Study on *Verticillium longisporum* of Canola from the First Reported Farm in North America

By

Abhishek Agarwal

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

Master of Science

Department of Soil Science

University of Manitoba

Winnipeg, Manitoba, Canada

ACKNOWLEDGMENTS

This research study would not have been possible without the constant support of several people involved in this project. Firstly, I am extremely thankful to my supervisor Dr. Mario Tenuta for his advice, guidance and enthusiasm all throughout my project. I admire his words that are a vast pool of knowledge and have always made me challenge myself and achieve the desired outcome. I would also like to thank my committee members Dr. Teresea de Kievit and Dr. Paul Bullock who guided me with valuable inputs and feedback and helped in improvement of the project analyses. Several results obtained in the project would not have been possible without the help from three excellent technicians Teri Fairman, Brad Sparling and Marlese Pedersen. Teri Fairman and Fernanda Gouvea have been a constant support during this journey and have always been available to help even on short notice. A special vote of thanks also goes to M.Sc. Soil Science, Megan Westphal for sharing her vast experience in sampling and writing. On the other side of the research team making all the paper work extremely easy, the office team Lynda Closson, Jennifer Henderson and Martha Blouw deserve special thanks as well. Pillars of my personal life all my friends, my parents Neena and Rajeev and my sister Shivangi, I would not be where I am today without their unconditioned love and sacrifices. Finally, my partner Maria Rodrigo, I would have never been able to complete my thesis without your constant support during difficult times.

ABSTRACT

Agarwal, Abhishek. M.Sc., The University of Manitoba, July, 2018. Study on *Verticillium longisporum* of Canola from the First Reported Farm in North America. Professor; Dr. Mario Tenuta.

Verticillium longisporum (Stark) Karapapa et al. 1997, is a soil-borne pathogen of several agricultural crops but most virulent on crops of the Brassicaceae family. It is responsible for the economically important disease, Verticillium stripe of canola. As the only known diploid hybrid of the Ascomycota phylum, *V. longisporum* occurs in three distinct hybrid types that differ in pathogenicity and virulence. Hybridization enabled the pathogen to acquire a broad host range and enhanced pathogenicity when compared to its parent *V. dahliae* Kleb. *Verticillium longisporum* of canola is distributed all across Europe but in North America, *V. longisporum* is only observed on cabbage, cauliflower and horseradish. This changed dramatically, when in 2014 for the first time, canola infected with *V. longisporum* was detected during combining at a research farm in Canada, The pathogen concentration, its distribution and the hybrid type at the farm were unknown. Moreover, the crop (canola) found infected by the pathogen is of extreme economic importance to Canada. Therefore, in 2016, 194 soil samples from the farm were analyzed using real-time PCR analysis to determine pathogen concentration per gram of dry soil. Of the 194 soil samples, the real-time assay reported that 39% of the samples tested positive for the pathogen, *V. longisporum*. Using a multiplex PCR, this study also added information on the pathogen's hybrid lineage at the farm helping to understand pathogen virulence and susceptible hosts. This study therefore, confirms the presence of *V. longisporum* of canola in the first reported farm in North America and its hybrid lineage to be the most aggressive type on canola.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

1. Introduction

1.1 History and Classification

The genus *Verticillium* is comprised of a group of fungal phytopathogenic species that are soil-borne and can cause vascular wilts in over 200 hosts (Klosterman et al. 2009; Fradin and Thomma 2006). With the first ever species being discovered in 1816 by Nees von Esenbeck (*V. tenerum* isolated from a stem of hollyhock), the genus, *Verticillium* has been recognized for over two centuries with approximately 190 species described (Inderbitzin et al. 2011a; Inderbitzin and Subbarao 2014).

The genus has a controversial history when it comes to classification, with many species being confused with one another (Inderbitzin and Subbarao 2014). The *Verticillium* genus has been classified into two categories based on phylo-genetics and economic importance. Phylo-genetic categorization is based on presence and absence of yellow coloured hyphal pigments. *Verticillium* species that produce yellow pigmented hyphae were classified as one group named as Clade Flavexudans; examples include *V. albo-atrum, V. tricorpus, V. zaregamsianum, V. isaacii* and *V. klebahnii.* Species classified as Clade Flavnonexudans, in contrast, do not produce yellow-pigmented hyphae; examples include *V. nubilum, V. dahliae, V. longisporum, V. alfalfae* and *V. nonalfalfae* (Inderbitzin et al. 2011a; Inderbitzin and Subbarao 2014).

The second categorization of Verticillium is based on economic importance. Economically important species that cause vascular wilt in several crops and ornamentals leading to high yield losses have been grouped as *Verticillium* sensu stricto. This group includes; *V. longisproum, V. dahliae, V. tricorpus, V. albo-atrum, V. alfalfa*, *V. nonalfalfae*, *V. zaregamsianum*, *V. nubilum*, *V. isaacii* and *V. klebahnii*. Economically unimportant and residual

species of the genus like *V. theobromae* that need to be reclassified under a different genus have been excluded from the *Verticillium* sensu stricto group (Inderbitzin et al. 2013; Inderbitzin and Subbarao 2014).

As members of the Ascomycota phylum, most *Verticillium* species are haploid with vertically branched conidiophores. (Inderbitzin et al. 2011a). *Verticillium longisporum* is a diploid hybrid of the genus and was first reported as a variant of *V. dahliae* (*V. dahliae* var. *longisporum*) by Stark, cited by Inderbitzin et al. (2011a) and then as a variant of *V. dahliae* with uncommonly long conidia by Ingram (1968). Three decades later, for the first time, *V. longisporum* was proposed as an individual species based on morphological differences from *V. dahliae* by Karapapa et al. (1997). The differences were based on three morphological characters: *V. longisporum* was found to have elongated resting structures (microsclerotia), long asexual spores (conidia) (7.1-8.8 μm) and three phialides per node on conidiophores, whereas *V. dahliae* had spherical microsclerotia, short conidia (3.5-5.5 μm), and 4-5 phialides per node on conidiophores (Karapapa et al. 1997).

