiorum* DNA per canola petal as measured by qPCR over the flowering period, as well as between fields and between sampling sites within fields (Figs. 3.1 – 3.3). When the data were separated by province, none of the relationships between the qPCR results and DI were significant at either sampling date in 2011 (data not shown). In 2012, however, the relationship between the early bloom petal infestation level determined by qPCR and DI for the fields in Alberta was found to be significant, with the early bloom qPCR results accounting for 59.1% of the variation in DI (regression coefficient (R^2)= 0.591; p = 0.0073; CV = 42.694). The regression models for the Alberta fields at full bloom and for the Saskatchewan and Manitoba fields at both sampling dates were not significant (data not presented).

When data from Exp.1 were separated by average annual precipitation, none of the relationships were significant for the locations with 400-500 mm annual precipitation in 2011 and 2012, or >500mm of precipitation in 2012. The regression analysis for locations with >500mm of annual precipitation at full bloom in 2011 could not be conducted using a quadratic model because of the large number of fields with a DI of 0%. For this group of locations, the simple linear regression was analyzed and found not to be significant (P>0.05). For locations in 2011 with <400 mm of average annual precipitation, the relationship between full bloom petal infestation levels as determined by qPCR analysis and DI was found to be significant, with the full bloom qPCR results accounting for 57.6% of the variation in DI (R^2 = 0.576; p = 0.0356; CV = 95.559). At early bloom in 2011, and at both early bloom and full bloom in 2012, the

relationship between petal infestation levels determined by qPCR analysis and DI for locations with <400 mm of average annual precipitation was not found to be significant.

When the data were separated by average summer precipitation, none of the regression models were significant in 2011 ($p > 0.05$). In 2012, the regression models for the group of locations with average summer precipitation levels > 300 mm were significant at both early bloom ($R^2 = 0.263$; $p = 0.035$; CV = 95.116) and full bloom ($R^2 = 0.244$; $p = 0.0459$; CV 96.301).

3.3.2 Experiment 2

Sclerotinia stem rot incidence, PPI and the estimates of petal infestation based on qPCR analysis were variable over the three years of the study (Table 3.2). Mean DI across all fields, presented as mean DI ± standard deviation, was lower in 2011 (7.8% ± 6.6%) than in 2012 and 2013 (64% ± 23.2% and 39.4% ± 24.8% respectively). For both sampling dates, petal infestation levels determined through qPCR analysis also were lowest in 2011 (early bloom 5.0×10^{-3} ng± 5.0×10^{-3} ng; full bloom 1.7×10^{-2} ng ± 51.3×10^{-2} ng). On a field scale, petal infestation levels determined by qPCR analysis were higher at full bloom than at early bloom for all 9 fields in 2011, for 5 of 10 fields in 2012, and for 5 of 11 fields in 2013. Variability in petal infestation levels was observed over the flowering season, as well as between and within fields as in Exp. 1. Mean PPI, as assessed by the agar plate test (Morrall and Thompson 1991), was similar across both sampling dates in 2011 (early bloom 35.9% ± 22.3%; full bloom 40.1% ± 6.8%) and 2012 (early bloom 34.3% ± 17.1%; full bloom 41.2% ± 15.1%). In 2013, mean PPI was generally higher than in the previous years (although no statistical analysis was conducted to determine if this difference was significant), but was still fairly similar across the two sampling dates (early bloom 43.9% ± 18.2%; full bloom 51.0% ± 18.5%). A comparison of petal infestation levels as

determined by qPCR analysis versus the agar plate test revealed similar results in 2011 and 2012: both methods indicated that petal infestation levels were lower at early bloom than at full bloom. In 2013, however, the different methods yielded different results. Petal infestation was found to be lower at early bloom when evaluated by the agar plate test and lower at full bloom when evaluated by qPCR analysis.

3.3.3 Relationship between DI and petal infestation levels determined by qPCR analysis

In each year of Exp. 2, three quadratic regression models were analyzed to determine the relationship between petal infestation levels, as determined by qPCR analysis, and final DI in the field. In all three years, DI increased as the amount of *S. sclerotiorum* DNA per canola petal increased (Figs. 3.4 and 3.5). However, the strength of the relationship varied with year and was not always significant. In all years, there was a high degree of variation with respect to both the final DI and petal infestation levels in the sampled fields. In 2011, none of the regression models were significant at $p = 0.05$ when all fields were included in the analysis. However, in that year, the field with the highest DI had a very low level of petal infestation (Fig. 3.4). This data point at full bloom fell outside of the predicted 95% confidence interval, and as a result was removed from the analysis as an outlier. When this outlier was removed, the relationship between DI and the level of petal infestation at full bloom was found to be significant ($R^2 = 0.919$; $p = 0.002$; CV = 27.21). Similarly, in 2012, there also was no significant statistical relationship between petal infestation and DI at early bloom, but a significant relationship existed between these parameters at full bloom ($R^2 = 0.602$; $p = 0.017$; CV = 22.91) (Fig. 3.4; Table 3.3).

In 2013, there was no significant statistical relationship at either early bloom or full bloom when all fields were included in the analysis. However, as in 2011, there was one outlier field. This data point also fell outside of the predicted 95% confidence interval and was removed

from the statistical analysis. When this outlier was removed, the relationship between petal infestation levels at full bloom and DI became significant ($R^2 = 0.605$; $p = 0.039$; CV = 40.79) (Fig. 3.4; Table 3.3). Some fields were seeded later in 2013 (May 9 to May 20) versus 2011 (May 6 to May 17) and 2012 (May 2 to May 16). When only the early seeded fields (i.e., those seeded on or prior to May 15) were included in the analysis, the regression was found to be significant at full bloom ($R^2 = 0.9204$; $p = 0.0063$; CV = 20.83) (Fig. 3.5; Table 3.3).

3.3.4 Relationship between PPI and transformed DI (TDI)

In Exp.2, in both 2011 and 2013, there was not a significant statistical linear relationship between PPI and TDI at either early bloom or full bloom. This was still true when late seeded fields in 2013 were excluded from the analysis. In 2012, the relationship was significant only at full bloom ($R^2 = 0.682$; $p = 0.003$; CV = 16.19).

3.3.5 Relationship between PPI and qPCR estimates of petal infestation

Correlation analysis was used to determine the relationship between the level of petal infestation, as assessed by qPCR analysis, and PPI as assessed by the agar plate test (data not shown). In 2011, the only significant correlation was at early bloom (Correlation coefficient (r) = 0.71; $p = 0.03$). In 2012, the correlation was not significant at early bloom, but was significant at full bloom ($r = 0.80$; $p = 0.0056$). In 2013, there was no significant correlation at either early bloom or full bloom.

3.5 Discussion

In Canada, risk assessment tools for Sclerotinia stem rot of canola have focussed on a variety of factors known to influence the incidence of the disease in the field. However, only one of these risk assessment tools, the agar plate test developed by Morrall and Thompson

(1991a), involves a quantitative estimation of the amount of inoculum present during a critical period of the disease cycle. This test provides an estimate of petal infestation, which has been demonstrated to be related strongly to Sclerotinia stem rot DI at the end of the growing season (Turkington and Morrall 1993; Turkington et al. 1991a). Fungicides are applied for control of Sclerotinia stem rot during flowering. An estimation of petal infestation should provide an indication of disease risk during the period over which fungicide spray decisions need to be made. However, the 3-5 day incubation period associated with the traditional petal testing procedure means that the results may become available only after the optimal time for fungicide application has passed (Wallenhammar et al. 2007). The correct identification of *S. sclerotiorum* colonies from plated petals also may prove difficult for farmers or crop consultants lacking expertise in mycology. In contrast, an evaluation of field collected canola petals by qPCR analysis can provide an accurate estimate of inoculum pressure within a single day.

In all fields and locations included in this study, there was variation in both the amount of *S. sclerotiorum* DNA detected by qPCR analysis and in the final DI. There also was significant year-to-year variation in these parameters, most likely reflecting differences in environmental conditions, which in turn influence carpogenic germination of the sclerotia and the timing of ascospore release (Abawi and Grogan 1979; Schwartz and Steadman 1978; Wu and Subbarao 2008). Variation in DI and petal infestation levels between fields also could reflect differences in crop stand density, seeding date, seeding rate, and crop and disease history, all of which can influence Sclerotinia stem rot development (Jurke and Fernando 2008; Turkington and Morrall 1993; Twengström et al. 1998). In addition to field-to-field variation, variation across sampling sites within particular fields also was observed. This variation likely reflected differences in crop stand and microclimate at different points within the same field, as well as possible

inoculum gradients if populations of germinated sclerotia varied spatially within fields or in adjacent fields. Five sites were sampled per field, since this was reported to be an accurate sampling size for evaluating the incidence of petal infestation (Turkington et al. 1988). The large amount of variation observed in this study, however, indicates that inclusion of more sampling sites would be able to provide a more reliable estimate of petal infestation in a particular field.

The amount of *S. sclerotiorum* DNA quantified per canola petal varied across the flowering season. These findings are consistent with previous reports that found that inoculum pressure is not consistent across the flowering period or between fields (Almquist and Wallenhammar 2015; Turkington and Morrall 1993). Differences in the level of infestation over the growing season will influence the strength of the statistical relationship between quantifications of petal infestation and DI when evaluated across multiple fields. Monitoring changes in petal infestation over the flowering period may serve to identify the best timing for fungicide application in a particular field, but such an approach would have to be balanced by cost and other practical considerations. Nonetheless, closer monitoring of infestation levels on a temporal scale could prove useful to better understand the epidemiology of Sclerotinia stem rot of canola.

The relationship between PPI as determined by the agar plate test and petal infestation as assessed by qPCR analysis was not always linear. An analysis of correlation indicated a significant amount of shared variation between the two methods only at early bloom in 2011 and at full bloom at 2012. Similarly, Almquist and Wallenhammar (2015) reported no correlation between the results of the agar plate test and a qPCR-based *S. sclerotiorum* detection method. There are several possible explanations for these differences. For example, the presence of a few

highly infected canola petals in a sample would be reflected in a higher concentration of *S. sclerotiorum* DNA in the qPCR analysis, but would not be reflected in the agar plate test results (Almquist and Wallenhammar 2015). Furthermore, while both the number of ascospores and the amount of mycelium present in a sample can be measured by qPCR analysis, the agar plate test can only indicate whether or not a petal is infested. Finally, when levels of *S. sclerotiorum* infestation are low on infected petals, other fungal species (e.g. *Rhizopus* spp., *Mucor* spp., *Trichoderma* spp.) that are present on the petal tissue may outgrow colonies of *S. sclerotiorum*, thus masking the presence of *S. sclerotiorum*. Given the increased sensitivity of the qPCR-based method, detection and direct measurement of *S. sclerotiorum* by qPCR analysis are more likely to be influenced by the environment in which those petals were produced.

The strength of the statistical relationship between qPCR-based measurement of petal infestation and final stem rot incidence in the field is critical to assessing the suitability of the former for predicting the risk of disease. In Exp. 1, the strength of the relationship between the qPCR results and disease incidence across the Prairies was variable across the three years of the study. Moreover, when the data were analyzed separately based on the historical 10 year average annual or summer precipitation levels in a particular region, the strength of the relationship was not increased. However, small-scale variation in precipitation levels due to localized rainfall or thundershowers could result in differences in risk within a region, and thus will need to be accounted for in a Sclerotinia stem rot forecasting system. It is important to note that, with the exception of the fields in the Edmonton region, there was no information available regarding fungicide application in the fields included in Exp. 1. This could have had a large impact on the ability to detect a significant relationship. For instance, a canola field in which there was heavy petal infestation may have been treated with fungicide, preventing or greatly

reducing Sclerotinia stem rot development. As such, the results of Exp. 1 must be treated with caution. In Exp. 2, all sampling was carried out in check-strips that were not treated with fungicide. Therefore, the results of this experiment may provide a better indication of the true relationship between DI and petal infestation as measured by qPCR analysis.

In Exp. 2 the relationship between these two parameters was stronger, with Sclerotinia stem rot incidence generally found to be higher in fields where petal infestation (*S. sclerotiorum* DNA per canola petal) also was higher. Nonetheless, the strength of this relationship varied across the years, and was found to be significant at full bloom, but not at early bloom in all three years of study, following the removal of any statistical outliers from the data set. In both 2011 and 2013, one field was identified as an outlier and was removed from the analysis. In both cases the outlier had a low level of petal infestation but a high DI. The existence of such outliers is important to consider if a forecasting model is to be developed based on qPCR assessment of petal infestation. The occurrence of a high DI at harvest time in fields that had a low level of petal infestation at full bloom could reflect peaks in *S. sclerotiorum* inoculum that took place outside of the petal sampling dates, perhaps due to differences in seeding date or microclimate within a particular field. To better understand the reason for the occurrence of such outliers, the influence of seeding date and crop canopy characteristics on petal infestation levels and final DI needs to be investigated further.

Seeding of some fields occurred later in 2013 than in either 2011 or 2012, and this was found to influence the statistical relationship in Exp. 2. When only the early seeded fields were included in the analysis, the relationship was significant at full bloom, which was consistent with the results in 2012. Furthermore, the results of this study suggest that differences in seeding dates may influence the risk of the development of Sclerotinia stem rot. This is supported by the

findings of Morrall and Dueck (1982), who found that differences in seeding dates between fields may result in differences in Sclerotinia stem rot incidence, as a result of differences in the crop stage and canopy closure when the environment is conducive to apothecial development and ascospore release.

Variation in petal infestation and Sclerotinia stem rot incidence across the sampling years also suggests that measures of environmental conditions should be included in the model using multiple regression analysis, to fully account for year to year variation in weather. More broadly, comparison of the results obtained in Exp. 1 and Exp. 2 suggests that qPCR-based assessments of Sclerotinia stem rot risk are more reliable on a smaller regional scale, and that differences in environment between locations should be considered when setting risk assessment thresholds. Furthermore, the qPCR-based assessments of petal infestation levels at full bloom were more consistently related to final disease incidence than petal infestation levels determined with the agar plate test. While the qPCR assay described in Chapter 2 may serve as the basis for a risk assessment system, it is important to emphasize that any forecasting system based on petal infestation should be linked to environmental conditions, as well as to cropping history, seeding date and crop canopy conditions, which may influence Sclerotinia stem rot development and the need to spray a fungicide.

