DEVELOPING A BLACKLEG MANAGEMENT PACKAGE FOR NORTH DAKOTA

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Blackleg, caused by *Leptosphaeria maculans*, inflicts greatest canola yield losses when plants are infected before reaching the six-leaf growth stage. Studies were conducted to model pseudothecia maturation and ascospore dispersal to help growers make timely foliar fungicide applications. Pseudothecia maturation occurred mostly during the second half of June or in July in 2017 and 2018 in North Dakota and ascospores concentrations peaked during mid to late June in both years. A logistic regression model developed using temperature and relative humidity predicted the maturation of pseudothecia and ascospore dispersal with approximately 74% and 70% accuracy respectively. In addition, trials to evaluate the efficacy of five seed treatment fungicides were conducted under greenhouse and field conditions. All treatments reduced (P = 0.05) disease severity on seedlings in greenhouse trials, but not in field trials. Seed treatments, while a valuable tool, should not be used as the only means to manage blackleg.

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CHAPTER 1: GENERAL INTRODUCTION

Canola (*Brassica napus* L.) is an oilseed crop bred from rapeseed with reduced erucic acid and glucosinolates contents (Lin et al. 2013). It is the world's second and third largest source of animal feed and vegetable oil, respectively. Australia, Canada, the European Union, and China are the world's major canola producers whereas the United States only produces one-fourth of its actual demand (US Canola Association). North Dakota leads the United States in canola production with its 2017 harvest valued at US$444 million (USDA-NASS 2018).

Blackleg disease is one of the major production constraints in canola-producing countries worldwide. It is caused by the dothideomycete, *Leptosphaeria maculans* (Desm.) Ces. & de Not [anamorph = *Phoma lingam* (Tode:Fr.) Desm.] (West et al. 2001; Howlett 2004). The virulence of *L. maculans* populations in North Dakota has changed in recent years with new virulent races threatening the canola industry in the state (Bradley et al. 2005; Nepal et al. 2014). Generally, yield losses attributed to blackleg range between 5 and 20%; however, under optimum conditions, losses greater than 50% have been recorded in North Dakota (del Río Mendoza et al. 2012) and up to 90% in South Australia (Van De Wouw et al. 2016).

Blackleg management practices can be grouped in three sections: 1) protection of seeds and seedlings until the 5-leaf growth stage; 2) deploy cultivars with effective levels of resistance against predominant blackleg strains; and, 3) establishment of crop rotation schemes that lower selection pressure on the pathogen. In the first group, seed treatments, fungicide applications and disease-warning models that help optimize fungicide applications are included. In the second group, the identification of new sources of resistance, characterization of markers associated with the trait, and transfer of resistance into modern breeding lines are included. In the third group, characterization of the prevalence of avirulence genes within *L. maculans* isolates from ND and

of the resistance genes in canola cultivars planted in ND are included. Activities conducted as part of this thesis address the first section.

Leptosphaeria maculans survives saprophytically as sexual fruiting body, pseudothecium, and asexual fruiting body, pycnidium, on blackleg-infected residues. These pseudothecia and pycnidia release ascospores and pycnidiospores, respectively, which serve as sources of inoculum for blackleg infection (West et al. 2001; Rouxel and Balesdent 2005). Timing of ascospore maturation and release varies between locations and seasons due to variation in weather conditions (Salam et al. 2003; Salam et al. 2007). Temperatures between 15 and 20°C (Toscano-Underwood et al. 2003) and frequent rainfall events or high humidity conditions favor pseudothecia maturation (Peres et al. 1999). Similarly, environmental conditions with high rainfall (>1mm), moderate relative humidity (>80%) and cool temperatures (13°C to 18°C) trigger the release of spores (Guo and Fernando 2005). The released spores initially infect the cotyledon and true leaves and then the fungus moves into the petiole and the stem. In the stem, the fungus continues moving towards the crown region where it produces cankers that could girdle the stem and cause lodging of the plant (Hammond and Lewis 1987).

Fungicide applications are currently the best alternative to manage blackleg disease in North Dakota. Most cultivars currently available to growers in North Dakota carry resistance genes *Rlm1, Rlm2,* and/or *Rlm3* and are susceptible to races of blackleg prevalent in the region (del Río et al. 2012). Fungicides are applied as seed-dressing and foliar sprays. In general, fungicide spray is recommended to be made between the two- and four-leaf growth stages in North Dakota (Markell et al. 2008). However, a study conducted by del Río Mendoza (2014) reported that foliar sprays made within eight days after *L. maculans* inoculation significantly control disease suggesting timing of foliar application is key for effective control of the disease.

Thus, a warning-system that alerts growers when pseudothecia matures and ascospores are released could help growers time their fungicide applications better.

Seed treatments help eliminate the seed-borne inoculum and can provide protection against infection during the early seedling stage (Gugel and Petrie 1992; Mancini and Romanazzi 2014). Most canola growers in the United States use fungicide-treated seeds. With the increased importance of blackleg, new fungicide seed treatments have been registered in North Dakota in recent years (Friskop et al. 2017). However, the effectiveness of these new seed treatments has not been explored well. The study of the efficacy of recently registered seed treatment fungicides would help growers make appropriate selections.

Therefore, the objectives of this study were;

1. To study the role of weather variables on pseudothecia maturation under controlled and field condition
2. To develop a warning model for *Leptosphaeria maculans* ascospores dispersal
3. To evaluate the efficacy of fungicide seed treatments against blackleg of canola

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CHAPTER 2: LITERATURE REVIEW

Canola

Canola (*Brassica napus* L.) is an important oilseed crop belonging to Brassicaceae family. The crop was bred from the rapeseed or oilseed rape nearly 40 years ago. Canola gained popularity in a short period of time becoming the third largest source of vegetable oil and second largest source of feed meal after soybean meal (USDA-ERS 2018). Rapeseed has been cultivated in Asia and the Mediterranean region since 2000 BC and in Europe since the 13th century for cooking and lighting purposes. Later, during the 18th century, rapeseed oil was used as lubricant in steam engines in Europe and North America (Busch et al. 1994). The high levels of glucosinolate, erucic acid and chlorophyll contents in rapeseed oil made it undesirable for human consumption. During the mid-1970s, plant breeders from the Universities of Manitoba and Saskatchewan, Canada, developed rapeseed lines with low erucic acid (< 2%) and glucosinolate contents (<30 µmoles) using traditional breeding techniques (Rempel et al. 2014). These new lines were named 'Canola' in 1979 referring to 'Canadian oil low acid' (Canola Council of Canada). Today, canola oil is one of the healthiest edible oils with low erucic acid and low glucosinolate compounds (Lin et al. 2013).

Economic importance

In 2016/17, canola was cultivated on 34.1 million hectares of land in the world with production of 71.3 million metric tons. Canada, China, the European Union, and Australia are the leading producers of canola in the world. In 2017, the United States produced nearly 1.2 million metric tons of canola from 0.6 million hectares of land (USDA-NASS 2018). North Dakota is the largest canola-producing state in the United States, contributing around 86% of its

production with a market value of US$ 444 Million (U.S. Canola Association, NASS-USDA-2018).

Blackleg of Canola

Introduction

Blackleg (Phoma stem canker), caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not [anamorph = *Phoma lingam* (Tode:Fr.) Desm.] is one of the most serious problems in major canola growing regions of the world. *L. maculans* is pathogenic on most Brassica crops (*B. napus*, *B. rapa*, *B. juncea*, *B. oleracea*) including wild cruciferous species. The dothideomycete fungus, *L. maculans* is widespread in Canada, Europe (UK, Germany, France, Poland), Australia, and North America (Howlett et al. 2001; West et al. 2001; Rimmer 2006) causing up to US$900 million per year in yield losses (Fitt et al. 2008). Generally, the estimated yield loss caused by blackleg is 5% to 20% however, it can be greater than 50% under a severely infected condition in North Dakota (Markell et al. 2008; del Río Mendoza et al. 2012).

Taxonomy and classification of *Leptosphaeria maculans*:

Kingdom: Fungi

Phylum: Ascomycota

Class: Dothideomycetes

Order: Pleosporales

Family: Leptosphaeriaceae

Genus: *Leptosphaeria*

Species: *maculans* (Desm.) Ces. & De Not.

Morphological features of *L. maculans*

L. maculans survives saprophytically on the infected canola stubbles in the fruiting bodies, pseudothecia and pycnidia. Pseudothecia are sexual fruiting bodies formed on the epidermis of the stem. They are round to oval shaped with a diameter ranging between 300 and 400 µm. The fruiting bodies have a small orifice at center and are flattened at the bottom. Pseudothecia contain numerous cylindrical and thick-walled asci of 100-120×18-21 µm size (Fig 2.1a) (Kaczmarek and Jedryczka 2011). Each ascus contains eight ascospores of 35-70× 5-8 µm size, 5-septate, cylindrical to an ellipsoidal shape, yellow to brown color and with several drops of fat or small oil drops with rounded ends (Fig 2.1b) (Williams 1992). On the other hand, pycnidia (150-400 µm diameter) of *L. maculans* are thick walled, black, round and are devoid of paraphyses. A pycnidium produces several million 4-5 × 1.5-2 µm single-celled pycnidiospores of hyaline to light brown color and cylindrical shape with blunt ends (Shoemaker and Brun 2001).

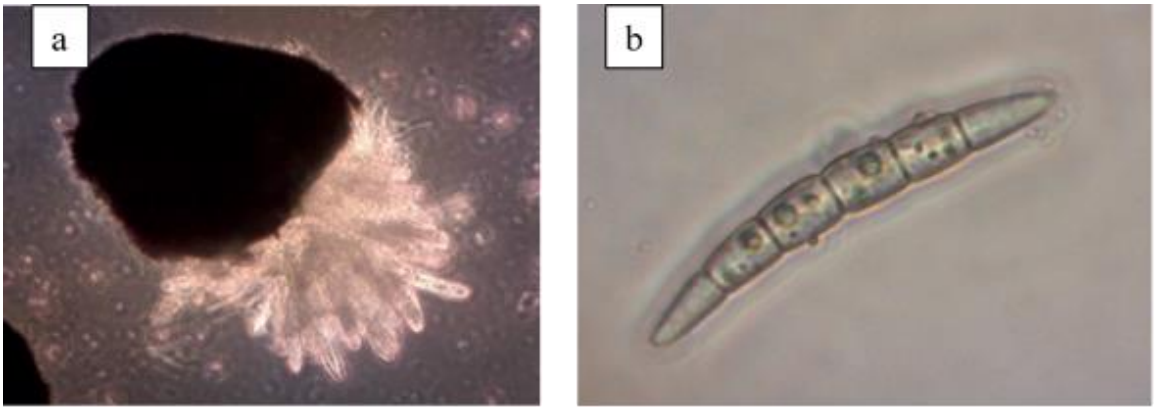

Figure 2.1. Mature pseudothecium releasing ascospores (a); and closeup of ascospore of *L. maculans* (b).

History of blackleg disease

In 1791, the fungus was first reported on dead red cabbage stems and named *Sphaeria lingam*. Fifty-eight years later, the same fungus was isolated from living cauliflower plants (*Brassica oleracea*) and renamed to *Phoma lingam* (reviewed in Rouxel and Balesdent 2005). The sexual stage of *P. lingam* (Tode ex Fr.) Desm. was first described in 1957 and was confirmed to be anamorph of *L. maculans* (Desm.) Ces & De Not.. Although stem canker was epidemic in cruciferous vegetables since the end of 19th century, in Australia, North America and Europe, the disease became economically important only after oilseed rape or canola became popular during the mid-20th century (reviewed in Rouxel and Balesdent 2005). Serious blackleg epidemics occurred in Western Australia in the early 1970s causing severe yield losses and reducing the planted acreage (Salisbury et al. 1995). In North America, virulent strains were first reported in Saskatchewan, Canada in 1975 and subsequently the disease became an economic threat in all canola growing regions except Asia (Rouxel and Balesdent 2005).

Leptosphaeria maculans species complex

Until 2001, *L. maculans* strains had been broadly classified into two groups, 'Group A' and 'Group B'. 'Group A' isolates were characterized as highly virulent, aggressive and non-host specific phytotoxin-sirodesmin producing isolates whereas, 'Group B' isolates were characterized as weakly virulent, non-aggressive and phytotoxin nonproducing strains. In 1997, Somda et al. observed reproduction incompatibility between group A and B isolates. Later, Shoemaker et al. (2001) solved the confusion and classified 'Group B' isolates into separate species, *Leptosphaeria biglobosa* based on distinct morphological features of pseudothecia and ascospores. Generally, *L. maculans* produces pale brown or grayish brown symptoms on the leaves and colonizes on the crown region of stem resulting in stem cankers whereas, *L. biglobosa*

produces small dark lesions on leaves and brown lesions on the upper stem. Further, gene-for-gene interaction is common in *L. maculans* isolates with Brassica hosts, but such interaction has not been observed in *L. biglobosa* yet (Delourme et al. 2004). Furthermore, *L. maculans* ascospores survive longer and *L. biglobosa* pseudothecia mature at a slower rate at temperature less than 10°C (Toscano-Underwood et al. 2003). Even though both species share same host and widely distributed worldwide, *L. maculans* is dominating over *L. biglobosa* causing major yield losses.

Pathogenicity groups and avirulence genes of *L. maculans*

L. maculans strains have been further divided into different pathogenicity groups (PGs) based on differential reactions on cotyledon leaves of *B. napus* cultivars, Westar (spring type), Glacier, and Quinta (winter types) (Koch et al. 1991). Strains non-virulent on Westar were classified as PG-1 (now *L. biglobosa*) while virulent strains on all differential cultivars were grouped under PGs 2-4. Isolates from PG-2 are virulent on Westar, which does not carry known resistance genes, but not on Glacier, which carries resistance genes *Rlm2* and *Rlm3*, or Quinta, which carries resistance genes *Rlm1* and *Rlm3*. Isolates virulent on Westar and Glacier but intermediate on Quinta were classified as PG-3. Isolates virulent on Westar and Quinta but intermediate on Glacier were grouped under PG-T (Kutcher et al. 2007). Isolates from PG-4 are virulent on all three differentials.

Avirulence genes present in *L. maculans* interact in a gene-for gene manner with resistance genes in the plants (Balesdent et al. 2005). The first avirulence gene, *AvrLm1* was discovered and characterized genetically in France in 1995 (Ansan-Melayah et al. 1995). To date, at least 14 additional avirulence genes have been identified in *L. maculans* (Fernando et al. 2018) and eight of those genes have been cloned (Van de Wouw et al. 2018). Typically, *L.*

maculans isolates have more than one avirulence genes. These new system of classification of *L. maculans* variability is used by most scientists while the use of pathogenicity group classification has decline and is quickly becoming obsolete.

Overview of blackleg disease in North Dakota

In the United States, the earliest stem canker epidemic caused by *L. maculans*, was documented in 1918 in Wisconsin where it caused up to 90% yield losses on cabbage (Henderson 1918). Virulent isolates of *L. maculans* were first reported on canola in 1989 from a field in SE Kentucky (Mengistu 1990). In North Dakota, the first blackleg epidemic, caused by PG-1 and PG-2 isolates of *L. maculans*, was recorded in 1991 with incidences ranging between 8 and 69% (Lamey and Hershman 1993). In 2003, surveys conducted in North Dakota canola fields detected the presence of strains of PG-3 and PG-4 at low population levels (Bradley et al. 2005). Since then, the prevalence of virulent strains (PG-4) of the fungus in North Dakota suggest that blackleg disease is a major constraint in canola production in the United States (Nepal et al. 2014). In addition, a study conducted in 2016, revealed the prevalence of *AvrLm4, AvrLm6, AvrLm7,* and *AvrLm11* genes in *L. maculans* populations in North Dakota (Mansouripour et al. 2016).

Epidemiology

Life cycle of *L. maculans*

L. maculans is a heterothallic fungus. The sexual reproduction between mating type-1 and mating type-2 of *L. maculans* produce sexual fruiting bodies, pseudothecia on infected canola residues. The pathogen also survives as pycnidia in the infected canola stubbles and seeds (Williams and Fitt 1999; Rouxel and Balesdent 2005). Rainfall events and high relative humidity favors the release of ascospores from pseudothecia and pink masses of pycnidiospores from

pycnidia that act as sources of primary inoculum. (Howlett et al. 2001; West et al. 2001). In Australia and Europe, ascospores are considered as a primary source of inoculum (West et al. 2001) whereas in Canada and USA both types of spores (ascospores and pycnidiospores) are important for the initiation of epidemics. The released ascospores are dispersed to canola plants by wind for several kilometers whereas pycnidiospores are disseminated over relatively shorter distances by rain-splash. Ascospores and conidia land on cotyledons and lower leaves and germinate to produce hyphae under humid and wet condition. The fungus generally enters the leaves via stomata and wounds and grows intercellularly between mesophyll cells (Howlett et al. 2001).