Verticillium longisporum also differ genetically from other *Verticillium* species. *Verticillium longisporum* species have a fairly large group-1 intron (839bp) inserted in the SSU-rRNA gene, not observed in other species. As a result of morphological and genetic differences, *V. longisporum* is considered a species of *Verticillium* (Karapapa and Typas 2001). The taxonomic classification of *V. longisporum* is as follows: Super Kingdom- Eukaryota, Kingdom- Fungi, Subkingdom- Dikarya, Phylum- Ascomycota, Subphylum- Pezizomycotina, Class- Sordariomycetes, Subclass- Hypocreomycetidae, Order- Glomerellales, Family- Plectosphaerellaceae, Genus- Verticillium, and Species- longisporum (Depotter et al. 2016; Dessimoz et al. 2012).

1.2 Hybridization in *V. longisporum*

The process of hybridization in fungi has been imperative in species variation development, transfer of important genes, and evolution (Morales and Dujon 2012; Nelson 1963). Hybridization is a source for development of new fungal species with enhanced pathogenicity and increased host range (Newcombe et al. 2000). Hybridization is less restricted in allotropic species (species that have not co-existed in the past), which is encouraged by agricultural trade that allows for interspecific hybridization between various plant pathogens.

Existence of stable hybrids in nature can be a rarity and this is mainly due to a phenomenon called Dobzhansky-Muller interaction (negative epistatic interaction) (Kondrashov et al. 2002). This interaction describes the genetic incompatibility of the hybrid so formed to sustain in the environment, especially the one with its parents (Barton 2001). Presence of the stable hybrid *V. longisporum* therefore means that the Dobzhansky-Muller fitness and genetic incompatibilities were overcome by this pathogen (Greig et al., 2002).

Contrary to other members of Ascomycota, *V. longisporum* is a diploid that has a genome roughly twice the size of its close relative *V. dahliae* (Ingram 1968; Typas and Heale 1980). Initially it was believed that *V. longisporum* hybridized via parasexual recombination, which results in restoration of the haploid state in hybrids following the fusion of parental hyphae and nuclei (Karapapa et al. cited by Inderbitzin et al. 2011b). However, now it is known that *V. longisporum* did not follow parasexual recombination after hybridization as the pathogen has two alleles at all six non-ribosomal loci and thus maintains a stable allodiploid state (Inderbitzin et al. 2011b).

Parents of *V. longisporum* are reported to be *V. dahliae* and *V. albo-atrum* in several studies (Clewes et al. 2008; Collado-Romero et al. 2010) and as diploid species of *V. dahliae* in another study (Collins et al. 2003). On the contrary, the latest findings indicate that *V. longisporum* hybridized from four parent lineages across three parent species, including *V. dahliae* lineage D2, *V. dahliae* lineage D3, species A1 and species D1 (Heale 2000). Species A1 and D1, yet to be isolated, are non-*Verticillium* species and have been arbitrarily named so (Heale 2000). Hybridization events in the polyphyletic pathogen (Heale 2000) occurred at least three different times (Collins et al. 2003). Species A1 was involved in all of the three hybridization events. Species A1 hybridization with Species D1 resulted in *V. longisporum* hybrid A1/D1. Species A1 hybridization with *V. dahliae* lineage D2 resulted in *V. longisporum* hybrid A1/D2 and species A1 with *V. dahliae* lineage D3 resulted in *V. longisporum* hybrid A1/D3. The above notion was confirmed by Inderbitzin et al. (2011b), based on non-ribosomal locus examination of *V. longisporum* that revealed the presence of species A1 allele across all strains along with the presence of D1, *V. dahliae* D2 and D3 alleles in combination with A1 allele. Geographical distribution of the three *V. longisporum* hybrid types varies from hybrid to hybrid. Hybrid A1/D1 is most prevalent in Japan, Europe and North America. Hybrid A1/D2 is mostly restricted to Illinois, USA whereas hybrid A1/D3 is has been reported in Japan and Europe (Inderbitzin et al. 2011b).

In terms of evolution and adaptation, hybridization events leading to the development of *V. longisporum* provided the pathogen with expanded host range (crucifers) and larger conidia, as compared to its parents (Heale and Karapapa 1999; Inderbitzin et al. 2011b). Changes in gene expression, phenotype, morphology, and regulatory mechanisms caused by hybridization are documented in depth in plants. Hybridization in fungi is yet to be studied in detail but it has

surely played an important role in the evolution of plant pathogen *V. longisporum* (Osborn et al. 2003; Soltis and Soltis 1999).

1.3 Morphology of *V. longisporum*

Verticillium longisporum colonies grown in the dark for 10 days can range from 2-3 cm in diameter and can be light grey in colour. On Potato Dextrose Agar (PDA) plates, hyphae can be fairly dispersed and usually short, brown, and septate. Colonies are dominated by elongated microsclerotia (30-250 µm in diameter) but rounded microsclerotia can also be observed and in some cases can even equal the number of elongated microsclerotia. *Verticillium longisporum* strain PD348 has been observed with approximately the same number of rounded and elongated microsclerotia. Microsclerotia formation also varies from one isolate to other. *Verticillium longisporum* isolate PD687 has been observed not to form microsclerotia (Inderbtizin et al. 2011a).

