Improving *Verticillium longisporum* inoculation protocols and quantifying canola yield losses

by

Ji Cui

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Plant Science

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Verticillium stripe, caused by *Verticillium longisporum*, is an emerging soilborne disease of the Canadian canola (*Brassica napus*) crop. This study aimed to refine techniques for inoculating the pathogen on canola and to quantify its impact on hybrid canola yield under greenhouse and field conditions in western Canada. Two inoculation methods, root-dip and grain inoculation, were compared in greenhouse experiments with three canola genotypes. Symptoms of Verticillium stripe appeared at early growth stages following root-dip inoculation, resulting in seedling mortalities ranging from 13% to 24% at 35 days post-inoculation. Mortality was significantly greater at higher inoculum concentrations using the root-dip method. Host age at inoculation did not significantly affect disease development in most cases. The grain inoculation method did not cause early-stage mortality, but disease severity at the adult stage differed significantly between control and high inoculum treatments for all host genotypes, with decreases in plant dry weight and height corresponding to increasing inoculum concentration. Grain inoculation may be preferable for large-scale resistance screening or field studies.

The relationship between Verticillium stripe severity and yield was investigated in two canola hybrids at two infested field sites near St. Albert, Alberta, in 2020 and 2021. Moderate levels of disease were observed in both hybrids in 2020, while symptoms were milder in 2021. Regression analysis indicated a decline in seed yield per plant with increasing Verticillium stripe severity in both years, best described by second-degree quadratic equations for both hybrids. At one site in 2020, yield loss per plant exceeded 60% in both hybrids when Verticillium stripe severity was > 3 on a 0-4 scale. Similarly, yield loss per plant often exceeded 50% in both hybrids

at both sites in 2021 when disease severity was ≥ 1. These findings suggest that *V. longisporum* can cause significant yield losses even with relatively mild disease severity.

Preface

This thesis represents the original contributions of Ji Cui. I conducted and analyzed all of the experiments and wrote the first drafts of each chapter. Dr. Stephen E. Strelkov and Dr. Sheau-Fang Hwang then reviewed and edited the chapters; I incorporated their suggestions into the finalized versions included in this thesis. Dr. Rudolph Fredua-Agyeman (Senior Research Associate, U of Alberta) provided assistance with the setup of my field trials and guidance on the use of molecular techniques. Mr. George D. Turnbull (Technician, U of Alberta) helped in the collection of canola tissue samples and weather data. Summer students working with the U of Alberta Plant Pathology Group provided support in setting up and maintaining field and greenhouse experiments, as well as in collecting canola samples. Drs. Hwang and Strelkov developed the original research concept, and secured funding from the Canola Agronomic Research Program (Alberta Canola, SaskCanola, and the Manitoba Canola Growers) Project No. 2019.34.

A version of Chapter 2 has been published as:

J. Cui, S. E. Strelkov, R. Fredua-Agyeman & S. F. Hwang (2022): Development of optimized *Verticillium longisporum* inoculation techniques for canola (*Brassica napus*), Canadian Journal of Plant Pathology, 45:92-102. DOI: 10.1080/07060661.2022.2120913.

Dedication

I dedicate the successful completion of my MSc program and thesis to my beloved family, with special gratitude to my husband. Tim, your unwavering support, encouragement, and understanding have been the pillars that sustained me throughout this challenging journey. Your belief in my abilities, together with the countless sacrifices you made, have enabled me to achieve this milestone. For that, I am forever grateful. A special thank you goes to my parents for being my source of strength and inspiration; I am profoundly grateful for the love and encouragement you have showered upon me.

Acknowledgements

I would like to express my deep gratitude to Dr. S.F. Hwang and Dr. Stephen E. Strelkov, who have played a pivotal role in the completion of this MSc thesis. Their expertise, patience, and encouragement have been instrumental in shaping the quality of this thesis. I would also like to acknowledge Mr. George D. Turnbull for his support in the collection of canola samples and weather data. I would like to thank Dr. Rudolph Fredua-Agyeman for teaching me new research techniques and for his assistance with the field set up. I extend my thanks to the staff members and summer students of the Plant Pathology Group at the University of Alberta for their assistance in the establishment of field and greenhouse experiments as well as data collection. I would also like to thank Dr. Boyd Mori for reading this thesis and serving as my University Examiner.

Special thanks are due to my family and friends for their unwavering support, understanding, and encouragement. Their belief in my abilities and constant encouragement have been a source of motivation and strength.

Finally, I would like to acknowledge the financial support provided by the Canola Agronomic Research Program (Alberta Canola, SaskCanola, Manitoba Canola Growers, and the Canola Council of Canada), without which I would never have been able to complete this work, as well as the farmers and industry collaborators who helped with the research.

Table of Contents

List of Tables

List of Figures

Chapter 1. General introduction and literature review

1.1 Introduction

Verticillium stripe, caused by the fungal pathogen *Verticillium longisporum* (C. Stark) Karapapa, Bainbridge and Heale is an emerging disease of canola (syn. oilseed rape, *Brassica napus* L.) and other cruciferous crops worldwide (Depotter et al. 2016). Although the detrimental effects of this disease on agricultural production have been well-documented in numerous regions (Zeise and Tiedemann, 2002), our understanding of *V. longisporium*, including its epidemiology and management, remains limited. In Canada, the fungus was first detected in Manitoba in 2014 (Province of Manitoba – Agritulture n.d.). In subsequent surveys organized by the Canadian Food Inspection Agency, the presence of *V. longisporum* was also confirmed in Alberta, Saskatchewan, Ontario and Quebec, suggesting a widespread distribution across the country (Government of Canada 2015). Given the importance of canola to the Canadian economy (Canadian Canola Growers Association 2022) the occurrence of this pathogen has raised concerns within the agricultural community. Further research is imperative to improving knowledge of the biology of *V. longisporum* and to formulating effective strategies for mitigating its potential impact on canola production.

1.2 Biology of *V. longisporum*

1.4.1 The fungus

Verticillium longisporium is an anamorphic, ascomycete fungus that infects canola and other cruciferous species (Zhang et al. 2006; Depotter et al. 2016). The phylogenetic classification of this and other species within the genus *Verticillium* is based on the presence or absence of yellow-colored hyphal pigments (Depotter et al. 2016). Verticillium species that produce yellow-

pigmented hyphae are grouped together in the Clade Flavexudans. This group includes *V. albo-atrum, V. tricorpus, V. zaregamsianum, V. isaacii*, and *V. klebahnii* among others. Conversely, species falling in the Clade Flavnonexudans do not produce yellow-pigmented hyphae. Examples of such species are *V. nubilum, V. alfalfae, V. nonalfalfae, V. dahliae* and *V. longisporum* (Inderbitzin et al. 2011; Inderbitzin 2014).

Verticillium longisporum is very closely related to *V. dahliae*, which exhibits a much wider host range than the former and causes Verticillium wilt on many crops. Isolates of *V. longisporum* were originally regarded as variants of *V. dahliae*, and the fungus was not reclassified as its own species until the 1990s (Karapapa et al. 1997; Depotter et al. 2016). Several morphological traits formed the basis for the differentiation of these species. *Verticillium longisporum* is characterized by elongated microsclerotia, elongated conidia ranging in size from 7.1 to 8.8 μm, and the presence of three phialides per node on the conidiophores. In contrast, *V. dahliae* exhibits spherical microsclerotia, shorter conidia measuring between 3.5 to 5.5 μm, and 4-5 phialides per node on the conidiophores (Karapapa et al., 1997).

The fungus *V. long*isporum is an amphidiploid hybrid that arose via interspecific hybridization of four distinct parent lineages across three parent species. These include *V. dahliae* lineages D2 and D3, as well as species A1 and species D1, which are non-*Verticillium* species and yet to be isolated (Inderbitzin et al. 2011; Depotter et al. 2016). At least three hybridization events occurred, and species A1 was a parent in all three, as it hybridized with D1, D2, and D3, forming the *V. longisporum* lineages A1/D1, A1/D2, and A1/D3, respectively (Novakazi et al. 2015). The geographic range of the three *V. longisporum* lineages differs, with the A1/D1 lineage predominant in Japan, Europe, and North America, A1/D2 primarily occurring in Illinois, USA, and the A1/D3 lineage documented in Japan and Europe (Inderbitzin et al. 2011).

1.4.2 Disease cycle

In the absence of a host, *V. longisporum* survives as microsclerotia (Heale and Karapapa 1999). These black-coloured, thick-walled, and melanized resting bodies (Issac 1953; Inderbitzin and Subbarao 2014) allow the pathogen to remain viable in the soil for more than a decade (Wilhelm 1955). Studies show that a density as low as 1 colony forming unit (cfu)/g soil of *V. longisporum* microsclerotia can initiate infection once conditions are favourable (Johansson 2006).

Germination of *V. longisporum* microsclerotia is enhanced in the presence of root exudates, producing hyphae that directly penetrate the epidermal cells of lateral roots (Zhou et al. 2006; Eynck et al. 2007; Depotter et al. 2016). Once inside the host, the fungus spreads through the root cortex to the xylem, where it produces conidial spores. The conidia are carried up the vascular system by the transpiration stream, which can result in colonization of the foliage (Iven et al. 2012). Proliferation of the host in the xylem results in the blockage of vascular flow, disrupting water transport through the plant (Depotter et al. 2016). As infected plants mature, the fungus spreads from the xylem into non-vascular tissues, colonizing the stem parenchyma and forming microsclerotia in the dying stem and root tissue (Depotter et al. 2016). The fungus survives on infected plant debris, with the microsclerotia returned to the soil as the host tissues decompose. Verticillium stripe is regarded as a monocyclic disease, with a single turn of the disease cycle per growing season (Verticillium - Canola Council of Canada n.d.).

1.4.3 Signs and symptoms of infection

Infection of *B. napus* by *V. longisporum* presents symptoms that are notably different from those induced by the closely related *V. dahliae*. While *V. dahliae* infection typically results in wilting of canola (Hwang et al. 2017), such wilting is not observed in cases of *V. longisporum* infection (Depotter et al. 2016). Under field conditions, symptoms and signs of *V. longisporum*

infection do not develop until later in the growing season, when dark necrotic stripes (which give rise to the disease name 'Verticillium stripe') appear, along with shredding of the stem and the formation of dark microsclerotia (Heale and Karapapa 1999; Depotter et al. 2016; Knüfer et al. 2017). In contrast, under greenhouse conditions, disease symptoms, including foliar chlorosis, vascular discoloration and a reduction in plant height, can be observed much earlier, including at the seedling stage (Zeise and Tiedemann 2001; Eynck, Koopmann, and von Tiedemann 2009). A significant reduction in plant biomass, particularly in the roots, is often observed in infected plants (Keunecke 2009).

1.4.4 Hosts

Most plants in the family *Brassicaceae* are susceptible to infection by *V. longisporum*, including cabbage (*Brassica oleracea* L. var. *capitata* L.) (Subbarao et al. 1995; Inderbitzin, Davis, et al. 2011), cauliflower (*Brassica oleracea* L. var. *botrytis* L.) (Koike 1994; Debode et al. 2005), Brussels sprouts (*Brassica oleracea* L. var. *gemmifera*) (Isaac 1957), and Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) (Narisawa et al. 2004). However, canola or oilseed rape is the preferred host (Zeise and Tiedemann 2002; Johansson et al. 2006). In some cases, such as broccoli grown in *V. longisporum*-infested soil, infection may not progress beyond the root surface (Shetty et al. 2000; Njoroge et al. 2011).

A study conducted via a root-dip inoculation method suggested that various non-cruciferous species, such as oat (*Avena sativa* L.), spring wheat (*Triticum aestivum* L.), and scentless mayweed (*Tripleurospermum inodorum* L., can also be infected by *V. longisporum*, but not as severely as oilseed rape or other Brassicas (Johansson et al. 2006). Similarly, the virulence of *V. longisporum* on eggplant (*Solanum melongena* L.), tomato (*Solanum lycopersicum* L.), lettuce (*Lactuca sativa* L.), and watermelon (*Citrullus lanatus* (Thunb.) Matsum. &Nakai) was

found to either match or exceed that of *V. dahliae* on these hosts (Novakazi et al. 2015). However, natural infection caused by microsclerotia may differ from studies performed by artificial inoculation, and the epidemiological significance of these other hosts, if any, remains unknown.

1.3 Yield losses

Yield losses caused by *V. longisporum* have been reported to be as high as 80% on a per plant basis on oilseed rape when disease severity is high (Dunker et al. 2008). Losses of 10% to 50% have also reported in other studies, but this has not yet been experimentally verified (Dunker et al. 2008; Günzelmann and Paul 1990; Paul 2003). The impact of *V. longisporum* on the yield of canola has not been evaluated under field conditions in Canada, although one field study conducted in Europe suggested that the yield of oilseed rape is not consistently reduced by Verticillium stripe, even under high disease severity (Depotter et al. 2019). Similarly, artificial inoculation of the soil under field conditions did not have any significant effect on the thousand-seed-weight (TSW) or oil content of the crop (Dunker et al. 2008). Anecdotal reports suggest that because the onset of Verticillium stripe symptoms occurs late in the growing season, it is less damaging than other diseases, such as blackleg or stem rot (Verticillium Stripe - Canola Encyclopedia n.d.). However, hard data on the effect of *V. longisporum* infection on canola yields, particularly under field conditions, are needed for accurate estimates of the true impact of this pathogen in western Canada.

1.4 Verticillium stripe management

Given the recent emergence of *V. longisporum* as an issue in Canadian canola production, few approaches are available for the management of Verticillium stripe. Nonetheless, information from northern Europe, where the pathogen has occurred on oilseed rape for several decades (Debode et al. 2005; Depotter et al. 2016), can provide some indications as to effective

management practices. Similarly, control methods for the closely related fungus *V. dahliae* may also serve as the basis for the development of strategies to manage Verticillium stripe in Canada.