3.6 Tables

Table 3.1. Average, minimum and maximum values for measurements of Sclerotinia stem rot disease incidence (DI) on canola and petal infestation levels at early bloom (EB) and full bloom (FB) across multiple fields at various locations in Alberta (AB), Saskatchewan (SK), and Manitoba (MB), in 2011 and 2012 (Experiment 1).

Location	Descriptive statistics	Number of fields	2011			Number of fields	2012		
			DI (%)[a]	Petal infestation level (ng)[b]			DI (%)[a]	Petal infestation level (ng)[b]	
				EB	FB			EB	FB
Edmonton, AB	Average		8.67	0.005	0.017		64.8	0.012	0.02
	Minimum		1.00	0	0.003		29	0.006	0.001
	Maximum		21.00	0.013	0.038		92	0.034	0.080
	Sample size[c]	9				10			
Lacombe, AB	Average		11	0.001	<0.001		15.5	0.001	0.016
	Minimum		0	0	0		0	0	0.001
	Maximum		40	0.002	0.001		35	0.003	0.475
	Sample size	5				4			
Melfort, SK	Average		28.860	0.012	0.025		1.8	0.037	0.030
	Minimum		4	0.001	0.005		0	0.002	0.001
	Maximum		92	0.017	0.047		4	0.087	0.130
	Sample size	7				5			
Saskatoon, SK	Average		6	0.001	0.009		34.4	0.046	0.382
	Minimum		3	0	0.004		26	0.004	0.017
	Maximum		11	0.004	0.018		38	0.190	0.893
	Sample size	4				5			
Brandon, MB	Average		0	0.002	0.012		2.4	0.007	0.004
	Minimum		0	0	0		0	0.002	0.002
	Maximum		0	0.006	0.082		11	0.016	0.006
	Sample size	4				5			
Carman, MB	Average		10.667	0.001	0		5	0.005	0.001
	Minimum		5	0	0		1	0.004	0
	Maximum		20	0.003	0		11	0.006	0.001
	Sample size	3				3			
Morden, MB	Average		12	0.003	0.001		14.5	0.008	0.004
	Minimum		1	0.001	0		11	0.004	0.001
	Maximum		21	0.006	0.002		18	0.011	0.007
	Sample size	3				2			
All fields	Average		11.89	0.005	0.013		27.2	0.017	0.068
	Minimum		0	0	0		0	0	0
	Maximum		92	0.017	0.082		92	0.190	0.893
	Sample size								

[a] DI was defined as the percentage of plants with Sclerotinia stem rot symptoms.

[b] Petal infestation level represents the amount of *S. sclerotiorum* DNA (ng) per canola petal as determined by qPCR analysis according to the protocol described in Chapter 2.

[c] Sample size refers to the number of fields samples at each location

Table 3.2. Average, minimum and maximum values for Sclerotinia stem rot disease incidence (DI) on canola, and petal infestation level across 9, 10 and 11fields in central Alberta, in 2011, 2012 and 2013 respectively (Experiment 2).

Year	Descriptive statistics	Number of fields	DI[a]	Petal infestation level (ng)[b]		PPI[c] (%)	
				Early bloom	Full bloom	Early bloom	Full bloom
2011	Average		7.78	0.005	0.017	35.9	40.11
	Minimum		1	0	0.003	13	33.5
	Maximum		20	0.013	0.038	68.5	52
	Sample size[d]	9					
2012	Average		64	0.012	0.020	34.3	41.2
	Minimum		29	0.006	0.003	21	19
	Maximum		92	0.034	0.080	74.5	63.5
	Sample size	10					
2013	Average		39.64	0.081	0.051	43.9	51
	Minimum		12	0.006	0.014	18.0	25.4
	Maximum		88	0.259	0.181	73.1	79
	Sample size	11					

[a]DI was defined as the percentage of plants with Sclerotinia stem rot symptoms rated as ≥2 on the 5 point scale scale of Kutcher and Wolf (2006) where 0 = no symptoms and 5 = a main stem lesion with the potential of affecting seed formation and willing of the entire plant.

[b]Petal infestation level represents the amount of *S. sclerotiorum* DNA (ng) per canola petal as determined by qPCR analysis according the protocol described in Chapter 2.

[c]PPI is the percent petal infestation, as determined by culturing canola petals on solid agar medium according to Morrall and Thompson (1991).

[d] Sample size refers to the number of fields samples at each location

Table 3.3. Significant regression models for the relationship between the amount of *Sclerotinia sclerotiorum* DNA per canola petal, as determined by qPCR analysis at early bloom (EB) and full bloom (FB), and the disease incidence (DI) of Sclerotinia stem rot for fields located near Edmonton, AB (Experiment 2).

Year	Regression equation	Coefficient of determination (R^2)	Model Significance (p-value)	Coefficient of variation
2011	DI[d]=3.72-248.73MFB_q[a] +14874.0MFB_qsq[b]	0.919	0.002	27.22
2012	DI= 41.37+3137.45MFB_q - 39660.0MFB_qsq	0.602	0.017	22.91
2012	TDI[e]=0.37+0.01MFBPPI[c]	0.682	0.003	16.19
2013	DI= 14.66+495.68MFB_q - 1193.20MFB_qsq	0.605	0.039	40.79
2013ES[f]	DI=1.49+1103.11MFB_q – 4181.96MFB_qsq	0.920	0.006	20.83

[a]MFB_q = Mean petal infestation measured as ng of *Sclerotinia sclerotiorum* DNA per canola petal as determined by qPCR analysis at FB according to Chapter 2.

[b]MFB_qsq = Mean petal infestation squared

[c]MFBPPI = is the mean percent petal infestation, as determined by culturing canola petals on solid agar medium according to Morrall and Thompson (1991).

[d]DI = the percentage of plants with Sclerotinia stem rot symptoms rated as ≥2 on the 5 point scale scale of Kutcher and Wolf (2006) where 0 = no symptoms and 5 = a main stem lesion with the potential of affecting seed formation and willing of the entire plant.

[e]TDI = arcsine transformed DI.

[f]2013ES = includes only those fields that were seeded on or before May 15th, 2013

3.7 Figures

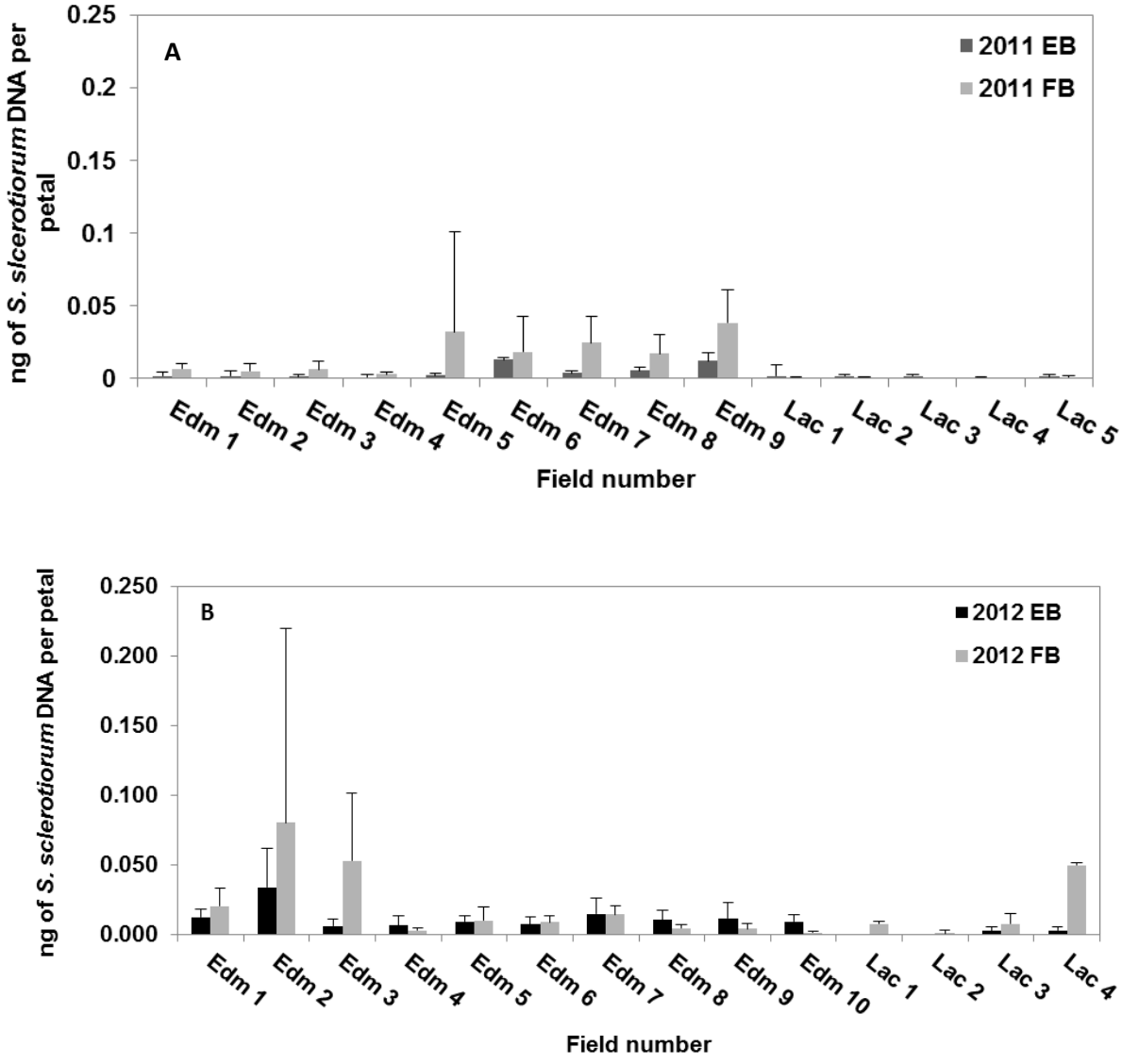


Fig. 3.1. Amount of *Sclerotinia sclerotiorum* DNA per canola petal, as determined by qPCR analysis, in fields near Edmonton (Edm) and Lacombe (Lac), Alberta, at early bloom (EB) and full bloom (FB) in (A) 2011 and (B) 2012 for Experiment 1. The error bars represent the standard deviation of the mean for each field.

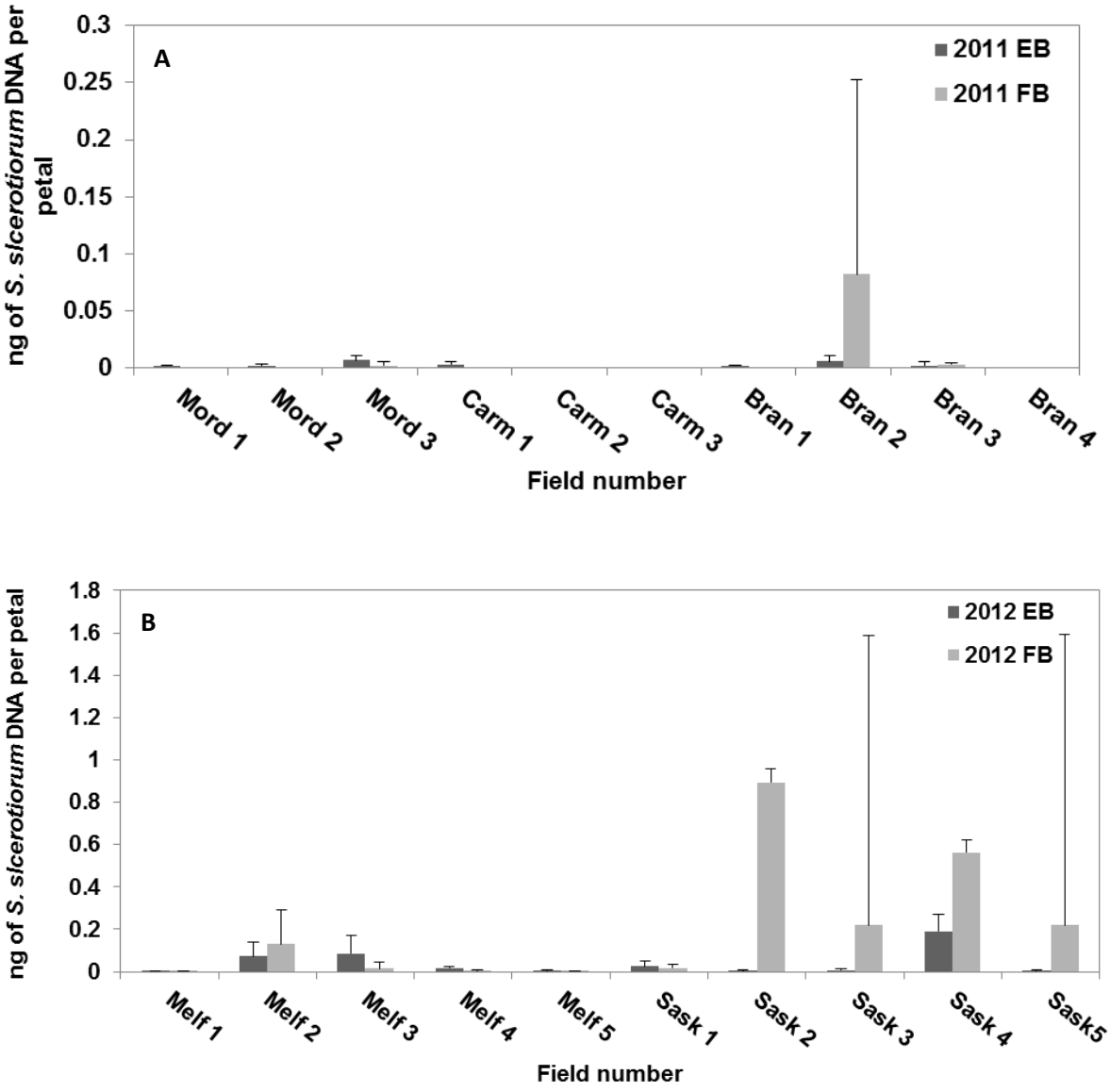


Fig. 3.2. Amount of *Sclerotinia sclerotiorum* DNA per canola petal, as determined by qPCR analysis, in fields near Melfort (Melf) and Saskatoon (Sask), Saskatchewan, at early bloom (EB) and full bloom (FB) in 2011 (A) and 2012 (B) for Experiment 1. The error bars represent the standard deviation for each field.