Conidia of *V. longisporum* are hyaline and elongated and on average are 8.5×3.5 µm in size. In recent studies it has been observed that *V. longisporum* and *V. dahliae* isolates can have 2-5 and 2-4 phialides per node on conidiophore, respectively (Inderbtizin et al. 2011a). These numbers do not support the studies conducted by Karapapa et al. (1997) that reported 4-5 phialides per node on conidiophores for *V. dahliae* and three phialides per node on conidiophores for *V. longisporum*. Based on varying microsclerotial and conidial structures, *V. longisporum* identification solely based on morphological structures can be challenging (Inderbtizin et al. 2011a; Yu et al. 2016).

1.4 Resting Structure and Disease Cycle

Resting structures of *Verticillium* species were first observed in *V. albo-atrum* and named as Dauermycelien, literally translated which means resting mycelium (Inderbitzin and Subbarao 2014; Isaac 1949). Another type of resting structure was observed in *V. nubilum*, known as chlamydospores, which are brown, rounded cells that occur in short chains (Inderbitzin et al. 2011a).

Microsclerotia in contrast are rounded, brown to black cells that occur in clusters and are observed in *V. dahliae* and *V. longisporum* (Isaac 1953; Inderbitzin and Subbarao 2014). Microsclerotia are black coloured, melanized, masses of hyphae that are highly compacted which allows the pathogen to withstand extreme environmental stress such as UV exposure, enzymatic degradation, desiccation and high temperature but can be susceptible in wet, warm soil (Bell et al. 1976; Butler and Day 1998; Heale and Karapapa 1999). In the absence of a host, produced as part of a survival strategy, microsclerotia of *V. dahliae* (parent of *V. longisporum*) can survive to up to 14 years being dormant in soil (Short et al. 2015). On return of favourable conditions, a density as low as 1 cfu/g soil of *V. longisporum* microsclerotia is known to be enough to initiate infection (Johansson 2006).

The melanin in *Verticillium* has been identified as dihydroxynaphthalene (DHN)-melanin. Essential enzymes and by-products in the synthesis of DHN melanin include polyketide synthase (PKS) that produces 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). 1,3,6,8-THN is reduced to scytalone by reductase T4HR, which followed by a series of downstream reactions results in the production of DHN melanin (Fan et al. 2017).

The *V. longisporum* disease cycle is asexual and monocyclic in nature which means the fungus produces only one inoculum during a growing season. The disease cycle can be split into

three phases: the dormant phase, parasitic phase and saprophytic phase. During the dormant phase, the resting structures, microsclerotia, are dormant and inactive in soil. In the absence of a host, microsclerotia do not germinate because of the process of microbiostasis. Rhizosphere rich with root exudates of a host stimulate microsclerotia to initiate germination, leading to the parasitic phase of the disease cycle (Mol and van Riessen, 1995; Huisman 1982; Inderbitzin et al. 2013).

The germinating hyphae initiate infection (penetration) at the epidermal cells of lateral roots and root hairs of the host, which is encouraged by the host entering a susceptible flowering stage (Eynck et al. 2007). The first site of infection for *V. longisporum* is different from *V. dahliae* for it infects host roots instead of lateral roots or root hairs (Zhou et al. 2006; Fradin and Thomma 2006).

Hyphae begin to multiply in the roots and infection spreads through the root cortex to the xylem where conidia (asexual spores) are produced and multiply. As a defence strategy, the host blocks its xylem tissues but conidia are capable of breaking through these blockages and continue the colonization process (Johansson, 2006; Eynck et al. 2007; Eastburn and Paul 2007).

Verticillium longisporum colonizes the host xylem vessels and derives essential nutrients like water, amino acids, sugars and organic acids (Lopez-Millan et al. 2000). The pathogen blocks the host xylem vascular system by preventing vascular flow, altering the host membrane permeability and increasing its hyphal growth. The flow of nutrients is thus disrupted which causes the xylem to turn black and lose its functional capability (Eastburn and Paul 2007).

During the parasitic phase *V. longisporum* secretes a wide range of enzymes such as: peroxidases, proteases, defense-related proteins, lectins, and remodeling proteins (Kehr et al.

2005). The enzyme catalase peroxidase, transcribed from the genes *vlcpeA-1* and *vlcpeA-2* is one of the most important enzymes up-regulated by *V. longisporum* during the late parasitic phase. The pathogen employs catalase peroxidase as a defence mechanism against the reactive oxygen compounds (hydrogen peroxide) produced by the host as a defence response (Wysong et al. 1998; Singh et al. 2012).

Following the colonization of the xylem, the diseased plant deprived of nutrients begins to senesce and the disease cycle enters the saprophytic phase where the pathogen relocates from the xylem to the neighbouring parenchymatic tissues (Mol and Scholte, 1995). The pathogen produces microsclerotia (budding of mycelia) in the dying tissues of host stems and roots giving a dark grey to black appearance (Eastburn and Paul 2007; Zhou et al. 2006; Goud et al. 2003). The decaying dead plant tissues thus return microsclerotia to the soil (Jiménez-Díaz et al. 2012). Microsclerotia therefore are found in higher densities in the top-soil from where they can be easily dispersed by tillage, wind and runoff (Sinclair and Lyon 2005), movement of farm equipment and infested soil and crop debris (Canadian Food Inspection Agency, 2017).