1.4.1 Inoculum control strategies

The melanized microsclerotia produced by *V. longisporum* may persist in the soil for up to 10-15 years in the absence of a host, serving as primary inoculum for subsequent infections (Verticillium - Canola Council of Canada n.d.). Therefore, successful management of Verticillium stripe should include an emphasis on reducing the level of this inoculum below thresholds conducive to disease development. This should be coupled with practices aimed at limiting infection and pathogen progression within the host plant. Options for control include non-chemical strategies such as the implementation of biosecurity measures, soil and equipment sanitation, and diversified cropping systems (Panth et al. 2020). Crop rotation is crucial for preventing the accumulation of soilborne pathogens in other pathosystems, but often proves ineffective against Verticillium wilt due to the broad spectrum of hosts susceptible to *Verticillium* spp. (Bhat and Subbarao 1999; Klosterman et al. 2009). Nevertheless, a few studies with *V. dahliae* have highlighted the benefits of crop rotation for long-term reductions in microsclerotia populations in cauliflower and eggplant (Xiao et al. 1998; Ikeda et al. 2015). Other research demonstrated that the incorporation of ryegrass and corn into soil could reduce the levels of microsclerotia by 50% or more in an European cauliflower field (Debode et al. 2005).

Since *V. longisporum* has a more restricted range of preferred hosts than *V. dahliae,* rotation out of cruciferous crops may prove more helpful in mitigating the impact of Verticillium stripe. A report from Belgium found that the density of microsclerotia in the soil fluctuated seasonally over a 4-year fallow period following a long history of cauliflower farming, consistent with fluctuations under continuous cauliflower (França et al. 2013). However, there is a limited

number of studies evaluating the effect of cropping systems on Verticillium stripe severity (Subbarao et al. 1999; França et al. 2013a; Cwalina-Ambroziak et al. 2016), and a review of the literature during preparation of this thesis indicates a gap in research concerning the efficacy of crop rotation in managing Verticillium stripe in Canada. This suggests a need for further evaluation of rotation as tool to control the disease.

Biosecurity practices can also contribute to the management of soilborne diseases by preventing pathogen spread and introduction to new fields. These practices include sanitation of farm equipment, monitoring off-farm traffic, tracing sources of seeds, feed, and fertilizer, developing on-farm biosecurity plans, and adopting measures such as wearing plastic boots and sanitation of small tools (Amass et al. 2001; Canola Council of Canada n.d.). Although seedborne transmission of *V. longisporum* has not been documented in Canada, it has been confirmed in greenhouse and field studies in Germany (Zheng et al. 2019). This suggests that the implementation of seed testing for this pathogen before or after harvest could prove beneficial. Nonetheless, the apparently widespread occurrence of *V. longisporum* in Canada (Government of Canada 2015) means that such strategies are unlikely, on their own, to prevent the development of disease.

1.4.2 Chemical control and soil amendments

No seed or foliar fungicides are currently registered for *V. longisporum* in Canada. Moreover, the efficacy of chemical control is limited, because the pathogen colonizes the xylem of its hosts, where few fungicides can reach without damaging the plant (Johansson 2006). Nevertheless, some inorganic chemicals, such as elemental sulfur and methyl bromide, were employed for over 50 years to reduce microsclerotia levels by directly applying them to the soil. Unfortunately, methyl bromide was identified as a stratospheric ozone-depleting agent, leading to

its complete discontinuation in many countries in 2015 (Powelson and Carter 1973; Subbarao 2002; Cooper and Williams 2004). Organic, carbon-containing compounds, such as nitrous acid, ammonia, and volatile fatty acids, have also shown significant efficacy in decreasing microsclerotia levels and reducing disease severity in acidic soils through the conversion of NH_4^+ to nitrite (NO_2^-). In soils with a pH < 5.5, NO_2^- is further converted to HNO_2 (nitrous acid), which is highly toxic to the microsclerotia (Tenuta and Lazarovits 2002; Klosterman et al. 2009).

The application of manure as a strategy for the control of *V. dahliae* and other soilborne pathogens has also been explored (Conn et al. 2005). The benefits of manure stem from the release of volatile fatty acids during its decomposition process. Volatile fatty acids can include the short-chain fatty acid acetic acid, along with propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids, which also contribute to pathogen suppression (Conn et al. 2005). These molecules are toxic to microsclerotia and many other pathogen structures (Conn et al. 2005; Klosterman et al. 2009). An evaluation of liquid swine mature indicated that it could significantly reduce the germination of *V. dahliae* microsclerotia by volatile fatty acid and nitrous acid toxicity in acid soils, and by ammonia toxicity in alkaline soils (Conn et al. 2005).

The effectiveness of *V. longisporum* control also seems to be influenced by the lignin content of the host crop residues. The 'lignin-melanin hypothesis' suggests that enzymes responsible for lignin biodegradation may also contribute to the breakdown of fungal melanin (Debode et al. 2005). Melanin provides protection to microsclerotia against both biotic and abiotic stresses during the periods between hosts (Bell and Wheeler 1986; Depotter et al. 2016). Hence, soil amendments containing a relatively elevated lignin content might encourage microbial organisms involved in lignin decomposition, thereby diminishing the viability of the microsclerotia.

1.4.3 Biological control

Biocontrol agents, including bacteria and fungi, can inhibit host colonization by *V. longisporum* under certain conditions, and help to reduce inoculum (microsclerotia) levels in the soil. Several strains of bacteria have been reported to protect oilseed rape against *V. longisporum*. For example, the strains UCMB5036, UCMB5113, and UCMB5033 of *Bacllius amyloliquefaciens* ssp. *plantarum* Priest et al. effectively inhibited growth of *V. longisporum in vitro* (Danielsson et al. 2007). Similarly, the application of *Serratia olymuthica* HRO-C48 to seeds of oilseed rape through bio-priming and pelleting techniques significantly reduced symptoms of *Verticillium* infection (Müller and Berg 2008).

Several fungal species have also been evaluated as potential biocontrol agents for *V. longisporum*. Research conducted in Germany showed that *Metarhizium brunneum* Petch, a soilborne fungal entomopathogen, could inhibit *V. longisporum* through competition and antibiosis in the root zone. Moreover, it could trigger a systemic response in oilseed rape, leading to enhanced plant growth and reduced disease symptoms. These effects were observed both *in vitro* and in greenhouse experiments (Posada-Vergara et al. 2023). In another study, the fungus *Microsphaeropsis ochracea* (Speg.) Höhn significantly reduced the viability of *V. longisporum* microsclerotia *in vitro* and under sterile soil conditions (Stadler and von Tiedemann 2014). Unfortunately, poor efficacy was observed under field conditions, due to the greater complexity of the soil microbial community, where *M. ochracea* appeared to be out-competed (Stadler and von Tiedemann 2014). Perhaps most interestingly, a non-pathogenic isolate of *Verticillium*, termed Vt305, could successfully reduce colonization of and symptom development on cauliflower tissue by *V. longisporum* in a controlled environment (Tyvaert et al. 2014). The isolate suppressed symptom development when inoculated 1 week prior to inoculation with *V. longisporum* (Tyvaert

et al. 2014). However, the mechanism by which it afforded this protection was not determined and requires further study (Tyvaert et al. 2014).

Despite promising outcomes from certain experiments, there are currently no commercially available biological control agents for managing Verticillium stripe in Canada or elsewhere. This could be due to inconsistent results observed under field conditions and/or cost considerations. Factors such as large-scale production, formulation, preservation conditions, shelf life, and application methods need to be carefully considered early in the selection process of biological control agents targeting *V. longisporum* (Deketelaere et al. 2017).

1.4.4 Genetic resistance

The development of host varieties that are genetically resistant to Verticillium stripe would represent one of the most effective and feasible means to manage this disease (Depotter et al. 2016). Given the limited genetic diversity of current canola/oilseed rape cultivars, it may be necessary to explore other Brassicas and non-canola types of *B. napus* as potential sources of resistance (Eynck et al., 2009; Happstadius et al., 2003; Depotter et al., 2016).

In a study evaluating resistance to Verticillium stripe, the majority of tested *B. napus* accessions were found to be susceptible to moderately resistant (Eynck et al. 2009). Accessions of *B. rapa* were largely susceptible, whereas significant resistance was observed in the *B. oleracea* group (Eynck et al. 2009). An earlier study also identified several *B. oleracea* accessions exhibiting strong resistance to *V. longisporum*, suggesting that this species could serve as an effective source of Verticillium stripe resistance (Happstadius et al. 2003). Nonetheless, significant variation has been reported in the reactions of specific *B. oleracea* genotypes (Debode et al. 2005), and careful selection of potential resistance donors will be needed when breeding for enhanced resistance. In a re-synthesized *B. napus* line obtained by crossing white cabbage (*B. oleracea* ssp. *oleracea*

convar. *capitata*) and winter turnip rape (*B. rapa* ssp. *oleifera*), two significant quantitative trait loci (QTLs) associated with resistance to *V. longisporum* were identified on chromosomes C4 and C5 (Rygulla et al., 2008). In another study, three QTLs for resistance to this pathogen were found in a moderately resistant oilseed rape. One QTL was located on chromosome C1, while two were on chromosome C5. Collectively, these findings suggest that the Brassica C-genome represents an important pool of quantitative Verticillium stripe resistance (Obermeier et al., 2013).

In addition to conventional breeding, transgenic approaches have also been suggested for enhanced resistance to *V. longisporum*. For instance, the *BvGLP-1* gene from sugar beet (*Beta vulgaris* L.) was found to improve resistance to this pathogen when expressed in Arabidopsis (Knecht et al. 2010). This gene shows high sequence homology to a set of germin-like proteins and is significantly upregulated in sugar beet plants after nematode (*Heterodera schachtii*) infection (Knecht et al. 2010). However, given the restrictions placed on genetically modified crops in some markets, and public concerns regarding the safety of such traits in the food supply, the practical application of transgenic approaches may encounter significant resistance.

1.5 Inoculation and disease rating techniques

The recent detection of *V. longisporum* in Canada has sparked considerable interest in developing Verticillium stripe-resistant canola germplasm. A critical prerequisite for the success of a resistance-breeding program is the establishment of effective inoculation protocols for screening germplasm, and accurate rating scales for assessing resistance phenotypes.

Several greenhouse inoculation methods for *Verticillium* spp. have been documented in the past. The most common method involves immersing seedling roots in a conidial suspension, then immediately planting them in soil or potting mix (Koike 1994; Zeise and Tiedemann 2002; Depotter et al. 2017). Alternatively, in other protocols, microsclerotia are directly blended into the

soil as inoculum (Zeise and Tiedemann 2002; Debode et al. 2005; Dunker et al. 2008). In some studies, a mixture of sand and cornmeal has been utilized for inoculating *Verticillium* spp., including *V. tricorpus* and *V. dahliae* (Paplomatas 1991; Tahmatsidou et al. 2006). In one report, a method called the 'box-test' was suggested. This involved placing the lower part of the stem and roots, contained within two magenta boxes, in direct contact with a pathogen suspension and the growing medium (Steventon et al. 2002). Many of these methods have not been assessed for their effectiveness in inoculating canola with *V. longisporum*, and some come with specific limitations. For instance, the need to submerge seedlings in a conidial suspension is time-consuming and impractical for large-scale resistance screening. Likewise, utilizing microsclerotia as inoculum demands the generation of large quantities of these structures, which in turn requires significant space, time, and materials.

It is important not only to ensure successful inoculation of host genotypes, but also to develop disease-rating scales that are easy to use and accurately represent the host's response to infection. Various rating scales have been proposed for assessing Verticillium wilt in many crops, including canola/oilseed rape. For instance, in one study, the severity of Verticillium wilt in *B. napus* was determined based on the percentage of wilted leaves and/or stunting of plants, assessed 25 days after inoculation (Steventon et al. 2002). Other approaches for disease assessment have involved quantifying microsclerotia present on the stubble, serving as an indirect measure of the host response (Dunker et al. 2008; Knüfer et al. 2017). However, methods for evaluating Verticillium stripe severity in canola are limited. Since this disease does not cause wilting, assessing wilt symptoms would not be relevant for scoring disease development. In addition, establishing rating scales for Verticillium stripe at both maturity and the seedling stage would facilitate studies on quantitative and qualitative resistance.

1.6 Research objectives

With the emergence of Verticillium stripe in the Canadian canola crop, it is crucial to develop enhanced protocols for managing this disease and its causal agent, and to gain a deeper understanding of the threat it poses to yields. In this context, the research presented in this dissertation aimed to achieve three specific objectives:

1. To refine techniques for inoculating *V. longisporum* on canola, including assessment of various types of inoculum and determining the optimal host age for inoculation
2. To generate scales for assessing Verticillium stripe severity at the seedling and adult plant stages in canola
3. To evaluate the impact of *V. longisporum* infection on the yield of hybrid canola under field conditions in western Canada, and to determine the relationship between Verticillium stripe severity and inoculum concentration.

Chapter 2: Development of optimized *Verticillium longisporum* inoculation techniques for canola (*Brassica napus*)

2.1 Introduction

The soilborne fungus *Verticillium longisporum* (C. Stark) Karapapa, Bainbr. & Heale causes Verticillium stripe, an important vascular wilt disease of canola/oilseed rape (*Brassica napus* L.). In Europe, yield losses as high as 50% have been reported in *V. longisporum*-infected crops (Novakazi et al. 2015). In Canada, Verticillium stripe was first identified in the province of Manitoba in 2014 (Province of Manitoba – Agriculture). This was a cause of concern, since canola is a major crop contributing $29.9 billion CAD annually to the Canadian economy, with about 20 million tonnes of canola produced each year (Canadian Canola Growers Association.). Surveys coordinated by the Canadian Food Inspection Agency (Government of Canada 2015) confirmed the presence of *V. longisporum* in Manitoba, as well as in other Canadian provinces including Alberta, Saskatchewan, Ontario, and Quebec.

The life cycle of *V. longisporum* begins in the soil, where the fungus can survive as long-lived microsclerotia (Heale and Karapapa 1999) that germinate in response to root exudates, producing hyphae that directly infect the epidermal cells of lateral roots (Zhou et al. 2006; Eynck et al. 2007; Eynck et al. 2009b; Depotter et al. 2016). Once inside the roots, the fungus grows inter- and intracellularly, spreading to the vascular tissue (Eynck et al. 2009a; Depotter et al. 2016). Conidia are produced in the xylem and can spread up the vascular system via the transpiration stream, leading to the colonization of the foliage (Iven et al. 2012). As the plants begin to mature, the fungus spreads from the xylem into non-vascular tissues, colonizing the stem parenchyma and forming microsclerotia in the stem pith and under the epidermis (Depotter et al. 2016). External

symptoms and signs of infection present later in the growing season, and include necrosis, shredding, and the appearance of microsclerotia on the stem (Depotter et al. 2016). The microsclerotia survive on infected residues and in the soil following decomposition of the host tissues, serving as inoculum for future infections (Heale and Karapapa 1999).