L. maculans is a hemi-biotrophic pathogen. The initial symptoms of infection appear on cotyledons or lower leaves with formation of gray green to ash-green lesions of 1-2 cm diameter. As tissues die, small, black colored pycnidia can be produced on the center of the lesion which makes the disease easily identifiable (Ash 2000). As they mature, pycnidia release pycnidiospores which could serve as secondary inoculum. The pathogen grows asymptomatically down the petiole through intercellular spaces and into the stem towards the crown region (Hammond et al. 1985). Colonization of the crown region results in canker formations. Generally, the stem cankers are visible on mature plants and results in lodging, premature ripening and death of the plant (West et al. 2001; Markell et al. 2008). On the stem cankers abundant pycnidia will be formed. Pseudothecia, which could serve as the source of primary inoculum is formed on the cankers at a later time. The fungus survives saprophytically on infected stubbles in the field for up to five years or longer (McGee, 1977).

Inoculum

L. maculans survives saprophytically on dead plant tissues as pseudothecia or pycnidia (Williams 1992). Pseudothecia are formed on canola stubbles by sexual recombination between two opposite mating types and produce ascospores upon maturation (McGee and Petrie 1979). Ascospores are the primary source of inoculum primarily in areas where winter canola is planted. Ascospores germinate, penetrate and cause disease symptoms faster compared to pycnidiospores (Li et al. 2005). Stem canker severity, type of cultivars, age of the stubbles, and climatic condition during the growth of crop affect the amount of primary inoculum production (Petrie 1995; Marcroft et al. 2003; J. Marcroft et al. 2004; Lô-Pelzer et al. 2009). The stem canker severity in the a given year has a direct relation to the production of primary inoculum in following year (Lô-Pelzer et al. 2009). Similarly, residues of susceptible cultivars discharge more ascospores than resistant cultivars.

Pycnidia are asexual fruiting bodies that are formed on the surface of blackleg-infected stems and can survive for at least three years in field conditions (Petrie 1995; Baird et al. 1999). Gosende et al. (2003) observed stable numbers of pycnidia in three-year-old stubbles with significantly higher germination ability compared to ascospores. In North America and Australia, pycnidiospores serve as a primary source of inoculum in previously blackleg infected fields with short crop rotation (Ghanbarnia et al. 2011). Pycnidia produced on leaf lesions serve as sources of secondary inoculum and help to increase the pathogen population in subsequent seasons (West et al. 2001). In addition, in low pathogen density areas, pycnidiospores play a vital role in pathogen population buildup leading to the sexual reproduction between spatially isolated opposite mating types. The sexual reproduction results in emergence of new strains of pathogen and production of pseudothecia which in turn release ascospores (Travadon et al. 2007).

The pathogen could survive on canola residues for five years (McGee 1977); however, its survival depends on how quickly the stems degrade. Wet weather with high temperatures helps to decompose canola stubbles rapidly whereas dry and low temperature conditions slow the process and favor the long-term survival of *L. maculans* (Sosnowski et al. 2006). In France, Gosende et al. (2003) observed that 90% of the stubble broke down by the end of the second season with reduced frequency of mature pseudothecia. In addition, germination ability of the ascospores produced on old stems also declined.

Blackleg disease is seed transmissible (Petrie and Vanterpool 1974). *L. maculans* can survive inside the seed coat as dormant mycelium and outside the seed as pycnidia in crucifer seeds for more than 10 years (Jacobsen and Williams 1971; Gabrielson 1983). In Canada, oil seed rape seed contamination is correlated with incidence and severity of blackleg disease resulting in yield losses of 1-2% (Hall et al. 1996). In contrast, McGee (1977) reported no significant role of seed inoculum on blackleg incidence and severity in Australia. Despite this, in recent years the disease impacted global trade of canola seeds resulting in temporary halt of canola export to China from Canada and Australia due to potential risk of seed contamination and spread (Fernando et al. 2016).

Pseudothecia maturation and ascospore formation

Pseudothecia at maturity release fully developed six-celled ascospores which serve as primary inoculum (Petrie 1994). The time required for pseudothecia to mature varies between locations and seasons and depend on external environmental factors like, temperature, rainfall, and relative humidity. Although, ascospore production can occur throughout the growing season, favorable weather conditions during cotyledon and seedling stage of canola draw economic concern. In Australia, 73 to 192 days were required for pseudothecia maturation (Khangura et al.

2007) whereas it took only 16 to 51 days in France (Peres et al. 1999). In Ontario Canada, ascospores were produced on the stubbles one month after the harvest of canola (Rempel and Hall 1993); however, in Western Canada, a few months were required due to extreme winter following canola harvest (Petrie 1995).

In England, pseudothecial maturity occurred faster at 20°C compared to 5°C under controlled condition (Toscano-Underwood et al. 2003). Similar observations were made in Canada where 15°C temperature favored ascospore production in naturally infected debris (Petrie 1994). In France, the average temperature of 14°C and rainfall of 2.5 mm in 3-4 days interval was favorable for pseudothecia maturation under controlled conditions. In the absence of rainfall, days with nearly 100% relative humidity complemented the rainfall requirement (Peres et al. 1999). In addition, Salam et al. (2003) reported the need of 43 favorable days (10-day average temperature $< 22^{o}$C and weekly rainfall $\geq$ 4mm) for ascospores production since harvest in Western Australia. Petrie (1995) and Peres et al. (1999), emphasized the importance of frequency of rainfall rather than total amount of rainfall in ascospore production. Erratic release of ascospores was observed at high temperature and low moisture condition. In support of this, at 15^{o}C, mature pseudothecia were produced on stubbles sprayed with water two or three times a day but no pseudothecia were observed with one water-spray per day (Naseri et al. 2009).

Release and dispersal of ascospores and pycnidiospores

The ascospores release period differed between countries and locations due to the differences in pseudothecia maturation timing. In most canola growing regions, the ascospores discharge coincides with the canola seedling stage, which is highly prone to infection (West et al. 2001). Ascospores releases peaked during September to November in western and central

Europe (Gladders and Musa 1980) and during June, July and August in Australia (McGee 1977) and Western Canada (Petrie 1995; Guo and Fernando 2005).

Rainfall triggered the release of pycnidiospores from pycnidia and of ascospores from mature pseudothecia (West et al. 2001; Salam et al. 2003). Major ascospore showers were often associated with the rainfall events during canola growing season (McGee and Petrie 1979; Gladders and Musa 1980). In France, the decrease in post-summer temperatures and cumulative rainy period favored the release of ascospores from pseudothecia (Peres et al. 1999). Similarly, Marcroft et al. (2003) observed significantly higher ascospore discharge in high rainfall environment followed by medium and low rainfall environment respectively. Although, rainfall >1mm favored the heavy discharge of ascospores, light rain events and dew also facilitated ascospore release in smaller number (McGee 1977). In Saskatchewan, Canada, increased ascospores releases were observed during June and July due to presence of high frequency of moistened stubbles (Petrie 1994). In addition to rainfall, relative humidity also plays an important role in spore dispersal. Daily maximum ascospores and pycnidiospores dispersal occurred in Canada from 9 pm to 4 am when the relative humidity was above 80% and temperatures ranged between 13 to 18°C (Guo and Fernando 2005). No consistent ascospore release pattern have been reported in *L. maculans*. In Canada, ascospores and pycnidiospores were captured during night (Guo and Fernando 2005); however, no such pattern was observed in Australia (Khangura et al. 2007).

The ascospores are disseminated mainly by wind. Wind speed, wind direction, type of crop canopy, and the topography of the area affect the distance travelled by ascospore (Salam et al. 2003; West and Fitt 2005). *L. maculans* ascospores can travel long distance and remain viable for up to one month in dry conditions at temperatures ranging between 5 and 20°C (Huang et al.

2003). Because of its ability to move long distances, in Australia it is recommended that fields be separated by distances of at least 5 to 8 km (Bokor et al. 1975) with fields located within 500 m from the source of inoculum being at the greatest risk (Barbetti and Khangura 2000).

Under field conditions, pycnidiospores could be released immediately after rainfall events but ascospores could be released nearly three days after the rainfall events (Guo and Fernando 2005). Once released, pycnidiospores (conidia) can be dispersed by rain-splash to short distances that range between 45 and 216 cm (Travadon et al. 2007; Barbetti 1976; Hall et al. 1996). Spore dispersal distance is affected by size of rain splash with larger droplets causing greater dispersal distance (Huber et al. 1996; Geagea et al. 2000).

Spore germination and initial plant infection

Germ tubes enter the leaves through stomata or wounds. Under appropriate moisture and temperature conditions, germination of ascospores starts within two hours and reach nearly 90% within 6-8 hours (Li et al. 2004). Pycnidiospores take up to 24 hours to start germinating. Ascospore germination occur at temperatures ranging between 5 and 20°C with, significantly higher germination occurring at 20°C (Huang et al. 2001). Further, optimum condition for leaf lesion production was 20°C temperature and 48 h leaf wetness period (Biddulph et al. 1999). Ascospores took 5 days at 20°C and 15 days at 8°C to develop leaf lesions (Biddulph et al. 1999) whereas pycnidiospores took 6 days at 23°C and 25 days at 8°C to develop leaf lesions (Sosnowski et al. 2005). In addition, high relative humidity favored the germination of pycnidiospores in inoculated cotyledon leaves (Vanniasingham and Gilligan 1989).

Management

Cultural practices

To reduce the amount of incoming inoculum into a new field, buffer distances greater than 400 m are recommended to canola growers in Australia (Marcroft et al. 2004). However, in Canada, that distance is between 50 to 100 meters (Guo and Fernando 2005).

In addition, adaptation of effective crop rotation programs helps break the disease cycle of diseases and pests. Diversification of crops helps in reducing *L. maculans* inoculum and may delay the breakdown of disease resistance genes in commercial cultivars (Kutcher et al. 2011). Thus, four-year rotations are recommended to canola growers in North Dakota to minimize the risk of blackleg disease (Markell et al. 2008). Control of wild mustard and volunteer canola plants is also important between the rotation with other non-host crops.

In addition, management of infected stubbles by raking, burying, or burning is recommended to reduce blackleg inoculum potential. Burying residues speeds up their decomposition and interfere with the release of spores; despite this, minimum tillage is generally practiced in Europe, America and Australia. In contrast, while labor intensive, removal of debris before planting makes blackleg disease less problematic in Asian countries (West et al. 2001). Other common cultural management strategies that could be used include, adjustment of sowing date of canola to avoid peak ascospores release and use of disease-free seeds (Ash 2000; West et al. 2001).

Host resistance

Host resistance is one of the most effective, inexpensive and environmentally friendly management strategies against blackleg disease. Generally, two types of resistance are present in the host, quantitative and qualitative. Quantitative resistance is also known as polygenic,

horizontal or durable resistance which is conferred by multiple genes. In contrast, qualitative resistance is mediated by single genes and provides complete resistance. The resistance gene in *Brassica* spp. interacts with its corresponding avirulence gene in the pathogen to have a resistant reaction (Balesdent et al. 2000). To date, 18 blackleg resistance genes have been identified in *Brassica* spp. They are, *Rlm1* to *Rlm11*, *LepR1* to *LepR4*, *BLMR1* and *BLM2* but only one gene *LepR3* has been cloned (Marcroft et al. 2012; Van de Wouw et al. 2014). Continued cultivation of the same varieties or hybrids can result in the selection of new virulent races making resistance genes no longer effective. Therefore, rotation of the resistance genes is recommended to the growers for the durability of the resistance genes (Kutcher et al. 2007; Marcroft et al. 2012). To our knowledge, most hybrids planted in North Dakota are susceptible to strains capable of infecting plants carrying resistance genes *Rlm1*, *Rlm2* and/or *Rlm3*.

Chemical control

Due to the unavailability of effective levels of resistance to current blackleg strains in commercial cultivars planted in North Dakota, canola growers will rely more on chemical control measures. Generally, fungicides are applied as foliar sprays, coated fertilizer granules, and seed treatments for the management of blackleg disease. Fungicide application is uneconomical in fields with disease resistant cultivars, or with susceptible cultivars with low yield potential and low disease pressure. In Europe foliar application is commonly practiced due to high yields of canola (West et al. 2001); however, fungicides do not provide economic return in Canada (Kutcher et al. 2011). Propiconazole has been commonly used as foliar spray in Canada whereas in Europe difenoconazole, carbendazim or flusilazole alone or in mixtures were used during early 1990's (Gladders et al. 1998). In early 2000's the strobilurins, e.g. azoxystrobin and pyraclostrobin, were introduced in Canada (Kutcher et al. 2011). Currently in

the United States, azoxystrobin, benzovindiflupyr, fluxapyroxad, pyraclostrobin, prothioconazole, and picoxystrobin fungicides are registered for control of blackleg disease (Friskop et al. 2017).

Foliar sprays provide limited protection due to the quick degradation of the chemical on the plant surface and because of the growth of new leaves and leaf expansion (West et al. 2001). Protection of canola plants during the 2 to 4 leaf growth stage is crucial (McGee and Petrie 1979); however, the efficacy of these fungicides diminishes as the interval between the time of inoculation and that of the application increase beyond eight days (del Río Mendoza 2014). A disease forecasting model based on weather conditions that predicts ascospores release would help time fungicide applications better (Salam et al. 2007).

Fungicide seed treatments

Use of fungicide treated canola seed is very common in the United States. It is used as an insurance against blackleg, and Fusarium and Pythium seedling blight diseases of canola (Marcroft and Potter 2008; Hwang et al. 2015). Fungicides used to treat seeds can be of broad-spectrum activity (effective against broad range of fungus) or of narrow spectrum of activity (protection against few species fungus). Similarly, systemic fungicides provide early protection against *L. maculans* spores infection during seedling stage of plant. In contrast, contact fungicides eliminate the seedborne inoculum, avoid the spread of the disease into new areas and also the introduction of new virulent races of *L. maculans* through seeds (Gugel and Petrie 1992; Mancini and Romanazzi 2014). Fungicide treatment is more effective in controlling seed-borne inoculum in comparison to physical treatments, plant extract and essential oil treatments, and biopesticide treatments (Mancini and Romanazzi 2014).

Canola is most susceptible to blackleg infection at the seedling stage. *L. maculans* infection after five-leaf growth stage does not result in stem canker development (Marcroft et al. 2005). According to Elliott et al. (2011), seed treatment fungicide (fluquinconazole) protect canola up to six weeks against blackleg. Therefore, use of treated seed could eliminate the need of foliar spray during early growth stage (Mancini and Romanazzi 2014). However, the Canada Council of Canada, reported that seed treatments could effectively reduce seed-borne inoculum but do not protect from infections that take place at later stages of development.

In Australia, treating seeds from susceptible canola cultivars with fluquinconazole reduced blackleg severity and plant mortality and increased canola yield under high-disease pressure conditions but it was not economical in low disease-pressure areas (Khangura and Barbetti 2004; Marcroft and Potter 2008) or when used on resistant cultivars. Under high disease pressure condition, plants from moderately resistance cultivars that had their seeds treated with fungicides performed like resistant cultivars (Marcroft and Potter 2008). Similarly, canola seeds imbibed in suspension of systemic fungicide, flutriafol (6 g/kg seed) reduced the disease severity at cotyledon stage, two leaf and four-leaf growth stages (Sprague and Burgess 2001).

In Canada, carbathin, thiram, and iprodione and in Europe thiram and iprodione were commonly used as a seed-treatments against blackleg (West et al. 2001). In North Dakota, as of 2018, eight seed treatment fungicides have been registered for use against blackleg diseases (Friskop et al. 2017). They are: 1) Dynasty (9.6% azoxystrobin), 2) Rancona V RS (6.78% carboxin, 0.73% ipconazole), 3) Prosper EverGol (22.32% clothianidin, 0.82% penflufen, 0.55% trifloxystrobin, 0.55% metalaxyl), 4) Maxim 4FS (40.3% fludioxonil), 5) Allegiance FL (28.35% metalaxyl), 6) Helix Vibrance ((0.26% sedaxane, 1.25% difenoconazole, 0.40% mefenoxam 0.13% fludioxonil, 20.7% thiamethoxam), 7) Thiram 480 DP (42% thiram), and 8) Obvius

(1.58% fluxapyroxad, 1.58% pyraclostrobin, 1.26% metalaxyl). Of these, Helix Vibrance is the most widely used seed treatment and provides systemic protection against insects as well.

Even though, new seed treatment compounds are registered in North Dakota, the effectiveness of these newly registered treatments have not been explored well. The study of the efficacy of recently registered seed treatment fungicides would help growers make appropriate selections.