1.5 Hosts and Disease Symptoms

The distribution of *V. longisporum* has spread rapidly in the past decade. In 2005 its distribution was prominently restricted to European countries (Dixelius et al. 2005). This information was updated in 2014 with a total of ten countries that reported economic losses due to the pathogen. These included, Belgium, France, Germany, Italy, Japan, the Netherlands, Russia, Sweden, the UK, and the USA (Inderbitzin and Subbarao 2014).

Currently, *V. longisporum* is prominently distributed across three continents which include the countries of Belgium, the Czech Republic, Denmark, France, Germany, Italy, the

Netherlands, Poland, Russia, Sweden, the United Kingdom (England and Wales), Ukraine, Japan, the USA (Illinois and California), China and Canada (Manitoba, British Columbia, Alberta, Saskatchewan, Ontario and Quebec) (Canadian Food Inspection Agency, 2017).

The preferred hosts of *V. longisporum* belong to the Brassicaceae family (Zeise and von Tiedemann 2002), the most common being broccoli, cabbage, cauliflower, field mustard, oilseed rape, turnip (Horiuchi et al. 1990), brussels sprouts (Canadian Food Inspection Agency 2017), horseradish, sugar beet and wild radish (Stark 1961). Other non-Brassica hosts, such as wheat, pea and oats can also be infected by the pathogen but not as aggressively as canola (oilseed rape). Microsclerotia formation in these crops is four times less than that in canola; therefore those non-Brassica crops are candidates serving as reservoirs for pathogen inoculum (Johansson et al. 2006). A similar phenomenon is also witnessed in *V. dahliae*, which is concealed in the roots of wheat and oats (Mathre 1989).

The disease symptoms of parent *V. dahliae* on canola include stunting, yellowing of leaves, senescence and most importantly, wilting (following colonization of the xylem) (Hwang et al. 2017). On the contrary, the disease caused by *V. longisporum* in canola has been recently renamed as Verticillium stripe of canola instead of Verticillium wilt. The pathogen does not display the symptoms of wilting in canola (Hornig 1987 cited by Knüfer 2011). The symptoms are observed to be more like stripes, as seen on infected stems (Clint J. cited by Hein 2017; Depotter et al. 2016). This is mainly because, unlike its parent *V. dahliae*, *V. longisporum* only colonizes individual xylem vessels in canola, such that adjacent vessels are un-infested and the host therefore, does not show symptoms of wilting (Eynck et al. 2007).

First signs of *V. longisporum* infection in a host include early ripening with lower leaves turning yellow and infection spreading gradually to the upper leaves (Heale and Karapapa 1999;

Canadian Food Inspection Agency, 2017). As the infection progresses, due to reduced water intake, the overall growth of the host suffers resulting in stunting and senescence (Johansson et al. 2006). Stems display brown to light black stripes running parallel to the plant. A peeled back epidermis of the stem reveals blackening on the inside of the stem and gradual production of microsclerotia in/on the pith later in the season. Microsclerotia serve two functions in the stem: firstly under moist conditions they germinate and produce conidia which make the stem more powdery. Secondly they serve as resting structures upon return of the pathogen to the soil once the host starts to decay (Heale and Karapapa, 1999; Dixelius et al. 2005; Hein 2017).

To better understand the disease stages of an infected crop, an infection assessment key was designed by Zeise (1990). It classifies infection advancement of *V. longisporum* into four stages: low infestation, medium infestation, strong, and very strong infestation. For low infestation, common symptoms observed are discoloured stem, stripping of epidermis and up to 25% of stem epidermis colonized by microsclerotia. For medium infestation, visible symptoms include an easy to peel stem epidermis and up to 50% of stem epidermis colonized with microsclerotia. For strong infestation, the host stem is completely discoloured and up to 75% of the stem is colonized with microsclerotia. A host entering the very strong infestation stage shows signs of premature death and has more than with 75% of stems colonized by microsclerotia.

Verticillium stripe owing to its similarities in symptoms can be confused with Sclerotinia stem rot and blackleg disease. Verticillium stripe and Sclerotinia stem rot both cause discolouration of the stem. However, the latter forms large sclerotia inside the stem as compared to the former that forms smaller microsclerotia. Blackleg in contrast can be confused with Verticillium stripe based on the similar symptoms of premature ripening. The former turns the

inside of the stem black which on cross sectioning is not observed in a stem infected with Verticillium stripe (Canola Council of Canada 2017).

1.6 Control Methods for *V. longisporum*

In the past decade, with the distribution of *V. longisporum* spreading across continents and in the absence of an effective fungicide, it has become crucial to develop control methods for the pathogen (Lopisso et al. 2017). These control methods are discussed in the following sub-sections.

1.6.1 Inoculum Control in Soil- Physical Methods

Studies on inoculum control of *V. longisporum* are limited but some control methods can be similar to those used for its close relative *V. dahliae*. These include approaches like increased crop rotation of non-host crops and trap crops, weed management, increased soil fertility, destruction of infected crops, green manures, fumigation and incorporation of high lignin substrates like cauliflower and corn leaves in soil (Debode et al 2005; Canola Council of Canada 2017).

The control methods for *V. longisporum* on farms include protocols to prevent inter and intra spread of the pathogen via dispersal of its resting structure. To achieve this, some common biosecurity practices include sanitation of farm equipment and tools, monitoring of off-farm traffic, monitoring the source of seed and fertilizer (Canola Council of Canada 2017), use of plastic boots and cleansing of small tools with virkon (Amass et al. 2001).