The effective management of Verticillium stripe requires an integrated plan incorporating multiple strategies, such as chemical and biological control, crop rotation, proper weed management, and host resistance (Depotter et al. 2016). While genetic resistance is one of the most effective and environmentally friendly methods for plant disease control, knowledge of *V. longisporum* resistance in Canadian canola cultivars is limited. Moreover, given the fact that the pathogen was not detected in Canada until recently, development of resistant germplasm has not been a priority until now. A prerequisite to the development of a successful resistance-breeding program, however, is the availability of effective inoculation protocols for germplasm screening and evaluation of resistance phenotypes. The main goal of this study was to develop optimized *V. longisporum* inoculation techniques for canola, including comparison of inoculum types and host age at the time of inoculation.

2.2 Materials and methods

2.2.1 Root-dip inoculation

Isolate VL43 of *V. longisporum*, collected from diseased *B. napus* plants sampled near Edmonton, Alberta, was grown in 9-cm diam. Petri dishes filled with potato dextrose agar (PDA). Cultures were incubated under darkness at room temperature for 14 days. A conidial suspension was prepared by adding 10 mL of sterile distilled water to each Petri dish and gently dislodging the spores by rubbing a glass rod over the culture. The spore suspension was filtered through four layers of sterile cheesecloth, with the filtrate collected in a 100 mL beaker and the spore

concentration estimated in a hemocytometer (Hausser Scientific, Horsham, Pennsylvania, USA) and adjusted to 1×10^6 spores/mL or 1×10^7 spores/mL with sterile distilled water. Conidial suspensions were used immediately or stored at 4°C for a maximum of 72 h prior to use.

Seeds of the canola cultivars '45H31', 'CS2000', and 'Westar' were surface-disinfected in 1% (v/v) sodium hypochlorite for 1 min followed by 1 min in 70% ethanol, and then rinsed twice for 30 sec in sterile water. The disinfected seeds were placed on moistened filter paper in Petri dishes for 7 days to allow germination. The 1-week-old seedlings were then transferred carefully to a piece of sterile paper towel and the roots were wounded with a pair of sterile scissors. The wounded roots were placed in the low or high concentration conidial suspensions (1×10^6 spores/mL and 1×10^7 spores/mL, respectively) for 45 min. Non-inoculated controls were placed in water instead of a conidial suspension. The seedlings were then transplanted into 32 L plastic tubs (30 seedlings per tub) filled with Sunshine Mix #4 growing mix (Sun Gro Horticulture, Vilna, Alberta) and transferred to a greenhouse. The plants were maintained under an 18 h photoperiod (22°C day/16°C night) with natural light supplemented by artificial lighting. The experiment was arranged in a split-block design with five replicates (tubs) assigned to each treatment and independently repeated.

2.2.2 Grain inoculum

Grain inoculum was produced (Hwang et al. 2014). Briefly, colonies of *V. longisporum* were cut into ~100 small pieces and mixed with sterilized, water-soaked wheat grain in a mushroom bag containing 950mL of wheat grain per Petri dish of fungal culture. The inoculated grain was incubated at room temperature for 4 weeks, and then dried at 30°C in a drying oven for 2 days. After drying, the grain was ground and passed through a 2-mm mesh sieve. Seeds of the canola cultivars '45H31', 'CS2000', and 'Westar' were surface-disinfected as described above,

and sown in 450 mL cups filled with Sunshine Mix #4 growing mix (Sun Gro Horticulture) at a density of 10 seeds per cup. Two inoculum levels were prepared by mixing 1.25 mL or 5 mL of infested grain with sterilized playground sand to achieve a total volume of 15 mL grain-sand inoculum mixture. Sand that had received 3.125 mL of non-infested grain inoculum served as a control. The grain-sand mixture was applied to the top of the potting mix after seeding, with the seeds and inoculum covered with 40 mL of Sunshine Mix #4 growing mix. Five replicates (cups) were assigned to each treatment for each cultivar. The cups were placed on a greenhouse bench in a split-block design and the experiment was repeated once.

2.2.3 Inoculation at different growth stages

One hundred and twenty seeds each of 'CS2000' and 'Westar' were surface-disinfected as described above and sown in two rows in 32 L tubs filled with 25 L of Sunshine Mix #4 growing mix (Sun Gro Horticulture), with seven tubs per cultivar. The tubs were incubated in a greenhouse with an 18 h photoperiod at temperatures of 22 °C (day) and 16 °C (night). Seedlings were thinned to a density of 80 plants per tub 5 days after planting. Four plants from each tub were carefully removed from the soil at 1-week, 2-weeks, and 3-weeks after planting, rinsed under tap water for 1 min, wounded with a pair of sterile scissors, and placed in a suspension of *V. longisporum* conidia (1×10^7 spores/mL) for 45 min. The inoculated seedlings were transplanted into 450 mL cups filled with Sunshine Mix #4 growing mix (Sun Gro Horticulture) at a density of four plants per cup. The experiment was arranged in a split plot design with seven replicates (cups) assigned to each growth stage treatment and repeated independently.

2.2.4 Disease assessment

Separate disease severity scales were developed to rate Verticillium stripe severity at the seedling versus adult plant stage. Disease severity (DS) at the seedling stage was assessed at 10

days post-inoculation (DPI) on a 0 to 6 scale, where: 0 = no symptoms; 1 = yellowing of cotyledons; 2 = yellowing and partial necrosis of cotyledons, some reduced growth of first true leaves; 3 = death of cotyledons, seedling stunted; 4 = death of cotyledons, seedling stunted, true leaves yellow; 5 = death of cotyledons, seedling stunted, little to no growth of true leaves; and 6 = seedling entirely necrotic (Fig. 2.1). In adult plants, DS was assessed 105 days after planting on a 0-4 scale, where: 0 = no symptoms or signs of disease; 1 = some discolouration of main stem, with dark unilateral stripe; 2 = discolouration of main stem, microsclerotia on ≤ 25% of the surface, slight stunting of entire plant; 3 = discoloration of main stem, some shredding of the epidermis, microsclerotia on ≤ 75% of the surface, stunting of entire plant; and 4 = stem entirely necrotic and covered with microsclerotia, shredding of the epidermis, plant severely stunted (Fig. 2). In plants inoculated by the root-dip method, 10 randomly selected plants from each replicate were evaluated for DS. In plants inoculated with grain inoculum or inoculated at different growth stages, all plants were evaluated from each replicate. Plants were photographed and monitored weekly for disease progression.

2.2.5 Plant growth parameters and seed yield assessment

Plant mortality was assessed at 35 days following root-dip inoculation by counting the number of dead plants out of the 30 initially transplanted into each tub. For plants inoculated with grain inoculum, plant emergence and height were recorded 21 days after sowing. Total dry weights (including roots and shoots) were assessed for plants harvested 105 days after sowing. Mean seed yield at maturity was evaluated for 10 plants from each tub in the root-dip experiment, and for all plants in each replicate for experiments testing the grain inoculum and inoculation at different growth stages.

2.2.6 Statistical analysis

Mean Verticillium stripe DS, seedling emergence, plant height, and seed yield in response to inoculum levels and cultivar were analyzed using R (R core Team, R Foundation for Statistical Computing, Vienna, Austria, 2013). For the root-dip and grain inoculum experiments, canola cultivars, inoculum levels, and their interactions were considered as fixed effects. Random effects were inoculum levels nested within cultivars (whole-plots), and replications within inoculum levels and inoculum levels interaction (split-plot). For the experiment comparing inoculation at different growth stages, cultivars, plant age (weeks), and their interactions were considered as fixed effects. Weeks nested within cultivar (whole-plots) and replications within weeks and weeks interactions were considered as random effects. A split plot design was used for analysis of the data set and the data were normally distributed.

2.3 Results

2.3.1 Root-dip inoculation

At the seedling stage, inoculation of canola with *V. longisporum* resulted in foliar yellowing, necrosis, and stunting, while no symptoms or signs of infection were observed on non-inoculated plants (Fig. 2.3A). Yellowing of the cotyledons was evident on all of the seedlings 1 week after inoculation (Fig. 2.3B, C). In some cases, plants recovered somewhat once the true leaves formed, while in other cases, development of the true leaves was impaired and the entire seedling was stunted (Fig. 2.3D, E, F). Disease severity on all canola cultivars was significantly greater following inoculation with the high versus low concentration of inoculum (Table 2.1), but there was no significant difference among cultivars. Average seedling DS ratings at the low and high inoculum concentrations were, respectively, 1.64 and 2.57 on '45H31', 1.97 and 2.71 on 'CS2000', and 1.82 and 2.78 on 'Westar'. At 35 DPI, plant mortality was greater for all cultivars

when they received the high versus low inoculum concentration. In both the high and low inoculum treatments, mortality was lowest for ‘CS2000’ and highest for ‘45H31’ (Table 2.1).

Symptoms and signs of *V. longisporum* infection on mature canola plants included stunting and necrosis, discolouration, and shredding of the stem, as well as the presence of microsclerotia (Fig. 2.4A-F). The DS was significantly milder in plants inoculated with the low versus high inoculum concentration. Significant differences were also observed with respect to DS among cultivars. The mildest disease was observed on ‘45H31’, with average severity ratings of 1.71 and 2.26 at the low and high inoculum concentrations, respectively, while the most severe disease was observed on ‘Westar’, with ratings of 3.23 and 3.32 at the adult plant stage (Table 2.1). While plant mortality at 35 DPI was highest for ‘45H31’, at maturity, the surviving plants of this cultivar appeared most resistant to *V. longisporum*. Significant declines were observed in the yield of all cultivars relative to non-inoculated controls, but no differences were detected among cultivars or inoculum concentrations (Table 2.1). In the inoculated treatments, yields for ‘45H31’, ‘CS2000’, and ‘Westar’ were 63% to 72%, 67% to 76%, and 76% to 80% lower, respectively, than in non-inoculated controls.

2.3.2 Grain inoculum

The application of *V. longisporum* grain inoculum resulted in reduced plant emergence as measured at 21 days (Table 2.2). Emergence declined significantly for all three canola cultivars relative to the non-inoculated controls at both the low and high inoculum concentrations. Moreover, while emergence was similar for ‘CS2000’ and ‘Westar’ at both inoculum concentrations, there was significantly lower emergence for ‘45H31’ at the high concentration (Table 2.2). Significant reductions in plant height also were observed for all cultivars at 21 days, although no difference was found in height at low or high inoculum concentrations (Table 2.2).

Symptoms and signs of Verticillium wilt at the adult plant stage (105 days) following grain inoculum application resembled the symptoms obtained via the root-dip method, including stunting and necrosis, discolouration, and shredding of the stem, as well as the presence of microsclerotia (Fig. 2.2). There was no disease observed on the non-inoculated controls, while DS was similar for '45H31' and 'CS2000' at both the low and high inoculum concentrations (Table 2.2). The DS on 'Westar' was significantly greater at the high inoculum concentration. Relative to the root-dip inoculation method, the DS obtained on all three canola cultivars using grain inoculum was milder. Dry weight at maturity was reduced significantly across cultivars relative to the non-inoculated controls, although there were no significant differences with respect to weight at low versus high inoculum concentrations (Table 2.2). Yields were also significantly reduced in the grain-inoculated treatments relative to the non-inoculated controls (Table 2.2). However, while yields for '45H31' were further reduced at the high versus low inoculum concentration, there was no significant difference for 'CS2000' or 'Westar'.

2.3.3 Inoculation at different growth stages

Disease severity was similar for 'Westar' regardless of plant age at the time of inoculation (Table 2.3). In contrast, DS was significantly greater when 'CS2000' was inoculated at 3-weeks versus 1- or 2-weeks of age. As expected, the non-inoculated controls did not develop any signs or symptoms of disease. In 'Westar', yield was lowest when plants were inoculated at 1- or 3-weeks of age; the yield for plants inoculated at 2-weeks, while numerically lower than the non-inoculated control, was not significantly different. In the case of 'CS2000', yields were significantly lower than the control when plants were inoculated at 2- or 3-weeks of age, but not when inoculated at 1-week.

2.4 Discussion

The recent emergence of *V. longisporum* as a pathogen of canola in Canada has generated interest in the development of Verticillium stripe-resistant cultivars of this crop. The generation of such cultivars, however, requires effective resistance screening, including optimized inoculation methods and timing. Many previously reported greenhouse inoculation techniques for *Verticillium* spp. involve immersion of the host roots in a conidial suspension, followed by planting in soil or a potting mix (Koike 1994; Zeise and Tiedemann 2002; 2002; Debode et al. 2005; Dunker et al. 2008; Eynck et al. 2009b; Depotter et al. 2017). In other cases, microsclerotia are mixed into the soil to serve as inoculum (Zeise and Tiedemann 2002; Debode et al. 2005; Dunker et al. 2008). Root-dip inoculation methods usually generate more consistent results than the use of microsclerotia (Eynck et al. 2009b), presumably because the seedling rootlets receive a uniform coating of inoculum in the former method. In addition to root-dip inoculation and soil infestation with microsclerotia, Steventon et al. (2002) described a 'box-test' inoculation method, wherein the base of the stem and roots placed in double magenta boxes were in direct contact with a pathogen suspension and growing medium. In order reports, seeds were germinated on growth medium followed by addition of a conidial suspension *in vitro* (Debode et al. 2005; Eynck et al. 2007). In the study presented here, we compared the inoculation of canola via a root-dip method versus the application of grain inoculum, at various timings and inoculum concentrations.

Root dip inoculation methods can be time-consuming, with the host seedlings often grown until the development of the first true leaves (approximately 14 days) prior to inoculation (Steventon et al. 2002; Depotter et al. 2017). Seeds are often sown directly into a sterilized growth substrate before the emerging seedlings are dipped in a conidial suspension and then transplanted into fresh potting mix (Steventon et al. 2002; Eynck et al. 2009b), increasing time, labor, and

material requirements. A modified root-dip method was developed to shorten the plant growth period to 10 days before inoculation, but which necessitated wounding of the roots before inoculation and a 30 min immersion in the inoculum suspension for consistent results (Eynck et al. 2009a). Furthermore, at least two weeks (Heale and Karapapa 1999; Debode et al. 2005; Eynck et al. 2007), and sometimes as many as six weeks (Depotter et al. 2017), are needed for disease development following inoculation. In the current study, seedlings were germinated on moistened filter paper in Petri dishes on a lab bench, under continuous light, to reduce the germination period to 7 days, saving time and greenhouse space. At this point, the seedlings were still at the cotyledon stage. While the rootlets were wounded as per Eynck et al. (2009b), the period over which they were immersed in the conidial suspension was increased from 30 to 45 min, to maximize the time of pathogen-host contact.