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CHAPTER 3: ROLE OF WEATHER VARIABLES ON PSEUDOTHECIA MATURATION UNDER CONTROLLED AND FIELD CONDITIONS

Abstract

The effect of weather variables on *Leptosphaeria maculans* pseudothecia maturation was studied under controlled and natural conditions in North Dakota. Under controlled conditions, blackleg-infected canola stems were incubated at 8, 13, and 18ºC, in growth chambers and inspected weekly for the presence of pseudothecia. Under field conditions, one-year old blackleg infected stems were collected weekly between May and July of 2017 and 2018 from four North Dakota locations and inspected for presence of pseudothecia. Logistic regression analysis showed a significant association ($P = 0.0157$) between heat-unit accumulation and pseudothecia maturation under controlled conditions. Under natural conditions, mature pseudothecia were first observed during the third week of May in 2017 and the second half of June in 2018 when canola plants were at seedling to early bud stage, respectively. Logistic regression analyses revealed the association between pseudothecia maturation, air temperature (expressed as cumulative heat units), and relative humidity (expressed as number of hours with RH $\geq$ 75%) in North Dakota. The resulting model has high predictive power with >70% sensitivity and specificity; however, data from additional location-years are needed for further validation of the model.

Introduction

Blackleg disease is one of the major production constraints in canola producing countries worldwide. Blackleg is caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. & de Not [anamorph = *Phoma lingam* (Tode:Fr.) Desm.] (West et al. 2001; Howlett 2004; Chen and Fernando 2006). In North Dakota, yield losses caused by this disease generally range between 5% to 20%; however, losses of up to 50% have been reported in some areas under severely

infected conditions (Markell et al. 2008; del Río Mendoza et al. 2012). The disease is reemerging as a threat in North Dakota because virulent strains belonging to pathogenicity group (PG) 4 have increased in prevalence and are capable of infecting most commercial hybrids planted in the state (Marino 2011; Nepal et al. 2014). Strains belonging to this group are virulent to cultivars carrying resistant genes *Rlm1*, *Rlm2* or *Rlm3* (Balesdent et al. 2002).

L. maculans overwinters in blackleg infected residues from previous year and produces sexual ascospores and asexual pycnidiospores. Ascospores serve as primary inoculum and are produced on pseudothecia. Ascospores are released into air and are dispersed by the wind sometimes to distances of up to a few kilometers. The time of year when spores disperse varies among countries and between regions within a country due to differences in climatic conditions, oilseed rape type planted, and agricultural practices (Fitt et al. 2006; West et al. 2001). Ascospore maturation and release usually coincide with the emergence of canola seedlings. Ascospores release peak between September and November in western and central Europe (Gladders and Musa 1980) and during June, July and August in Australia (McGee 1977) and Western Canada (Kharbanda 1993). The dynamics of *L. maculans* ascospore dispersal in North Dakota has not been characterized.

Mature pseudothecia produce six-celled ascospores and its maturation is dependent on environmental conditions like temperature, moisture, relative humidity. In Australia, 73 to 192 days were required for pseudothecia to mature (Khangura et al. 2007) whereas in France it took between 16 and 51 days (Peres et al. 1999). Salam et al. (2003) reported that 43 favorable days, (days with average temperature $< 22^{o}$C and weekly rainfall $\geq$ 4mm) were required for pseudothecia maturation in Western Australia. In France, average temperature of 14°C and rainfall of 2.5 mm in 3-4 days interval was favorable for the maturation of pseudothecia under

controlled conditions (Peres et al. 1999). In UK, pseudothecia matured nearly 34 days earlier at temperature 20°C compared to 5°C (Toscano-Underwood et al. 2003). Similarly, in Canada, 15°C temperature was optimum for ascospores formation in naturally infected debris compared to 10, 15, 24, 28 and 32°C temperatures (Petrie 1994).

Association of temperature, rainfall and relative humidity on *L. maculans* pseudothecia maturation and ascospore release has been studied in Australia, Canada and Europe (Guo and Fernando 2003; Salam et al. 2003; Huang et al. 2005). However, there is no information available on the timing and optimum climatic conditions required for pseudothecia maturation in the United States. The development of a model for pseudothecia maturation of *L. maculans* would help canola growers in North Dakota to make fungicide spraying decisions.

The objectives of this study were, to characterize the effect of temperature on pseudothecia maturation under controlled condition and to determine the timing of pseudothecia maturation and its association with weather variables under field conditions in North Dakota.

Materials and Methods

Controlled condition study

Stubble preparation

Single-spore cultures of *Leptosphaeria maculans* isolates collected from North Dakota fields were prepared and kept at -80^{o}C. Isolates BL46, BL425, BL428, and BL436 belonging to Mating type-1 and BL422, BL423, and BL424 belonging to Mating type-2 were retrieved from the freezer and cultured in V8 medium in 9 mm Falcon Disposable Petri Dishes (BD Biosciences, Northbrook, Illinois) separately. The inoculated plates were incubated under continuous white light for 12 days at room temperature (21^{o}C). After 12 days each plate was flooded with 5 ml sterile distilled water and scrapped with sterile glass rod to dislodge conidia

from pycnidia. The suspension was collected on the plastic vials and concentration was estimated using hemocytometer and adjusted to 10^7 pycnidiospores/ml. Equal volumes of suspensions from each isolate were mixed and immediately taken to the greenhouse to be used.

In greenhouse, 400 plastic pots were filled with soilless potting mix (PRO-MIX BX, Premier Tech Horticulture, Quakertown, PA) and two seeds of cv. Westar were planted at the center of each pot. Pots were kept in greenhouse rooms maintained at 20 ± 2°C with 16 hours of photoperiod regulated by 600-Watt high pressure sodium-lamps (P.L. Light Systems, Inc., Beamsville, Ontario, Canada). Five days after planting, the plants were thinned to one seedling per pot. Ten days after planting, both cotyledon leaves of seedlings were pricked once in the center of each leaf with a tweezer and 10 µl of the spore suspension were deposited on each wound. The inoculated plants were incubated in dark mist chambers for 24 hours to facilitate the infection and then moved back to the greenhouse room and allowed to grow until maturity. Upon maturity, cankered plants were uprooted from the pot and its lower portion cut into four-inches long pieces that included the crown region. These stem pieces were air-dried for two days and stored in the fridge at -20°C for three months to emulate winter conditions.

Experiment setup

Svea-Barnes loam type soil was collected from the Cavalier County, ND and uniformly mixed by passing it through 2-mm mesh sieves. Water holding capacity (WHC) was determined using the funnel test method using five 50 gm samples as described by Noggle and Wynd (1941) with some modifications. Briefly, the soil samples were poured onto Whatman filter #1 paper that was folded and placed in the funnel. Water was poured on each sample until saturation and after five hours, when water had stopped dripping out of the funnel, the soil was weighted. The

same sample was oven dried for 24 h at 105ºC and weighed. The water holding capacity was calculated as (weight of wet soil- weight of dry soil)/ weight of dry soil.

Plastic trays, 15×10×5 cm, were wrapped with aluminum foil and filled with soil. Soil moisture content of each tray was adjusted to 75% of WHC. In each tray, five cankered stem pieces were placed on the soil surface and pressed lightly into it. Each tray was considered a replication. Trays were incubated at either of three-temperature regimes, 10ºC at day and 5ºC at night (daily average 8ºC); 15ºC at day and 10ºC at night (daily average 13ºC); or 20ºC at day and 15ºC at night (daily average 18ºC). The experiment was conducted using a complete randomized experimental design and the study was conducted three times.

Data collection

Two weeks after the setup of the experiment, each stem was examined under dissecting microscope for presence of pseudothecia. Pseudothecia samples were placed on a glass slide, squashed, and observed under the compound microscope at 400× magnification for presence of ascospores. This process was repeated on a weekly basis for the next eight weeks. Stems without mature pseudothecia were placed back in the soil tray in the growth chamber.

Field study

Location and data collection

Timing of pseudothecia maturation on blackleg infected stem debris was studied under natural condition in 2017 and 2018 in North Dakota. For the year 2017, blackleg-infected canola stems from 2016 were collected from commercial canola field during first week of May and deployed at the NDSU Research Extension Center at Langdon, ND and fifty stems were collected each week starting from May 31 to June 30. For the 2018 field study, the cankered stems were collected from a commercial canola field in Cavalier County at the end of the 2017

growing season. The stems were cut in six cm long pieces containing the crown area and placed in nylon pouches in groups of 12 stubbles. The residues were placed in a field to overwinter and during the 2018 season they were deployed during first week of May in wheat or canola fields in Fargo, Langdon, Minot, and Hettinger, ND. At each location, ten pouches were deployed by placing them on the soil surface. One pouch from each location was collected every week starting on May 9th and was placed in a freezer at -20^{o}C until it was inspected in the lab. Like in the controlled-condition study, pseudothecia were periodically examined under the microscope for the presence of mature ascospores.

Hourly data on air and soil temperature (^{o}C), precipitation (mm) and relative humidity (%) during the canola growing season at Langdon in 2017 and in Langdon, Hettinger, Minot, and Fargo in 2018, were retrieved from weather stations deployed by the North Dakota Agricultural Weather Network (https://ndawn.ndsu.nodak.edu//index.html). The downloaded data were processed further to calculate the average air and soil temperature, total rainfall, numbers of hours with $\geq$ 75% relative humidity for a one-week period. In addition, cumulative heat units (with bases at 0, 5, 8, and 13^{o}C) accumulated at the end of each week were calculated.

Data analyses

Logistic regression analysis was used to evaluate the association between weather variables and pseudothecia maturation under controlled and field conditions. The weekly recorded data on pseudothecia maturation was converted into the dichotomous form 0 and 1. Under controlled condition if percentage of stems with mature pseudothecia was < 10% and $\geq$ 10% the days were designated as ‘0’ and ‘1’ respectively. For field study, ‘0’ and ‘1’ represented absence and presence of pseudothecia, respectively. For controlled condition study, total heat-unit accumulation at all sampling dates were calculated for each incubation temperature by

multiplying the average daily temperature in the growth chamber by the numbers of incubation days. These units were used as predictors an independent variable. Similarly, for the field study, weekly weather variables calculated for temperature, relative humidity, rainfall were used. Logistic regression analysis was performed separately for controlled and field condition study using SAS version 9.4 (SAS Institute, Inc., Cary, NC). The non-significant ($P > 0.05$) weather parameters were eliminated and significant ($P \leq 0.05$) ones were used to develop the model. The statistical significance of the models was evaluated based on the Likelihood ration test ($P > \chi^2$) and quality of models were compared using Akaike information criterion (AIC) and Schwarz criterion (SC) values. Similarly, Hosmer-Lemeshow goodness-of-fit test was used to evaluate fit of model and *c* statistic was used to determine the predictability of the model. Sensitivity (true positive proportion) and specificity (true negative proportion) of the models were calculated and used to estimate the overall accuracy of the models using the following equation:

$$Accuracy = Sensitivity * \frac{Observed\ Cases}{N} + Specificity * \frac{Observed\ Controls}{N}$$

Where observed cases are the number of observations considered "diseased"; observed controls are the number of observations considered "not diseased"; and *N* is the total number of observations.

Results

Effect of temperature on pseudothecia maturation under controlled environment condition

Mature pseudothecia were observed at all temperatures evaluated but the timing and rate of maturation differed for each temperature conditions. Pseudothecia were observed earlier and matured at significantly faster ($P = 0.05$) rate at 18°C compared to lower temperatures (Fig 3.1). Mature pseudothecia were detected on first sampling date, two weeks after experiment setup, at 18 and 13°C temperatures but at 8°C it took another two weeks to first

appear. After ten weeks of incubation, almost three-fourths of the stems incubated at 18°C had mature pseudothecia on them, but only one-fifth of those incubated at 13 or 8°C had mature pseudothecia on them. Production of mature pseudothecia increased linearly over time at all three temperatures ($R^2 \geq 0.85$). Based on these, pseudothecia on stems incubated at 18°C matured at a rate four times faster than those incubated at lower temperatures (Fig 3.1).

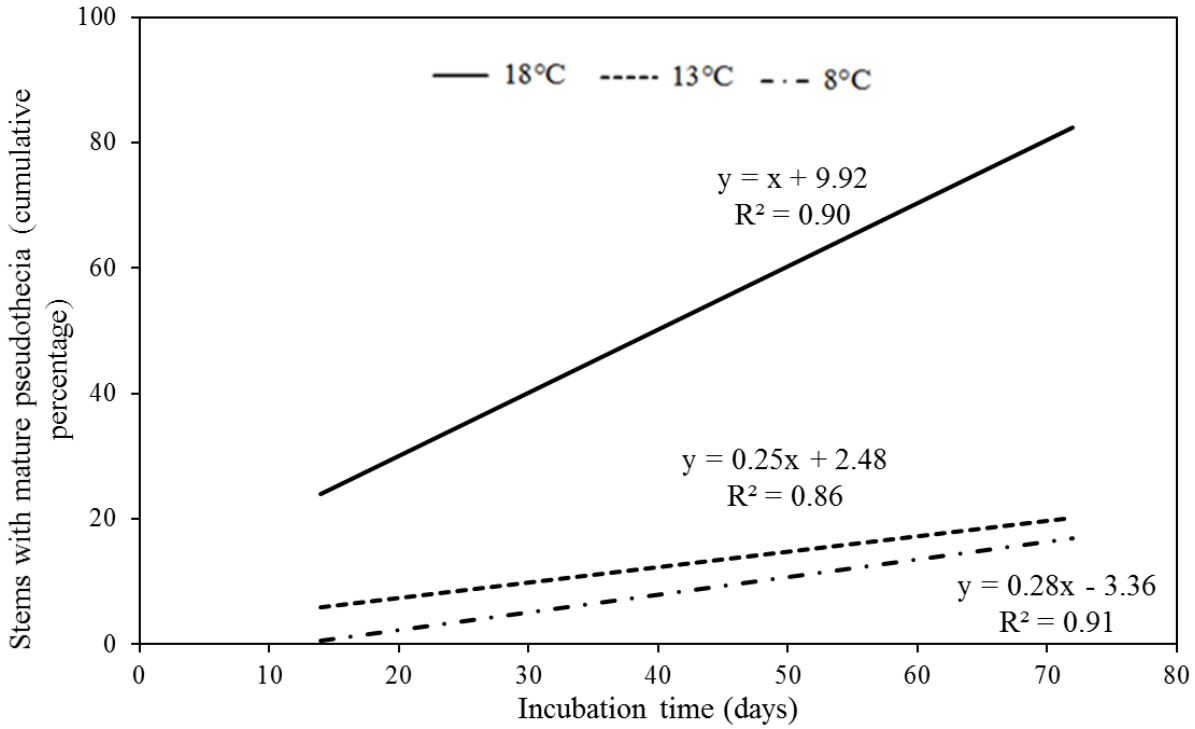

Figure 3.1. Linear regression models for rate of pseudothecia maturation at different temperatures under controlled condition.

Model to predict the probability of pseudothecia maturation under controlled condition

The logistic regression analysis showed significant positive association ($P < 0.05$) between heat-unit accumulation and pseudothecia maturation at all incubation periods after 35 days. Although models developed with fewer than 35 days had lower AIC and SC values, the value of the *c*-statistics and the sensitivity and accuracy of models improved when models were developed with data from more than 35 days (Table 3.1). The model with data from 70 days was

selected as having the best combination of predictive power with a sensitivity of 88% and an overall accuracy of 85%. The model was,

$$P = -6.14 + 0.016 * \text{accumulated heat-units}$$

Where, P is logit of odds of pseudothecia being mature. Based on the above model, the chances of pseudothecia maturation are > 50% when total heat-unit accumulation is more than 400 in a 70-day period; however, when the accumulated heat-units reached 756, the chances increased to 100% (Fig 3.2).

Table 3.1. Logistic regression models for pseudothecia maturation based on heat-unit accumulation at different days after experiment setup.

Model[a]	AIC[b]	SC[c]	Model significance (*P*)	*c*-value	Hosmer & Lemeshow test	TPP[d] (%)	TNP[e] (%)	Model accuracy (%)
28-days	11.3	11.7	ns					
35-days	14.7	15.6	ns					
42-days	15.8	17.2	0.0453	0.89	0.71	67	89	80
49-days	17.0	18.8	0.0305	0.90	0.48	75	90	83
56-days	18.0	20.1	0.0252	0.92	0.82	82	80	83
63-days	18.4	20.8	0.0200	0.94	0.91	86	80	84
70-days	18.6	21.1	0.0157	0.95	0.80	88	80	85

[a]Models based on heat-unit accumulation per time period.
[b]AIC = Akaike information criterion
[c]SC = Schwarz criterion
[d]TPP = True positive proportion (Sensitivity) expressed as percentage
[e]TNP = True negative proportion (Specificity) expressed as percentage
ns = not significant at $P = 0.05$

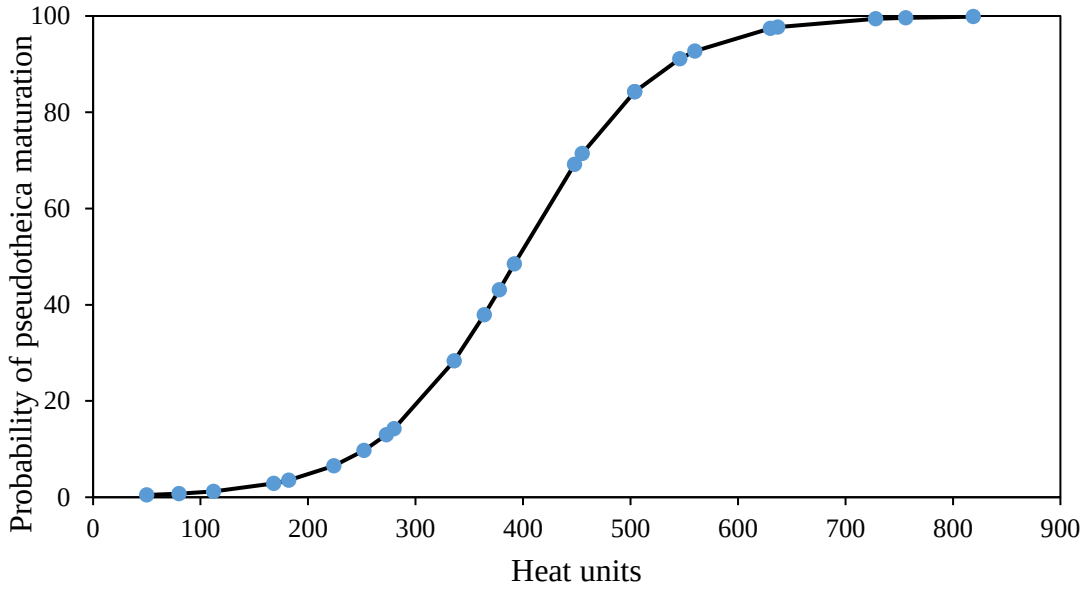

Figure 3.2. Probability of pseudothecia maturation as a function of heat-unit accumulation during a 70-days period estimated from data collected in controlled condition trials.