An effective method of controlling *V. dahliae* inoculum in soil was presented by Katan et al. (1976). The study involved mulching soil with polyethylene sheets to increase net soil temperature via solar heating. Results revealed an immense reduction in the soil-borne pathogen

level as compared to the control soil. This was mainly due to three mechanisms; fungistasis at high temperature by lytic microbes in the soil, weakening of resting structure and decomposition by other microbes, and rise in numbers of heat resistant saprophytes. Soil mulching is inexpensive, safe, chemical-free, and does not require any machinery for application; therefore, it is a prospective control method for *V. longisporum*.

1.6.2 Inoculum Control in Soil- Chemical Methods

One of the main reasons behind absence of any effective fungicide against *V. longisporum* is because the pathogen colonizes in the xylem of its host where no fungicide can reach without collaterally killing the host (Schnathorst, W. C. cited by Johansson 2006). Therefore chemical control is more effective when applied to soil directly, in order to reduce the resting inoculum. Chemicals like elemental sulphur and methyl bromide are used for this purpose to eradicate microsclerotia from the soil (Cooper and Williams, 2004; Debode et al. 2005). As pathogen concentration as low as one microsclerotia per gram soil is enough to cause disease incidence in susceptible hosts (Debode et al. 2011), efficacy of these chemicals is questionable and needs further research. On the other hand, chemical control of microsclerotia of *V. dahliae* in soil has been documented with high success. Compounds like nitrous acid, ammonia, and volatile fatty acids are known to be poisonous to microsclerotia resting in soil (Conn et al. 2005). These chemicals therefore could prove equally effective in control of *V. longisporum*.

1.6.3 Natural Resistance in Host

In the absence of an efficient fungicide and resistant oilseed rape cultivar (Eynck et al. 2009), *V. longisporum* is considered most vulnerable to resistance breeding due to lack of a sexual stage (McDonald and Linde 2002). Therefore breeding programs set through the above

mentioned species could be a possible solution to tackle this economically crucial phytopathogen (Eastburn and Paul 2007; Heale and Karapapa 1999; Eynck et al. 2009).

Susceptibility to the pathogen varies across different varieties of the same host. Although there is no *V. longisporum* resistant oilseed rape cultivar, cultivars Express and RBN 03 were found least susceptible to Verticillium stripe infection in a study conducted in Romania, by Burlacu et al. (2012).

Recent findings suggest three Brassica species, *B. carinata* (field mustard), *B. rapa* (turnip) and *B. oleraceae* (cabbage) to be least susceptible to Verticillium stripe infection (Happstadius et al., 2003; Dixelius et al. 2005). Two *B. napus* (oilseed rape) genotypes SEM 05-500256 and AVISO are also documented to be resistant to Verticillium stripe infection. The resistance is expressed only after the roots are penetrated (Lopisso et al. 2017); therefore, these genotypes are still vulnerable to *V. longisporum* infestation in the roots. A study conducted by Eynck et al. (2009) documented the role of phenols, lignin, and plant hormones auxin and ethylene in imparting resistance against *V. longisporum* in the *B. napus* genotype SEM 05-500256. Phenols were suspected to play a role in maintaining plant redox state under infection. Lignin was involved in sealing off penetration sites at the lateral roots or root hair and auxin and ethylene in the release of secondary plant metabolites. These factors together might have been responsible for imparting resistance in genotype SEM 05-500256.

1.6.4 Biological Control (Biocontrol)

Biocontrol of fungal phytopathogens by bacterial agents has been reported in other microbial relationships in the past. One of the most common biocontrol examples is that of *Sclerotinia sclerotiorum* by *Pseudomonas chlororaphis* PA23 (Duke et al. 2017). Although

biocontrol of *V. longisporum* is quite uncommon, there are a few examples of bacterial species that are known to be effective against *V. dahliae*. Examples of bacterial species that control fungal growth by the means of antibiotics, lytic enzymes and siderophores include *Talaromyyces flavus* (Nagtzaam et al. 1998), *Bacillus subtilis*, *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia* (Berg et al. 1994).

Serratia plymuthica C48 (Berg et al. 1999) and *Paenibacillus alvei* K165 (Tjamos et al. 2004) are bacterial species more specific in control of *V. longisporum*. The rhizospshere bacteria *S. plymuthica* C48 produces the hydrolytic enzymes chitobiosidase and chitobiase, which are examples of chitinases that digest the *V. longisporum* cell wall and prevent growth (Berg et al.1999). *P. alvei* K165, in contrast, does not interact with the pathogen directly but induces systemic resistance (ISR) in host plant species (Tjamos et al. 2005). Induced systemic resistance is a salicylic acid independent cycle that involves jasmonic acid and ethylene signalling hormones used to induce defence hormones across the plant. Therefore the objective of ISR is to prime the plant's defence system by rhizobacteria (plant growth promoting rhizobacteria), before the onset of actual phytopathogenic infection (Choudhary et al 2007). In the absence of a resistant cultivar, efficient fungicide, and given the deteriorating effects of fungicides on plants (Petit et al. 2012) the use of biocontrol agents in preventing *V. longisporum* infection is highly desirable (Fravel 1988).

1.6.5 Enhanced Host Resistance

Oilseed rape (*B. napus)* is the preferred host of *V. longisporum* and this is primarily due to the lack of resistant genes in its gene pool (Happstadius et al. 2003). Therefore interspecific transfer of genes from other related species is one of the methods of enhancing resistance in vulnerable host species. Ancestral parents of *B. napus* and members of the same genus, *B. rapa*

and *B. oleracea* are typically resistant to *V. longisporum* infection (Parkin et al. 1995; Happstadius et al. 2003).