Generally, previous studies have used *V. longisporum* inoculum concentrations of 1×10^6 conidia/mL or higher to achieve sufficient disease pressure (Heale and Karapapa 1999; Zeise and Tiedemann 2002; Debode et al. 2005; Eynck et al. 2007; Eynck, et al. 2009b; Depotter et al. 2017). Thus, in this study, two inoculum concentrations (1×10^6 conidia/mL and 1×10^7 conidia/mL) were compared. While significant disease development resulted from inoculation with either concentration, mean disease severity was more consistent across growth stages at the higher concentration. The symptoms of infection can appear as early as 2 weeks after seedling inoculation via the root-dip method. Heale and Karapapa (1999) observed chlorosis on the cotyledons at 11-15 days, with disease symptoms gradually moving upward, resulting in chlorosis and some necrosis of the adult leaves at 15-18 days after inoculation. Disease severity ratings, however, are often conducted later; for example, symptom severity on *B. napus*, based on the percentage of wilted leaves and/or stunting, was assessed 25 days after inoculation with *V. longisporum*

(Steventon et al. 2002). Therefore, despite various protocols for inoculation of *Verticillium* spp. at the seedling stage, most studies have conducted disease assessments on older plants. To our knowledge, the 0-6 scale presented in this paper is the first to evaluate Verticillium stripe development on seedlings.

The development of Verticillium stripe was assessed 10-days post-inoculation, and was based on development of chlorosis, reduced growth of the true leaves, stunting, and plant necrosis. In addition, plant mortality was evaluated at 35 days, by which time the most highly susceptible seedlings had died. Thus, the modified root-dip inoculation protocol presented in this study, together with the seedling rating scale and measure of mortality, enabled characterization of seedling resistance within a fairly short timeframe. In addition, disease development can continue to be monitored on adult plants following root dip inoculation. Adult plants were assessed for symptoms of Verticillium stripe 98 days after the root-dip inoculation. Symptoms and signs of infection on the adult plants were distinct from those on the seedlings, and included a unilateral dark stripe on the main stem, the presence of microsclerotia and the shredding of the stem. In previous studies, disease severity on mature plants was often rated after harvest and was based on the abundance of microsclerotia on the stubble (Dunker et al. 2008; Knüfer et al. 2017; Zheng et al. 2019). In the current study, a rating scale of adult plants was developed that can be used to rate disease severity before harvest. This scale could facilitate resistance phenotyping for quantitative resistance in canola breeding programs.

A drawback of root-dip inoculation methods is that seedlings must be immersed in a conidial suspension, making such an approach unsuitable for large-scale resistance screening or evaluation under field conditions. As such, the use of *V. longisporum*-infected wheat grain was also tested in the current study. While the use of a sand-corn meal mixture has been reported for

other *Verticillium* spp., including *Verticillium tricorpus* and *Verticillium dahliae* (Paplomatas 1991; Tahmatsidou et al. 2006), to our knowledge, this is the first reported use of grain inoculum for *V. longisporum*. An advantage of the grain inoculum was that, while it required an incubation period of 4 weeks to produce, large quantities could be generated in one round and it could be applied rapidly once prepared. In addition, the *B. napus* seeds did not need to be pre-germinated (nor the rootlets wounded), since the grain inoculum could be applied directly to the pots containing the seeds. Treatment with grain inoculum significantly reduced seedling emergence and plant height at both rates evaluated, compared with the non-inoculated control, and while no yellowing of the cotyledons was observed, the application of grain inoculum resulted in the development of Verticillium stripe in the adult plants. These results suggest that grain inoculum could be useful for field screening or evaluation of large numbers of plants under greenhouse conditions. Additional testing of *V. longisporum* grain inoculum under field conditions, in biosecure disease nurseries, would be needed to confirm its utility in field experiments.

Both the root-dip method and application of grain inoculum could cause significant Verticillium stripe severity at different growth stages of canola. Furthermore, the consistency of the host reactions suggested that either inoculation method should be effective in screening for resistance to *V. longisporum*, particularly at high inoculum concentrations. Although the purpose of this study was not to evaluate the level of resistance in Canadian canola genotypes, it is worth noting that the three cultivars included in the tests showed differential reactions to *V. longisporum*, and were ranked in the same order in terms of resistance (‘Westar’, ‘CS2000’, and ‘45H31’) when inoculated at the high inoculum concentration. This indicates that there are differences in resistance in Canadian *B. napus* germplasm, although additional testing of a much larger number of genotypes will be required to assess the situation more fully.

In previous reports, host plants have been inoculated at various growth stages, ranging from 10 days to 4 weeks, under greenhouse conditions. (Eynck et al. 2009b; Zou et al. 2020). Therefore, in this study, the effect of plant age at the time of exposure to *V. longisporum* was also compared using the root-dip method. In general, there did not seem to be a significant impact of host age at inoculation on disease development, although later inoculations resulted in higher Verticillium stripe severity and yield reductions in one of the hosts, 'CS2000'. In terms of time and costs, the inoculation of 1-week-old seedlings seems preferable, particularly since seedlings cannot be maintained in Petri dishes for much longer than a week and would have to be grown elsewhere for inoculation of older plants.

The results of this study indicate the potential utility of the root-dip and grain inoculation methods for screening for *V. longisporum* resistance and other experiments in canola, while the seedling and adult plant rating scales presented could facilitate evaluation of resistance. Such work will be necessary for the development of canola with Verticillium resistance, which may become important in managing this emerging disease in Canada.

Table 2. 1 Effect of root-dip inoculation of canola with different concentrations of *Verticillium longisporum* on disease severity at the seedling and adult plant stages, plant mortality, and yield.

Treatment	Disease severity at seedling stage (0-6 scale)			Mortality (%)			Disease severity at the adult plant stage (0-4 scale)			Yield (g/10 plants)		
	'45H31'	'CS2000'	'Westar'	'45H31'	'CS2000'	'Westar'	'45H31'	'CS2000'	'Westar'	'45H31'	'CS2000'	'Westar'
Low inoculum concentration	1.64b	1.97b	1.82b	17.1b	8.3b	14.1b	1.71b	2.54b	3.23b	2.97b	3.40b	1.26b
High inoculum concentration	2.57c	2.71c	2.78c	39.5c	19.7c	33.3c	2.26c	2.87b	3.32b	2.18b	2.40b	1.14b
Control	0a	0a	0a	0a	0a	0a	0a	0a	0a	7.86a	10.17a	5.54a

Assessments of disease severity at the seedling and adult plant stages were made at 10 days and 98 days after inoculation, respectively. Plant mortality was evaluated at 35 days after inoculation, while yields were measured at 98 days after inoculation. Means in a column and category followed by the same lowercase letter do not differ based on the Tukey–Kramer's honest significant difference test at $p \leq 0.05$. Data are the least-square means of five replications.

Table 2. 2 Effect of inoculation of canola with grain inoculum of *Verticillium longisporum* on disease severity at the adult plant stage, plant height, dry weight, and yield.

Treatment	Emergence %			Plant Height (cm)			Dry Weight (g)			Disease severity at the adult plant stage (0-4 scale)			Yield (g/rep)		
	'45H31'	'CS2000'	'Westar'	'45H31'	'CS2000'	'Westar'	'45H31'	'CS2000'	'Westar'	'45H31'	'CS2000'	'Westar'	'45H31'	'CS2000'	'Westar'
Low inoculum concentration	41 b	51 b	35 b	11 b	12 b	9 b	10 b	10 b	7 b	0.26ab	1.36b	1.28b	2.32b	2.01b	1.18b
High inoculum concentration	27 c	50 b	31 b	10 b	11 b	9 b	7 b	8 b	7 b	0.75b	1.74b	2.66c	1.68c	1.65b	0.85b
Control	70 a	84 a	61 a	14 a	16 a	16 a	13 a	13 a	10a	0a	0a	0a	2.89a	2.74a	2.51a

Assessments of plant emergence and plant height were made at 21 days after inoculation, while disease severity, dry weight, and yield were recorded at 105 days after inoculation. Means in a column and category followed by the same lowercase letter do not differ based on the Tukey–Kramer's honest significant difference test at $p \leq 0.05$. Data are the least-square means of five replications.

Table 2. 3 Effect age of canola plants at the time of inoculation with *Verticillium longisporum* on yield and disease severity at the adult plant stage.

	Disease severity at the adult plant stage (0-4 scale)		Yield (g/rep)	
Treatment	'CS2000'	'Westar'	'CS2000'	'Westar'
1-week	1.03b	2.88b	1.88ab	0.47b
2-week	1.18b	2.62b	1.62b	0.87ab
3-week	2.09c	3.14b	1.38b	0.78b
Control	0a	0a	2.28a	1.39a

Plants were inoculated at 1-week, 2-week, and 3-weeks after sowing. Controls did not receive inoculum. Assessments of disease severity and yield were made at 105 days after planting. Means in a column and category followed by the same lowercase letter do not differ based on the Tukey–Kramer's honest significant difference test at $p \leq 0.05$. Data are the least-square means of seven replications.

Fig 2. 1 Disease rating scale for canola seedlings infected by *Verticillium longisporum*, where: 0 = no symptoms; 1 = yellowing of cotyledons; 2 = yellowing and partial necrosis of cotyledons, some reduced growth of first true leaves; 3 = death of cotyledons, seedling stunted; 4 = death of cotyledons, seedling stunted, true leaves yellow; 5 = death of cotyledons, seedling stunted, little to no growth of true leaves; and 6 = seedling entirely necrotic.

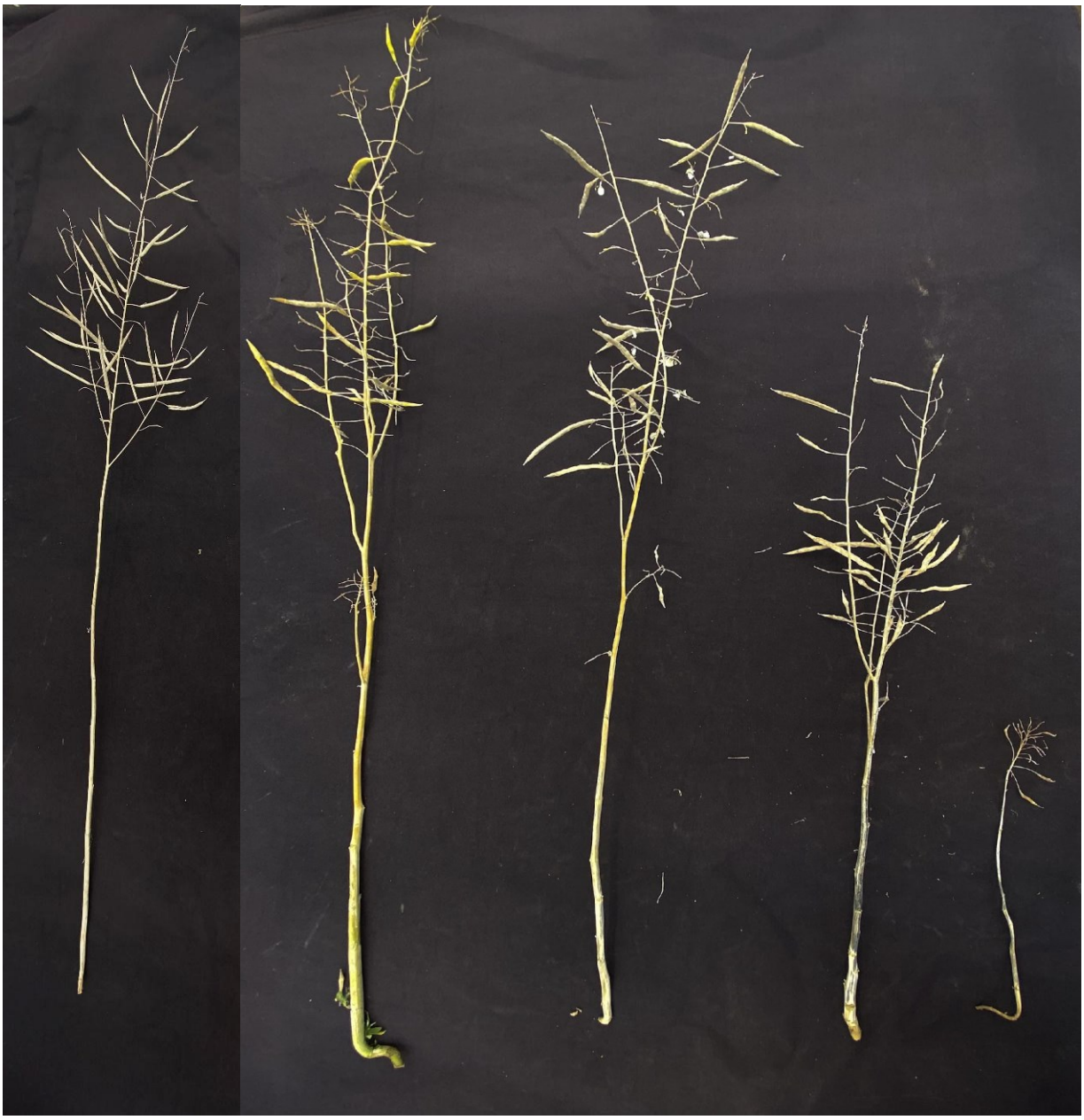

Fig 2. 2 Disease rating scale for adult plants infected by *Verticillium longisporum*, where: 0 = no symptoms or signs of disease; 1 = some discolouration of main stem, with dark unilateral stripe; 2 = discolouration of main stem, microsclerotia on ≤ 25% of the surface, slight stunting of entire plant; 3 = discoloration of main stem, some shredding of the epidermis, microsclerotia on ≤ 75% of the surface, stunting of entire plant; 4 = stem entirely necrotic and covered with microsclerotia, shredding of the epidermis, plant severely stunted.