Pseudothecia maturation under natural condition

In Langdon 2017, mature pseudothecia were first detected during the third week of May on nearly 5% of the canola residues. Number of stems with mature pseudothecia increased with incubation time and was at its highest at the end of June with nearly 12% of the samples having mature pseudothecia. In 2018, mature pseudothecia were observed nearly 40 days later than in 2017; however, the numbers of stems with mature pseudothecia was higher in 2018. Stems incubated at Hettinger and Minot in 2018, produced mature ascospores one week earlier than those incubated at Fargo and Langdon. Samples from Hettinger had higher frequency of mature pseudothecia on all dates followed by Langdon, Fargo and Minot (Fig 3.3).

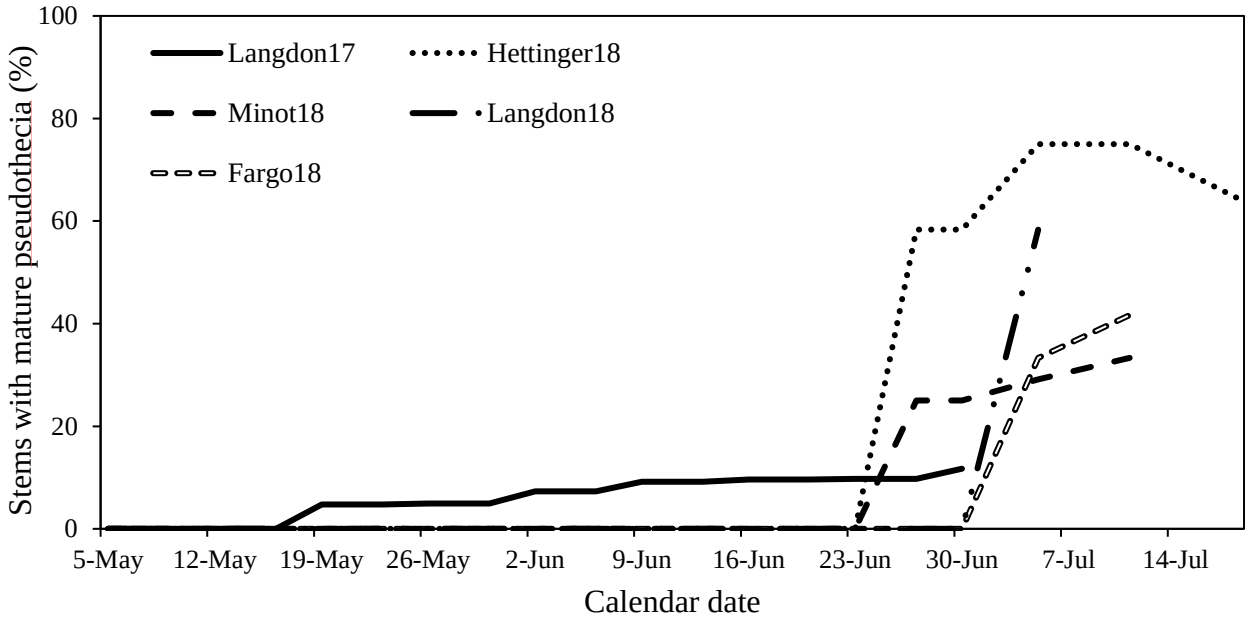

Figure 3.3. Temporal dynamics of pseudothecia maturation at different North Dakota locations in 2017 and 2018.

Weather variables

Langdon had higher average air temperature and total rainfall in 2018 compared to 2017. The temperature ranged between -1 and 30°C and between -4 and 31°C during the period between May 1 and July 31 of 2017 and of 2018, respectively. In 2018, Langdon received nearly 37 mm more rainfall compared to 2017, however, the number of rainy days with >1mm rainfall were similar, 21, for both years. At Langdon, the month of June had the highest numbers of rainy days with seven in 2017 and nine in 2018. The average interval between the rainy days were three and two for 2017 and 2018 respectively. The average daily relative humidity for the three-month period was similar for both 2017 and 2018 in Langdon (Table 3.2). The month of May had the lowest average relative humidity of the three months with only six and four days with daily mean relative humidity of 75% or higher in 2017 and 2018 respectively. The average air

Table 3.2. Mean daily temperature (°C), mean daily relative humidity (%), and total precipitation (mm) during May, June and July month at four locations of ND in 2017, 2018 and 20-year average.

Station	May			June			July		
	T_{av}[a]	RH_{av}[b]	P_{tot}[c]	T_{av}[a]	RH_{av}[b]	P_{tot}[c]	T_{av}[a]	RH_{av}[b]	P_{tot}[c]
Langdon-2017	11.1	57.7	24.6	16.5	67.8	74.7	19.2	76.4	48.8
Langdon-2018	14.6	54.1	38.9	18.6	69.9	85.9	19	75.8	61
Fargo-2018	18	54.6	43.6	21.4	65.7	123.2	21.9	70.7	80.9
Hettinger-2018	14.8	69	41.7	18.5	71.2	93.5	20.7	65.8	69.1
Minot-2018	16	52.5	16.4	19.4	68	125.1	20.3	68	36.6
Langdon- 20-year average	11.0	65.6	70.4	16.5	72.2	91.7	19.2	75.8	67.4

[a]T_{av} = Average air temperature for a month
[b]RH_{av} = Average relative humidity for a month
[c]P_{tot} = Total amount of rainfall for a month
Source: NDAWN

temperature at Langdon in 2017 and 2018 was similar to the 20-year average but the total rainfall in both years were lower than the 20-year average. In addition, the relative humidity during May and June in both years was also lower than the 20-year average (Table 3.2). In 2018, Fargo was comparatively hotter with lower relative humidity and highest amount of total rainfall than the other locations used in the study. However, the frequency of rainfall was higher in Hettinger in both May and June month with highest average relative humidity (Table 3.2).

Role of weather variables in pseudothecia maturation under natural condition

The average temperature and relative humidity during mature pseudothecia observation period were 14.5°C and 66.2% in 2017 and 21.6°C and 72.3% in 2018. Mature pseudothecia were first observed in Langdon in 2017 when average air temperature for the previous 7-day period was 10.7°C with a single rainfall event that dropped > 2mm rainfall in a day and 37 hours of > 75% RH (Table 3.3). Afterwards, the values for all weather variables during previous 7-day period were improved during rest of the sampling period with highest during mid-June. However, in 2018, mature pseudothecia were observed when average temperature was 19.6°C, number of rainfall events > 2 mm/day were four and number of hours with >75% RH were 106 per week (Table 3.3).

Logistic regression analyses showed a significant (P = 0.0025) positive association between the number of hours with ≥75% relative humidity per week and cumulative heat-units (base =13°C) with pseudothecia maturation. The Akaike information criterion (AIC) and Schwarz criterion (SC) values for the model were 57 and 63 respectively. The *c*-statistic value for the model was 0.79 and Hosmer & Lemeshow test was also not significant (P = 0.47). The model was 74% accurate with 71% sensitivity and 75% specificity. The proposed predictive model was, P = -3.42 + 0.0049 (cumulative heat units) + 0.028 (no. of hours with ≥ 75% RH)

Table 3.3. Average air temperature, average relative humidity, total rainfall, number of hours with ≥75% relative humidity, number of rainfall events with ≥1mm rainfall, cumulative heat units during one-week period in Langdon, Fargo, Hettinger and Minot in 2017 and 2018.

Station	Weeks since April 1	Average air temperature (°C)	Average relative humidity (%)	Total rainfall (mm)	Number of hours with ≥75% RH	Number of rainfall events with ≥1mm rainfall	Cumulative heat units (base=13°C)
Langdon-2017	6	12	44	0	11	0	34
	7	11	56	5	38	1	4
	8	11	53	3	37	0	10
	9	11	69	13	77	6	10
	10	16	59	9	63	1	31
	11	19	62	31	41	3	75
	12	16	73	31	98	10	100
	13	14	74	3	92	1	109
	14	16	71	5	78	2	127
Langdon-2018	6	12	42	4	16	2	11
	7	13	55	27	49	12	25
	8	20	55	1	38	1	46
	9	19	73	27	90	5	106
	10	18	65	1	54	0	126
	11	18	71	21	79	9	165
	12	20	62	5	47	1	208
	13	20	76	37	109	4	264
	14	20	78	19	106	4	313

Whole number was used for each data point
Source: NDAWN

Table 3.3. Average air temperature, average relative humidity, total rainfall, number of hours with ≥75% relative humidity, number of rainfall events with ≥1mm rainfall, cumulative heat units during one-week period in Langdon, Fargo, Hettinger and Minot in 2017 and 2018 (continued).

Station	Weeks since April 1	Average air temperature (°C)	Average relative humidity (%)	Total rainfall (mm)	Number of hours with ≥75% RH	Number of rainfall events with ≥1mm rainfall	Cumulative heat units (base=13°C)
Fargo-2018	6	14	53	8	36	4	35
	7	18	52	28	37	5	69
	8	22	54	0	23	0	95
	9	22	67	19	49	6	178
	10	21	60	36	30	2	226
	11	21	70	35	69	9	278
	12	22	61	21	40	2	342
	13	23	70	20	67	2	414
	14	29	72	39	72	7	494
	15	25	70	10	60	4	565
Hettinger-2018	6	11	75	23	98	7	23
	7	14	73	3	85	1	33
	8	18	62	3	47	1	50
	9	19	71	12	77	5	104
	10	21	59	3	36	2	150
	11	18	66	16	68	6	195
	12	16	79	22	109	5	221
	13	19	82	51	114	8	259
	14	21	70	21	72	2	308
	15	25	64	0	54	0	382
	16	21	68	22	74	9	445

Whole number was used for each data point
Source: NDAWN

Table 3.3. Average air temperature, average relative humidity, total rainfall, number of hours with ≥75% relative humidity, number of rainfall events with ≥1mm rainfall, cumulative heat units during one-week period in Langdon, Fargo, Hettinger and Minot in 2017 and 2018 (continued).

Station	Weeks since April 1	Average air temperature (ºC)	Average relative humidity (%)	Total rainfall (mm)	Number of hours with ≥75% RH	Number of rainfall events with ≥1mm rainfall	Cumulative heat units (base=13ºC)
Minot-2018	6	14	45	1	16	0	28
	7	15	52	4	32	1	49
	8	22	51	0	29	0	71
	9	19	74	33	90	13	132
	10	20	60	1	46	0	164
	11	19	64	58	52	12	216
	12	20	67	4	71	1	255
	13	20	78	27	104	3	307
	14	21	72	6	87	2	356
	15	23	68	12	63	2	419

Whole number was used for each data point.
Source: NDAWN

Based on this model, the chances of pseudothecia maturation increases with increase in cumulative heat-units and numbers of hours with ≥ 75% RH (Fig 3.4).

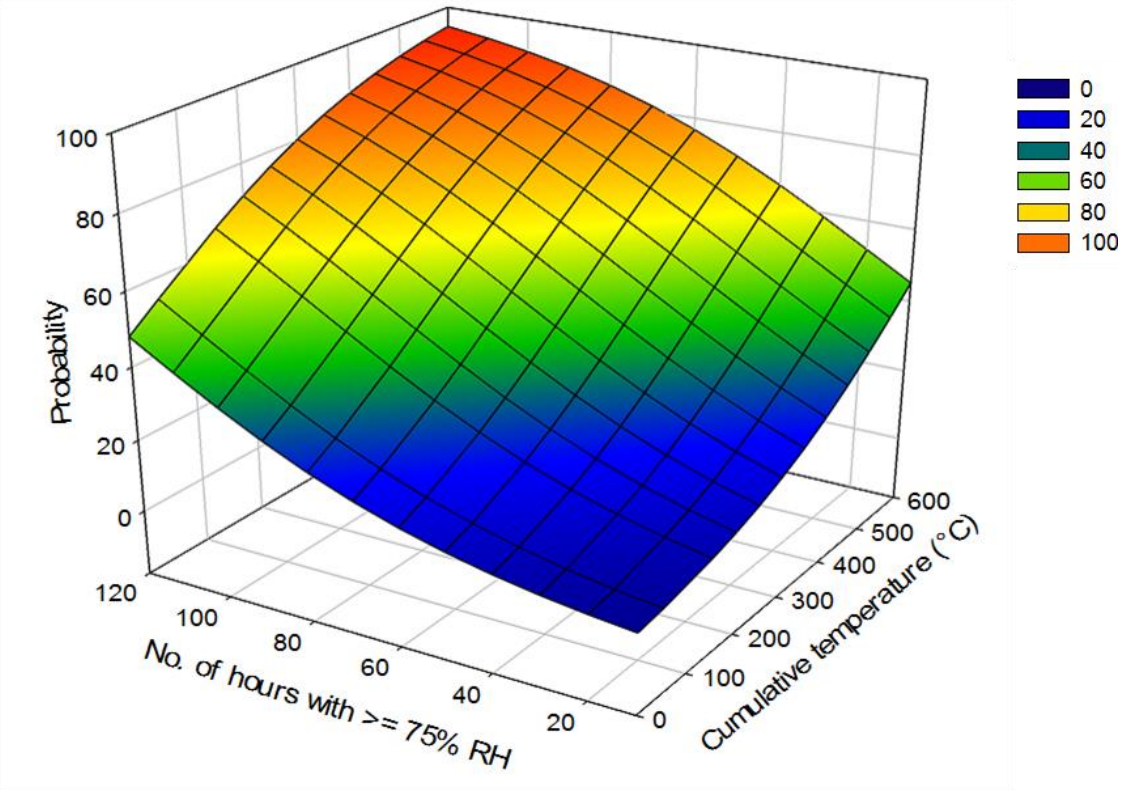

Figure 3.4. Probability of pseudothecia maturation as a function of relative humidity (no. of hours with ≥75% RH) and cumulative air temperature (base=13°C) under field condition in North Dakota.

Discussion

Two studies were conducted to identify the role of weather variables on pseudothecia maturation. Results from the first study confirmed the significant positive association (P = 0.0157) between heat-units and pseudothecia maturation under controlled condition. The second study, conducted under natural conditions, revealed the occurrence of pseudothecia maturation during mid-May to late July in North Dakota and produced a model that identified high relative humidity (≥ 75%) and heat units as being significantly associated with this phenomenon.

The importance of temperature in *L. maculans* pseudothecia maturation has been highlighted by researchers in other countries. Studies conducted in the United Kingdom and Australia reported 15 to 20^{o}C as an optimum temperature condition for pseudothecia maturation under controlled environment conditions (Toscano-Underwood et al. 2003; Naseri et al. 2009). Similarly, in Canada, 15^{o}C temperature was identified as favorable for pseudothecia maturation on naturally infected debris (Petrie 1994). In agreement with our findings, mature pseudothecia were observed within 2-3 weeks at 15-20^{o}C and after 40 days at 10^{o}C (Toscano-Underwood et al. 2003). Similarly, in France, pseudothecia developed earlier at 14^{o}C temperature with high frequency of rainfall or 100% relative humidity under controlled condition (Peres et al. 1999). In different studies, Petrie (1995) and Peres et al. (1999) did not observe mature pseudothecia below 10^{o}C and above 30^{o}C temperature (Petrie 1995; Peres et al. 1999). Their results are in contrast to the findings in our study where mature pseudothecia were observed after four weeks of incubation at 8^{o}C temperature under controlled condition. However, the rate of pseudothecia maturation was significantly lower than higher temperature condition (18^{o}C).