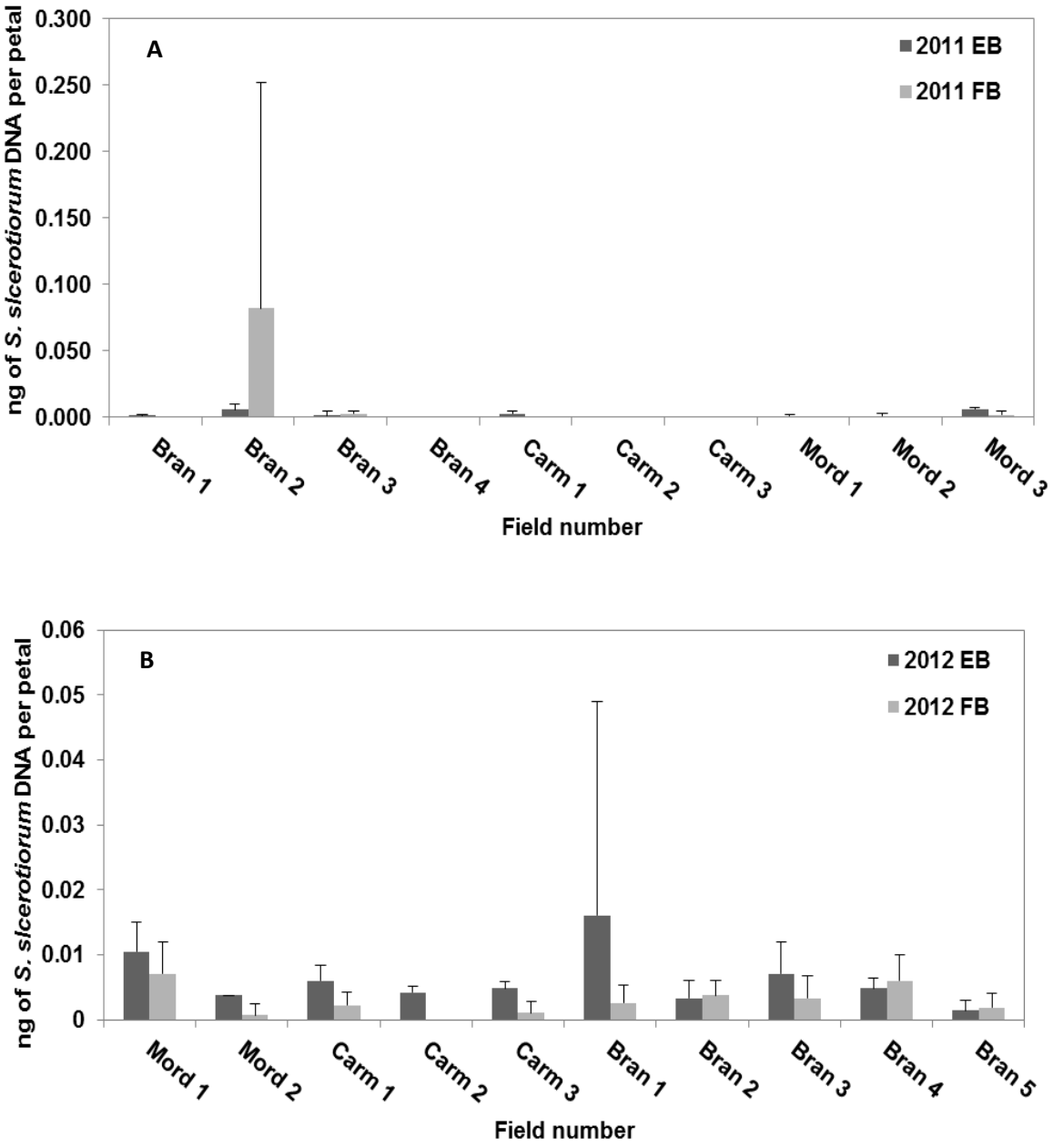
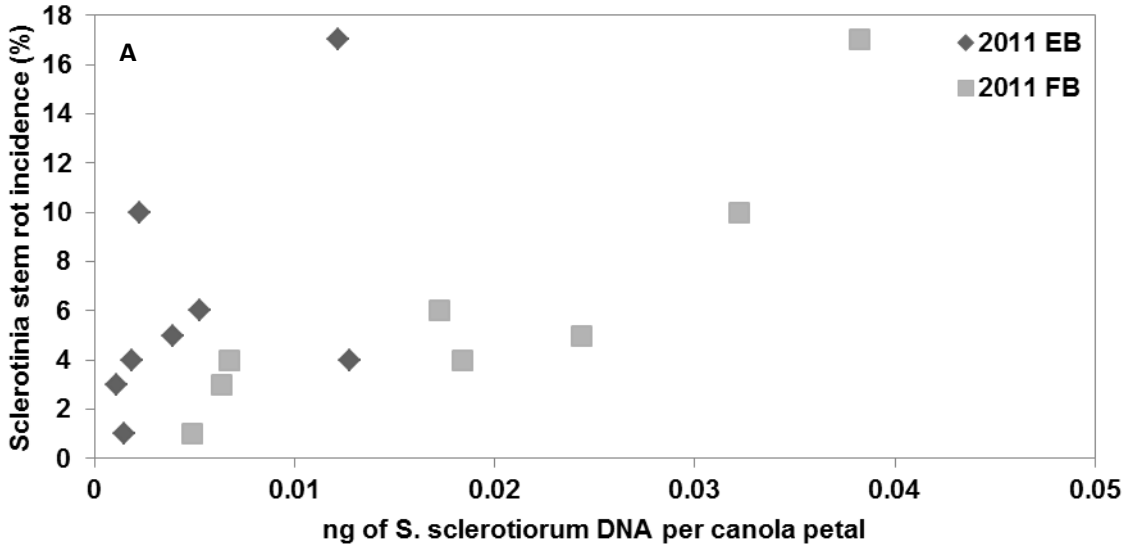
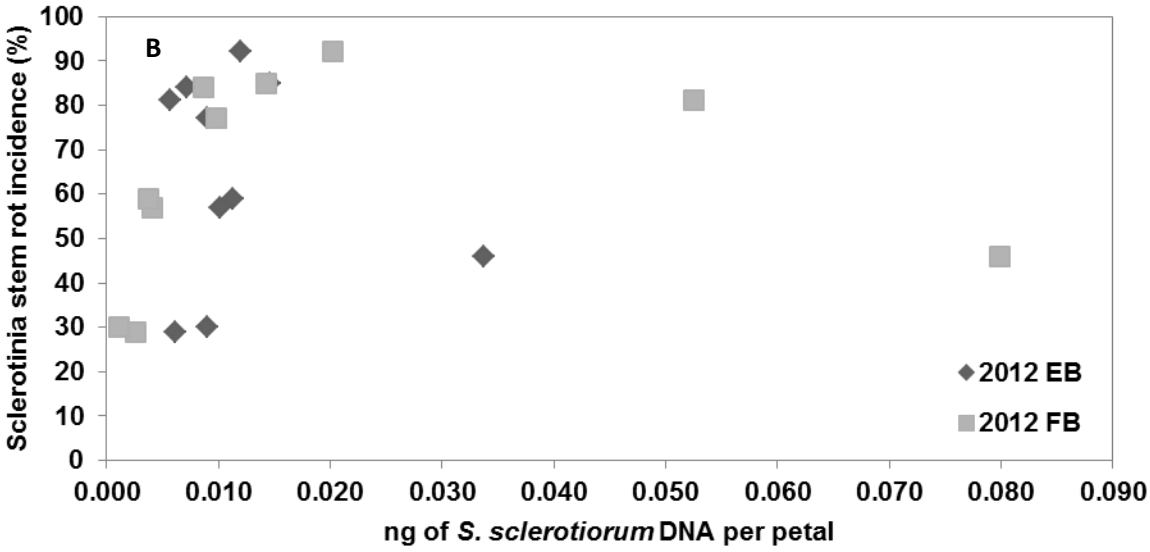


Fig. 3.3. Amount of *Sclerotinia sclerotiorum* DNA per canola petal, as determined by qPCR analysis, in fields near Morden (Mor), Carman (Carm) and Brandon (Bran), Manitoba, at early bloom (EB) and full bloom (FB) in 2011 (A) and 2012 (B) for Experiment 1. The error bars represent the standard deviation for each field.


A
2011 EB
2011 FB
Sclerotinia stem rot incidence (%)
ng of S. sclerotiorum DNA per canola petal
0
2
4
6
8
10
12
14
16
18
0
0.01
0.02
0.03
0.04
0.05


B
2012 EB
2012 FB
Sclerotinia stem rot incidence (%)
ng of S. sclerotiorum DNA per petal
0
10
20
30
40
50
60
70
80
90
100
0.000
0.010
0.020
0.030
0.040
0.050
0.060
0.070
0.080
0.090

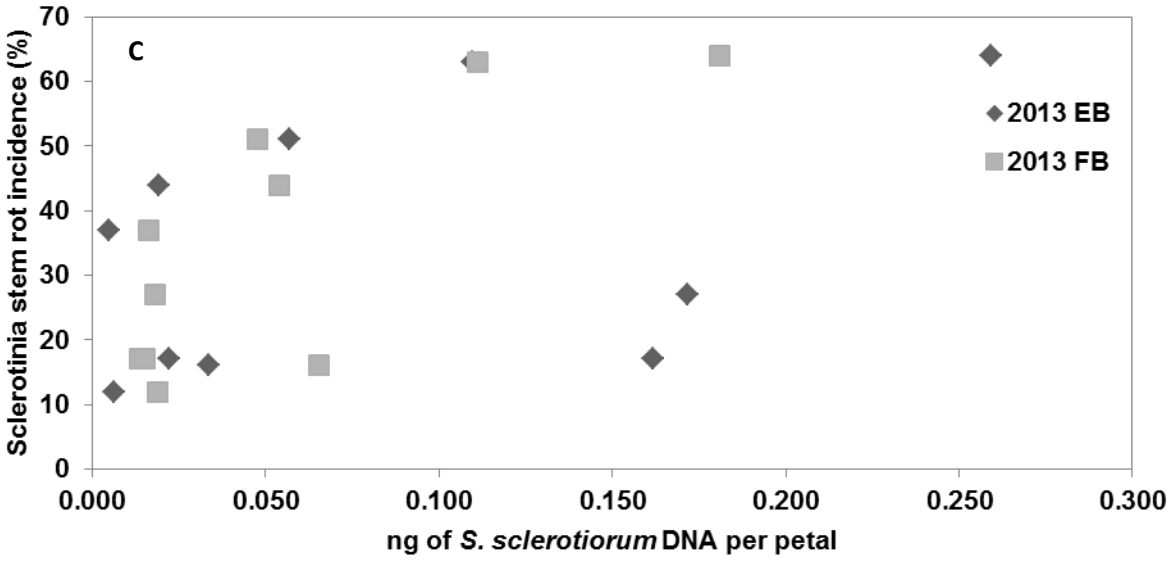


Fig. 3.4. Relationship between the amount of *Sclerotinia sclerotiorum* DNA per canola petal, as measured by qPCR analysis, and the incidence of Sclerotinia stem rot in fields in the Edmonton, Alberta, region in (A) 2011, (B) 2012 and (C) 2013 (Experiment 2). Measurements of petal infestation were made at early bloom (EB) and full bloom (FB). The relationship between petal infestation and disease incidence was found to be significant only at FB in 2011 ($R2 = 0.919$; $p = 0.002$; Coefficient of variation (CV) = 27.22), 2012 ($R2 = 0.602$; $p = 0.017$; CV = 22.91) and 2013 ($R2 = 0.605$; $p = 0.039$; CV = 40.79). In both 2011 and 2013, one field was removed from the data set as an outlier and is not shown.

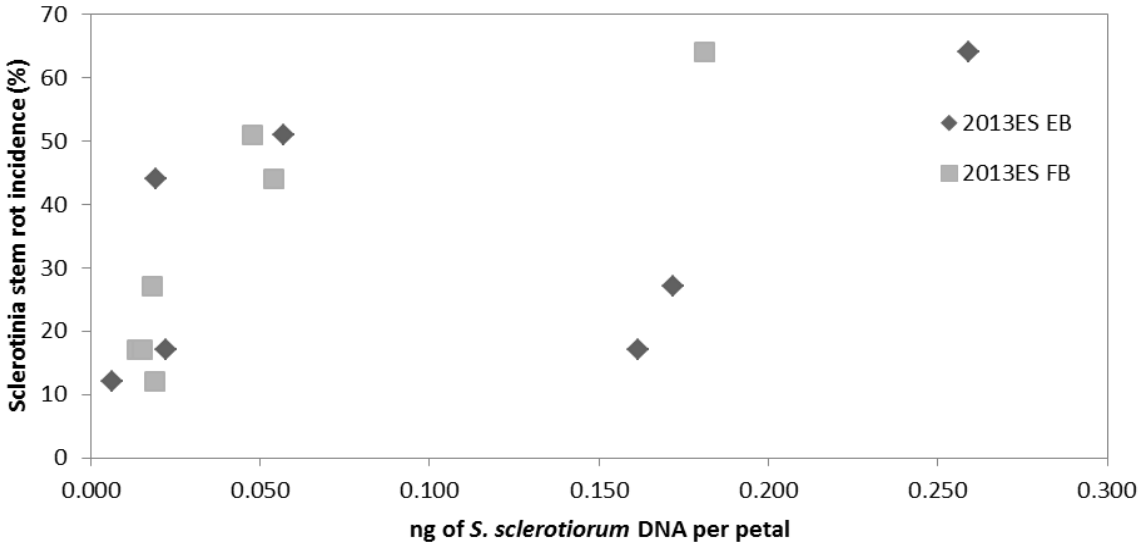


Fig. 3.5. Relationship between the amount of *Sclerotinia sclerotiorum* DNA per canola petal, as determined by qPCR analysis, and the incidence of Sclerotinia stem rot in fields near Edmonton, Alberta, which were early seeded (ES; i.e., seeded prior to May 15) in 2013 (Experiment 2). Measurements of petal infestation were made at early bloom (EB) and full bloom (FB). There was a significant relationship between the level of petal infestation (amount of *S. sclerotiorum* DNA) and final Sclerotinia stem rot incidence at full bloom (FB) ($R2 = 0.920$; $p = 0.006$; $CV = 20.834$).