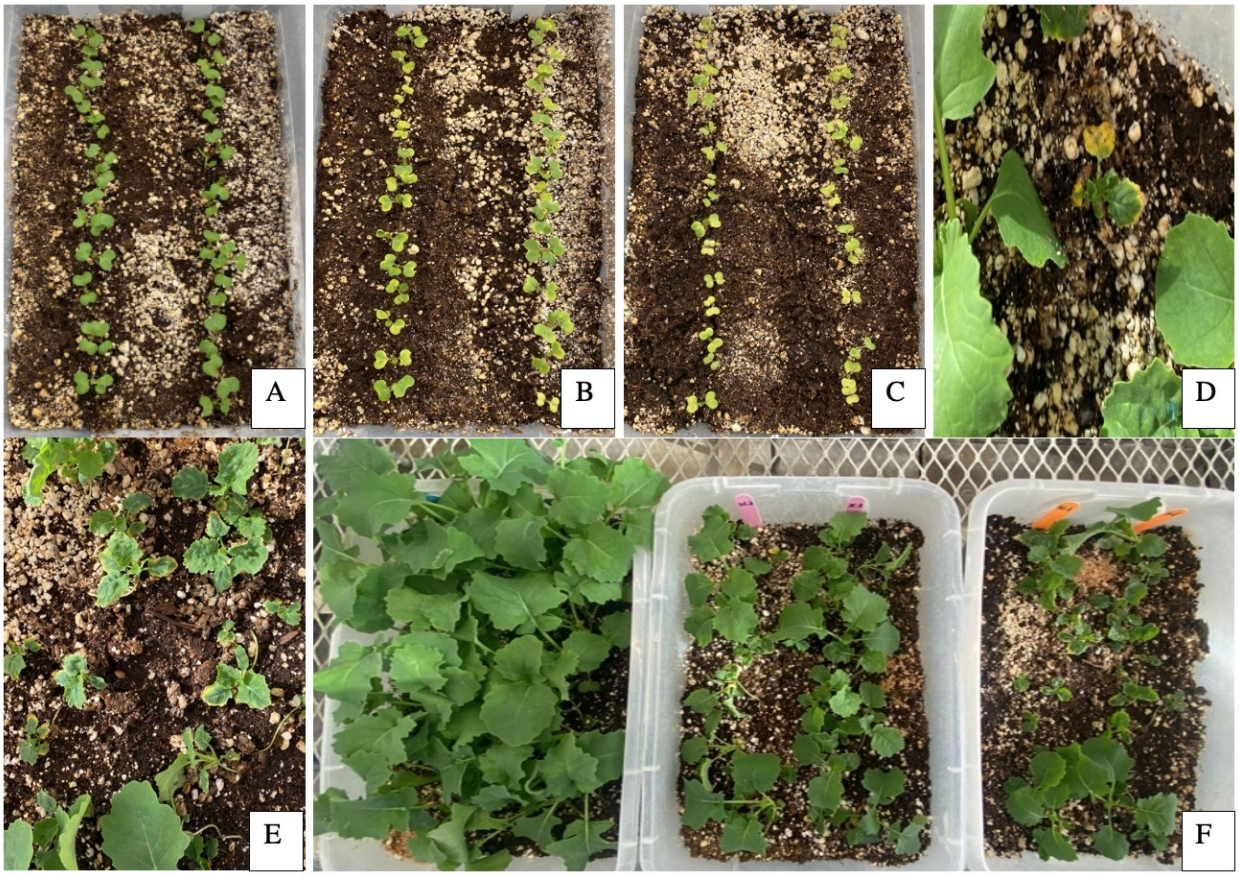

Fig 2. 3 Symptoms of infection by *Verticillium longisporum* on canola at the seedling stage inoculated by the root-dip method. (A) Non-inoculated control 7 days post-inoculation (DPI). (B) Appearance of seedlings at 7 DPI with a low concentration of inoculum. (C) Appearance of seedlings at 7 DPI with a high concentration of inoculum. (D, E) Close-ups showing yellowing. (F) Appearance, from left to right, of the non-inoculated control, low inoculum treatment, and high inoculum treatment at 21 DPI.

Fig 2. 4 Symptoms and signs of infection by *Verticillium longisporum* on canola at the adult plant stage following inoculation by the root-dip method. (A). Plants of the cultivar 'Westar' 98 days post-inoculation (DPI) with a high inoculum concentration. (B, C). Shredding of the stem, presence of black microsclerotia, and discolouration and necrosis resulting from infection. (D). Comparison of non-inoculated (left) and inoculated (right) plants.

Chapter 3: Quantifying yield losses in canola (*Brassica napus*) caused by *Verticillium longisporum*

3.1 Introduction

Verticillium stripe is a vascular disease caused by the soilborne fungus *Verticillium longisporum* (C. Stark) Karapapa, Bainbr. & Heale, which mainly affects cruciferous hosts including canola (oilseed rape; *Brassica napus* L.) (Depotter et al. 2016; Fröschel 2021). In contrast to the wilt symptoms caused by the closely related fungus *Verticillium dahliae* (Koike et al. 1994), infection by *V. longisporum* does not typically result in wilting in B. napus (Heale and Karapapa 1999; Depotter et al. 2019). Instead, dark unilateral striping develops on the main stem approximately 3-4 weeks prior to harvest; this is followed by the formation of fungal microsclerotia below the epidermis and in the stem pith (Depotter et al. 2019; Zheng et al. 2019). The infected tissues take on a shredded, whitish-grey appearance, with peeling back of the stem epidermis at plant maturity. The microsclerotia can survive on infected residues and in the soil following decomposition of the host tissues, and may remain viable for more than 10 years, serving as inoculum for future infections (Heale and Karapapa 1999; Depotter et al. 2016).

Serious losses in oilseed rape attributed to *V. dahliae* were first reported in Sweden in the 1960s (Karapapa et al. 1997). Several other studies found significant variation in yield losses resulting from infection by Verticillium species, ranging from 10% to 50% (Daebeler et al. 1988; Paul 2003; Dunker et al. 2008). Losses on a single plant were reported to be as high as 80% for *V. longisporum*-infected winter oilseed rape in Germany, but only when disease incidence and severity were high (Dunker et al. 2008). In contrast, a more recent study found insignificant or inconsistent yield losses despite increased disease incidence, suggesting the need for further study of the effect of *V. longisporum* on yields (Depotter et al. 2019).

In Canada, Verticillium stripe is a new disease, having first been identified on canola in the Prairies in 2014 (Province of Manitoba - Agriculture n.d.). No fungicides are registered for control of the disease, and little is known regarding the Verticillium stripe resistance of Canadian canola cultivars. While research on the development of disease management strategies has been initiated, there is no information on the relationship between Verticillium stripe severity and yields in hybrid canola. Such information is needed to determine the extent of the threat posed by this disease, particularly given the importance of canola to the Canadian economy (Canadian Canola Growers Association 2022). The objective of this study was to evaluate the impact of *V. longisporum* infection on the yield of hybrid canola, including the relationship between pathogen virulence and inoculum concentration on disease severity and yield under field conditions in western Canada.

3.2 Materials and methods

3.2.1 Grain inoculum preparation

Wheat grain inoculum was prepared following Hwang et al. (2014). In brief, *V. longisporum* isolate VL43, originally collected from diseased canola plants sampled around Edmonton, Alberta, was grown in 10-cm diam. Petri dishes filled with potato dextrose agar (PDA). Fresh cultures were incubated in darkness at 22 °C for 14 days, at which time the colonies were cut into about 100 small pieces and mixed with water-soaked, sterilized wheat grain (950 mL grain per culture) in an autoclaved Hi-Patch mushroom grow bag (Western Biologicals, Aldergrove, BC). The inoculated wheat grain was incubated in darkness at 22°C for 4 weeks and then placed in a drying oven at 30°C for 2 days. The grain was then ground and separated through a 2-mm mesh sieve using a grain mill.

3.2.2 Field experiments

Field experiments were conducted at the St. Albert Research Station (53.6951° N, 113.6327° W), University of Alberta, in 2020 and 2021. Two trials were conducted each year at two different locations (designated as Site 1 and Site 2 in both the 2020 and 2021 trials) at the research station. The two sites were adjacent to one another in both years. The canola hybrids '45H31' and 'CS2000' were used for the experiments and evaluated at four different inoculum levels (see below); control treatments did not receive any inoculum. The experiments were arranged in a split-plot design with four replicates per treatment for each canola hybrid. Individual plots were 9 m^2 (6 m × 1.5 m) in 2020 and 4.5 m^2 (3 m × 1.5 m) in 2021 and consisted of four rows spaced 0.3 m apart with individual plots spaced at 0.6 m. There was a 2-m tilled buffer between replicate treatments. The various inoculum levels were generated by manually applying grain inoculum at specific rates: 25 mL per 3-m row or 50 mL per 6-m row (low inoculum), 50 mL per 3-m row or 100 mL per 6-m row (medium-low), 75 mL per 3-m row or 150 mL per 6-m row (medium), and 100 mL per 3-m row or 200 mL per 6-m row (high inoculum). Plots were seeded at the time of inoculation using a push seeder at a rate of 0.35 g per 3-m row or 0.7 g per 6-m row. The trials were planted on 17 May 2020 and 26 May 2021. Rainfall in 2020 was higher than average from May to August (Fig. 3.1), resulting in flooding in one replicate at Site 2. Consequently, this replicate was excluded from the analysis. Precipitation in 2021 was below average, resulting in drought-like conditions (Fig. 3.1).

3.2.3 Assessments of disease severity and seed yield

Ten plants were selected randomly from each plot at maturity, uprooted, and placed in paper bags. They were then transported to the laboratory, where the plants were dried for 5 days at 30°C in a drying oven. Each plant was rated for Verticillium stripe severity on a 0 – 4 scale

according to (Cui et al. 2022), where: 0 = no symptoms or signs of disease; and 4 = stem entirely necrotic and covered with microsclerotia, shredding of the epidermis with most pods lost. Seed yield per plant was also recorded for each sampled plant. The remaining plants in each plot were harvested using a small plot combine on 13 October 2020 and 30 September 2021 to assess the total yield per plot.

3.2.4 Statistical analysis

To evaluate the effects of hybrid type and inoculum level on Verticillium stripe severity and single plant seed yield, an analysis of variance (ANOVA) was conducted. The data sets from the two years of the study were examined separately, without considering the site effect. An adjusted R^2 was utilized to estimate the fit of the regression model. Regression equations evaluated seed yield with increases in disease severity. Differences were considered significant at $p \leq 0.05$ unless otherwise noted. The percentage yield loss at each disease severity rating (0-4) was calculated based on yield loss, with no disease = 0% yield loss. The regression equations estimated the percentage of yield loss for each unit increase in disease severity. All analyses were conducted with R v. 4.3.3 (R core Team, R Foundation for Statistical Computing, Vienna, Austria, 2013).

3.3 Results

3.3.1 Field experiments in 2020

An early symptom of Verticillium stripe, half-sided yellowing of the leaves, was observed by early July in most of the plots treated with *V. longisporum* at both sites in 2020. Symptoms and signs of infection on the mature canola plants included discolouration and shredding of the stem, necrosis and the presence of microsclerotia. No symptoms were noted on plants in the non-inoculated plots at any time, resulting in disease severity ratings of zero (Table 3.1). In general, mean Verticillium stripe severity increased with higher inoculum level in 2020 (Table 1). On

‘45H31’, the most severe disease was observed in the medium (mean rating = 1.1) and high inoculum (1.4) treatments, which were significantly greater than observed in the non-inoculated control (Table 3.1). On ‘CS2000’, the severity of Verticillium stripe was significantly greater in the medium-low (mean rating = 1.3), medium (1.9) and high inoculum treatments (1.5) relative to the control (0.0) (Table 3.1).

A trend of decreasing single plant yield with increasing inoculum was observed in 2020. The mean single plant yield for ‘45H31’ in the non-inoculated control treatment was 4.2 g, significantly greater than observed in the medium (1.7 g) and high inoculum (1.8 g) treatments (Table 3.1). Values for the low (2.8 g) and medium-low (2.1 g) inoculum treatments were intermediate within this range. Single plant yield for ‘CS2000’ was 4.7 g in the non-inoculated control, significantly greater than in the medium-low (2.2 g), medium (1.6 g) and high (2.3 g) inoculum treatments (Table 3.1). No significant differences in total plot yields were detected for any of the treatments in either hybrid, with these values ranging from 576 g to 752 g and from 564 g to 797 g for ‘45H31’ and ‘CS2000’, respectively (Table 3.1).

3.3.2 Field experiments in 2021

No early symptoms of Verticillium stripe were observed in 2021, and disease severity at maturity on both hybrids remained mild. Additionally, weak symptoms of Verticillium stripe (mean rating = 0.1) were noted in the non-inoculated plots of ‘45H31’. No significant differences in disease severity were detected in either hybrid, although the numerically highest mean disease severities were found in the medium-low inoculum treatment for both '45H31' (0.4) and 'CS2000' (0.3) (Table 3.1).

In 2021, no significant differences in mean seed yield per plant were noted between inoculum treatments with the exception of the non-inoculated control (6.2 g) vs. the high inoculum treatment (13.2 g) for 'CS2000' (Table 3.1). The mean seed yield per plant was 6.0 g and 6.2 g for '45H31' and 'CS2000', respectively, in the non-inoculated controls. This was not significantly ($p \leq 0.05$) different from the seed yields per plant for these hybrids in the low ('45H31' = 7.5 g, 'CS2000' = 8.0 g), medium-low (8.9 g, 8.7 g), medium (9.1 g, 9.6 g), or high inoculum treatments (9.3 g, 13.2 g) (Table 3.1). No trends were observed for total plot yield for either of the hybrids, and any differences between treatments were not significant (Table 3.1).

3.3.3 Regression models

In 2020, regression analysis indicated that the relationships between disease severity and seed yield per plant at the two sites were best described by quadratic equations (Fig. 3.2A, B). In the case of '45H31', the regression model was $y = 3.5 - 0.33x - 0.12x^2$, with the expected average seed yield ranging from 0.26 g to 3.5 g per plant at Site 1 (Fig. 3.2A). At Site 2, the expected average seed yield ranged from 0.792 g to 1.8 g per plant with a regression model $y = 1.8 + 0.052x - 0.076x^2$ (Fig. 3.2B). For 'CS2000', the regression model at Site 1 was $y = 4.6 - 1.3x + 0.08x^2$ and the expected average seed yield ranged from 0.68 g to 4.6 g per plant. At Site 2, the regression model for this hybrid was $y = 2.7 - 0.023x - 0.14x^2$ with the expected average seed yield ranging from 0.368 g to 2.7 g per plant (Fig. 3.2A, B).