According to the model developed under controlled-conditions, there was 50% chance that mature pseudothecia could be observed as soon as 396^{o}C heat-units were accumulated. Similar findings were observed by Toscano-Underwood et al. (2003) who estimated the requirement to produce mature pseudothecia to be 420 heat-units.

In contrast to the previous studies where stems were kept on wet sand (Toscano-Underwood et al. 2003; Naseri et al. 2009), in this study stems were kept on soil surface and moisture was supplied through soil. The intention was to emulate the field condition in North Dakota where conventional tillage practice is common, and stems are in direct contact with soil.

Canola is cultivated during the summer season in North Dakota. Canola harvest is followed by cold winter when pathogen withstands the subzero temperature and produce pseudothecia during next canola growing season. The significant differences observed between 2017 and 2018 in the timing when first mature pseudothecia were detected, highlight the contribution of other factors in addition to temperature, e.g. relative humidity which was also comparatively lower during May in 2018. Previous studies have reported differences in the timing of pseudothecia maturation between years within the same locations (Salam et al. 2003; Huang et al. 2005; Dawidziuk et al. 2012). While the model produced in this study was elaborated using observations from five-location years, there is no doubt that additional data collected from different locations in future years would strengthen the model; this is especially true when considering that the years 2017 and 2018 were relatively drier than average.

In Australia, 73 to 192 days were required to produce mature ascospores (Khangura et al. 2007) whereas in France it took nearly 16 to 51 days (Peres et al. 1999). In western Canada, which borders North Dakota, mature ascospores were produced nine months after the harvest of canola due to long winter (Petrie 1995). However, in Kentucky, United States, pseudothecia developed nearly one week after the harvest of winter canola (Hershman and Perkins 1995). The significant variation in onset of pseudothecial maturity between the regions and seasons was driven by differences in weather conditions, canola growing seasons, and canola type grown in the region (Salam et al. 2007). Despite significant variation in timing of pseudothecia maturation and release between the locations, the spore release often coincides with seedling stage of the canola causing economic damage to the crop (West et al. 2001).

Pseudothecia maturation under natural condition was predicted using logistic regression. Relative humidity during one-week period and total accumulated temperatures were found to be

significantly associated to pseudothecia maturation. Different studies have confirmed the role of relative humidity and temperature accumulation in ascospore maturation and release in different pathosystems (Llorente and Montesinos 2004; Eikemo et al. 2011; Qandah and del Río Mendoza 2011). Based on model, high heat unit accumulation (> 250 units, base=13^{o}C) and extended hours of high relative humidity (≥ 75%) increases the chances of pseudothecia maturation in North Dakota. The model had satisfactory accuracy with good sensitivity and specificity values.

Previous studies have shown significant positive effect of temperature and moisture in *L. maculans* pseudothecia maturation (Petrie 1995; Peres et al. 1999; Salam et al. 2003; Toscano-Underwood et al. 2003; Huang et al. 2005). In addition, various studies have signified the importance of moisture in pseudothecia maturation process (Petrie 1995; Peres et al. 1999; Salam et al. 2003; Khangura et al. 2007). Frequent rainfalls of >1mm rainfall were found to be enough to accelerate the pseudothecia maturation. Although in present study, pseudothecia maturation was not related to the rainfall but its role cannot be overlooked. North Dakota had at least six rainfall events per month with > 1 mm rainfall during canola growing season in both 2017 and 2018. According to McGee (1977), in the absence of rainfall, high relative humidity and heavy dews help to moisten the stems. Mature pseudothecia were observed in France at 100% relative humidity in the absence of rainfall events (Peres et al. 1999).

For the greenhouse and field studies, one-year-old stubbles were examined for the presence of pseudothecia. Almost 80% of the stems obtained in greenhouse study and between 30 to 75% of the field-collected stems produced pseudothecia in this study. The greater percentage observed in the controlled conditions studies suggest that when appropriate temperature and moisture conditions provided, the fungus will able to produce the pseudothecia in short period. However, under field condition, no consistent weather conditions are observed.

Though, temperature was somewhat consistent throughout the growing season, the longer dry period under natural condition make not conducive as that of the controlled condition study.

Mature pseudothecia release ascospores which are the major source of inoculum of the blackleg disease. This two-year study provided a basic understanding on dynamics of pseudothecia maturation and relationship with weather variables in North Dakota. While the strength of the models developed here could be improved by adding more location-years, once validated, these models could be implemented in North Dakota to alert growers when *L. maculans* ascospore showers are imminent, so they can take appropriate measures to protect their crops.

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CHAPTER 4: MODELING THE TEMPORAL DYNAMICS OF LEPTOSPHAERIA MACULANS ASCOSPORE DISPERSAL IN NORTH DAKOTA

Abstract

Blackleg, caused by *Leptosphaeria maculans*, inflicts greatest canola yield losses when plants are infected before reaching the six-leaf growth stage. During this period, fungicide applications made more than one week after initial infection provide significantly less protection. The objectives of this study were to characterize the dynamics of *L. maculans* ascospore dispersal and to identify environmental factors associated with this phenomenon. Airborne ascospores concentrations were monitored using Burkard 7-day volumetric samplers between mid-May and mid-July in Langdon, ND in 2017 and 2018. Ascospores were identified and counted under compound microscope. Concentrations > 5 ascospores/m^3 of air were considered peaks. Ascospores concentrations peaked during mid to late June in both years when canola plants were at four leaf stage to early bud stage. Logistic regression analysis indicated there was a significant association between ascospore peaks and seven-day moving averages of daily mean relative humidity and soil temperature. In this way, there is a 50% probability that ascospores peaks would occur when the daily mean relative humidity is at 50% and the soil temperature is at 15^{o}C. These results suggest that a warning-system could be developed; however, additional locations-years are needed to strengthen it before it is deployed.

Introduction

Blackleg (Phoma stem canker, stem canker), caused by the ascomycete fungus, *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph *Phoma lingam* (Tode:Fr.) Desm.) is a major constraint for canola production in North Dakota (Markell et al. 2008). Under severely

infected conditions, the disease can reduce yield by 45% or more (del Río Mendoza et al. 2012). *L. maculans* survives saprophytically as fruiting bodies, pseudothecia and pycnidia, on crop residue. These structures release ascospores and pycnidiospores, respectively, under favorable environmental condition to start the disease cycle. The spores infect the leaves and produce gray-green to ash-green lesions at the infection site. The fungus colonizes the stem crown region to form cankers that result in premature ripening, lodging and death of the plant (Markell et al. 2008; West et al. 2001). Integration of management practices like crop rotation, weed and volunteer plant control, use of resistant cultivars, and fungicide application can be used to effectively manage the disease (Kharbanda and Tewari 1996; Fitt et al. 2006; Kutcher et al. 2011).

Previous studies have reported the role of environmental factors e.g. temperature, rainfall, and relative humidity on *L. maculans* pseudothecia maturation. In France, studies in controlled environment showed mature pseudothecia could be produced within 13 to 16 days when stems were incubated in average daily temperatures of 14°C with simulated rainfalls of 2.5 mm at 3-4 days intervals; reducing rain frequency to one event every 8 days increased the time to maturity to 26 days but when no moisture was provided, pseudothecia did not mature within 30 days independently of the incubation temperature (Peres et al. 1999). In England, 50% of pseudothecia matured within 29 days when stems were incubated at 15^{o}C on sand held at constant moisture saturation but within 41 days when incubated at 10^{o}C (Toscano-Underwood et al., 2003). In Australia, at 15 ^{o}C, mature pseudothecia were produced on stubbles sprayed with water two or three times a day but no pseudothecia were observed with only one water-spray per day (Naseri et al. 2009). In addition, Salam et al. (2003) reported the need of 43 favorable days (10-day average temperature < 22^{o}C and weekly rainfall ≥ 4mm) for pseudothecia to mature and

produce ascospores in Western Australia. In Ontario, Canada, mature pseudothecia were detected on stubbles one month after the harvest of canola (Rempel and Hall 1993); however, in Western Canada, a few months were required for pseudothecia to reach maturity due to extreme winter following canola harvest (Petrie 1995).

After maturation, favorable weather conditions trigger the release of ascospores. Ascospores showers have been associated with seasonal rainfall in major canola growing regions of the world (McGee and Petrie 1979; Gladders and Musa 1980). Significantly higher numbers of ascospores discharge occurred at high rainfall environment (604 mm average annual rainfall) followed by medium (428 mm average annual rainfall) and low rainfall environment (292 mm average annual rainfall) respectively (Marcroft et al. 2003). Similarly, rainfall > 1 mm/day favored the heavy discharge of ascospores however light rain event, high relative humidity and dew also facilitated the ascospore release in smaller number (McGee 1977). The pattern of ascospore release varied between the locations. In Canada, maximum ascospores and pycnidiospores dispersal occurred from 9 pm to 4 am when the relative humidity was above 80% and temperature ranged between 13 to 18°C (Guo and Fernando 2005). However, in Australia no such diurnal pattern of ascospores release was observed (Khangura et al. 2007).

Development of a disease-warning system against blackleg could help growers reduce losses caused by blackleg and increase their net return. Blackleg causes the greatest economic losses when seedlings are infected before the plants reach the 3-5 leaf growth stage (Marcroft et al. 2005). In general, fungicide sprays are recommended to be made when canola plants are at the two to four leaf growth stage in North Dakota (Markell et al. 2008). However, a study conducted by del Río Mendoza (2014) reported that foliar sprays made within eight days after *L. maculans* inoculation significantly control disease suggesting timing of foliar application is key

for effective control of the disease. Although, various epidemiological studies have been conducted around the world on *L. maculans* (Salam et al. 2007), there are no studies on spore dispersal pattern and its relation to environmental condition in North Dakota.

The objectives of the study were to characterize ascospore dispersal patterns during the spring canola growing season in North Dakota and to determine the environmental conditions conducive for dispersal.

Materials and Methods

Sampling location

Burkard 7-day recording volumetric spore samplers (Burkard Manufacturing, Rickmansworth, United Kingdom) (Fig 4.1a) were used to monitor *Leptosphaeria maculans* spore dispersal. Two samplers were operated in canola fields with blackleg-infested stubbles starting from mid-May to early July at North Dakota State University Research Extension Center at Langdon, in Cavalier County, North Dakota (lat. 48.758562, long. -98.339699) in 2017 and 2018.

Ascospore count

The samplers drew air at the rate of 10 liters/min and revolved once in 7 days. A 336 mm long Melinex tape (cellophane), was wrapped around each drum and uniformly coated with a thin layer of gel mix composed of 50 g petroleum gel, 6 g paraffin wax, 0.75 ml phenol and 71 ml toluene. The drums were loaded in the samplers and ran powered by single 12 v car batteries. The tapes and batteries were replaced every week. The collected tapes were labeled with start and end, date and time and were taken to the laboratory for processing. In the lab, the tapes were cut into seven 48 mm-long parts, each representing a 24 h sampling. Each piece was placed on a microscope glass slide and stained with lactophenol cotton blue before being examined using a

compound light microscope at 400x magnification. Ascospores of *L. maculans* were identified based on their morphological features, 35-70× 5-8 µm size, 5-septate, cylindrical to ellipsoidal shape, yellow to brown color and with several drops of fat or small oil drops with rounded ends (Fig 4.1b) (Williams, 1992). The number of *L. maculans* ascospores were counted on an hourly basis and converted into the number of ascospores per cubic meter of air per day.

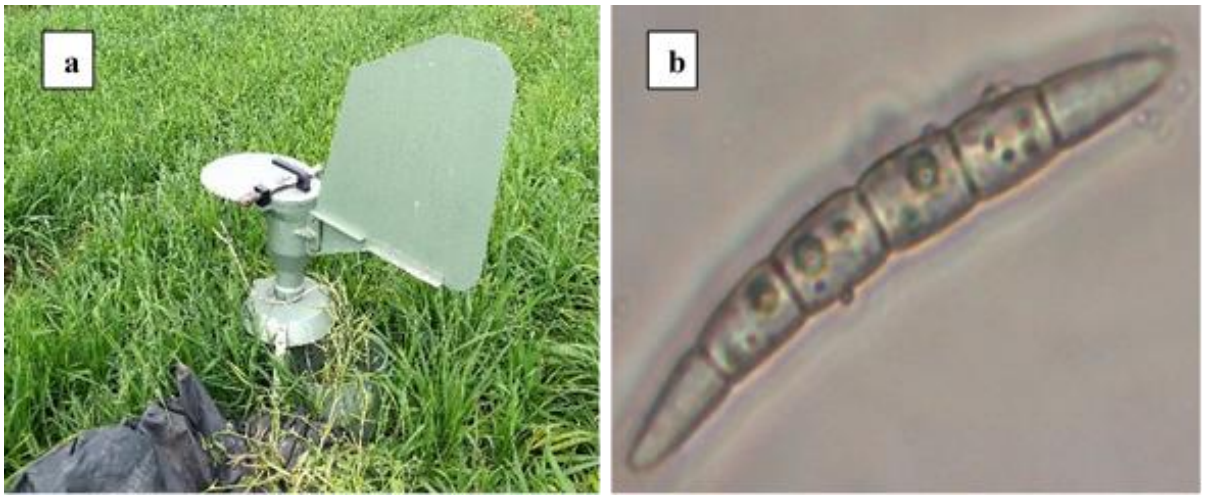

Figure 4.1. Burkard 7-day recording volumetric spore sampler (a); and *L. maculans* ascospore (b).

Meteorological data

Air temperature (°C), precipitation (mm), air relative humidity (%), and soil temperature (°C) at 10 cm depth on soil with a bare surface or a surface covered with turf grass and air dew point temperature (°C) recorded every hour during May and June, 2017 and 2018 at Langdon weather station were retrieved from the North Dakota Agricultural Weather Network (https://ndawn.ndsu.nodak.edu//index.html). The weather station was within 500 m distance from the canola field where samplers were deployed.

Data analyses

To study hourly ascospore dispersal pattern, the number of ascospores observed on each hour on days with >10 ascospores/m^3/day was expressed as a percentage of daily total and was plotted against hours of a day. In addition, to study the association of ascospore dispersal with weather variables, data on daily ascospore concentrations were converted to binary values considering five ascospore m^{-3} day^{-1} was threshold to separate the peak-days from the regular days. Days were designated as 0 if no peak ascospores were observed or as 1 if peak ascospore concentrations were higher than the threshold and used as dependent variables. Hourly weather data retrieved from NDAWN were processed further to calculate daily means, and maximum and minimum values. Running averages for periods of three, five and seven consecutive days were calculated for each weather parameter and Pearson correlation analysis was conducted among the variables using SAS version 9.4 (SAS Institute, Inc., Cary, NC). The weather variables significantly correlated with each other ($P < 0.05$ and $r > 0.40$) were excluded from use as predictors for model development. Logistic regression analysis with stepwise selection was performed on SAS to develop models that associated peak-days to weather parameters for each time period. The models were compared using the Akaike information criterion (AIC); Schwarz criterion (SC) values; Hosmer-Lemeshow goodness-of-fit test; and *c* statistic. Sensitivity (true positive proportion) and specificity (true negative proportion) were calculated for each model and used to estimate the overall model accuracy using the following equation.

$$Accuracy = Sensitivity * \frac{Observed\ Cases}{N} + Specificity * \frac{Observed\ Controls}{N}$$

Where observed cases are the number of observations considered “disease”; observed controls are the number of observations considered “not diseased”; and *N* is the total number of observations.

Results

Hourly ascospore dispersal pattern

Leptosphaeria maculans ascospores were detected at all hours of the day at Langdon, ND in both years 2017 and 2018 with no consistent dispersal patterns. In 2017, the highest numbers of ascospore concentrations were detected at noon whereas in 2018 they were observed at midnight. In 2017, ascospore concentrations were relatively uniform throughout the day (Fig 4.2a); however, in 2018, comparatively higher numbers of ascospores were observed between midnight and 4 am (Fig 4.2).

Interestingly, in peak-days, the air temperature reached its maximum at 3 pm and its minimum between 4 and 5 am in both years. The daily average temperatures during peak-days was 17.6^{o}C in 2017 and 24^{o}C in 2018 (Fig 4.3a and b). During peak-days, the relative humidity was >80% between 11 pm and 9 am in 2017 and between 9 pm and 8 am in 2018 (Fig 4.3b). The lowest relative humidity levels were recorded during the period when air temperatures were at their lowest daily. In 2017, airborne ascospores were detected at average air temperatures between 9.3^{o}C and 17.6^{o}C when relative humidity was between 61% and 92%. In 2018, nearly 51% of the ascospore releases occurred between 11 pm and 5 am. During this period, the temperature was between 16 and 18^{o}C and the relative humidity was between 86% and 91%.