3.8 References

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Chapter 4: The influence of sampling date, time, petal age and environmental conditions on canola petal infestation by *Sclerotinia sclerotiorum* and development of Sclerotinia stem rot

4.1 Introduction

Sclerotinia sclerotiorum is the causal agent of Sclerotinia stem rot, an economically important disease of canola. In the Prairies region of Canada, the incidence of Sclerotinia stem rot has been reported to be as high as 92% (Chapter 2). Infection of host petals by ascospores of the pathogen, produced via the carpogenic germination of sclerotia, has been demonstrated to be an important stage in the disease cycle and initiation of infection in canola (Bom and Boland 2000; Gugel 1986; Jamaux et al. 1995; Turkington and Morrall 1993; Turkington et al. 1991a). Sclerotinia stem rot incidence in the field is sporadic and varies from year-to-year, region-to-region, field-to-field and site-to-site within fields (Chapter 2; Morrall and Dueck 1982; Turkington et al. 1988; Turkington et al. 1991a, 1991b). This sporadic occurrence reflects the influence of environmental conditions on carpogenic germination of the sclerotia, ascospore production and release, as well as on the infection of canola leaves and stems (Abawi and Grogan 1979; Clarkson et al.2003; Willets and Wong 1980). Fungicides are often applied for the management of Sclerotinia stem rot in canola crops, usually without any indication of disease risk. To assist canola producers in making informed fungicide application decisions, a reliable and functional risk assessment tool is required.

An agar plate-based method was developed by Morrall and Thompson (1991a) to obtain estimates of the incidence of petal infestation by *S. sclerotiorum*. This method was based on the visual identification of the fungus, and was shown to be able to forecast final Sclerotinia stem rot disease incidence (DI) in the field (Turkington and Morrall 1993; Turkington et al. 1991a). However, although a strong relationship between the incidence of petal infestation and DI was

evident, the strength of the relationship was influenced by the amount of rainfall and the density of the crop canopy, resulting in year-to-year variation in the accuracy of Sclerotinia stem rot risk forecasts (McLaren et al. 2004; Turkington and Morrall 1993; Turkington et al. 1991a). Variation in DI between and within fields also occurs and likely reflects differences in seeding rate, plant density and the degree of lodging (Jurke and Fernando 2008; Turkington and Morrall, 1993). This suggests that a reliable forecasting system for Sclerotinia stem rot of canola should include a field level quantification of petal infestation, but also needs to account for environmental conditions and crop canopy characteristics that are known to influence disease development.

Recently, a hydrolysis probe-based quantitative polymerase chain reaction (qPCR) assay was developed to quantify the amount of *S. sclerotiorum* DNA in field-collected canola petals (Chapter 2). The assay was very sensitive and highly specific for *S. sclerotiorum* quantification, and estimates of petal infestation could be obtained within a day after collection of samples from the field. The rapid turn-over time from sample collection to the availability of results was in contrast with the traditional agar plate-based petal test, which requires a 3-5 day incubation period to allow for fungal growth. Evaluation of canola petal samples collected from the Edmonton, Alberta, region with the qPCR assay indicated that Sclerotinia stem rot incidence tended to increase as the amount of *S. sclerotiorum* DNA per canola petal increased (Chapter 3). The relationship was significant at full bloom, following the removal of statistical outliers, with petal infestation levels accounting for between 60 and 92% of the variation in DI over the three years of the study (Chapter 3). This year-to-year variability in the strength of the relationship was consistent with results obtained using the traditional agar plate test (McLaren et al. 2004; Turkington and Morrall 1993; Turkington et al. 1991a), indicating that there is a strong influence

of the environment on the relationship between petal infestation and final DI. Interestingly, no consistent correlation was observed between the amount of *S. sclerotiorum* DNA per canola petal, as measured by qPCR analysis, and the incidence of petal infestation as determined with the agar plate test (Chapter 3). The poor correlation between the two methods could result from differences in their capacity to distinguish between dead and living pathogen propagules (ascospores and mycelia), misidentification of infested petals using the agar plate test, and/or competition with other fungi present on the petals which may preclude the growth and identification of *S. sclerotiorum* in culture.

The timing of petal collection, the age of the petals, and environmental conditions may influence the amount of *S. sclerotiorum* DNA detected on field collected petals. A diurnal fluctuation in ascospore release has been documented and may affect the level of petal infestation at different times of the day (Qandah and del Río Mendoza 2011; Turkington et al. 1991b), which may in turn influence the relationship between petal infestation and final DI in the field. Jamaux et al. (1995) found that *S. sclerotiorum* ascospores germinated 3 h after inoculation of the adaxial surface of canola petals, and that hyphae were evident on the other side of the petal after 20-30 h. Both sides of the canola petals were covered in hyphal growth 72 h after inoculation. This suggests that younger, newly emerged petals will contain less *S. sclerotiorum* DNA than older petals, which may have been infected earlier. Thus, the timing of sampling and the age of the petals collected may influence the consistency and strength of the relationship between petal infestation levels, as determined by qPCR analysis, and final DI in the field.

To minimize year-to-year variation in the accuracy of Sclerotinia stem rot risk assessments, a forecasting system based on qPCR quantification of petal infestation should be

linked to environmental conditions, as well as to cropping history, seeding date and crop canopy conditions. The purpose of this study was to evaluate the impact of environmental conditions (temperature, humidity and precipitation), crop canopy characteristics (plant density and lodging), the timing of sampling (morning vs. afternoon) and petal age on the level of petal infestation as determined by qPCR analysis. Specifically, the objectives of this study were to: 1) assess if there is a difference in the amount of *S. sclerotiorum* DNA on canola petals collected in the morning vs. the afternoon; 2) determine if petal age has an influence on the amount of *S. sclerotiorum* DNA detected by qPCR and whether this affects the observed relationship between petal infestation and DI; 3) investigate the influence of temperature and relative humidity (RH) on petal infestation over the flowering period; 4) identify the optimal petal age and sampling time for qPCR-based assessments of Sclerotinia stem rot risk; and 5) use multiple regression analysis to determine the influence of precipitation, plant density and lodging on the relationship between petal infestation levels determined by qPCR analysis and final DI in the field.

4.2 Materials and methods

4.2.1 Field selection and sample collection

In 2014, intensive sampling was conducted in five commercial canola crops near Edmonton, AB. Each crop was located in a field with a history of Sclerotinia stem rot, but which in at least four cases had not been seeded to canola in the previous growing season. The five crops were planted between May 8th and May 20th, 2014 (field 1 seeded on May 8th, fields 2 and 3 seeded on May 20th, field 4 seeded on May 16th, and field 5 seeded on May 17th). Fields 1 to 4 had been last seeded to canola in 2012 (Chapter 2), while the last year in which canola had been grown in field 5 was not known. A total of 15 sampling sites were selected per field. These included 10 sampling sites located in a linear fashion along the centre of a fungicide-free check-

strip in the centre of each field, and an additional 5 sampling sites situated outside the check-strip, but parallel to the 5 first sampling sites in the check-strip. All sampling sites were located at least 60 m from the field edge and at least 50 m apart from each other. The sampling sites were marked with a flag and the global positioning system (GPS) coordinates were recorded. Sampling was carried out at three different stages: 10-20% flowering (early bloom), 30-40% flowering (mid-bloom), and >50% flowering (full to late bloom), as determined by visual assessment (Harper and Berkenkamp 1975). Field 5 was sampled only at mid-bloom and full to late bloom, since the flowering period progressed quickly and the crop could not be sampled at early bloom. To examine the influence of diurnal fluctuation of ascospore release on the level of petal infestation, samples were collected both in the morning (before 11:00 am) and afternoon (after 12:00 pm). Sampling was conducted as described in Chapter 2, with the top 20-30 cm of the inflorescence from each of 20 randomly selected plants collected and placed in labelled plastic bags. The samples were kept in a cooler on ice during transport back to the laboratory, where they were stored at 4˚C for 4-5 days prior to processing.

4.2.2 Monitoring environmental conditions

Temperature and RH were monitored using WatchDog A150 Temp/RH loggers mounted inside a radiation shield (Spectrum Technologies Aurora, IL, USA). Conditions were monitored both within and outside the crop canopy in each field. For measurements within the canopy, a logger was placed on a metal pole about 0.25 m above the ground in an area with dense foliage, situated at the second or third petal sampling site in the fungicide-free check-strip. For measurements outside the canopy, the data logger was mounted on a metal pole at a height of approx. 1.5 m in an open area at the edge of each field. Temperature and RH measurements were recorded every 30 min starting on May 12th, 2014, and ending on the first or second week

of August, for the loggers positioned at the field edges, and on the first sampling date for the loggers in the crop canopy. Data loggers were removed from within the crop canopy on August 6th and from the field edge around August 19th, 2014. Rainfall also was monitored with a rain gauge placed adjacent to the temperature/RH logger at the edge of each field. Tall grasses and weeds were cut as needed to prevent obstruction of the rain gauge. The rain gauges were placed on May 28th, 2014, and the amount of precipitation was recorded weekly until August 6th.

As a measure of the favourability of the environment for disease development, the number of hours with RH >80% and the number of hours with temperatures between 15 and 22ºC were calculated for the period from early bloom to 1 week after late bloom. These conditions represent the RH threshold (>80%) and optimal temperature range for infection identified by Koch et al. (2007). For fields 1 to 4, the period from early bloom to 1 week after late bloom spanned 15 days (about 360 h). For field 5, sampling was delayed and the hours of optimal RH and temperature conditions were recorded for a 10 day period (about 240 h) corresponding from mid-bloom to 1 week after late bloom.

4.2.3 Measurements of crop canopy characteristics

To determine the influence of crop canopy characteristics on the level of petal infestation and disease development, measurements of crop lodging and plant density were recorded. Lodging was recorded as the ratio of the height of the canopy relative to the average height of 10 randomly selected plants at each sampling site within the fungicide-free check-strip. For crops with no lodging this ratio would be 1, while for crops with the most severe lodging (i.e., completely flat) it would be 0. Plant density (plants/m^2) was measured at each of four randomly selected areas in each of the 10 sampling sites located within the fungicide free check-strip. Plant density counts were calculated by counting the number of plants along 1 m of a row. That

number was then multiplied by 100 and divided by the row space distance (cm). The four points where measurements were taken were located in close proximity to the flag marking the centre of the sampling site. The density measurements were averaged to give a single plant density per sampling site. Measurements of lodging and plant density were recorded at the end of the growing season, but prior to swathing or straight combining.

4.2.4 Disease incidence

At the end of the growing season, but prior to swathing or straight combining, each sampling site was revisited to determine the final incidence of Sclerotinia stem rot. As in Chapter 2, plants with minor upper plant infection that would likely not result in any major yield loss were not included in the DI counts. DI was defined as the percentage of plants assessed with symptoms of Sclerotinia stem rot having a severity rating of ≥ 2 on a five point rating scale, where: 0 = no symptoms and 5 = a main stem lesion with the potential of affecting seed formation and wilting of the entire plant (Kutcher and Wolf 2006). Twenty plants were randomly selected and evaluated for each sampling site. For each field, DI values were averaged for the 10 sampling sites within the fungicide free check-strip and for the 5 sites located outside of the check-strip.

4.2.5 Sample processing

Due to the large number of samples collected, processing was delayed and petal samples for all fields were stored at 4˚C for 4-5 days. Each plastic bag containing 20 inflorescences was then shaken and two distinct samples representing young and old petals were collected. Young petals were defined as those remaining attached to fully opened flowers after shaking, while old petals were those that fell to the bottom of the bag. For each of the young and old samples, 20 petals were placed in labelled 2.5 ml tubes (Eppendorf, Hauppage, NY) and 100 petals were

selected at random, placed in separate labelled plastic bags, and stored at -80˚C prior to DNA extraction. The 100 petal sample was kept in case samples had to be reanalyzed.

An aliquot consisting of 20 petals was homogenized with a single 5 mm-diameter stainless steel bead in a TissueLyser II (Qiagen, Toronto, ON, Canada) as described in Chapter 2. Each sample was then spiked with 2×10^6 pSilent1 plasmids (Nakayaahiki et al. 2005) containing the *ToxB* gene from *Pyrenophora tritici-repentis* (Aboukhaddour et al. 2012), which served as an exogenous internal control (Chapter 2). Total genomic DNA was isolated from the petal homogenates following the protocol of Liang et al. (2013).