The regression models for percentage yield loss per plant vs. disease severity at Site 1 in 2020 were $y = 5.7 + 8.8x + 3.3x^2$ for '45H31' and $y = 6.1 + 27x - 1.6x^2$ for 'CS2000' (Fig. 3.2C). At Site 2, these models were $y = 9.2 - 2.7x + 3.8x^2$ for '45H31' and $y = 4.5 + 0.88x + 5.1x^2$ for 'CS2000' (Fig. 3.2D). Yield losses exceeding 60% were estimated for both hybrids at a Verticillium stripe severity ≥ 3 at Site 1. Furthermore, in the case of '45H31' at both sites, plants

with a disease severity rating of 1 showed a higher percentage yield loss compared with those with a severity rating of 2. However, as the severity increased further from 2 to 4, there was a subsequent decrease in yields.

As was found in 2020, the relationships between disease severity and seed yield per plant in 2021 were also best explained by quadratic equations. For hybrid '45H31' at Site 1, the regression model was $y = 10 - 7.1x + 1.3x^2$, with the expected average seed yield ranging from 0.4 g to 10 g per plant. At Site 2, the expected average seed yield ranged from 0.8 g to 7.4 g per plant with a regression model of $y = 7.4 - 6.7x + 1.5x^2$ (Fig. 3.3A, B). In the case of 'CS2000' at Site 1, the regression model was $y = 9.6 - 5.9x + 0.93x^2$ and the expected average seed yield ranged from 0.27 g to 9.6 g per plant. At Site 2 for this hybrid, the regression model was $y = 10 - 8.6x + 1.8x^2$ with the expected average seed yield ranging from 0.4 g to 10 g per plant (Fig. 3.3A, B).

In 2021, the regression models for percentage yield loss per plant vs. disease severity were $y = 1.2 + 68x - 13x^2$ and $y = 2.3 + 89x - 20x^2$ for '45H31' at Sites 1 and 2, respectively (Fig. 3.3C, D). For 'CS2000', the models were $y = -0.3 + 61x - 9.5x^2$ at Site 1 and $y = 1.5 + 85x - 18x^2$ at Site 2 (Fig. 3.3C, D). Both hybrids showed yield losses exceeding 50% at disease severities ≥ 1 at both sites in 2021. At Site 2, plants with a disease severity of 2 showed a greater percentage of seed yield loss than those with a severity rating of 3 for both hybrids (Fig. 3.3D).

3.4 Discussion

Verticillium stripe is an emerging disease of canola in Canada, leading to concern regarding its potential impact on the production of this crop (Dunker et al. 2008; Government of Canada 2015). A systemic understanding of the impact of Verticillium stripe on canola yields is critical for assessing the necessity and efficacy of various disease management approaches. In

order to investigate the relationship between disease severity, inoculum density, and seed yield, field trials were conducted with two canola hybrids, '45H31' and 'CS2000', over two years. To our knowledge, this is the first report on yield losses in canola caused by Verticillium stripe under Canadian conditions.

The results indicated varying effects of inoculum density on disease severity across the two years of the study. In 2020, significant differences were observed in Verticillium stripe severity between inoculum treatments. Plots treated with medium-low ('CS2000') or medium ('45H31') to high densities (both hybrids) of *V. longisporium* inoculum showed higher disease severity compared with non-inoculated controls. These findings align with an earlier greenhouse study, which indicated significant differences in Verticillium stripe severity between high inoculum and control treatments at the adult plant stage (Cui et al. 2022). In contrast, in 2021, no significant differences were found between any of the inoculum treatments or controls for either hybrid examined. An absence of a significant effect on disease severity from artificial inoculation of the soil, even at the highest inoculum density, was also reported in a European study with winter oilseed rape (Dunker et al. 2008). The authors suggested that, in years with unfavorable environmental conditions for disease development, delayed colonization of the host plant helps to mitigate the impact of infection on yield (Dunker et al. 2008).

Disease development caused by *V. longisporum* is reported to be favored by dry and increased temperature conditions (Soesanto and Termorshuizen 2001). In the current study, however, Verticillium stripe was milder under the warmer and drier conditions experienced in 2021 vs. 2020. This suggests possible variation between environmental conditions and pathogen development in the field and/or the influence of other factors. German studies have found that the

relationship between higher temperatures and increased levels of disease in oilseed rape is not consistently observed (Siebold and von Tiedemann, 2013).

In 2020, there was a clear trend of decreasing single plant seed yield with increasing Verticillium stripe severity. However, in 2021, there were generally no significant differences in single-plant seed yields, except for a significant increase detected only in the high inoculum treatment in 'CS2000'. This unexpected outcome could reflect a decrease in the number of plants surviving to maturity in 2021. This reduction likely led to less competition for resources among the remaining plants, potentially enabling them to compensate for losses associated with the disease. In canola, there is an established inverse relationship between plant density and yield (Shirtliffe and Hartman, 2009). While stand establishment appeared to be poorer in some of the high inoculum treatments, it was not quantified in this study. It is possible that inoculation with *V. longisporum* reduced seedling emergence, although there is limited research on the impact of this fungus on this parameter. Nevertheless, other research has demonstrated that soilborne diseases such as root rot and damping off, caused by *Fusarium* spp. and *Rhizoctonia solani* (Hwang et al. 2014; Yu et al. 2023), notably reduce seed emergence. Total plot yields were similar across treatments in both years of this study, further suggesting that losses experienced by individual plants were offset by reduced competition among the surviving plants.

In both years of the experiment, microsclerotia on infected tissues were detected in early September, near the end of the growing season in western Canada. This pattern mirrors findings from a German study, where late colonization of plant tissue by *V. longisporum* led to an exponential increase in disease at the pod filling stage in winter oilseed rape (Siebold and von Tiedemann 2013). The delayed onset of symptoms in the field has also been reported in cauliflower (*B. oleracea* var. *botrytis*) infected by Verticillium species (Koike et al. 1994). Infection by *V.*

longisporum involves the germination of the microsclerotia, penetration of lateral roots, and eventual spread into the vascular tissue (Eynck et al. 2007). The timing of these events can significantly affect yields, and environmental conditions may play an important role in determining this timing, as demonstrated for *V. dahliae* in cotton (Pullman and DeVay, 1982).

In both hybrids and across both years of the study, single plant seed yield showed a negative correlation with Verticillium stripe severity. Plants with severe infection (disease rating > 3) experienced reductions in single plant yields surpassing 60%. Similar observations were reported earlier in winter oilseed rape, where individual plant yields were negatively correlated with disease severity caused by *V. longsiporum* (Dunker et al. 2008). The current study suggests that the relationship between Verticillium stripe severity and yield loss is best described by quadratic equations. The expected average reductions in single plant yields ranged from 17.5% to 82.8% and from 61.8% to 92.3% across both hybrids in 2020 and 2021, respectively, surpassing previous European estimates of 10% to 50% in winter oilseed rape (Dunker et al., 2008). The differences in potential yield impact could arise from variations in the physiological and morphological traits between winter oilseed rape and spring canola, as well as the diverse environmental and soil conditions present in Europe and western Canada. Environmental factors such as soil temperature and humidity are indeed likely contributors to these yield effects (Siebold and von Tiedemann 2013).

This study provided an initial assessment of the relationship between Verticillium stripe severity and canola yields under conditions in western Canada. The results suggest the potential for significant yield reductions by *V. longisporum*, even in the absence of strong disease symptoms. Given the lack of registered fungicides for the control of this fungus, there is a need for enhanced

resistance in commercial canola hybrids. Improved resistance, combined with other strategies, may help to ensure the sustainable management of Verticillium stripe of canola.

Table 3. 1 Mean Verticillium stripe severity, mean single plant yield, and mean plot yield of the canola hybrids '45H31' and 'CS2000' in field experiments with different quantities of Verticillium longisporum inoculum in 2020 and 2021.

Hybrid	Treatment	Disease Severity		Mean single plant yield (g)		Mean plot yield (g)	
		2020	2021	2020	2021	2020	2021
'45H31'	Control	0.0 A	0.1 a	4.2 AB	6.0 a	752 A	387 a
	Low	0.3 AB	0.3 a	2.8 ABC	7.5 a	637 A	320 a
	Medium-Low	0.7 ABC	0.4 a	2.1 BC	8.9 a	576 A	339 a
	Medium	1.1 BCD	0.1 a	1.7 C	9.1 ab	596 A	408 a
	High	1.4 CD	0.1 a	1.8 C	9.3 ab	646 A	398 a
'CS2000'	Control	0.0 A	0.0 a	4.7 A	6.2 a	564 A	406 a
	Low	0.8 ABCD	0.0 a	2.4 ABC	8.0 a	797 A	360 a
	Medium-Low	1.3 BCD	0.3 a	2.2 BC	8.7 a	782 A	454 a
	Medium	1.9 D	0.1 a	1.6 C	9.6 ab	662 A	353 a
	High	1.5 CD	0.1 a	2.3 BC	13.2 b	690 A	485 a

Note: Field plots were located at two sites in the St. Albert Research Station, University of Alberta. Treatments refer to the relative amount of *V. longisporum* grain inoculum applied to the plots. Verticillium stripe severity was assessed on a 0–4 scale. Means in a column followed by the same letter are not significantly different at $p \leq 0.05$.

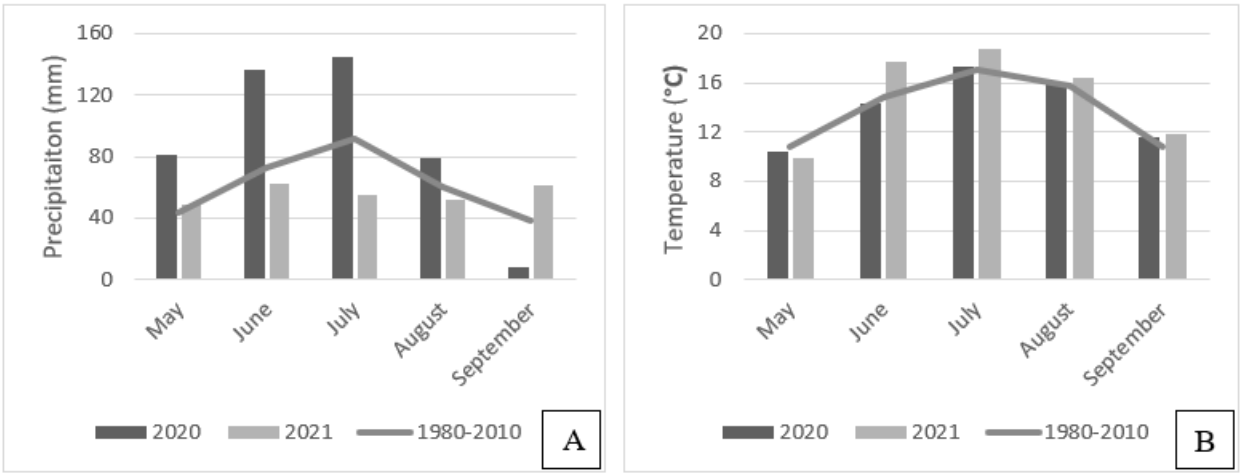

Fig 3. 1 Average monthly precipitation (A) and temperature (B) in 2020 and 2021 vs. the 30-year average (1980-2010) at the St. Albert Research Station, University of Alberta (A).

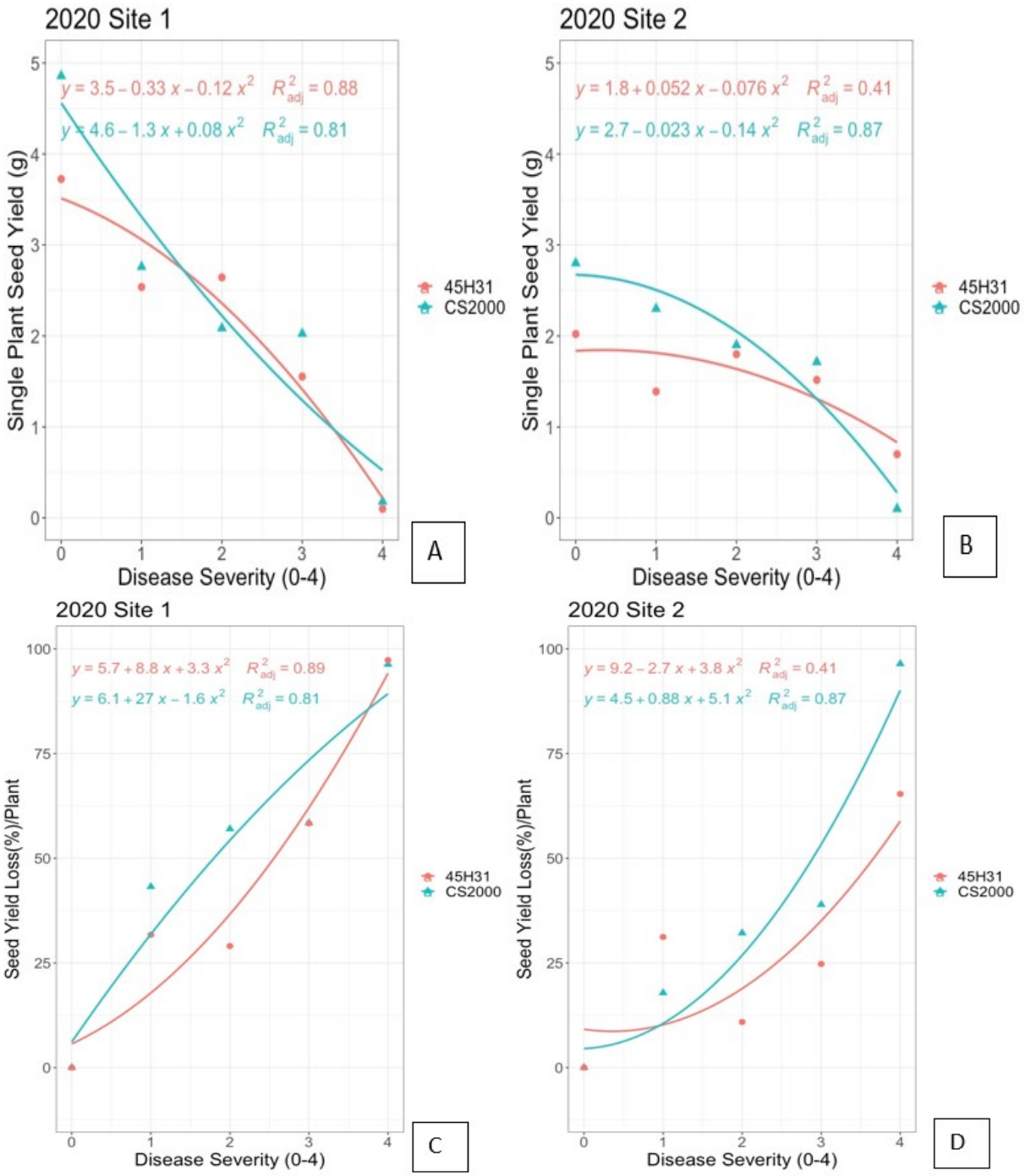

Fig 3. 2 Relationship between Verticillium stripe severity and single plant seed yield (A, B) and yield loss (C, D) in the canola hybrids '45H31' and 'CS2000' under field conditions at the St. Albert Research Station, University of Alberta, in 2020. Each point represents the mean of four (A, C) or three (B, D) replicates. Verticillium stripe severity was assessed on a 0–4 scale. The yield loss data were estimated using the y-intercept in the equation averaged over the replicates. The data points were transformed into a percentage of the maximum yield in (C, D).