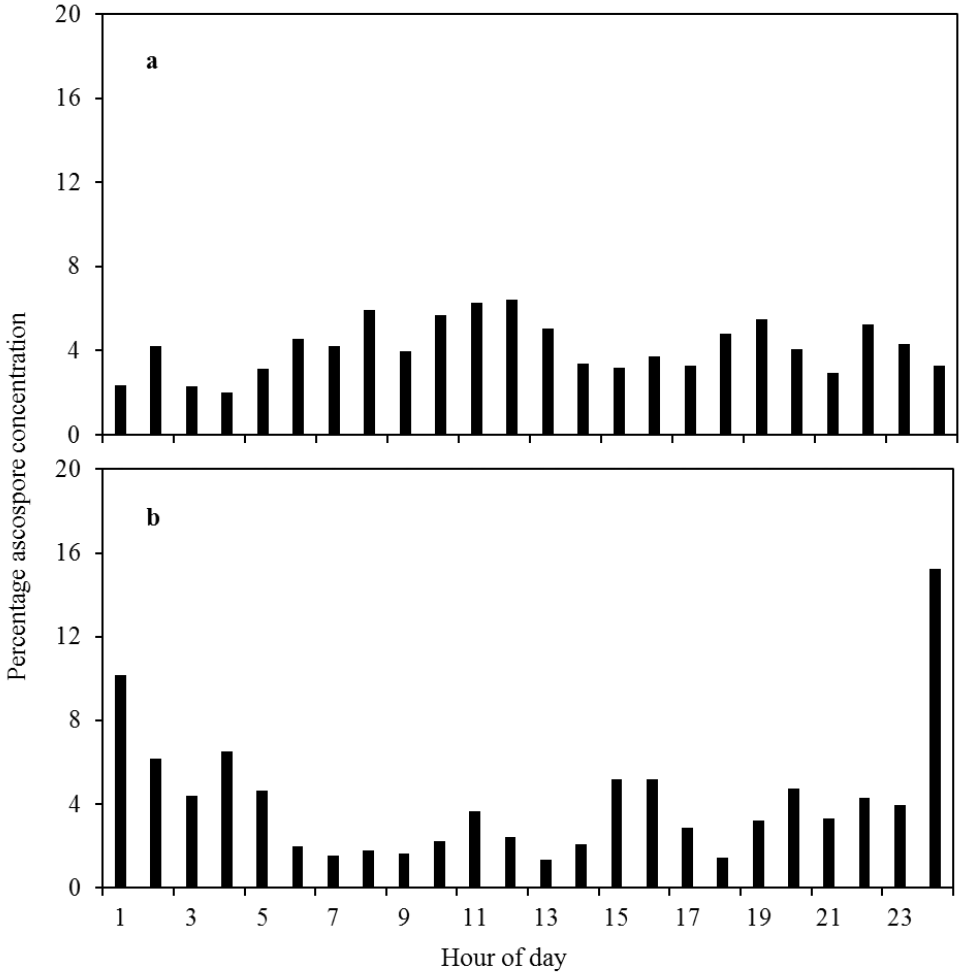

Figure 4.2. Hourly dispersal patterns of Leptosphaeria maculans ascospores on days with >10 ascospore/m3/day concentration in Langdon, North Dakota in 2017(a); and 2018(b).

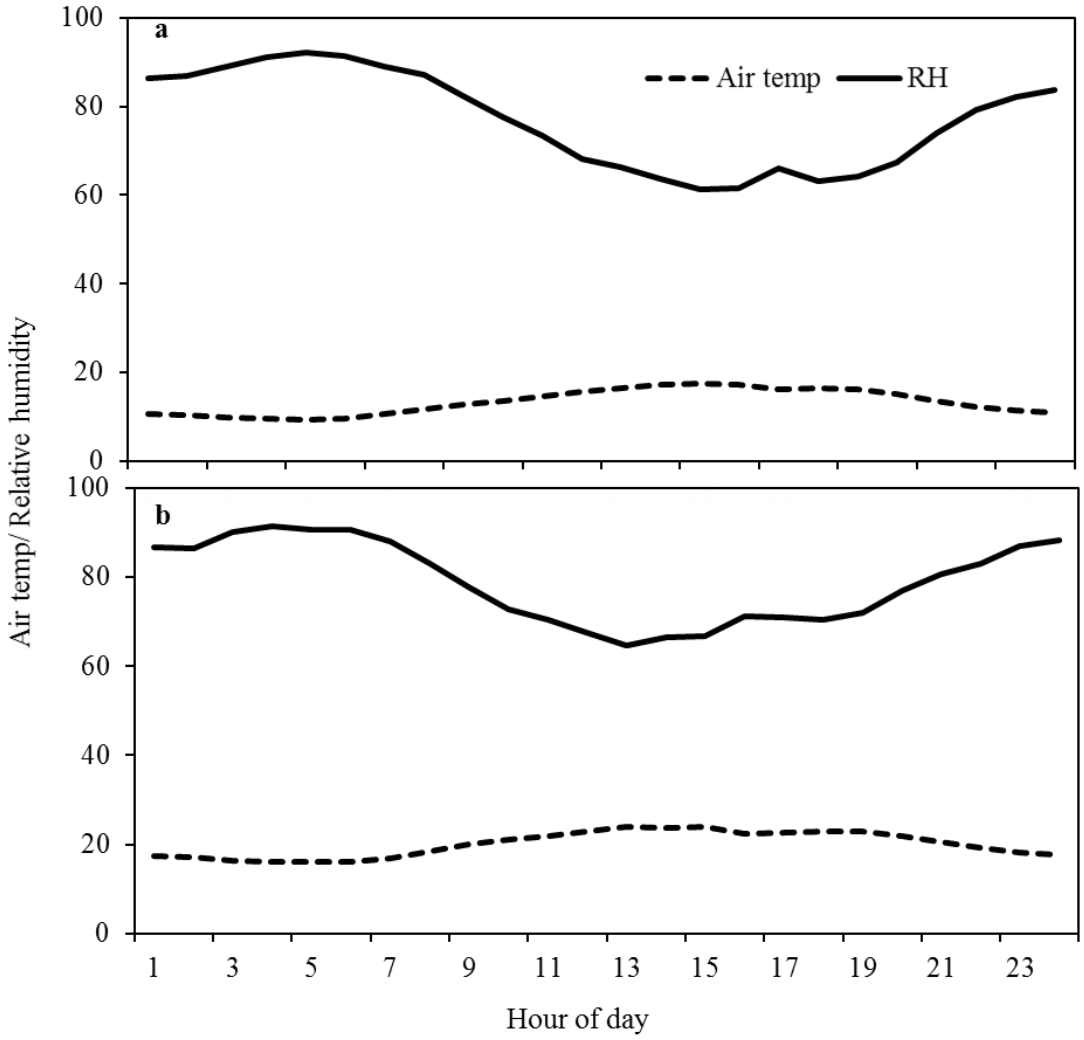

Figure 4.3. Hourly mean air temperature (°C) and mean relative humidity during 24-hour period on days with >10 ascospore/m^3/day concentration in Langdon, ND in 2017 (a); and 2018 (b).

Seasonal ascospore dispersal pattern

Leptosphaeria maculans ascospores were first detected on 3rd June and 7th June in 2017 and 2018 respectively, at Langdon, North Dakota. The number of ascospores trapped between 25th May and 30th June both years were summed to 390 ascospores m^{-3}, averaged to 5 ascospores m^{-3} day^{-1}, and ranged between 0 to 72 ascospores m^{-3} day^{-1}. The highest ascospore releases occurred on 21st June in 2017 with concentrations of 50 ascospores m^{-3} day^{-1} (Fig 4.4a) and on 24th June in 2018 with concentration of 72 ascospores m^{-3} day^{-1} (Fig 4.4b). A total of fourteen

peaks were observed in 2017 with the first peak occurring on 13th June. In contrast, only six peaks were observed in 2018 with the first occurring on 22nd June. All peak-days were observed only during June in both years when canola plants were at the four-leaf growth stage and early bud stage, respectively. The ascospores concentrations observed in 2017 were on average 1.5 times greater than that observed in 2018. No ascospores were detected after 13th July in 2017 and 27th June in 2018; at these dates, canola fields were starting to flower in both years.

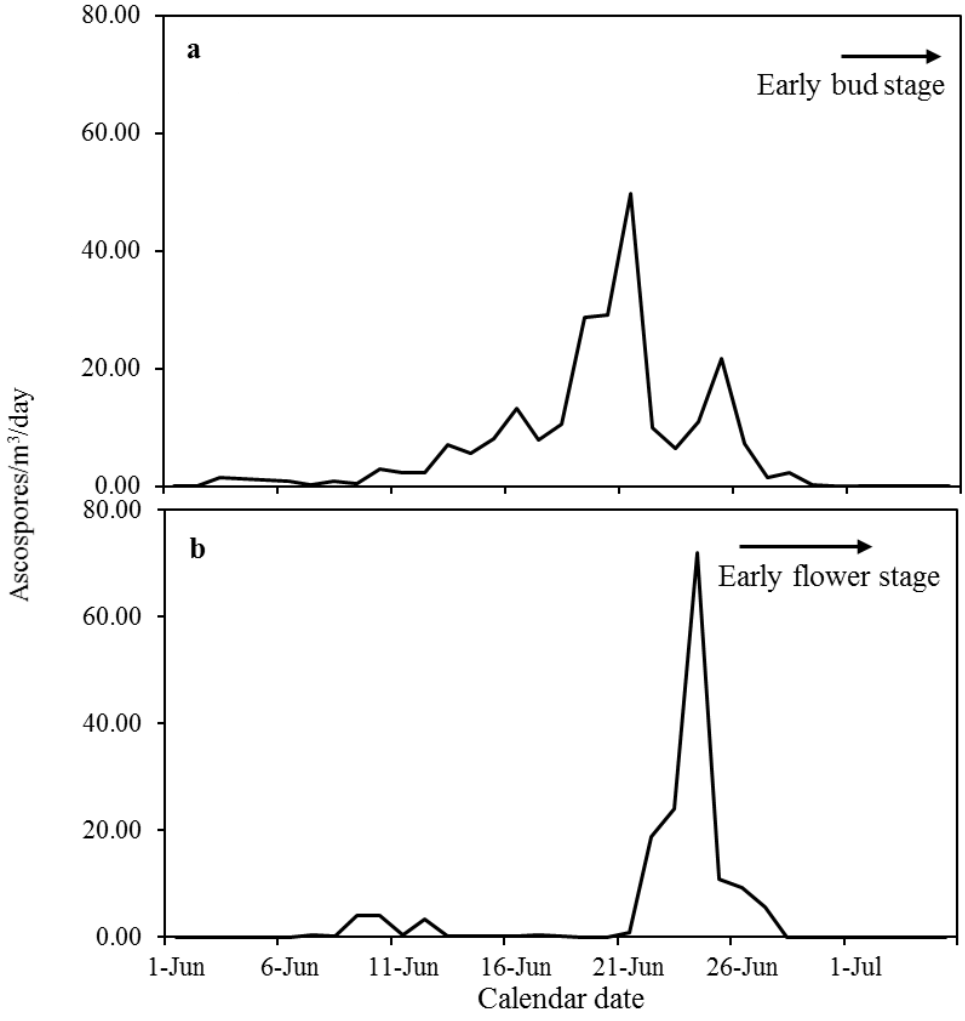

Figure 4.4. Seasonal dispersal patterns of *Leptosphaeria maculans* ascospores during June-July at Langdon, North Dakota in 2017 (a); and 2018 (b).

Weather variables

The average air temperature for June 2017 and June 2018 were 16.5°C and 18.6°C, respectively. The 2017 mean was similar to the 20-year average data while the 2018 mean was nearly 2^{o}C warmer (Table 4.1). The daily mean air temperature was 1°C higher during the first half of June in 2017 compared to the first half of June in 2018. However, in 2018, the second half of June was on average 5.7°C warmer than the second half of the June of 2017 (Fig 4.5a). In addition, both years got seven rainfall events, days with >2 mm total rainfall, in June (Table 4.1). The average interval between these events was approximately 3 and 4 days in 2017 and 2018, respectively. In general, June 2018 received 11 mm more rainfall than June 2017; and the distribution of rainfall also differed. In 2017, 84% of the rainfall received in June occurred in the first half of the month whereas, in 2018, equal amount of total rainfall occurred in first and second halves of June (Fig 4.5b). The average relative humidity during June 2017 and 2018 was 68% and 70% respectively which was lower compared to 20-year average relative humidity (Table 4.1). The average relative humidity was less during the first half of June in 2017 than in 2018 (Fig 4.5c). In contrast, the second half of the month was more humid in 2017 compared to 2018.

Table 4.1. Weather variables at Langdon, ND during June month of 2017 and 2018.

Year	Average air temperature (^{o}C)	Average relative humidity (%)	Total precipitation (mm)	Number of rainfall events with >2mm rainfall/day	Average interval between rainfall events with >2 mm rainfall/day (day)
2017	16.5	67.8	74.7	7	3.3
2018	18.6	69.9	85.9	7	3.7
20-year average	16.5	72.2	91.7	8	2.3

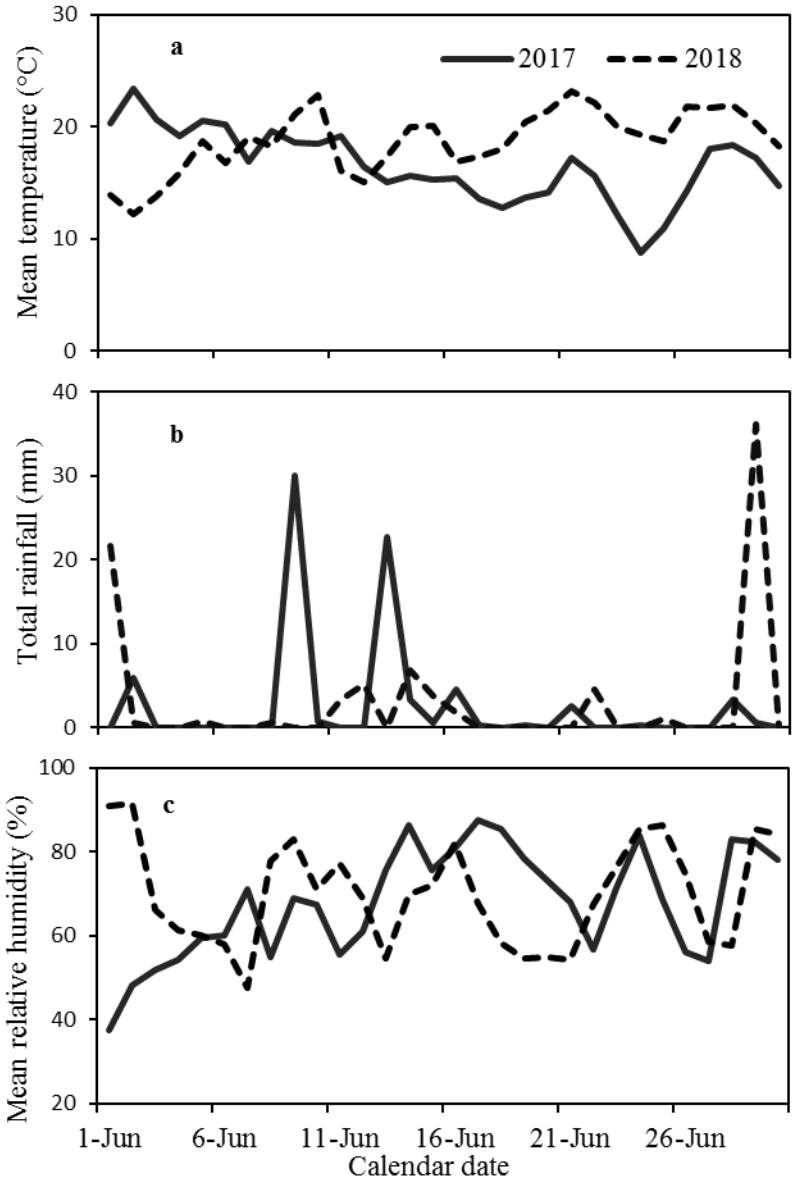

Figure 4.5. Daily mean temperature (a); total rainfall (b); and mean relative humidity (c) during June 2017 and 2018 at Langdon, North Dakota.

Role of weather variables in ascospores release

The three, five- and seven-days averages of maximum, mean and minimum air temperature, bare soil temperature, turf soil temperature, and dew temperatures were correlated with each other ($r \geq 0.40$) but they were not correlated with rainfall or relative humidity (Table 4.2). However, negative correlation was observed between relative humidity and air temperature in hourly data in 2017 ($P < 0.0001$, $r = -0.71$) and 2018 ($P < 0.0001$, $r = -0.60$) during June

month. Logistic regression analysis conducted for three time periods, three-, five-, and seven-days moving averages, revealed significant ($P < 0.05$) associations between ascospore dispersal and mean relative humidity and turf soil temperature in Langdon. The three-day and seven-day models were considered equally good; however, the seven-day model had slightly smaller AIC and SC values and thus it was selected as best model even though its overall accuracy was slightly lower than that of the three-day model (Table 4.3).

Table 4.2. Correlation coefficients among seven-day moving averages of weather variables during May 25 to June 30 in Langdon, ND in 2017 and 2018.