4.2.6 PCR analysis

The amount of DNA in each sample was analyzed by qPCR in a ViiA7 Real-Time PCR System (Life Technologies, Carlsbad, CA) (Chapter 2). The risk of a false negative due to failed DNA isolation or PCR inhibition was determined by analyzing each sample in a qPCR assay with the *ToxB* specific primers, ToxBF (5'CCATGCTACTTGCTGTGGCTAT3'), ToxBR (5'CGCAG TTGGCCGAAACA 3') and the hydrolysis probe ToxBP (3'CTCCCTGCTGCCCC3') as described in Chapter 2. The variation in the internal control and the risk of a false negative was considered to be low when the standard deviation of the average quantification cycle (C_q) values for a set of samples was close to or <1. A set of samples was defined to be all petal samples from either young or old petals collected at a single sampling date, time and bloom stage. When the risk of a false negative was confirmed to be low, qPCR analysis was carried out with the *S. sclerotiorum*-specific primers SSBZF(5'GCTCCAGCA GCCATGGAA3'), SSBZR (5'TGTTGAAGCAGTTGACGAGG TAGT3') and the hydrolysis probe SSBZP (5'CAGCGCCTCAAGC3') following the protocol in Chapter 2. A standard curve

was generated with 1:10 dilutions of purified *S. sclerotiorum* DNA, enabling quantification of sample DNA in a range from 8.0 ng to 8.0×10^{-4} ng. Any sample for which no *S. sclerotiorum* DNA could be detected, or that was below the range of the standard curve, was recorded as zero. Results are expressed in ng of *S. sclerotiorum* DNA on a per petal basis by first accounting for the dilution and then dividing the estimate by 20.

4.2.7 Statistical analysis

The data were analyzed using the Statistical Analysis System (SAS) version 9.3 (SAS Institute, Cary, NC, USA). Non-parametric, one-sided t-tests using the Wilcoxon-Mann-Whitney Two-Sample Test were conducted to determine if old petals had a higher level of petal infestation than young petals, and whether or not petals collected in the afternoon had a higher level of infestation than those collected in the morning. A non-parametric method of analysis was selected because the data were non-normally distributed. The comparison of young vs. old samples was conducted for each bloom stage independent of sample timing. The comparison of morning and afternoon sampling was carried out separately for young and old samples at each bloom stage.

Regression analysis was used to determine the relationship between petal infestation level, as determined with the qPCR assay, and final DI in the field. Given the relatively low number of fields included in this study, DI and petal infestation level were not averaged for each field. Instead, sampling sites were included in the analysis as replicates nested within field [sampling site(field)]. The results were analyzed with Proc Mixed to calculate the Akiake information criterion (AIC) and Bayesian information criterion (BIC) values to compare models. General linear model (GLM) analysis (Proc GLM) was used to calculate the regression coefficient (R^2) and determine the significance of each factor included in the regression model.

The effect of field and sampling site(field) was accounted for by including field in the class statement and both variables in the model statement. The R^2 value obtained through GLM analysis included the effect of field and over-estimated the strength of the relationship. The partial R^2, representing the variation accounted for only by the independent variable in question, was calculated manually by dividing the sums of squares for the independent variable by the total sums of squares of the model. For multiple regressions, the adjusted R^2 was calculated by dividing the total sums of squares for all of the independent variables of interest by the total sums of squares, while excluding the sums of squares values for field and sampling site(field). In comparisons of single and multiple regression models, the model with the lowest AIC and BIC was considered to have the best fit. Regression analyses were considered significant when the F-value of each independent variable was significant at $p = 0.05$. Quadratic regression analysis did not result in a better fit as determined by the AIC and BIC values, and the data did not appear to be non-linear. Therefore, linear regression analysis was conducted.

4.3 Results

4.3.1 Disease incidence

In 2014, DI ranged from 17.5% (field 3) to 49.5% (field 1) in the fungicide-free check-strips, and from 3.0% (field 2) to 42.0% (field 1) outside of the fungicide-free check-strips (Table 4.1). Fungicides were applied to all five fields, with the application dates recorded for fields 1 to 3 but unknown for fields 4 and 5 (spraying was carried out independently by the cooperating farmers). Field 1 was sprayed at about 20% bloom on July 4th, field 2 was sprayed at about 40% bloom on July 11th, and field 3 was sprayed at about 20% bloom on July 8th. The greatest difference (24.5%) in DI between a fungicide-free check-strip and the sprayed area was observed in field 2 (Table 4.1).

4.3.2 Precipitation

The total precipitation in the five fields ranged from 16.8 cm to 22.4 cm, with the highest amount of precipitation occurring in field 1 and the lowest in field 3 (Table 4.1). There was variation across the fields with respect to the timing and volume of precipitation over the growing season, but the largest precipitation event in all fields occurred around July 29, which is after the flowering period (Figs. 4.1-4.5). The statistical relationship between the amount of precipitation and DI was not investigated since there was only a single weekly measurement recorded for each of the five fields.

4.3.3 Plant density and lodging

The highest plant density (86 plants/m^2) was found in field 5, while the lowest was in field 1 (63 plants/m^2) (Table 4.1). The degree of lodging was similar in all fields, with the lodging ratios ranging from 0.65 (field 2) to 0.78 (field 5), indicating the occurrence of moderate to slight lodging, respectively (Table 4.1). There was no significant relationship between final DI and plant density or the degree of lodging when analyzed independently (data not presented).

4.3.4 Petal infestation levels over the flowering period

There was variation in the amount of *S. sclerotiorum* DNA per canola petal, as measured by qPCR analysis, across the five sampled fields and over the entire flowering period. In fields 1, 2 and 3, the highest level of petal infestation was observed at mid-bloom, while in fields 4 and 5, petal infestation was highest at late bloom (Table 4.2).

4.3.5 Young versus old petals

Canola petals were divided into young and old groups, respectively, based on whether or not they remained attached to the inflorescences when shaken. Quantitative PCR analysis

indicated that for all three bloom stages, there was a significantly higher level of infestation in old (average = 1.86 ng *S. sclerotiorum* DNA/petal) versus young petals (average = 0.19 ng *S. sclerotiorum* DNA/petal) ($p = 0.001$).

4.3.6 Morning versus afternoon sampling

At each bloom stage, samples were collected both in the morning and afternoon to determine if diurnal fluctuations in ascospore release influenced the level of petal infestation. In the young petals, infestation levels were significantly higher in the afternoon (0.06 ng *S. sclerotiorum* DNA/petal) vs. morning (0.02 ng *S. sclerotiorum* DNA/petal) samples during early bloom ($p = 0.001$), in the afternoon (0.47 ng *S. sclerotiorum* DNA/petal) vs. morning (0.14 ng *S. sclerotiorum* DNA/petal) samples during mid-bloom ($p = <0.004$), and in the afternoon (0.26 ng *S. sclerotiorum* DNA/petal) vs. morning (0.14 ng *S. sclerotiorum* DNA/petal) samples during late bloom ($p = 0.0318$). In contrast, no statistically significant difference in the level of infestation was observed between the old petals collected in the morning or afternoon at any flowering stage.

4.3.7 Relationship between petal infestation levels and DI

The relationship between DI and petal infestation level, as measured with the qPCR assay, was examined using single and multiple regression analysis to determine which combination of petal age, sampling time and bloom stage best predicted final DI. Partial regression coefficients (R^2) were calculated for each independent variable to determine how much of the variation in DI was accounted for by the independent variable while removing the effect of field. For young petals, significant relationships were found between DI and petal infestation in morning samples collected at early bloom (partial $R^2 = 5.4\%$), mid-bloom (partial $R^2 = 16.9\%$) and late bloom (partial $R^2 = 7.8\%$). For old petals, significant relationships between

DI and petal infestation were found for morning samples collected at early bloom (partial R^2 = 8.0%) and mid-bloom (partial R^2 = 14.2%), and for afternoon samples collected at early bloom (partial R^2 = 16.9%) and mid-bloom (partial R^2 = 13.0%).

The strongest regression model included both young and old petal samples collected at mid-bloom. Indeed, at mid-bloom, multiple regression models including both young and old petals were significant for both morning (partial R^2 = 31.9%) and afternoon samples (partial R^2= 30.9%). Plant density and lodging ratios were included in the two significant mid-bloom multiple regression models. The lodging ratios were found to account for a significant amount of additional variation when included in a multiple regression model with the petal infestation level for both young and old afternoon samples collected at mid-bloom, and accounted for 34.5% of the variation in DI. In contrast, including lodging ratios in the mid-bloom multiple regression model with young and old petal morning samples was not found to improve the strength of the model. The inclusion of plant density counts was not found to significantly improve the strength or fit of any other regression model in this study (data not presented).

4.3.8 Relative humidity

The daily average percent RH was higher within the crop canopy than at the field edge in all five fields (Figs. 4.1-4.5). In field 3, there was no consistent difference in the average daily minimum percent RH in the field edge vs. within the canopy. However, in the four other fields the minimum percent RH was higher within the canopy than at the field edge.

The number of hours with a RH >80% (favourable for disease) were calculated from early bloom to 1 week after late bloom (15 days; about 360 hours) for fields 1, 2, 3 and 4, and from mid-bloom to 1 week after late bloom (10 days; about 240 hours) for field 5 (Table 4.1).

The percentage of favourable (RH >80%) hours during these periods was used to make comparisons between field 5 and the four other fields. For all fields, there was a greater number of hours with RH >80% within the crop canopy than at the field edge. At the field edge, field 1 had the greatest proportion of time with RH >80% (55% of the time; 198 h). There were few differences between the other four fields, which had a RH >80% for about 45-48% of the monitored periods (162-167 h for fields 2, 3, 4; 115 h for field 5). There was slightly more variation in the proportion of time with favourable RH conditions within the crop canopy. Fields 1 and 3 had the fewest hours of RH >80% within the crop canopy (60 and 59% of the time; 216 and 214 h, respectively). Field 4 had the greatest number of hours with RH >80% within the crop canopy (68% of the time; 245.5 h), while field two and five were intermediate (66% and 65% of the time; 236.5 and 157 h respectively). There was a general trend of increasing DI with more hours of RH >80% within the crop canopy for fields 2, 3, 4 and 5, but this pattern was not confirmed through statistical analysis. Among these fields, field 3 had both the lowest DI and fewest hours of RH >80%, while field 4 had the highest DI and the highest proportion of time with RH >80%. Fields 2 and 5 had intermediate DIs and intermediate numbers of hours of RH >80%. In contrast, field 1 did not appear to follow this trend. Field 1 had the highest overall DI but had the second lowest RH within the crop canopy; however, it had the greatest amount of precipitation (Table 4.1).

4.3.9 Temperature

There were no consistent differences in the average temperature conditions between the field edge and within the crop canopy for any of the five fields monitored in this study. In four of the fields (1, 2, 3 and 5), higher minimum temperatures were recorded within the crop canopy than at the field edge (Figs. 4.1-4.5). There were no consistent differences in the average daily

maximum temperature at the field edge and within the crop canopy for three of the five fields. The exceptions were fields 2 and 3, in which the maximum temperatures were higher within the canopy than at the field edge.

The number of hours with temperatures between 15˚C and 22˚C were calculated for the period from early bloom to 1 week after late bloom (15 days; about 360 h) for fields 1 through 4 and from mid-bloom to 1 week after late bloom (10 days; about 240 h) for field 5 (Table 4.2). The percentage of hours within this temperature range (considered most favourable for Sclerotinia stem rot development) was used to make comparisons between field 5 and the other four fields. The proportion of time with temperatures of 15-22˚C was greater at the field edge than within the crop canopy in four of the five fields. The greatest proportion of time with temperatures in the 15-22˚C range occurred in field 2 (38%; 137 hours). In the other fields, this value ranged from 16% (66 h; field 3) to 31% (112 h; field 1). No consistent relationships were observed between temperature and RH or between temperature and DI across the five fields included in this study.

4.4 Discussion

Ascospore release by *S. sclerotiorum* over the course of a day is not constant. A diurnal pattern, resulting in different amounts of ascospore discharge from apothecia in the morning and afternoon, has been reported (Qandah and del Río Mendoza 2011). In wet years (consistent with the growing conditions in this study), the highest level of ascospore release was found to occur between late morning to early afternoon (Qandah and del Río Mendoza 2011). Using the agar plate test, Turkington et al. (1991b) demonstrated that a diurnal pattern also occurs with respect to canola petal infestation by *S. sclerotiorum*. The incidence of petal infestation was found to be generally greater in the afternoon than in the morning (Turkington et al. 1991b). The results of

the current study are consistent with these previous reports. Quantification of *S. sclerotiorum* DNA by qPCR analysis revealed that infestation levels in young petals were significantly higher in those petals that had been collected in the afternoon vs. the morning. The influence of diurnal fluctuation was not evident in old petals, however, and no significant differences were observed in the level of old petal infestation between morning and afternoon samples. It is likely that a greater proportion of the *S. sclerotiorum* DNA detected in young canola petals originated from ascospores that had been freshly deposited or had germinated recently. In contrast, a significant amount of the *S. sclerotiorum* DNA in older petals likely originated from hyphae that would have had time to develop over many hours or several days. As such, in older petals, the amount of pathogen DNA measured by qPCR analysis would be less likely to be influenced by ascospore release patterns in a given day, and instead reflect colonization of the petal tissue by *S. sclerotiorum*. This suggestion is supported by the significantly greater amounts of *S. sclerotiorum* DNA measured in old vs. young petals across all fields and flowering stages. Canola petals have been documented to remain in the inflorescence for up to 6 days, providing a relatively wide window for petal infection by *S. sclerotiorum* ascospores (Penaud 1984 cited in Turkington et al. 1991b). Ascospores can germinate within 3 h of adhering to a canola petal, and hyphal growth penetrating the other side has been documented to occur 20-30 h after initial infection (Jamaux et al. 1995). As a result, the greater infestation level found in older petals is likely due primarily to a greater amount of fungal mycelium, masking any effect associated with the time of ascospore release or deposition.