Hybridized *B. napus* progeny (genome AACC, 2n=38) re-synthesized from complete genomes of *B. rapa* (genome AA, 2n=20) and *B. oleracea* (genome CC, 2n=18) using an embryo rescue technique are found to be resistant to *V. longisporum* infestation. The highest resistance was demonstrated by a *B. napus* hybrid developed from *B. rapa* gene bank accession 56515 and *B. oleracea* gene bank accession 8207 (Rygulla et al. 2007b).

In terms of selection pressure imposed on *V. longisporum* to overcome host resistance, the strategy of using two resistant species to develop a resistant *B. napus* hybrid is extremely crucial. While this extremely promising strategy imparts long-term resistance in *B. napus* and prevents development of acquired resistance in *V. longisporum* lines (Fahling et al 2003) it has a serious economic drawback. The high erucic acid content in the seed of the *B. oleracea* parent is passed on to the *B. napus* hybrid making it unsuitable for economic use (Sahasrabudhe 1977). Therefore it becomes imperative to reduce the erucic acid in either the parent *B. oleracea* or the re-synthesized *B. napus* hybrid. The solution lies in *B. oleracea* gene accession Kashirka 202, which is a mutant of *B. oleracea* that cannot produce erucic acid but is still resistant to *V. longisporum*. Crossing of this mutant with a resistant *B. rapa* progeny results in a *B. napus* hybrid that is resistant to *V. longisporum* in addition to having a low erucic acid content (Rygulla et al. 2007a).

Another example of enhanced resistance is demonstrated by the β-aminobutyric acid (BABA) pathway that is responsible for chemically induced resistance across multiple plant species against a wide spectrum of plant pathogens (Cohen 2002). The actual BABA induced resistance pathway is yet to be discovered but has been reported to induce resistance in plants via

salicylic acid pathway (Zimmerli et al. 2001), jasmonic acid pathway (Hamiduzzaman et al. 2005) and via BABA mediated callose production (Ton and Mauch-Mani 2004).

Similar to the *B. napus* resistant genotype SEM 05-500256, BABA induced resistance is also expressed only after penetration of the host roots by the pathogen (Kambleab et al. 2013). BABA induced resistance against *V. longisporum* in susceptible *B. napus* cultivar involves an array of physical and chemical changes in the host tissues. On penetration of roots the first step towards resistance is repression of the pathogen in the hypocotyl and prevention of colonization in the shoot. β-aminobutyric acid also induces a rise in Phenylalanine Ammonia-lyase **(**PAL) activity (Kambleab et al. 2013); an enzyme of the phenylpropanoid pathway responsible for deamination of phenylalanine to trans-cinnamic acid, followed by lignification. Phenylalanine Ammonia-lyase activity induced by BABA is therefore directly responsible for initiating lignin and phenol pathway mediated inhibition of the *V. longisporum* pathogen (Bagal et al. 2012; Kambleab et al. 2013).

1.7 Detection of *V. longisporum*

Detection of fungal phytopathogens from plant and soil sources plays an important role in modern day research sectors of genetics, disease diagnosis, and disease forecasting (Kang et al. 2014). The following sub-sections describe some of the methods used for detection of *V. longisporum* from soil samples.

1.7.1 Detection on Selective media

Fungi very rarely exist in pure cultures especially in soil where organic matter, secondary colonizers and other microbes are present in abundant numbers. On non-selective media, bacteria,

actinomycetes and other fast growing fungi outcompete pathogenic fungal colonies on plates. Therefore use of selective media for isolation and detection of pathogenic fungi from plant tissues and soil samples has become extremely important (Tsao 1970).

Goud et al. (1997) used two selective media for isolation of *V. dahliae*. The media used were modified soil extract agar (MSEA) and ethanol agar medium (EA) spiked with 50 mg/L oxytetracycline as an antibacterial agent. Modified soil extract agar as selective media for detection of *V. longisporum* was successfully tested by Andersson (2003), in a study based on detection of the pathogen from soil.

Other examples of selective media not yet reported for *V. longisporum* that proved effective for *V. dahliae* include glucose concentration gradient based rearing (Hall and Ly 1972), ethanol-streptomycin agar based rearing (Pegg and Brady 2002), and ethanol, pentachloronitrobenzene and antibiotics based media rearing (Ausher 1975).

1.7.2 Microsclerotia Based Detection

Wet sieving to estimate the density of *V. longisporum* microsclerotia in soil was successfully used by Andersson (2003). Approximately 12.5 g of air-dried soil was passed through 106 µm and 20 µm mesh screens with running water. The trapped particles (mostly microsclerotia) were then suspended in 50ml of 0.08% sterile water agar and evenly distributed in aliquots of 800 µl (0.8 ml) across ten MSEA media plates per soil sample. Following air-drying, the plates were stored in plastic bags at 20°C for four weeks. The plates were then washed with water and microsclerotia colonies counted (ten plates) under a microscope (Andersson 2003).

In the wet sieving method for 12.5 g soil, the suspension volume was approximately 55 ml. Therefore the suspension volume on each plate can be expressed as mass of soil divided by

suspension volume for 12.5 g soil times volume plated, which in the above scenario equals 12.5/55 x 0.8 giving 0.182 g per plate. Thus total amount in a soil sample equaled 0.182 x 10 (ten plates), which gave 1.82 g total soil equivalent. Now for example if there were ten colonies across the ten plates, colony forming units (cfu) per gram would equal 10/1.82, which is 5.49 cfu per gram soil (Andersson 2003). All tools and equipment used in the wet sieving method were sterilized with 20% alcohol to kill any microsclerotia residue to avoid cross contamination across different samples (Andersson 2003).