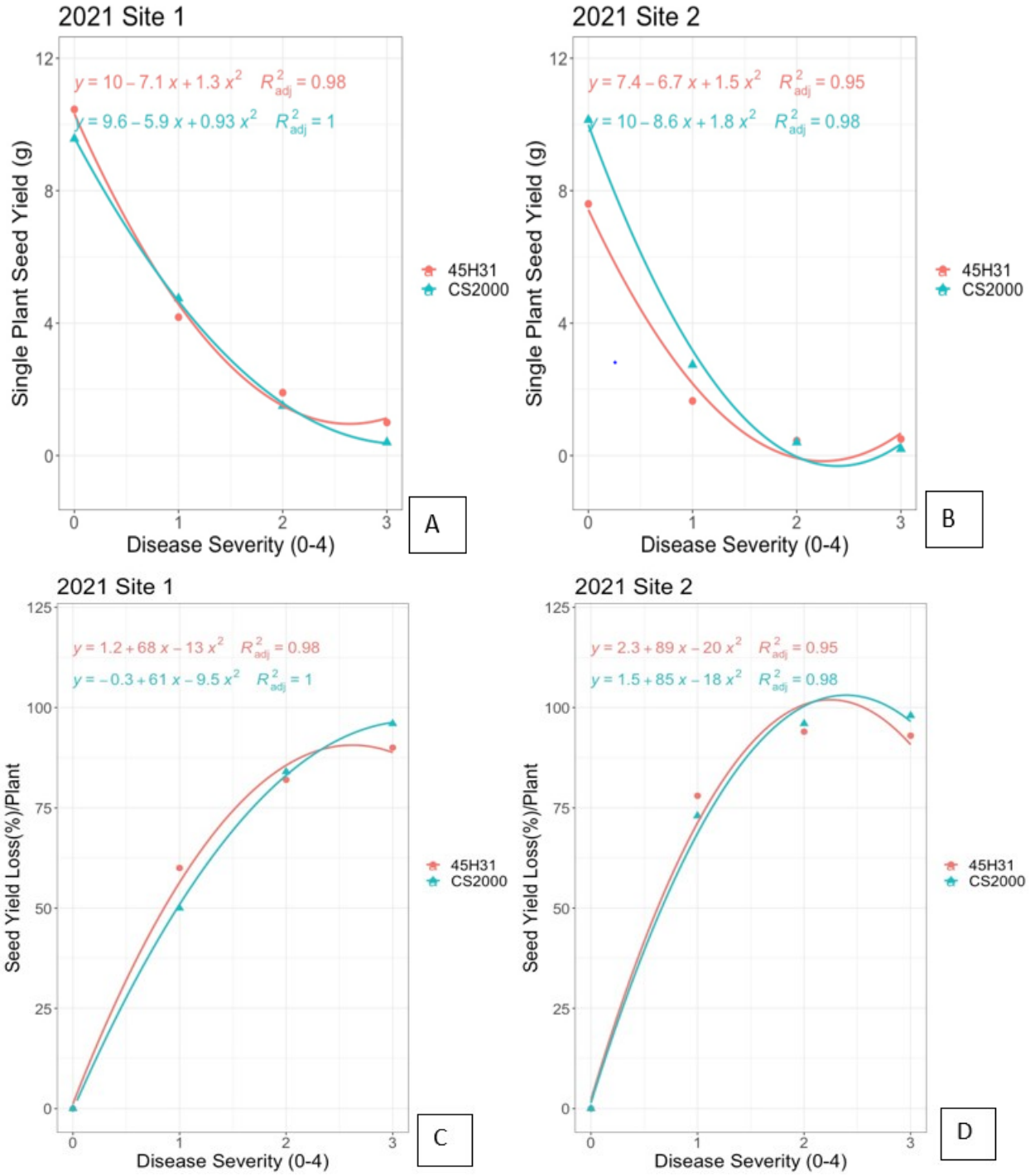

Fig. 3 Relationship between Verticillium stripe severity and single plant seed yield (A, B) and yield loss (C, D) in the canola hybrids '45H31' and 'CS2000' under field conditions at the St. Albert Research Station, University of Alberta, in 2021. Each point represents the mean of four (A, C) or three (B, D) replicates. Verticillium stripe severity was assessed on a 0–4 scale. The yield loss data were estimated using the y-intercept in the equation averaged over the replicates. The data points were transformed into a percentage of the maximum yield in (C, D).

Chapter 4: Conclusions

4.1 Key findings

The fungal pathogen *Verticillium longisporum* (C. Stark) Karapapa, Bainbr. & Heale is responsible for causing Verticillium stripe, a significant vascular disease affecting canola/oilseed rape (*Brassica napus* L.). Crops infected with *V. longisporum* have been reported to experience yield losses of up to 50% in Europe (Novakazi et al., 2015). Verticillium stripe was first identified in Canada in the province of Manitoba in 2014. Subsequent surveys conducted by the Canadian Food Inspection Agency (Government of Canada, 2015) confirmed the presence of *V. longisporum* not only in Manitoba, but also in other provinces, including Alberta, Saskatchewan, Ontario, and Quebec. In 2016, Hwang et al. (2017) also confirmed the first case of *Verticillium dahliae* (Kleb.) causing wilt symptoms in canola in Alberta. In this context, it appears that *Verticillium* spp. pose a serious threat to Canada's billion-dollar canola industry.

Chapter 1 of this thesis reviewed the biology of *V. longisporum*, disease management, and techniques for host inoculation and disease rating. Unfortunately, no fungicides are currently registered for the control of *V. longisporum* in Canada. Consequently, most research on Verticillium stripe at present focuses largely on assessing genetic resistance. The Brassica C-genome may serve as a significant reservoir of quantitative resistance to this disease (Obermeier et al., 2013). For example, Rygulla et al. (2007) demonstrated that interspecific hybridization of *B. rapa* (AA) and *B. oleracea* (CC) resulted in resynthesized lines of *B. napus* (AACC) with enhanced resistance to *V. longisporum*. This and similar studies suggest that developing improved canola cultivars resistant to Verticillium stripe holds promise for disease management. However, further research on the genetic mechanisms underlying resistance is necessary.

In Chapter 2 of this thesis, refined methods for the inoculation of *V. longisporum* on canola were developed. Collectively, these represent an important tool for screening germplasm and evaluating resistance phenotypes in this crop. Symptoms of Verticillium stripe were observed at the seedling stage as early as 2 weeks following root dip inoculation under greenhouse conditions. A 0-6 disease assessment scale was proposed to evaluate disease severity in seedlings, as most studies have focused on older plants. In addition, a 0-4 scale was developed for the evaluation of Verticillium stripe at the adult stage. The capacity to assess disease at different stages of plant growth is important for distinguishing between different types of resistance (such as quantitative vs. qualitative) that may be active at different times, and provides a quantitative measure of disease progression. Moreover, through the evaluation of different inoculum types—a conidial suspension applied by the root-dip method and grain inoculation—and comparisons of the timing of inoculation, this work established a foundation for more accurate evaluation of host resistance.

The primary objective of Chapter 3 was to establish the relationship between Verticillium stripe severity and canola yield under field conditions in western Canada. Prior to this research, there was a notable absence of information quantifying *V. longisporum*-induced yield losses in canola. Such data are critical for estimating the potential threat posed by this pathogen, particularly considering the significant role of canola in the Canadian economy (Canadian Canola Growers Association 2022). The results from these experiments, which were conducted over 2 years with two hybrids, indicated that the relationship between disease severity and yield was most accurately described by second-degree quadratic equations. In 2020, in the first year of the study, both cultivars experienced yield losses exceeding 60% at one of the sites when Verticillium stripe severity was > 3 on a 0-4 scale. In the second year of the study in 2021, single plant seed yield losses surpassed 50% for both hybrids at both sites when the disease severity was ≥ 1. These

results suggest that that *V. longisporum* has the potential to cause significant yield losses in canola, even when disease levels are relatively mild.

4.2 Future research

The root-dip and grain inoculation methods presented in this dissertation could serve as the basis for *V. longisporum* resistance screening in canola, while the seedling and adult plant rating scales introduced may enhance the assessment of resistance. Conducting such experiments is critical for the development of Verticillium stripe-resistant canola, which could represent the key to effective management of this emerging disease. However, root dip inoculation methods, which were used in this study for inoculating the seedlings, can be time-consuming and labor-intensive. As such, future studies could explore how to streamline seedling inoculation for large-scale screening of germplasm. The coordinated testing of much larger numbers of Brassica genotypes from seed banks and breeding programs could help to identify effective resistance for deployment in the Canadian Prairies and beyond.

The quantification of yield losses, as determined by the severity of Verticillium stripe, underscores the potential impact on productivity of *V. longisporum* infection in Canadian canola hybrids. Further exploration of the influence of this pathogen on canola yields, including field-level assessments and evaluation of a wider selection of hybrids, could lead to a more robust yield loss model to aid growers in establishing action thresholds for disease management. Similarly, understanding the effect of infection on canola seed quality and harvest timing, as well as its potential for seed transmission, would inform the development of knowledge-based strategies for the control of Verticillium stripe.

Bibliography

Bell AA, Wheeler MH. 1986. Biosynthesis and Functions of Fungal Melanins. Annual Review of Phytopathology. 24(1):411–451.https://doi.org/10.1146/annurev.py.24.090186.002211.

Bhat RG, Subbarao KV. 1999. Host Range Specificity in *Verticillium dahliae*. Phytopathology. 89(12):1218–1225. https://doi.org/10.1094/PHYTO.1999.89.12.1218.

Canadian Canola Growers Association. 2022. Canola: Growing Canadian Prosperity [Internet]. [accessed 2022 Apr 19]. https://www.ccga.ca/policy/Pages/AdvocacyPriorities.aspx.

Conn KL, Tenuta M, Lazarovits G. 2005. Liquid Swine Manure Can Kill *Verticillium dahliae* Microsclerotia in Soil by Volatile Fatty Acid, Nitrous Acid, and Ammonia Toxicity. Phytopathology. 95(1):28–35. https://doi.org/10.1094/PHYTO-95-0028.

Cooper RM, Williams JS. 2004. Elemental sulphur as an induced antifungal substance in plant defence. Journal of Experimental Botany. 55(404):1947–1953. https://doi.org/10.1093/jxb/erh179.

Cui J, Strelkov SE, Fredua-Agyeman R, Hwang SF. 2022. Development of optimized *Verticillium longisporum* inoculation techniques for canola (*Brassica napus*). Canadian Journal of Plant Pathology. 0(0):1–11. https://doi.org/10.1080/07060661.2022.2120913.

Daebeler F, Amelung D, Zeise K. 1988. Verticillium-Welke an Winterraps – Auftreten und Bedeutung. Nachrichtenblatt Pflanzenschutzdienst. D D R. 42:71–73.

Danielsson J, Reva O, Meijer J. 2007. Protection of Oilseed Rape (Brassica napus) Toward Fungal Pathogens by Strains of Plant-associated *Bacillus amyloliquefaciens*. Microb Ecol. 54(1):134–140. https://doi.org/10.1007/s00248-006-9181-2.

Debode J, Clewes E, De Backer G, Höfte M. 2005. Lignin is involved in the reduction of *Verticillium dahliae* var. *longisporum* inoculum in soil by crop residue incorporation. Soil Biology and Biochemistry. 37(2):301–309. https://doi.org/10.1016/j.soilbio.2004.08.001.

Debode J., Declercq B, Hofte M. 2005. Identification of Cauliflower Cultivars that Differ in Susceptibility to *Verticillium longisporum* using Different Inoculation Methods. J Phytopathol. 153(5):257–263. https://doi.org/10.1111/j.1439-0434.2005.00965.x.

Deketelaere S, Tyvaert L, França SC, Höfte M. 2017. Desirable Traits of a Good Biocontrol Agent against Verticillium Wilt. Front Microbiol. 8:1186. https://doi.org/10.3389/fmicb.2017.01186.

Depotter JRL, Deketelaere S, Inderbitzin P, Tiedemann AV, Höfte M, Subbarao KV, Wood TA, Thomma BPHJ. 2016. *Verticillium longisporum*, the invisible threat to oilseed rape and other brassicaceous plant hosts. Molecular Plant Pathology. 17(7):1004–1016. https://doi.org/10.1111/mpp.12350.

Depotter JRL, Rodriguez-Moreno L, Thomma BPHJ, Wood TA. 2017. The Emerging British *Verticillium longisporum* Population Consists of Aggressive *Brassica* Pathogens. Phytopathology®. 107(11):1399–1405. https://doi.org/10.1094/PHYTO-05-17-0184-R.

Depotter JRL, Thomma BPHJ, Wood TA. 2019. Measuring the impact of *Verticillium longisporum* on oilseed rape (*Brassica napus*) yield in field trials in the United Kingdom. Eur J Plant Pathol. 153(1):321–326. https://doi.org/10.1007/s10658-018-1537-1.

Dunker S, Keunecke H, Steinbach P, von Tiedemann A. 2008. Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. Journal of Phytopathology. 156(11–12):698–707. https://doi.org/10.1111/j.1439-0434.2008.01429.x.

Eynck C, Koopmann B, Grunewaldt-Stoecker G, Karlovsky P, von Tiedemann A. 2007. Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. Eur J Plant Pathol. 118(3):259–274. https://doi.org/10.1007/s10658-007-9144-6.

Eynck C, Koopmann B, Karlovsky P, von Tiedemann A. 2009. Internal Resistance in Winter Oilseed Rape Inhibits Systemic Spread of the Vascular Pathogen *Verticillium longisporum*. Phytopathology. 99(7):802–811. https://doi.org/10.1094/PHYTO-99-7-0802.