Weather variables	Correlation coefficients[a]					
	AT (°C)	RH (%)	BST (°C)	TST (°C)	Rain (mm)	DT (°C)
AT (°C)	1.00*	-0.36	0.85*	0.62*	0.09	0.72*
RH (%)	-0.36	1.00*	-0.02	0.24	0.21	0.39
BST (°C)	0.85*	-0.02	1.00*	0.89*	0.00	0.84*
TST (°C)	0.62*	0.24	0.89*	1.00*	-0.04	0.81*
Rain (mm)	0.09	0.21	0.00	-0.04	1.00*	0.23
DT (°C)	0.72*	0.39	0.84*	0.81*	0.23	1.00*

[a] AT= air temperature; RH= relative humidity; BST and TST= bare and turf-covered soil measured 10 cm deep; DT= dew temperature.
*** =** correlation coefficient significant at $P = 0.05$.

The seven-day model was,

P = -18.4 + 0.2 (RH) + 0.4 (ST).

Where P is logit of odds, RH is average relative humidity (%) of seven consecutive days and ST is the mean soil temperature of seven consecutive days (°C) four centimeters below soil surface covered with turf. Based on the seven-day predictive model, probability of ascospore release is 8% and 97% at 50% and 80% relative humidity respectively at 15°C soil temperature (Fig 4.6). However, at 20°C the probability increases to 40% at 50% relative humidity and to 100% at 80% relative humidity (Fig 4.6).

Table 4.3. Logistic regression models on relative humidity and bare soil temperature based on peak-days of *Leptosphaeria maculans* ascospore release in Langdon, North Dakota in 2017 and 2018.

Model[b]	AIC[c]	SC[d]	*c*-value	Hosmer & Lemeshow test	Sensitivity	Specificity	Model accuracy
3-day	73.6	80.5	0.82	0.61	70	75	73
5-day	71.8	78.8	0.82	0.03	70	71	71
7-day	71.2	78.1	0.83	0.58	75	67	69

[a]Peak -days = days with *L. maculans* ascospore concentrations ≥ 5 ascospores m^{-3} day^{-1}.
[b]Models based on moving averages of relative humidity and soil temperatures.
[c]AIC = Akaike information criterion; [d]SC = Schwarz criterion

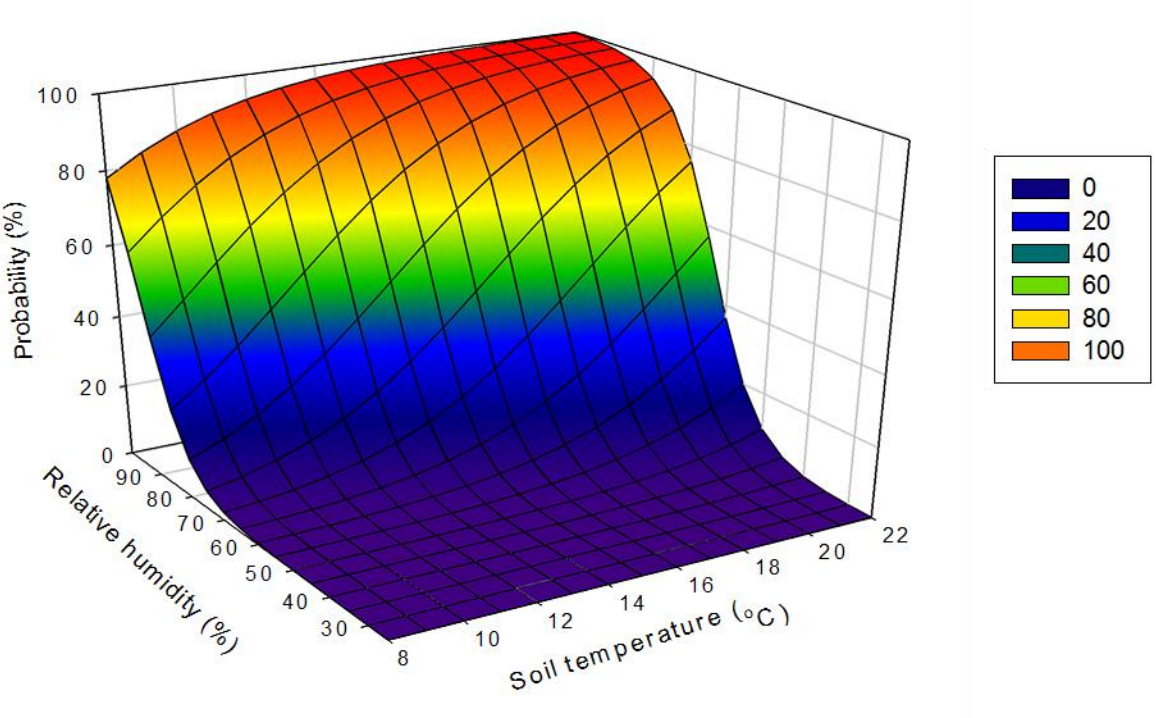

Figure 4.6. Probability of peak-days of *L. maculans* ascospores release based on the 7-days average of mean relative humidity and mean soil temperature.

Discussion

To our knowledge, this is the first study to reveal the seasonal pattern of *L. maculans* ascospore release in North Dakota. Major peaks of *L. maculans* ascospores were observed during mid to late June in 2017 and 2018 in Langdon North Dakota. Chances of peak ascospores

showers were associated with seven-day moving averages of mean relative humidity and mean bare soil temperature.

In North Dakota, the growing seasons are relatively short and thus spring canola is cultivated. Canola harvest is followed by long and cold winter conditions. In this region, fields are typically planted to canola in the second half of May and most hybrids enter the flowering period during the last week of June and first week of July. *L. maculans* survives as pseudothecia or pycnidia during the winter and release spores once environmental conditions become favorable. (West et al. 2001). In the two years of this study, ascospore releases were first noticed during the first week of June and peaked during the second half of the month. Similar results have been observed in Manitoba, Canada, where ascospore dispersal peaked during June and July month (Guo and Fernando 2005). Plants in fields sowed during the third week of May in 2017 and 2018 would have been at the fourth leaf growth stage and at early bud, respectively, by the time the largest peaks of ascospores concentrations were occurring. In support with these results, canola disease survey showed less prevalence of blackleg disease in 2018 compared to 2017 in Cavalier county. The disease incidence and prevalence in 2017 was 13 and 85 and in 2018, 14 and 60 respectively (del Río Mendoza et al., data not published). Further, in North Dakota, mature pseudothecia were first observed during late May in 2017 and one month later in 2018 (Chapter 3 of this thesis). Differences in the timing of pseudothecia maturation would explain in part the differential patterns of ascospore release observed in those years. Major yield loss occurs when spore infection coincides with cotyledon to 4-5 leaf stage. In this study, major ascospores discharge was observed during susceptible period of canola in 2017 and post susceptible period in 2018. In agreement with this data, canola survey conducted at four leaf stage of canola in Cavalier county North Dakota reported less disease prevalence in 2018

compared to 2017. Late release of ascospores in 2018 could have been resulted in less prevalence of the disease.

In agreement with Khangura et al. (2007), no consistent daily ascospore discharge patterns were observed in this study. In one year, most ascospores were released at night and in the next they were released during daytime. In UK and Canada, the diurnal periodicity of ascospore release have been reported (Huang et al. 2005; Guo and Fernando 2005). Savage et al. (2013) observed variation in ascospore release pattern between months and locations in Australia suggesting the role of weather variables in differential pattern of release.

Logistic regression analysis was used in this study to predict the peak periods of ascospore dispersal in relation to weather variables. The model found significant association of average relative humidity and soil temperature with peak ascospore release. Salam et al. (2003) used simple linear regression to predict the ascospore release in relation to rainfall in three locations of Western Australia. The R^2 value for prediction vs observation were 0.66 at Mount Baker and 0.93 at East Chapman. Similarly, Huang et al. (2005) studied release of first ascospore (>10 ascospore m^{-3}) in relation to total rainfall, and rainfall events. The predicted data fitted well with total rainfall and rainfall events with R^2 being 87% and 57% respectively. However, Guo and Fernando (2005) observed the visual pattern of ascospore release with rainfall events and relative humidity in Canada. The developed model in this study had 69% model accuracy. The model had pretty good sensitivity which allows to predict the occurrence of the ascospore release in 75% of the cases.

There was a 7°C difference in air temperature during peak ascospores release period in 2017 (13th June to 26th June) and 2018 (22nd June to 27th June). But interestingly, the mean relative humidity was 75% during both periods. In addition, 60% of peak-days had >75% mean

relative humidity. These results suggest that relative humidities $\geq$ 75% would contribute to ascospore release. These results are in agreement with those from Canadian researchers who found that relative humidities >80% and temperature between 13 to 18°C helped release ascospores (Guo and Fernando 2005). Unlike Salam et al. (2003), Guo et al. (2005), and Huang et al. (2005), however, significant associations between rainfall and ascospore dispersal could not be found in this study. Nevertheless, the average total rainfall during peak days in 2017 was 2.5 mm/day and 0.98 mm/day in 2018. The average interval between rainfall events with > 2mm rainfall per day was three and half days in both years. This suggest that role of the rainfall in ascospore dispersal cannot be ruled out here. In addition, rainfall was found to be essential component in maintaining relative humidity thus indirectly helping in the release of ascospores.

Overall, comparatively low ascospore concentration was observed in this study. The study conducted by Nepal (2013) observed the imbalance in the mating type population of *L. maculans* in North Dakota. Less sexual reproduction results in fewer pseudothecia being formed and ultimately fewer ascospores being released which explains the reason behind the less concentration of ascospores overall. Though, no pycnidiospores count was taken in this study; disease incidence before the release of ascospore in 2018 hints towards role of pycnidiospores as a primary source of inoculum. Pycnidiospores as a primary source of inoculum have been reported in Manitoba, Canada, which shares somewhat similar weather conditions as in North Dakota (Ghanbarnia et al. 2011). Even though, late released ascospores are not important from economic point of view; they help to evolve virulent races of the pathogen by sexual recombination.

The potential benefits of the model developed in this study go beyond indicating when ascospores would be released. The model indicated the chances of ascospore release were 100%

at 80% RH and at 20°C soil temperature. When this probability is reached, it is likely that ascospores would germination very quickly ensuring the start of the infection process (Li et al. 2004). The ascospore concentrations observed during this study suggest that weather conditions for two years may not have been too favorable for blackleg development. Thus, data from additional locations-years are needed to strengthen and validate the model before it is deployed. In addition, incorporation of pseudothecia maturation model would provide a complete prediction for ascospore release in North Dakota. In future, detailed studies in the role of pycnidiospores on blackleg disease development under field condition in North Dakota would help to widen the knowledge about epidemiology of blackleg of canola.

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CHAPTER 5: EFFICACY OF FUNGICIDE SEED TREATMENTS IN CONTROLLING BLACKLEG OF CANOLA

Abstract

The efficacy of five fungicide seed treatments as management tool against blackleg on spring canola was evaluated under greenhouse and field conditions in North Dakota. Blackleg, caused by *Leptosphaeria maculans*, inflicts greatest yield losses when infecting seedlings before they reach the six-leaf growth stage. In greenhouse studies, 10 days-old seedlings were inoculated with *L. maculans* spore suspensions and evaluated 12 days later and at maturity or inoculated 12, 20, or 28 days after planting and evaluated at maturity. In field trials conducted in 2017 and 2018, severity was assessed at maturity. In greenhouse, all seed treatments reduced ($P = 0.05$) disease severity at seedling stage but only the protection provided by Obvius (fluxapyroxad + pyraclostrobin + metalaxyl) and Helix Vibrance (mefenoxam + fludioxonil + sedaxane + difenoconazole + thiamethoxam) reduced ($P < 0.05$) severity at adult stage; however, none of them provide effective protection when plants were inoculated 20 days after planting or later. In field trials, none of the treatments significantly ($P < 0.05$) improved plant stand, yield or reduced disease incidence and severity. Although, seed treatment is a valuable tool, however it should not be used as the only method to manage blackleg disease.

Introduction

Blackleg of canola (Fig 5.1) is caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not [anamorph = *Phoma lingam* (Tode:Fr.) Desm.] and is one of the major production constraints in North Dakota as well as worldwide (del Río Mendoza et al. 2012; Howlett et al. 2001; Rimmer 2006; West et al. 2001). In North Dakota, the largest canola producing state in the United States, the first blackleg epidemic was detected in 1991 (Lamey and Hershman 1993). Since then, the

virulence profile of its *L. maculans* populations have changed (Bradley et al. 2005; Nepal et al. 2014) and currently prevalent strains are capable of infecting cultivars that carry resistance genes *Rlm1, Rlm2,* or *Rlm3*. Generally, in North Dakota, yield loss due to blackleg disease ranges between 5 and 20%; however, under severely infected conditions, estimated yield losses > 45% have been observed (del Río Mendoza et al. 2012).

Protecting canola seedlings from emergence until they reach the six-leaf growth stage is critical to avoid formation of yield-robbing blackleg cankers (Marcroft et al. 2005). This susceptible period could last, depending on temperature, between five and seven weeks (Miller et al. 2018). To protect the plants, canola growers in North Dakota are advised to make fungicide foliar applications when seedlings are between the two- and four- leaf growth stages period (Markell et al. 2008) which means seedlings could be exposed without protection to blackleg inoculum for three to four weeks. Some researchers believe the use of fungicide-treated seed could eliminate the need for fungicide foliar applications during the early growth stages (Mancini and Romanazzi 2014). The heightened threat of blackleg to the canola industry in the region has prompted the registration in recent years of several fungicide seed-treatments for its management (Friskop et al. 2017). Fungicide seed treatments in general, reduce the risk of seedborne inoculum being introduced to an area and protect seedlings from pathogens that could reduce germination and plant emergence from soil (Gugel and Petrie 1992; Mancini and Romanazzi 2014). However, their impact on chronic diseases, like blackleg, is less clear and seems to be influenced by the environment. Studies conducted in Australia indicated that under high disease pressure conditions, seed treatments reduced disease severity and plant mortality and increased yields in susceptible and moderately resistant canola cultivars but were not economical when used on resistant cultivar (Khangura and Barbetti 2004; Marcroft and Potter

2008). In contrast, Sprague et al. (2007) noted that treating seeds with Maxim® (fludioxonil + metalaxyl) and Jockey® (fluquinconazole) occasionally reduced blackleg severity and rarely increased yields. Interestingly, canola seeds imbibed with flutriafol (6 g/kg seed) reduced disease severity at cotyledon stage, two-leaf, and four-leaf stage (Sprague and Burgess 2001). The objectives of this research were to evaluate the efficacy of five fungicide seed treatments registered for use in North Dakota against blackleg of canola and to evaluate the longevity of the protection they provide.

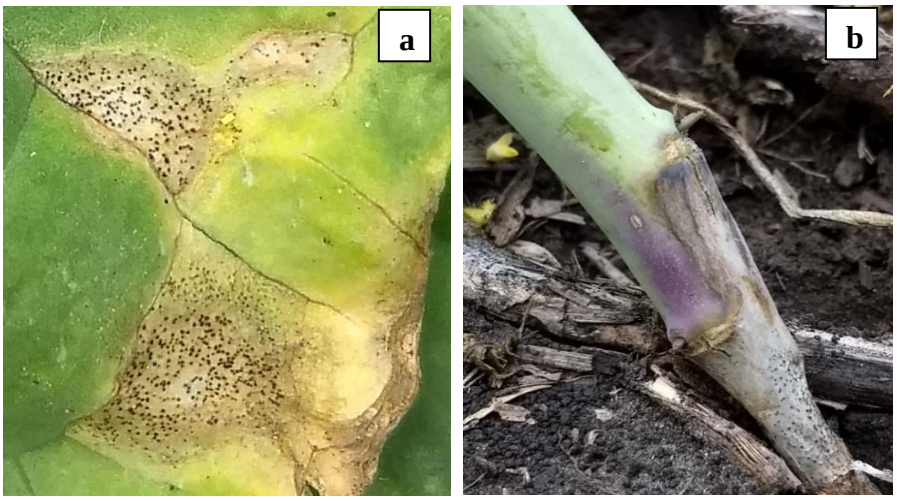

Figure 5.1. Blackleg lesion on canola leaf with pycnidia (a); Girdling of adult canola plant due to blackleg stem canker (b).

Materials and Methods

Greenhouse studies

Greenhouse studies were conducted to evaluate five commercial seed-treatment products (Table 5.1) for their efficacy and longevity of protection under controlled conditions. The seeds of blackleg susceptible cultivar, Westar were treated with each product separately at their recommended dosage and compared with the non-protected seeds. All products used in this study are registered in North Dakota for control of blackleg of canola (Friskop et al. 2017). Cultivar Westar was used because it does not have known major blackleg-resistance genes (Balesdent et

al. 2005) and would in this way provide us with a "worst-case scenario" that is plausible given the current status of blackleg in the state.