The level of petal infestation was not consistent over the flowering period. In three of the five fields, there was a higher level of petal infestation detected at mid-bloom, while in the two other fields, the highest level of petal infestation was found at late bloom. The variation in petal

infestation levels noted in this study is consistent with previous findings where petal infestation levels were assessed by qPCR analysis (Chapter 2) or the agar plate test (Turkington and Morrall 1993). This variation likely resulted from variation in crop growth stage and whether flowering coincided with weather conditions that were conducive for sclerotial germination, ascospore release, petal infection and the infection of leaf and stem tissues. In all fields, with the exception of field 1, the highest level of petal infestation occurred on July 10, which also occurred the week after the largest precipitation event during the flowering period. This suggests that the timing of precipitation plays an important role in determining when the highest level of petal infestation will occur.

Petal infestation levels as evaluated by qPCR analysis were found to have the strongest relationship with DI at mid-bloom, when the infestation levels were highest in three of the five fields. This is consistent with the earlier observation that petal infestation levels accounted for the highest variation in DI at 40-50% flower (Chapter 2). Thus, the assessment of Sclerotinia stem rot risk would be most accurate when inoculum pressure is greatest, which may also reflect when conditions are most conducive to ascospore production and release. The time of sampling did not influence the strength of the regression analysis at mid-bloom, but petal age did. Individually, both young and old petals accounted for a significant amount of DI at mid-bloom, but were most strongly related to DI when both were included in the regression analysis. Although the relationship was significant, the partial R^2 values accounted only for a small amount of the total variation in DI, with the independent variables accounting for 34.2% of this variation in the strongest model. The strength of the relationship was not as strong as seen in Chapter 3. This is likely due to differences in the experimental design and statistical analysis. In Chapter 3, a larger number of fields were included in the study, and a field average of petal

infestation was used for regression analysis. The method of analysis used in Chapter 3 will remove the effect of variation between sites and provide a better prediction of DI for the field as a whole. In addition, there may be more clustering of the data points given it comes from the same field and this will impact the strength of the relationship between these variables.

Plant canopy characteristics were not found to have a consistent influence on the development of Sclerotinia stem rot in this study. Plant density did not account for a significant amount of variation in DI on its own or when included in multiple regression analysis with petal infestation levels. The non-significant influence of plant density on Sclerotinia stem rot development likely reflects the consistently high RH level across all crops included in this study, with all fields having average RH values >80% within the crop canopy between early bloom and the end of the growing season. Plant density may have a significant influence on DI in years when moisture levels are limiting, or when extremely high temperatures occur during the flowering period (Morrall and Dueck 1982). Furthermore, the plant density measurements taken in this study did not take into account differences in canopy density such as the degree of canopy closure, which has been shown previously to be related to DI and may have a greater influence on the micro-environmental conditions within the crop canopy (Turkington and Morrall 1993; Turkington et al. 1991).

The degree of lodging was found to have a stronger influence on DI than density and accounted for a significant amount of variation in DI when included in multiple regression analysis with a combination of young and old petals collected in the afternoon at mid-bloom (R^2 = 34.5%). This is likely due to plant-to-plant disease transfer under lodging conditions, which would contribute to an increase in DI in the field (Morrall and Dueck 1982). These findings are consistent with those of Jurke and Fernando (2008), who found that field plots with lodged

canola plants had a greater DI than plots in which no lodging occurred. Including measurements of plant lodging would not be practical for a Sclerotinia stem rot risk assessment system, since most lodging occurs later in the growing season. However, the impact of lodging on disease development could be minimized by selecting canola varieties that are rated as less susceptible to lodging.

Relative humidity is known to influence the timing of ascospore release, ascospore survival and successful infection and mycelial growth (Abawi and Grogan 1979; Qandah and del Río Mendoza 2011). In this study, RH measurements suggest that micro-environmental conditions may partially account for the differences in DI in the fields examined. There was a trend of increased DI with increased hours of RH >80% within the crop canopy for fields 2, 3, 4 and 5. As the hours of favourable RH increased, DI increased as well. This indicates that RH may account for differences in DI in these four fields. However, field 1 did not follow this trend. It had the highest overall DI, but the second lowest RH within the crop canopy. It is important to note that although field 1 had the fewest hours of favourable RH conditions within the crop canopy, RH was still >80% for 60% of the time. Field 1 also had the highest number of hours with RH >80% at the field edge, with 7% more time with favourable RH conditions than the next highest field (field 5). The hours of RH >80% at the field edge were very similar for fields 2 through 5, suggesting that differences in RH within the crop canopy are most influential on disease development in these fields. Nonetheless, favourable humidity conditions outside of the crop canopy may contribute to a higher level of ascospore germination and mycelial growth on infected petals, leading to a higher potential infection of stem and leaf tissue from cast petals. Field 1 also received the most precipitation, which may have contributed to longer periods of leaf wetness than the other fields. Prolonged leaf wetness has been reported to be required for

infection by *S. sclerotiorum* (Abawi and Grogan 1979; Harikrashnan and del Río 2008; Tu 1989). Another important consideration may be the interaction between the timing of inoculum presence and the environmental conditions. Field 1 had relatively low levels of petal infestation at all three sampling dates, but had the highest levels of petal infestation at early bloom relative to the other fields. It is possible that inoculum arrived earlier in this field, before RH conditions within the crop canopy were measured. The results from this study represent only five fields and a single year, and therefore the data should be interpreted with caution. However, the importance of RH on disease development indicates that RH conditions need to be included in Sclerotinia risk assessment systems, as previously suggested by others (Clarkson et al. 2004; Clarkson et al. 2014; Koch et al. 2007).

For the five fields included in this study, temperature conditions did not appear to have as strong of an influence on DI as did RH. There were differences in the percent of time with optimal temperature conditions (16%-38%) at the field edge and within the crop canopy (16%-21%) for four of the five fields. However, no consistent trends were detected between the amount of time under optimal temperature conditions and either DI or RH across the five fields.

Sclerotinia stem rot of canola is a very complex disease that is known to be strongly influenced by environmental conditions, resulting in variation in disease levels from year-to-year. Petal infestation levels are also influenced by the age of the sample, the flowering stage, and the time of sample collection. Differences in sample collection and handling time will influence the level of petal infestation as measured by qPCR analysis and may influence estimates of disease risk. Petal infestation level has been demonstrated to be related to DI, and the strength of the relationship has been found in most fields to be highest when inoculum levels are highest and/or when weather and micro-environmental conditions are most conducive to

disease development. Given the monocyclic nature of Sclerotinia stem rot, when environmental conditions are favourable, final disease levels would be a function mainly of inoculum availability and the impact of the host. In the current study, RH appeared to play a more significant role in the development of Sclerotinia stem rot than temperature, and may be used to account for differences in DI between fields. This indicates that if qPCR-based petal infestation levels are used as a risk assessment tool, environmental conditions also need to be incorporated into the forecasting model. Further research should be conducted to explore the findings of this study and to better identify field-specific factors that influence Sclerotinia stem rot disease development and differences in DI between neighboring fields.

4.5 Tables

Table 4.1. The average lodging ratio, plant density, Sclerotinia stem rot disease incidence (DI), and number of hours of relative humidity (RH) >80% within and outside the crop canopy in five commercial fields of canola located near Edmonton, AB. Values for the lodging ratio, plant density, and disease incidence are reported as field averages ± the standard deviation.

	Lodging ratio[a]	Plant density (plant/m^2)[b]	Total Precip. (mm)[c]	Hours of RH >80% at field edge (% of time)[d]	Hours of RH >80% in crop canopy (% of time)[e]	Hours of Temp between 15 and 22˚C at field edge (% of time)	Hours of Temp between 15 and 22˚C in crop canopy (% of time)	DI [f]	DI-sprayed[g]
Field 1	0.70 ± 0.05	63 ± 8	22.4	198 (55%)	216 (60%)	112 (31%)	75 (21%)	49.5 ± 11.17	42.0 ± 8.37
Field 2	0.65 ± 0.07	71 ± 13	19.9	162 (45%)	237 (66%)	137 (38%)	88 (24%)	27.5 ± 8.6	3.0 ± 6.7
Field 3	0.69 ± 0.01	68 ± 26	16.8	167 (46%)	214 (59%)	66 (16%)	81 (22%)	17.5 ± 10.9	10.0 ± 5.0
Field 4	0.75 ± 0.02	79 ± 14	17.2	166 (46%)	246 (68%)	104 (29%)	87 (24%)	37.5 ± 7.69	6.0 ± 4.2
Field 5	0.78 ± 0.05	86 ± 13	18.4	115 (48%)	157 (65%)	57 (25%)	38 (16%)	22.5 ± 11.1	13.0 ± 12.0

[a]Lodging ratio was defined as the ratio of the height of the canopy relative to the average height of 10 randomly selected plants at each sampling site within the fungicide-free check-strip; no lodging = 1 while complete lodging = 0.

[b]Plant density was measured at 4 randomly selected areas at each of 10 sampling sites in the fungicide-free check-strip per field and averaged for each field.

[c]Total precipitation is the sum of the weekly precipitation amounts from July 2 until August 6, 2014.

[d]RH was monitored with a WatchDog A150 data logger mounted 1.5 m above-ground at the edge of the field. For fields 1-4, the number of hours with RH >80% was determined over the 15 day (~360 h) period from early bloom to 1 week after late bloom. For field 5, the number of hours with RH >80% was determined for the 10 day (~ 240 h) period from mid-bloom to 1 week after late bloom. To enable direct comparisons, the percentage of time during these periods with RH >80% was calculated and is presented in the table.

[e]RH as monitored with a WatchDog A150 data logger mounted 0.25 m above-ground within the crop canopy. For fields 1-4, the number of hours with RH >80% was determined over the 15 day (~360 h) period from early bloom to 1 week after late bloom. For field 5, the number of hours with RH >80% was determined for the 10 day(~240 h) period from mid-bloom to 1 week after late bloom. To enable direct comparisons, the percentage of time during these periods with RH >80% was calculated and is presented in the table.

[f]Average Sclerotinia stem rot DI in the fungicide-free check-strip. DI was defined as the percentage of plants assessed with symptoms of Sclerotinia stem rot that had a severity rating of ≥ 2 on a five-point rating scale, where: 0 = no symptoms and 5 = a main stem lesion (Kutcher and Wolf 2006).

[g]Average DI in the sampling sites where fungicides were applied for Sclerotinia stem rot management.

Table 4.2. Amount of *Sclerotinia sclerotiorum* DNA (ng) per canola petal in young and old canola petals at three flowering stages (early bloom (EB), mid-bloom (MB), and late bloom (LB)) and collected in the morning (AM) or afternoon (PM).

Field	Young-AM-EB	Old-AM-EB	Young-PM-EB	Old-PM-EB	Young-AM-MB	Old-AM-MB	Young-PM-MB	Old-PM-MB	Young-AM-LB	Old-AM-LB	Young-PM-LB	Old-PM-LB
1	0.011	1.637	0.141	0.025	0.618	0.051	0.450	0.587	0.022	0.093	0.205	0.407
2	0.022	0.036	0.033	0.071	0.085	1.191	0.777	4.499	0.102	0.029	1.044	0.133
3	0.015	0.023	0.023	0.107	0.338	4.774	0.977	3.571	0.072	0.011	0.0590	1.368
4	0.017	0.123	0.047	0.084	0.028	0.505	0.036	0.082	0.147	0.689	5.850	9.059
5					0.167	0.537	0.09	0.289	0.38	0.689	5.228	9.089

Where young petals were defined as those that remained attached to fully opened flowers when shaken in a sampling bag, and old petals were defined as those that fell to the bottom of the sampling bag.

4.6 Figures

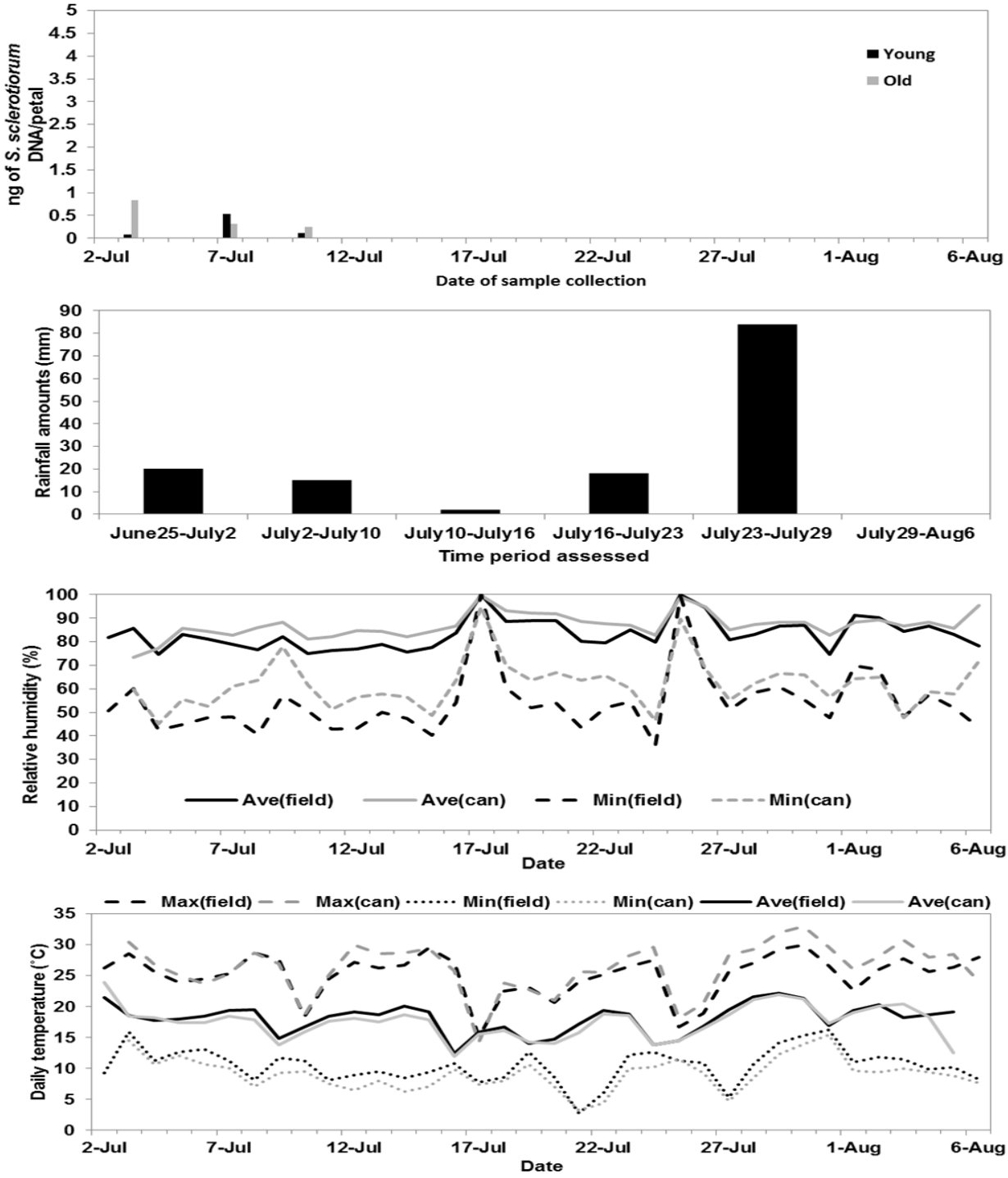

Fig. 4.1. Rainfall, relative humidity (RH), temperature, and canola petal infestation by *Sclerotinia sclerotiorum* in field 1, located near Edmonton, AB. Total weekly precipitation (mm)

was measured with a rain gauge. Petal infestation levels (ng *S. sclerotiorum* DNA/petal) were assessed by qPCR analysis for young and old petals, averaged for morning and afternoon sampling times. The average daily RH (%) and daily maximum (max), minimum (min) and average (ave) temperatures were measured at the field edge (field) or within the canopy (can) with a WatchDog A150 data logger.