Eynck C, Koopmann B, von Tiedemann A. 2009. Identification of *Brassica* accessions with enhanced resistance to *Verticillium longisporum* under controlled and field conditions. J Plant Dis Prot. 116(2):63–72. https://doi.org/10.1007/BF03356288.

França SC, Spiessens K, Pollet S, Debode J, De Rooster L, Callens D, Höfte M. 2013. Population dynamics of *Verticillium* species in cauliflower fields: Influence of crop rotation, debris removal and ryegrass incorporation. Crop Protection. 54:134–141. https://doi.org/10.1016/j.cropro.2013.08.008.

Fröschel C. 2021. In-depth evaluation of root infection systems using the vascular fungus *Verticillium longisporum* as soil-borne model pathogen. Plant Methods [Internet]. [accessed 2022 Apr 27] 17(1). https://doi.org/10.1186/s13007-021-00758-x.

Government of Canada CFIA. 2015. Verticillium stripe – *Verticillium longisporum* [Internet]. [accessed 2022 May 12]. https://inspection.canada.ca/plant-health/invasive-species/plant-diseases/verticillium-stripe/eng/1420746212959/1420746213803.

Happstadius I, Ljungberg A, Kristiansson B, Dixelius C. 2003. Identification of *Brassica oleracea* germplasm with improved resistance to Verticillium wilt. Plant Breeding. 122(1):30–34. https://doi.org/10.1046/j.1439-0523.2003.00774.x.

Heale JB, Karapapa VK. 1999. The verticillium threat to canada's major oilseed crop: canola. Canadian Journal of Plant Pathology. 21(1):1–7. https://doi.org/10.1080/07060661.1999.10600114.

Hwang SF, Ahmed HU, Turnbull GD, Gossen BD, Strelkov SE. 2014. The effect of seed size, seed treatment, seeding date and depth on Rhizoctonia seedling blight of canola. Can J Plant Sci. 94(2):311–321. https://doi.org/10.4141/cjps2013-294.

Hwang S-F, Strelkov SE, Ahmed HU, Zhou Q, Fu H, Fredua-Agyeman R, Turnbull GD. 2017. First report of *Verticillium dahliae* Kleb. causing wilt symptoms in canola (*Brassica napus* L.) in North America. Canadian Journal of Plant Pathology. 39(4):514–526. https://doi.org/10.1080/07060661.2017.1375996.

Ikeda K, Banno S, Furusawa A, Shibata S, Nakaho K, Fujimura M. 2015. Crop rotation with broccoli suppresses Verticillium wilt of eggplant. J Gen Plant Pathol. 81(1):77–82. https://doi.org/10.1007/s10327-014-0559-6.

Inderbitzin P, Bostock RM, Davis RM, Usami T, Platt HW, Subbarao KV. 2011. Phylogenetics and Taxonomy of the Fungal Vascular Wilt Pathogen Verticillium, with the Descriptions of Five New Species. Idnurm A, editor. PLoS ONE. 6(12):e28341. https://doi.org/10.1371/journal.pone.0028341.

Inderbitzin P, Davis RM, Bostock RM, Subbarao KV. 2011. The Ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. Litvintseva A, editor. PLoS ONE. 6(3):e18260. https://doi.org/10.1371/journal.pone.0018260.

Inderbitzin P, Subbarao KV. 2014. *Verticillium* Systematics and Evolution: How Confusion Impedes Verticillium Wilt Management and How to Resolve It. Phytopathology. 104(6):564–574. https://doi.org/10.1094/PHYTO-11-13-0315-IA.

Isaac I. 1957. Verticillium Wilt of Brussels Sprout. Annals of Applied Biology. 45(2):276–283. https://doi.org/10.1111/j.1744-7348.1957.tb00467.x.

Issac I. 1953. A further comparative study of pathogenic isolates of Verticillium: *V. nubilum Pethybr.* and *V. trichorpus* sp. nov. [Internet]. [accessed 2022 Jun 26]. http://www.cabi.org/ISC/abstract/20057007963.

Iven T, König S, Singh S, Braus-Stromeyer SA, Bischoff M, Tietze LF, Braus GH, Lipka V, Feussner I, Dröge-Laser W. 2012. Transcriptional Activation and Production of Tryptophan-Derived Secondary Metabolites in Arabidopsis Roots Contributes to the Defense against the Fungal Vascular Pathogen *Verticillium longisporum*. Molecular Plant. 5(6):1389–1402. https://doi.org/10.1093/mp/sss044.

Johansson A. 2006. *Verticillium longisporum*, infection, host range, prevalence and plant defence responses [Internet]. [accessed 2022 Jun 26]. https://pub.epsilon.slu.se/1119/.

Johansson A, Goud J-KC, Dixelius C. 2006. Plant Host Range of *Verticillium longisporum* and Microsclerotia Density in Swedish Soils. Eur J Plant Pathol. 114(2):139–149. https://doi.org/10.1007/s10658-005-2333-2.

Karapapa VK, Bainbridge BW, Heale JB. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb, nov., pathogenic to oilseed rape. Mycological Research. 101(11):1281–1294. https://doi.org/10.1017/S0953756297003985.

Keunecke H. 2009. Einfluss von Kohlfliegenbefall auf die Infektion und Schadwirkung von *Verticillium longisporum* und Phoma lingam an Raps [Internet]. [accessed 2022 Jun 26]. https://doi.org/10.53846/goediss-1914.

Klosterman SJ, Atallah ZK, Vallad GE, Subbarao KV. 2009. Diversity, Pathogenicity, and Management of *Verticillium* Species. Annual Review of Phytopathology. 47(1):39–62. https://doi.org/10.1146/annurev-phyto-080508-081748.

Knecht K, Seyffarth M, Desel C, Thurau T, Sherameti I, Lou B, Oelmüller R, Cai D. 2010. Expression of *BvGLP-1* Encoding a Germin-Like Protein from Sugar Beet in *Arabidopsis thaliana* Leads to Resistance Against Phytopathogenic Fungi. MPMI. 23(4):446–457. https://doi.org/10.1094/MPMI-23-4-0446.

Knüfer J, Lopisso DT, Koopmann B, Karlovsky P, von Tiedemann A. 2017. Assessment of latent infection with *Verticillium longisporum* in field-grown oilseed rape by qPCR. Eur J Plant Pathol. 147(4):819–831. https://doi.org/10.1007/s10658-016-1045-0.

Koike ST, Subbarao KV, Davis RM, Gordon TR, Hubbard JC. 1994. Verticillum wilt of cauliflower in California.

Müller H, Berg G. 2008. Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on Verticillium wilt in oilseed rape. BioControl. 53(6):905–916. https://doi.org/10.1007/s10526-007-9111-3.

Narisawa K, Usuki F, Hashiba T. 2004. Control of Verticillium Yellows in Chinese Cabbage by the Dark Septate Endophytic Fungus LtVB3. Phytopathology. 94(5):412–418. https://doi.org/10.1094/PHYTO.2004.94.5.412.

Njoroge SMC, Vallad GE, Park S-Y, Kang S, Koike ST, Bolda M, Burman P, Polonik W, Subbarao KV. 2011. Phenological and Phytochemical Changes Correlate with Differential Interactions of *Verticillium dahliae* with Broccoli and Cauliflower. Phytopathology®. 101(5):523–534. https://doi.org/10.1094/PHYTO-08-10-0219.

Novakazi F, Inderbitzin P, Sandoya G, Hayes RJ, von Tiedemann A, Subbarao KV. 2015. The Three Lineages of the Diploid Hybrid *Verticillium longisporum* Differ in Virulence and Pathogenicity. Phytopathology. 105(5):662–673. https://doi.org/10.1094/PHYTO-10-14-0265-R.

Panth M, Hassler SC, Baysal-Gurel F. 2020. Methods for Management of Soilborne Diseases in Crop Production. Agriculture. 10(1):16. https://doi.org/10.3390/agriculture10010016.

Paplomatas E. 1991. Biocontrol of damping-off of cotton seedlings by *Verticillium tricorpus*.pdf.

Paul V. 2003. Raps. In: [place unknown]: Krankheiten, Scha¨dlinge, Schadpflanzen, 3rd edn. Gelsenkirchen-Buer, Germany, Verlag Th. Mann,.

Posada-Vergara C, Vidal S, Rostás M. 2023. Local Competition and Enhanced Defense: How *Metarhizium brunneum* Inhibits *Verticillium longisporum* in Oilseed Rape Plants. Journal of Fungi. 9(8):796. https://doi.org/10.3390/jof9080796.

Powelson RL, Carter GE. 1973. Efficacy of soil fumigants for control of Verticillium wilt of potatoes. American Potato Journal. 50(5):162–167. https://doi.org/10.1007/BF02853206.

Province of Manitoba - Agriculture. n.d. Verticillium stripe of canola detected in Manitoba [Internet]. [accessed 2022 Jan 23]. https://www.gov.mb.ca/agriculture/.

Shetty KG, Subbarao KV, Huisman OC, Hubbard JC. 2000. Mechanism of broccoli-mediated verticillium wilt reduction in cauliflower. Phytopathology. 90(3):305–310. https://doi.org/10.1094/PHYTO.2000.90.3.305.

Soesanto L, Termorshuizen AJ. 2001. Effect of Temperature on the Formation of Microsclerotia of *Verticillium dahliae*. Journal of Phytopathology. 149(11–12):685–691. https://doi.org/10.1046/j.1439-0434.2001.00697.x.

Stadler M, von Tiedemann A. 2014. Biocontrol potential of *Microsphaeropsis ochracea* on microsclerotia of *Verticillium longisporum* in environments differing in microbial complexity. BioControl. 59(4):449–460. https://doi.org/10.1007/s10526-014-9586-7

Steventon LA, Happstadius I, Okori P, Dixelius C. 2002. Development of a Rapid Technique for the Evaluation of the Response of *Brassica napus* to Verticillium wilt. Plant Disease. 86(8):854–858. https://doi.org/10.1094/PDIS.2002.86.8.854.

Subbarao KV. 2002. Methyl bromide alternatives: meeting the deadlines – Introduction. Phytopathology®. 92(12):1334–1336. https://doi.org/10.1094/PHYTO.2002.92.12.1334.

Subbarao KV, Chassot A, Goron TR, Hubbard JC, Bonello P, Mullin R, Okamoto D, Davis RM, Koike ST. 1995. Genetic relationships and cross pathogenicities of *Verticillium dahliae* isolates from cauliflower and other crops. Phytopathology. 85:1105–1112.

Tahmatsidou V, O'Sullivan J, Cassells AC, Voyiatzis D, Paroussi G. 2006. Comparison of AMF and PGPR inoculants for the suppression of Verticillium wilt of strawberry (*Fragaria×ananassa* cv. Selva). Applied Soil Ecology. 32(3):316–324. https://doi.org/10.1016/j.apsoil.2005.07.008.

Tenuta M, Lazarovits G. 2002. Ammonia and Nitrous Acid from Nitrogenous Amendments Kill the Microsclerotia of *Verticillium dahliae*. Phytopathology®. 92(3):255–264. https://doi.org/10.1094/PHYTO.2002.92.3.255.

Tyvaert L, França S c., Debode J, Höfte M. 2014. The endophyte *Verticillium* Vt305 protects cauliflower against Verticillium wilt. Journal of Applied Microbiology. 116(6):1563–1571. https://doi.org/10.1111/jam.12481.

Verticillium - Canola Council of Canada. n.d. [accessed 2019 Dec 1]. https://www.canolacouncil.org/canola-encyclopedia/diseases/verticillium/#fnote1.

Verticillium Stripe - Canola Encyclopedia. n.d. Canola Council of Canada [Internet]. [accessed 2024 Feb 28]. https://www.canolacouncil.org/canola-encyclopedia/diseases/verticillium-stripe/.

Wilhelm S. 1955. Longevity of the Verticillium wilt fungus in the laboratory and field. [Internet]. [accessed 2022 Jun 26]. http://www.cabi.org/isc/abstract/19551102133.

Xiao CL, Subbarao KV, Schulbach KF, Koike ST. 1998. Effects of Crop Rotation and Irrigation on *Verticillium dahliae* Microsclerotia in Soil and Wilt in Cauliflower. Phytopathology. 88(10):1046–1055. https://doi.org/10.1094/PHYTO.1998.88.10.1046.

Yu H, Chang K-F, Hwang S-F, Strelkov SE. 2023. Characterization of the Virulence and Yield Impact of *Fusarium* Species on Canola (*Brassica napus*). Plants (Basel). 12(17):3020. https://doi.org/10.3390/plants12173020.

Zeise K, Tiedemann A von. 2001. Morphological and Physiological Differentiation among Vegetative Compatibility Groups of *Verticillium dahliae* in Relation to *V. longisporum*. Journal of Phytopathology. 149(7–8):469–475. https://doi.org/10.1111/j.1439-0434.2001.tb03879.x.

Zeise K, Tiedemann AV. 2002. Host Specialization among Vegetative Compatibility Groups of *Verticillium dahliae* in Relation to *Verticillium longisporum*. Journal of Phytopathology. 150(3):112–119. https://doi.org/10.1046/j.1439-0434.2002.00730.x.

Zhang N, Castlebury LA, Miller AN, Huhndorf SM, Schoch CL, Seifert KA, Rossman AY, Rogers JD, Kohlmeyer J, Volkmann-Kohlmeyer B, Sung G-H. 2006. An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. Mycologia. 98(6):1076–1087. https://doi.org/10.1080/15572536.2006.11832635.

Zheng X, Pfordt A, Khatri L, Eseola AB, Wilch A, Koopmann B, von Tiedemann A. 2019. Contrasting Patterns of Colonization with *Verticillium longisporum* in Winter- and Spring-Type Oilseed Rape (*Brassica napus*) in the Field and Greenhouse and the Role of Soil Temperature. Plant Disease. 103(8):2090–2099. https://doi.org/10.1094/PDIS-01-19-0236-RE.

Zhou L, Hu Q, Johansson A, Dixelius C. 2006. *Verticillium longisporum* and *V. dahliae*: infection and disease in Brassica napus. Plant Pathology. 55(1):137–144. https://doi.org/10.1111/j.1365-3059.2005.01311.x.

Zou Z, Bisht V, Fernando WGD. 2020. Identification and Characterization of *Verticillium longisporum* Lineage A1/D1 from Brassica Crops in Manitoba, Canada. International Journal of Molecular Sciences. 21(10):3499. https://doi.org/10.3390/ijms21103499.