In both studies, fungicide-treated seeds were planted in plastic pots filled with a soilless potting mix (PRO-MIX BX, Premier Tech Horticulture, Quakertown, PA) and the pots were arranged in randomized complete blocks. The plants were grown in greenhouse rooms maintained at 20 ± 2°C with 16 hours of photoperiod regulated by 600-Watt high pressure sodium-lamps (P.L. Light Systems, Inc., Beamsville, Ontario, Canada). Canola seedlings were inoculated using mixtures of five *L. maculans* isolates selected for being highly aggressive (Franceschi 2015). These isolates were cultured separately on dishes containing V8-agar medium. After 12 days, the dishes were flooded with 5 ml sterile distilled water and gently scrapped with sterile glass rod. The resulting spore suspensions were collected on plastic vials and their concentrations were estimated with help of a hemocytometer and adjusted to 10^7 pycnidiospores/ml. Equal amounts of these suspensions were mixed and used immediately for inoculations.

Table 5.1. Details on seed treatments, active ingredients, and application rates used in field and greenhouse studies.

Fungicide trade name	Company name	Active Ingredients	Application rate (L/100 kg seed)
Dynasty	Syngenta	Azoxystrobin (9.6%)	0.24
Prosper EverGol	Bayer	Clothianidin (22.32%) + Penflufen (0.82%) + Trifloxystrobin (0.55%) + Metalaxyl (0.55%)	1.4
Obvius	BASF	Fluxapyroxad (1.58%) + Pyraclostrobin (1.58%) + Metalaxyl (1.26%)	0.59
Helix Vibrance	Syngenta	Sedaxane (0.26%) + Difenoconazole (1.25%) + Mefenoxam (0.4%) + Fludioxonil (0.13%) + Thiamethoxam (20.7%)	1.5
Maxim 4FS	Syngenta	Fludioxonil (40.3%)	0.01

Efficacy studies

The protective activity of the seed-treatments was evaluated using four replications per trial and five plants per replication. Each trial was conducted twice and each time, the cotyledon leaves of ten-day old plants were slightly wounded once with a sharp tweezer and 10 µl of the spore suspension was deposited on each wound (Fig 5.2a). Plants were incubated in dark mist chambers for 24 h at 20^{o}C and 98% relative humidity and returned to the greenhouse room. Reaction to inoculations were evaluated at the seedling stage twelve days after inoculation (Fig 5.2b) and when plants reached maturity. At the seedling stage, disease severity was recorded using a modified 0-9 scale of Williams and Delwiche (1979). In the modified scale, a 0 represents no lesion development; while a 3, 5, 7, and 9, represent lesions with up to 3, up to 5, up to 7, and >7 mm in diameter, respectively. At maturity, plants were cut at the stem crown region and the percentage of internal discolored tissues was estimated between 0 to 100. An illustration of the severity scales used in this study are presented in Mansouripour and del Río Mendoza (2017).

Timing of inoculation study

To identify the length to which the protection provided by seed treatments extended, twelve-days old canola seedlings were inoculated with *L. maculans* on the cotyledons as described (Fig 5.2a) or on the petioles at 20 days, and 28 days after planting. Inoculated plants were incubated in a mist chamber as described and then returned to the greenhouse room. The study was conducted twice using a randomized complete block design with 6*3 factorial arrangement. At maturity, disease severities were assessed based on the percentage of internal discoloration of the stem crown region.

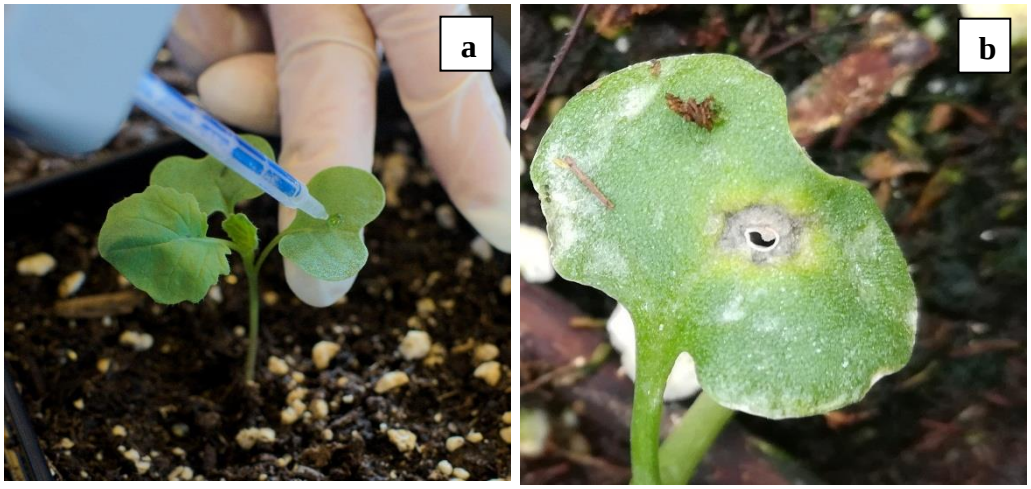

Figure 5.2. Inoculation of cotyledon leaves of ten-day old plant (Picture courtesy of K. Chittem (a); Blackleg symptom on cotyledon leaf in greenhouse trial twelve days after inoculation (b).

Data analyses

Seedling data was analyzed using the non-parametric approach suggested by Shah and Madden (2004) whereas data from adult plants was evaluated using parametric analysis of variance. Levene's test of homogeneity of variances performed across the trials to test whether both trials were homogenous ($P > 0.05$) and could be combined for analysis. For seedlings, the median disease severity was calculated for each replication and treatment using PROC MEANS of SAS 9.4 (SAS Institute Inc., Cary, NC). The medians were ranked using PROC RANK and anova-type analysis was performed using PROC MIXED. Treatment relative effects and their 95% confidence intervals were calculated using SAS macro LD_CI.sas (Brunner et al. 2002). The relative effect ranges from 0 to 1 with treatments providing the best protection having it closer to zero. For adult plants, Levene's test of homogeneity of variances was performed to determine whether trials could be combined for analysis. Upon confirmation, a combined analysis of variance was performed considering treatments as fixed effects and replications, trials and their interactions with treatments as random effects. Least significant difference (LSD) test was conducted to compare treatment means at $P = 0.05$.

For data analysis on timing of inoculation study, single-degree-of-freedom orthogonal linear contrast analysis was used in SAS 9.4 to make independent comparison between the treatments. Significance of each comparison was conducted at probability value $P = 0.05$.

Field study

Field trials were conducted in Langdon (Research Extension Center, North Dakota State University) North Dakota in 2017 and 2018 to evaluate the efficacy of the seed treatments under field conditions. Four treatments, Dynasty®, Helix Vibrance®, Maxim 4FS®, and Obvius® were applied to seeds from cultivar Westar and evaluated against non-treated controls using randomized complete blocks with four replications. The trials were established during mid-May. *L. maculans* inoculum was produced as described earlier and three spray-inoculations of a suspension containing 10^5 pycnidiospores/ml, were made when seedlings were between the cotyledon and 3-leaf growth stage. In addition, the plots were supplemented with blackleg-infected canola stubbles collected from commercial fields. Plant stand counts were taken from the central two rows of each plot when plants were at the 2-3 leaf growth stage. At physiological maturity, the plants were swathed, and 50 stubbles were uprooted from each plot for disease severity rating. The stubbles were cut perpendicular to the stem at the crown region. The disease severity was assessed on the scale of 0-100 where, 0 and 100 being no discoloration and complete discoloration of the internal tissue, respectively. In addition to disease severity, the yield data of each experimental unit was recorded.

Levene's test of homogeneity of variances, was conducted using the general linear model procedure of SAS 9.4. Treatment mean separation was performed using Fisher's protected least significant difference (LSD) at $P = 0.05$.

Results

Greenhouse studies

Efficacy studies

At the seedling stage, all seed-treatments significantly reduced the disease severity (P = 0.05) compared to the non-protected controls and had median disease severities < 5 whereas, the non-protected controls had median disease severity of 7.3 (Table 5.2). Obvius® had the strongest effect on disease severity while Prosper EverGol® had the lowest (Table 2). Disease severity on mature plant ranged between 39 and 100% on fungicide-protected plants and between 72 and 100% on non-protected plants. While Obvius® and Helix Vibrance® were the only products whose protection extended into significantly (P < 0.05) lower levels of disease severity at the adult plant stage compared to the non-protected controls (Table 5.2), the average severity for all treatments was 68% or higher.

Table 5.2. Blackleg disease severity observed in greenhouse trials at seedling and adult stage.

Seed treatments	Seedling stage			Adult stage
	Median[b]	Mean Relative effect[c]	95% Confidence Interval	Mean severity (%)[d]
Dynasty	4.5	0.48	0.36 – 0.60	78 abc
Helix Vibrance	4.3	0.45	0.30 – 0.60	74 bc
Maxim 4FS	4.0	0.44	0.32 – 0.57	91 a
Obvius	2.8	0.12	0.09 - 0.23	68 c
Prosper EverGol	5.0	0.64	0.52 – 0.74	83 ab
Control[a]	7.3	0.88	0.79 – 0.90	89 a

[a] Control= Westar seeds without fungicide seed treatment.
[b] Medians based on the disease severity on two cotyledons per plant; five plants per replication; four replications per trial and two trials. Disease severity ratings based on modified 0-9 scale of William and Delwiche (1979).
[c] Treatment with mean relative effect closest to 0 was the most effective.
[d] Mean severities based on percentage discoloration of internal stem tissues at crown region. Means with the same letter are not significantly different based on Fisher's protected least significant difference test (P = 0.05).

Timing of inoculation study

Overall, the seed treatments reduced disease severity compared to the non-protected controls. However, the protective effect of the treatments was evident only on plants inoculated 12 days after planting. In later inoculations, all seed treatments behaved in the same way as the non-protected controls. Among seed treatments, only Obvius® and Helix Vibrance® significantly ($P < 0.047$) reduced the mean disease severity compared to the non-protected control inoculated 12 days after planting (Table 5.3). None of the seed treatments reduced disease significantly ($P > 0.05$) when plants were inoculated 28 days after planting (Table 5.3).

Table 5.3. Linear contrast analyses of the effect of time of inoculation and of seed treatments on blackleg severity on canola cultivar Westar evaluated under greenhouse conditions.

Linear contrasts	Mean 1[a]	Mean 2	Significance (*P*)
All seed treatments vs. not treated seeds:			
Inoculated 12 days after planting	76	84	0.0244
Inoculated 20 days after planting	78	82	0.2481
Inoculated 28 days after planting	60	66	0.0796
Effect of time of inoculation on non-treated seeds:			
12 days vs 20 days	84	82	0.7402
20 days vs 28 days	82	66	0.0003
Obvius vs. no treatment:			
12 days after planting	69	84	0.0010
20 days after planting	77	82	0.2461
28 days after planting	59	66	0.1221
Helix Vibrance vs. no treatment:			
12 days after planting	75	84	0.0470
20 days after planting	82	82	0.9120
28 days after planting	60	66	0.2040

[a] Means 1 and 2 represent the values to the left and right of the "vs." term in the contrast.

Field study

Levene's test of homogeneity of variances, conducted using the general linear model procedure of SAS 9.4, indicated the trials could not be combined for analysis ($P < 0.05$). Therefore, the trials were analyzed separately (Table 5.4). Compared to year 2018, 2017 had

lower blackleg severity and higher yield. Statistical analysis indicated that none of the seed treatments improved ($P = 0.05$) plant stand count and yield, or reduced blackleg incidence and severity in both years.

Table 5.4. Effect of seed treatments on plant stand, disease incidence, disease severity, and yield in Langdon in 2017 and 2018.

Seed treatment	2017				2018			
	Plant stand[a]	Blackleg[c]		Yield (kg/ha)[d]	Plant stand	Blackleg		Yield (kg/ha)
		incidence (%)[b]	severity (%)[c]			incidence (%)	severity (%)	
Dynasty	37	96	72	1214	34	98	89	535
Helix Vibrance	44	97	70	1426	35	98	88	608
Maxim 4FS	36	93	65	1542	30	98	90	437
Obvius	40	95	74	1331	26	97	88	530
Non-protected control	30	97	68	1584	36	99	91	673
LSD[b]	NS	NS	NS	NS		NS	NS	NS

[a] Average number of plants per meter of row at 2-3 leaf growth stage.
[b] LSD= Least significant difference; NS= not significant at $P = 0.05$.
[c] Blackleg incidence calculated as percentage of symptomatic plants based on 200 plants per treatment (50 in each of four replications) per year. Severity based on percentage of discolored internal stem tissues at crown region based on 200 plants per treatment (50 in each of four replications) per year.

Discussion

Under controlled conditions, all seed-treatments evaluated in this study reduced the severity of blackleg infections compared to the non-protected control. However, none of them suppressed disease development completely or prevented the fungus from further colonizing the stems as the plants grew. At maturity, plants from all treatments had on average more than 50% of the stem internal tissues at the crown region affected by the pathogen, a condition that would result in severe yield reductions (Hwang et al. 2016). While incomplete, the protection offered by the seed treatments lasted only two weeks as plants inoculated after that time had blackleg

severities equal to that of the non-protected controls. A similar observation was made by Kharbanda (1992) who also reported that iprodione and prochloraz protected canola seedlings against blackleg for three weeks under controlled conditions. Iprodione is no longer available in the US market and prochloraz is not registered for use on canola in North Dakota (Friskop et al. 2017).

Under field condition, the beneficial activity of the seed treatments observed in the first two weeks in the greenhouse trials, did not translate into lower disease severity or higher yields. In contrast to greenhouse conditions, in the field there is no control over the frequency and timing of arrival of *L. maculans* inoculum, but plants may have access to better nutritional conditions and could benefit from changes in environmental conditions that help slow disease progress. These results are in contrast with those reported by Marcroft and Potter (2008) and Sprague et al. (2007) who saw yield advantages of seed treatments of susceptible cultivars planted under high blackleg pressure conditions. In our study we used cultivar Westar, which is known to have neither quantitative nor qualitative resistance to blackleg (Balesdent et al. 2005) and extremely high disease pressure. Thus, it is possible that materials that carry quantitative resistance may benefit more from the seed treatments if moderate disease pressure is present.

Nevertheless, results from this study suggest that seed treatments as an only management tool is not enough for the control of blackleg and that under high disease pressure, the application of foliar fungicides may be required.

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CHAPTER 6: GENERAL CONCLUSIONS

Blackleg of canola is caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not [anamorph = *Phoma lingam* (Tode:Fr.) Desm.] and is one of the major production constraints worldwide. Protecting canola seedlings during its earliest stages of development is critical to avoid formation of yield-robbing blackleg cankers. Although, *L. maculans* spores can infect canola plants at any growth stage; the greatest yield losses occur when plants are infected before reaching the six-leaf growth stage. The objectives of this study were to study the role of weather variables in pseudothecia maturation under controlled and field condition; to develop a warning model for *Leptosphaeria maculans* ascospore dispersal; and to evaluate the efficacy of fungicide seed treatments against blackleg of canola.

For the first objective, studies were conducted both in controlled and field conditions. Blackleg-infected canola stems were incubated at, 8, 13, and 18ºC, in growth chambers and inspected weekly for the presence of pseudothecia. Under field conditions, one-year old blackleg infected stems were collected weekly between May and July of 2017 and 2018 from four North Dakota locations and inspected for presence of pseudothecia. Results from the study under controlled conditions confirmed the significant positive association ($P = 0.0157$) between heat-units and pseudothecia maturation. Under field conditions, pseudothecia maturation occurred during mid-May to late July in North Dakota and produced a model that identified high relative humidity (≥75%) and heat-units as being significantly associated with this phenomenon.

For the second objective, *L. maculans* ascospores concentrations were monitored using Burkard 7-day volumetric samplers between mid-May and mid-July in Langdon, ND in 2017 and 2018. Ascospores were identified and counted under compound microscope. Concentrations > 5 ascospores/m^3 of air were considered peaks. Ascospores concentrations peaked during mid to late

June in both years when canola plants were at four leaf to early bud stage. Logistic regression analysis indicated that chances of peak ascospores showers were associated with seven-day moving averages of mean relative humidity and mean bare soil temperature.

For the third objective, five fungicide seed treatments were applied to seeds of susceptible cultivar Westar and evaluated in greenhouse and field trials. All treatments reduced disease severity compared to the non-protected control at seedling stage in greenhouse, but only Obvius (fluxapyroxad + pyraclostrobin + metalaxyl) and Helix Vibrance (Metalaxyl + fludioxonil + sedaxane + difenoconazole + thiamethoxam) showed significant ($P<0.05$) reductions in severity at harvest. None of the treatments provide effective protection to plants when inoculated 20 days after planting or later. In field trials, none of the treatments significantly ($P < 0.05$) improved plant stand, yield or reduced disease incidence and severity. Thus, seed treatments, while a valuable tool, should not be used as the only means to manage blackleg.

The information produced by these studies will contribute to the development and implementation of more effective management practices for blackleg.