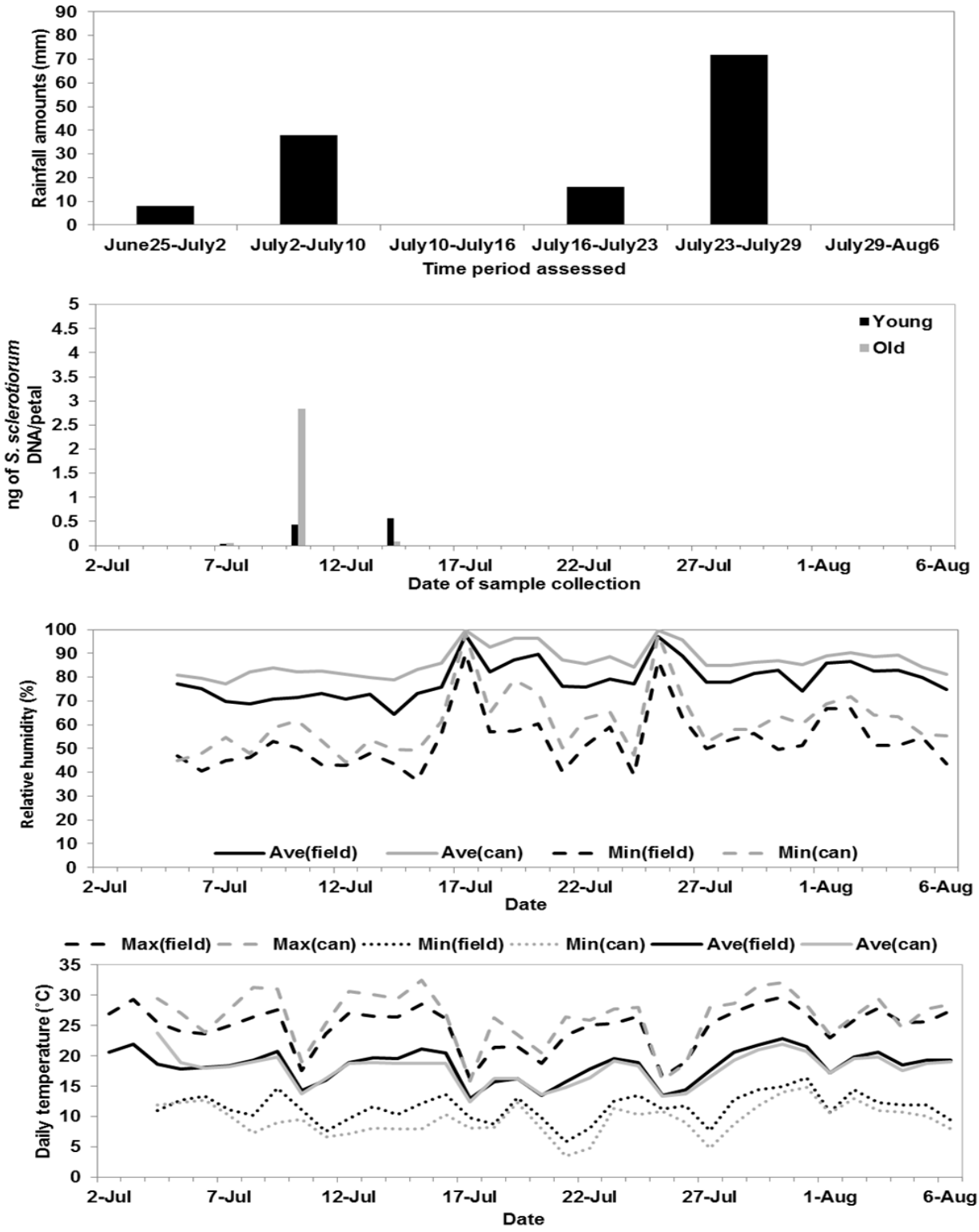

Fig. 4.2. Rainfall, relative humidity (RH), temperature, and canola petal infestation by *Sclerotinia sclerotiorum* in field 2, located near Edmonton, AB. Total weekly precipitation (mm)

was measured with a rain gauge. Petal infestation levels (ng *S. sclerotiorum* DNA/petal) were assessed by qPCR analysis for young and old petals, averaged for morning and afternoon sampling times. The average daily RH (%) and daily maximum (max), minimum (min) and average (ave) temperatures were measured at the field edge (field) or within the canopy (can) with a WatchDog A150 data logger.

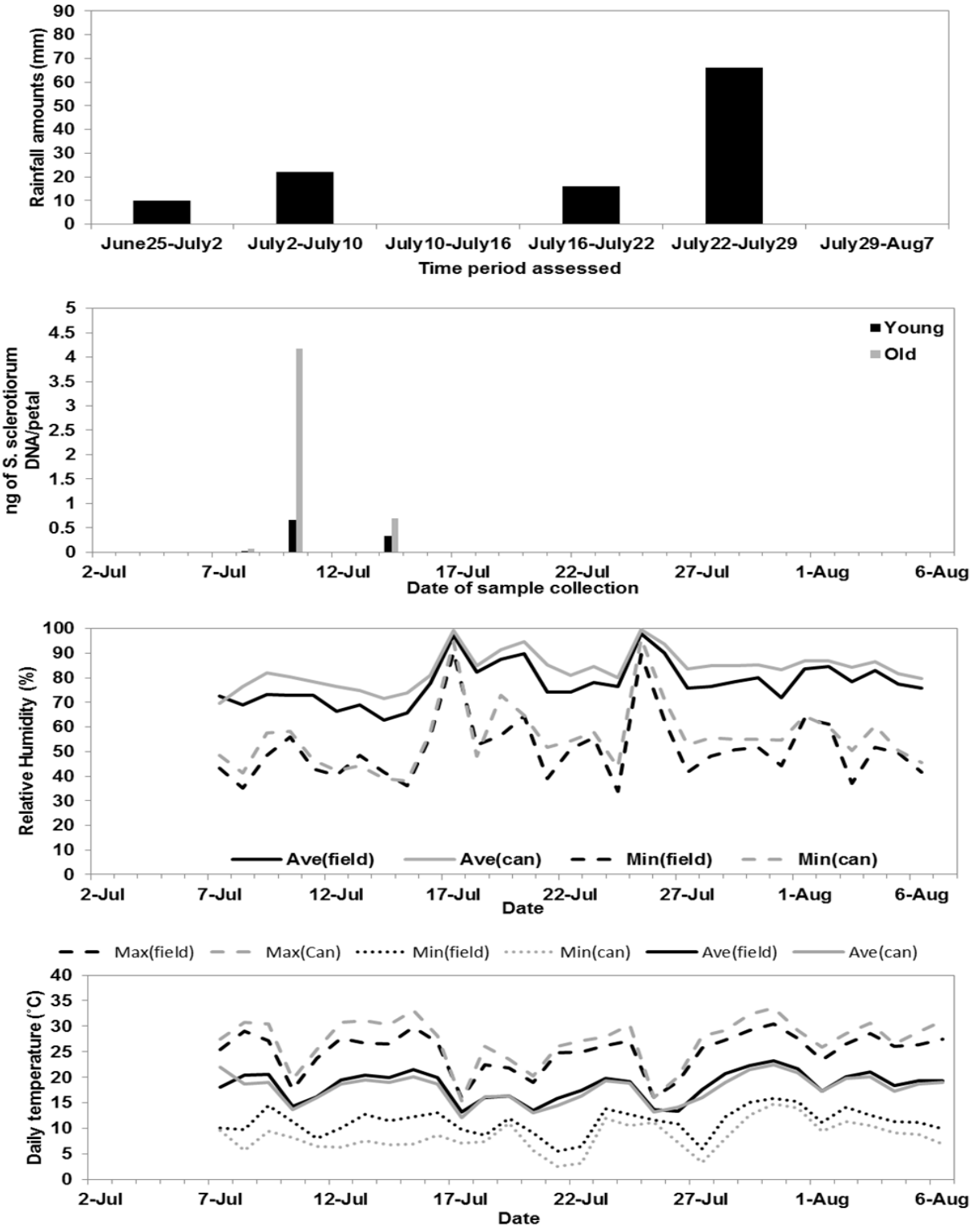

Fig. 4.3. Rainfall, relative humidity (RH), temperature, and canola petal infestation by *Sclerotinia sclerotiorum* in field 3, located near Edmonton, AB. Total weekly precipitation (mm)

was measured with a rain gauge. Petal infestation levels (ng *S. sclerotiorum* DNA/petal) were assessed by qPCR analysis for young and old petals, averaged for morning and afternoon sampling times. The average daily RH (%) and daily maximum (max), minimum (min) and average (ave) temperatures were measured at the field edge (field) or within the canopy (can) with a WatchDog A150 data logger.

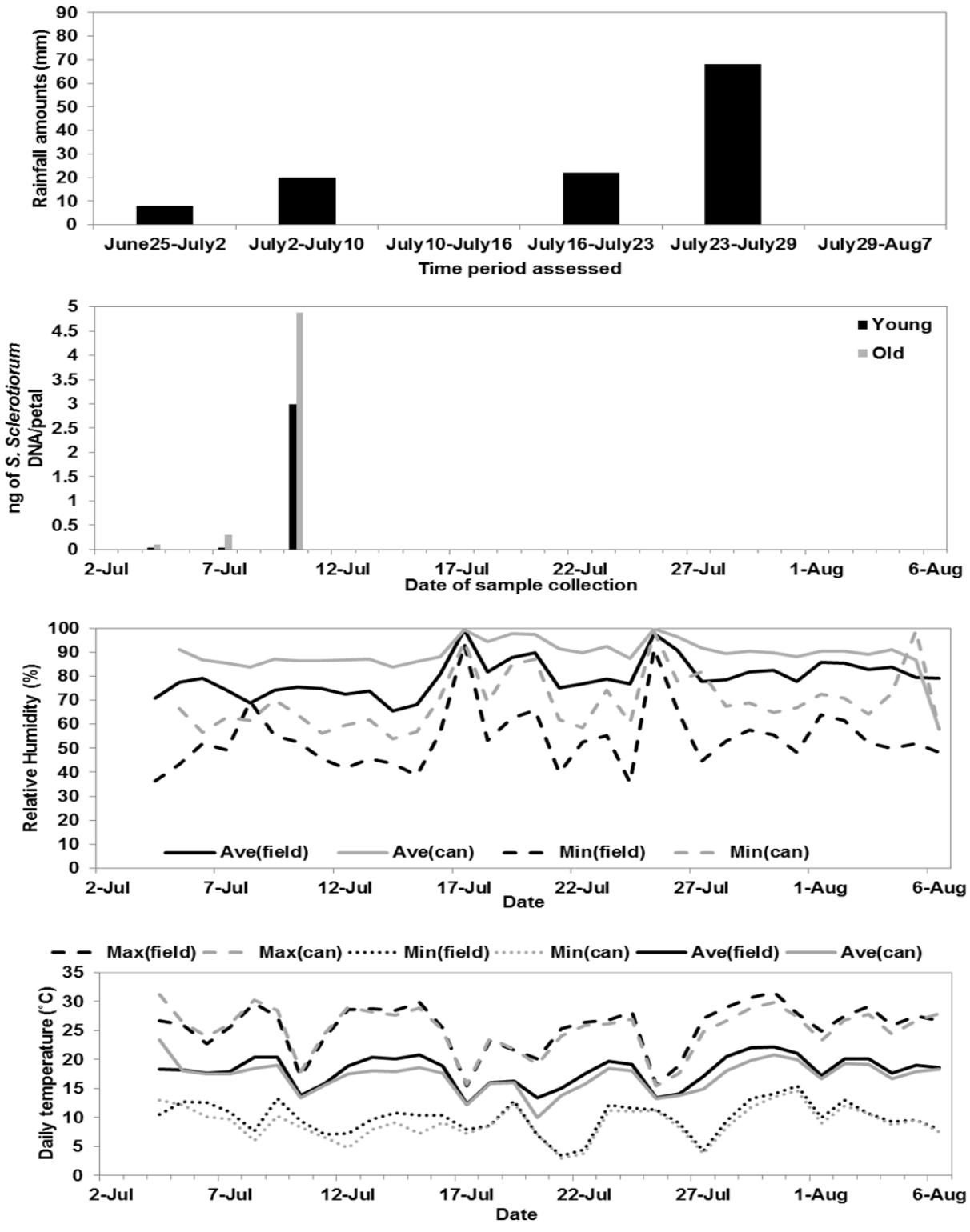

Fig. 4.4. Rainfall, relative humidity (RH), temperature, and canola petal infestation by *Sclerotinia sclerotiorum* in field 4, located near Edmonton, AB. Total weekly precipitation (mm)

was measured with a rain gauge. Petal infestation levels (ng *S. sclerotiorum* DNA/petal) were assessed by qPCR analysis for young and old petals, averaged for morning and afternoon sampling times. The average daily RH (%) and daily maximum (max), minimum (min) and average (ave) temperatures were measured at the field edge (field) or within the canopy (can) with a WatchDog A150 data logger.