Another more advanced method of quantifying *Verticillium* species microsclerotia is known as real-time polymerase chain reaction (PCR). The use of primers in PCR makes this assay specific to *V. tricorpus* (using primers designed to anneal to the ribosomal DNA internal transcribed spacer), and *V. dahliae* and *V. longisporum* (using primers designed to anneal to the beta-tubulin gene). Real-time PCR based assay (discussed later in detail) is more specific and faster than the conventional wet sieving method that fails to differentiate between species of the genus *Verticillium* (Debode et al. 2011).

1.8 Polymerase Chain Reaction (PCR)

In the past century out of the 585 Nobel Prizes awarded to individuals across different streams of Physics, Chemistry, Medicine, Literature, Peace and Economic Science (Nobel Prize Facts 2017), two prizes are of interest in this research. First being awarded to James Dewey Watson, Francis Harry Compton Crick and Maurice Wilkins for discovering the structure of DNA to be a double helix held by complementary base pairing (Watson and Crick 1953).

The second Nobel Prize being awarded to Kary Bank Mullis for inventing PCR, a technique that generates multiple copies of a specific region on a template DNA (Erlich 1989;

Hongbao 2005). Conceived by Mullis only three decades ago in 1983, PCR has emerged as one of the most important tools in molecular biology and biotechnology (Pray 2008; Walker, 2002) and also plays an important role in this research.

In vivo DNA replication requires multiple biological compounds. Therefore, PCR, an in vitro version of replication requires the following components: target DNA (template), a heat-stable DNA polymerase, forward oligonucleotide primer, reverse oligonucleotide primer, four deoxynucleoside triphosphates (dNTPs) (dATP, dCTP, dGTP, and dTTP), magnesium ions, buffer and sterile water along with a thermal cycler for heating and cooling the reaction mixture (Bermingham and Luettich 2003; Hongbao 2005).

PCR is split into cycles and each cycle has three steps. At the end of every cycle two copies of double stranded DNA are yielded from every parent copy in the reaction (Lipp et al. 2005). The first step in PCR is known as denaturation where the reaction mixture is heated to temperatures between 92ºC-98ºC for one to three minutes (Bermingham and Luettich 2003). In a positive correlation, the GC content and length of the template DNA determine the duration of this step (Lorenz 2012). This step causes reversible denaturation (separation) of the double stranded DNA into two single strands (Bermingham and Luettich 2003). This is because in the presence of low pH, low salt concentration or high temperature the double stranded DNA separates into single strands due to the loss of secondary hydrogen bonds between the nitrogen bases present in the DNA backbone (Thomas 1993).

The second step is known as the annealing step where the temperatures are reduced to 37ºC-55ºC for 10 to 30 seconds, which allows each of the short oligonucleotide primers (complementary to the target site) to bind to the single stranded DNA (Bermingham and Luettich 2003). Temperatures in the annealing step are not fixed as they are set 5ºC less than the melting

temperature (T_m) of the primers used; the T_m of the primers can range between 45ºC-65ºC (Lorenz 2012).

The last step in PCR is known as the elongation step where the temperatures are increased to between 60ºC-72ºC for one to two minute(s). This allows the DNA polymerase to extend the template-primer strand by using dNTPs as building blocks to synthesize a complementary strand to the template DNA; thus, restoring the double stranded structure of the DNA once again (Bermingham and Luettich 2003). The above-described steps are repeated for 20-30 cycles generating close to a million copies of the target site in a template DNA (Erlich 1989).

The elongation temperatures depend on the DNA polymerase used in the reaction mixture. Since the reaction goes up to high temperatures of 90ºC the polymerase being used should be heat stable. For this purpose in all PCR assays heat stable Taq polymerase isolated from hot spring bacterium *Thermus aquaticus* is used (Lorenz 2012; Brock 1997).

Ever since the advent of PCR there have been many advancements in its applications, one of which is real-time polymerase chain reaction, better known as quantitative polymerase chain reaction (qPCR) (Valasek and Repa 2005). As the name suggests, in real-time PCR the user can observe amplification of the template DNA in real time (Valasek and Repa 2005).

Shortly after the invention of PCR, Higuchi and Dollinger (1992) at Roche Molecular Systems and Chiron successfully performed qPCR. In their experiment they used a video camera to record the amplification of DNA strands as they fluoresced under UV light once bound to ethidium bromide (EtBr) dye. EtBr was used as fluorescing dye that binds only to double stranded DNA and fluoresces under UV light; every time a fluorescing event was recorded it meant that one cycle of PCR was completed and hence monitoring was performed in “real time”.

The working principle of qPCR is to detect a specific sequence in template DNA and measure the amplification progress in real time. The PCR component amplifies the target sequence using complementary designed primers and the real time component reports the growth in amplification by means of fluorescent signatures. Real-time PCR can also quantify the amount of template in the starting DNA sample. This is achieved by correlating the number of cycles at which the fluorescent signature was first registered to the amount of initial DNA in samples (Valasek and Repa 2005). qPCR uses a linear relationship between the Cq value and log10 of concentration of sample DNA to determine the initial target DNA in the sample. Therefore, the more the initial template, the lower is the Cq value required to register a fluorescent signature (Bustin 2005). One of the biggest advantages of qPCR over PCR is circumventing the gel electrophoresis step required to confirm the PCR product needed in conventional PCR (Bustin 2005).

The number of amplification cycles required to register a fluorescent signature was termed threshold cycle (Ct) (Bustin 2005). The term Ct has been recently replaced by quantitative cycle (Cq), which is now a more MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) accepted term (Bustin et al 2009).