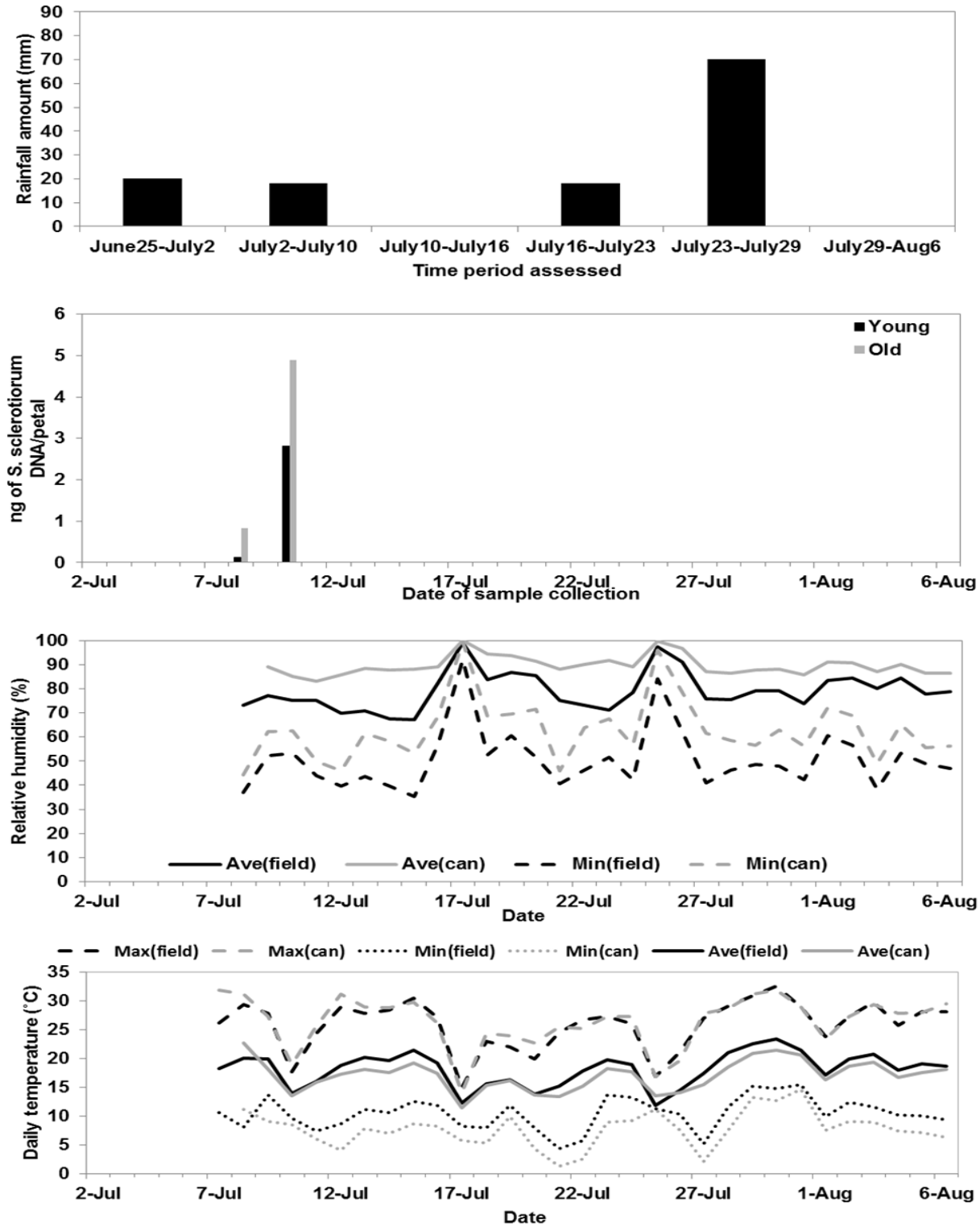

Fig. 4.5. Rainfall, relative humidity (RH), temperature, and canola petal infestation by *Sclerotinia sclerotiorum* in field 5, located near Edmonton, AB. Total weekly precipitation (mm)

was measured with a rain gauge. Petal infestation levels (ng *S. sclerotiorum* DNA/petal) were assessed by qPCR analysis for young and old petals, averaged for morning and afternoon sampling times. The average daily RH (%) and daily maximum (max), minimum (min) and average (ave) temperatures were measured at the field edge (field) or within the canopy (can) with a WatchDog A150 data logger.

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Chapter 5: General discussion

In western Canada, Sclerotinia stem rot of canola is managed primarily through the routine application of fungicides. To increase the accuracy, sustainability and economics of Sclerotinia stem rot management, a risk assessment system that can help guide fungicide application decisions is required. The overall goal of the work presented in this thesis was to develop a new method for quantifying petal infestation levels and to determine the potential of this method as a risk assessment tool. This was accomplished by addressing three specific objectives: (1) to develop a hydrolysis probe-based qPCR assay with an internal control to quantify *S. sclerotiorum* DNA on canola petals; (2) to examine the relationship between petal infestation estimates and final Sclerotinia stem rot incidence (DI) in the field; and (3) to determine the influence of sampling time, petal age, flowering stage, crop canopy characteristics and environmental conditions on petal infestation levels and the relationship between petal infestation and DI.

The qPCR assay developed in Chapter 2 could detect as little as 8×10^{-4} ng of *S. sclerotiorum* DNA in a highly specific manner. Petal infestation estimates based on this method were more rapid and accurate than estimates based on the traditional agar plate test (Morrall and Thompson 1991). The relationship between petal infestation estimates obtained by qPCR analysis versus the agar plate test method was not consistent between years or across the flowering period (Chapter 3). Similar results were reported by Almquist and Wallenhammar (2015), who found no correlation between the results of the agar plate test and a qPCR detection system for *S. sclerotiorum*. The poor and inconsistent correlation between the two methods likely results from differences in their capacity to differentiate between dead and live pathogen propagules (ascospores and mycelia), the misidentification of fungal colonies, and/or

competition with other fungi present on the petals which may preclude the growth and identification of *S. sclerotiorum* colonies when using the agar plate test. To determine if petal infestation levels were related to DI and could be used for risk assessment, in-field validation of the qPCR assay was also conducted.

Sclerotinia stem rot levels within a fungicide-free check-strip were found to be generally higher in fields where petal infestation levels also were high (Chapter 3). When statistical outliers were removed, petal infestation levels determined at full bloom were found to account for between 60 and 92% of the variation in DI. However, no significant statistical relationship was found between petal infestation and DI at early bloom. This suggests that the flowering stage at which petal samples are collected impacts the ability to accurately assess disease risk. Estimates of Sclerotinia stem rot risk based on petal infestation also appeared to be more accurate when inoculum pressure was high (Chapter 4). To provide reliable risk assessments, petal infestation levels may need to be assessed more than once over the flowering period, to enable an evaluation when inoculum pressure is at its highest (Turkington and Morrall 1993; Turkington et al. 1991a). Monitoring the risk of disease development over the flowering season can also be accomplished through spore trapping (Almquist and Wallenhammar 2015; Parker et al. 2014), or by monitoring the weather to determine when conditions are most suitable for carpogenic germination of sclerotia (Clarkson et al. 2014; Clarkson et al. 2004; Harikrishnan and del Río 2008; Koch et al. 2007; North Dakota state University 2009; Sharma et al. 2015). The early arrival of ascospores, as found by Almquist and Wallenhammar (2015), may be a possible explanation for the two statistical outliers that had high levels of DI, but low levels of petal infestation (Chapter 3). Spore trapping, monitoring of environmental conditions, and assessment

of petal infestation levels may be useful for estimating field-specific disease risk over the critical flowering period.

Although the relationship between petal infestation and final DI was significant at full bloom, the strength of the relationship was not consistent across study years (Chapter 3). Year-to-year variation in DI likely reflected differences in environmental conditions. This suggests the need to incorporate a measure of environmental conditions into the risk assessment system. This idea is supported by Bom and Boland (2000a), who suggested that accounting for soil moisture levels can improve risk assessments based on petal infestation levels. In the current study, the effects of the external environment (weekly precipitation, temperature and relative humidity (RH)), crop canopy characteristics (plant density and lodging), and the microenvironment within the crop canopy (temperature and RH) on petal infestation and DI were examined (Chapter 4). Relative humidity appeared to play a more significant role in the development of Sclerotinia stem rot than temperature. Since Koch et al. (2007) found that >80% RH is required for Sclerotinia stem rot development in winter oilseed rape, this threshold was selected to investigate the ability of RH conditions to explain differences in DI (Chapter 4). In most fields, DI increased as the proportion of time within the crop canopy during flowering with RH >80% also increased. These results indicate that monitoring RH conditions over the flowering period may increase the accuracy of risk assessments based on petal infestation. Additional experiments to further explore the relationship between RH and DI may be warranted, particularly over a greater number of fields and a bigger geographical area. Measures of precipitation were recorded weekly, but were not very informative for explaining differences in DI or petal infestation levels. Daily measures of the amount and frequency of precipitation

events over the growing season may be needed to further elucidate the relationship between rain and final DI.

Canopy density (Turkington et al. 1991a; Turkington and Morrall 1993) and the level of lodging within the crop canopy (Jurke and Fernando 2008) have been found to influence Sclerotinia stem rot development in the field. In the results presented in this thesis, however, plant density was not found to account for a significant amount of variation in DI (Chapter 4). This could be due, at least in part, to a low level of variation in plant density levels in the fields included in the analysis. Also, the measurement of plant density used in this study did not take into account differences in canopy closure, and may not have been as informative as other density measurements. Lodging within the canopy was found to have a stronger influence on disease development. When included in a multiple regression analysis with petal infestation levels, measurements of lodging were found to account for additional variation in DI and improved the strength of the statistical relationship.

The time of sample collection and petal age were found to influence the amount of *S. sclerotiorum* DNA present in the canola petals (Chapter 4). A diurnal pattern of ascospore release has been reported for *S. sclerotiorum*, with the highest levels of spore release occurring from late morning to early afternoon in wet years (Qandah and del Río Mendoza 2011). In the work presented in this thesis, a diurnal fluctuation in petal infestation levels was found in young petals, which was consistent with the findings of Turkington et al. (1991b). In old petals, however, there was no significant evidence of such a fluctuation (Chapter 4). As discussed earlier, this likely reflected the fact that a greater proportion of the *S. sclerotiorum* DNA on old petals originated from hyphae, rather than from freshly deposited, non-germinated ascospores. This greater overall biomass of hyphae would mask the contribution of additional DNA from

recently deposited spores that fell during the day. Indeed, significantly higher levels of *S. sclerotiorum* DNA were found on old versus young petals, supporting this hypothesis. As a result, a consistent sampling protocol would be required to avoid over- or under-estimating disease risk, and to make valid comparisons between fields.

The work presented in this thesis highlights the benefits and drawbacks of using qPCR-based petal infestation estimates for assessing the risk of Sclerotinia stem rot. Although a significant relationship was found between petal infestation at full bloom and final DI, the variation in the year-to-year strength of this relationship indicated that additional information should be incorporated into any predictive model. Since RH both within and outside the crop canopy could help to explain variation in DI, incorporating measures of this parameter into Sclerotinia stem rot forecasts based on petal infestation levels may improve their accuracy. Beyond its utility as a possible risk assessment tool, however, the qPCR assay described in this dissertation also represents a useful tool to study the epidemiology of Sclerotinia stem rot. It could be used in future studies to further explore the causes of field-to-field variation in DI, and to identify the most beneficial timing for fungicide application or other disease management activities.

There are a number of possibilities for further refinement and improvement of Sclerotinia stem rot risk assessments. One option may be to incorporate petal infestation levels, determined by qPCR analysis, into existing modeling systems based on environmental conditions, to determine if the reliability and accuracy of the risk assessments can be improved. Another option would be to develop and validate a field-specific modeling system that incorporates petal infestation level (or the level of airborne inoculum, as per Almquist and Wallenhammar 2015) and RH conditions within the crop canopy or at the field edge. For a risk assessment system to

be used by agronomists and producers, it must be reliable, accurate and easy to use. This needs to be considered in any further validation studies focused on using qPCR-based estimates of inoculum pressure for Sclerotinia risk assessment.

There are still gaps in our understanding of Sclerotinia stem rot epidemiology, and these gaps need to be addressed to improve the efficiency and accuracy of disease management. Work conducted by Almquist and Wallenhammar (2015) indicated that in some years, in Sweden, ascospore arrival may occur prior to the flowering period, suggesting that lower leaf infection rates may be used to provide a more accurate indication of disease risk. There is currently no evidence of this occurring in Canada, but this possibility should be investigated. In western Canada, fungicide application remains the primary method for managing Sclerotinia stem rot of canola, and fungicides are registered for application at 20-50% flowering. If significant levels of inoculum arrive outside of this period, fungicide applications at this stage may not be as effective in reducing disease levels. Monitoring changes in inoculum arrival over time will provide important information with regards to when the highest inoculum pressure occurs, and when fungicide applications would be the most effective for suppressing Sclerotinia stem rot. The qPCR-based assay presented in this thesis represents an important tool for conducting such research.

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