Currently, there are several other fluorescing technologies such as SYBR® Green dye 1, Taqman® probes, and molecular beacons (Walker 2002) that have replaced the conventional EtBr. The least expensive of the three, SYBR® Green dye 1 produces fluorescence once bound to a double stranded DNA at the end of every elongation step. The intensity of fluorescence is positively correlated to the DNA concentration and amplification progress in the reaction mixture (Arya et al. 2005).

SYBR® Green dye 1 binds non-specifically to any double stranded DNA (template DNA, primer dimers or other PCR products) making it susceptible to false fluorescence (Ririe et al. 1997). The thermal cycler software overcomes this issue by generating melting curves that report Tm of all amplicons, which makes it simpler to identify amplification of DNA of interest from non-specific PCR products (Arya et al. 2005).

In contrast, Taqman® probe is slightly more specific. A designed Taqman® probe is complementary to the template DNA and has the reporter dye (fluorescing) attached to one end and a quencher dye attached to the other. During the annealing step the probe binds to the template DNA, downstream of the annealed primer. Following the elongation step, the probe is cleaved by Taq polymerase, which separates the reporter dye from the quencher dye. The quencher dye's function is to absorb fluorescence from the reporter dye. As soon as these two components are separated by the polymerase, fluorescence signatures are detected. Similar to SYBR® Green dye 1 fluorescence is detected at the end of every of every cycle with a gradual increase in the fluorescence intensity as amplification progresses (Arya et al. 2005).

Molecular beacons function in a similar manner to Taqman® but are completely different in structure. Molecular beacons are synthesized in a hairpin-like structure to keep the quencher and reporter dye extremely close to each other, hence supressing fluorescence from the reporter dye (Tyagi and Kramer 1996). Similar to the Taqman® probe, during the annealing stage the molecular beacon binds to the complementary template that disrupts the hairpin structure causing both the dyes to move apart from each other losing suppression of fluorescence. Unlike the first two fluorescing dyes, molecular beacons register fluorescence during the annealing stage of the PCR (Arya et al. 2005).

Real-time PCR in the past couple of decades has emerged as one of the most important analytical tools in agriculture and plant pathology (Martin et al. 2000; Lipp et al. 2005). PCR quantifies the amount of fungi in samples of interest thereby purging the laborious conventional methods of isolation and culturing of fungal colonies (Zheng et al. 2005). Real-time PCR in identification and quantification of *V. longisporum* and its parent *V. dahliae* has been successful in recent studies. Real-time assay based detection of *V. dahliae* in California spinach seed was successfully tested by Duressa et al. (2011). Moreover, comprehensive studies conducted by Tzelepis et al. (2017) on Swedish soils and Banno et al. (2011) on cabbage fields in Japan proved extremely effective in identification and quantification of *V. longisporum*. Hence, PCR plays a vital role in the studies involved in this research.

Verticillium longisporum infestation in cabbage, cauliflower (California) and horseradish (Illinois) has been recorded in North America in the past (Novakazi et al. 2015). However for the first time in North America, in August 2014, infection in canola by *V. longisporum* was noticed at a Manitoba farm during end of season combining. The presence of the pathogen in the farm soil was then confirmed by a series of analysis by Manitoba Agriculture, the Agriculture-Agri Food Canada (AAFC) and the Canadian Food Inspection Agency (CFIA). Following the confirmation of *V. longisporum*, the farm was placed under quarantine by the CFIA for two growing seasons and since, has developed a stringent biosecurity protocol to prevent further spread of the pathogen by controlling movement of farm traffic, equipment, soil, personnel and sanitization practices.

1.9 Hypotheses

The first hypothesis for this research is that the *V. longisporum* pathogen will be widespread throughout the farm due to the inevitable dispersal of spores via wind, field

equipment traffic and other organisms. The second hypothesis is that *V. longisporum* hybrid lineage at the farm will be one of three lineages previously described.

1.10 Thesis Objectives

Since this is the first documented case of Verticillium stripe on canola in North America, very little is known about pathogen's spatial distribution within farms, ability to establish in soil, virulence, and hybrid lineage. To help address these uncertainties, the following are the objectives of this thesis:

1) To investigate the spatial variation of *V. longisporum* and infer to its ability to establish at a Manitoba (Canada) farm positive for the pathogen using analyses based on qPCR of the pathogen and cropping history of the fields;

2) To determine the hybrid origin of *V. longisporum* isolates in Manitoba using triplex conventional PCR and gel electrophoresis technique.

1.11 Structure of Thesis

This thesis begins with a general introduction chapter (Chapter 1) that describes various aspects of *V. longisporum* such as history and classification, morphology and hybridization, and disease cycle and symptoms of disease. The chapter also describes detection and prospective control methods for V. *longisporum*.

The samples for this study were collected during the fall of 2015 and analyzed during 2016-2017. Both the objectives of this thesis were accomplished and described in detail in the

following research chapter (Chapter 2). Following the research chapter is an overall synthesis chapter (Chapter 3) that discusses and concludes the contribution of this thesis towards the limited knowledge on *V. longisporum* of canola in North America.

1.12 Literature Cited

Amass, S.F., Ragland, D., and Spicer, P. 2001. Evaluation of the efficacy of a peroxygen compound, VirkonS, as a boot bath disinfectant. Swine Health and Production **9**:121-123.

Andersson, C. 2003. Detection methods of *Verticillium longisporum* in soil and in oilseed rape. M.Sc. Dissertation. Swedish University of Agricultural Sciences. Uppsala **45**: ISSN 1651-5196.

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., and Patel, H.R.H. 2005. Basic principles of real-time quantitative PCR. Expert Review of Molecular Diagnostics **5**:209-219.

Ausher, R. 1975. An improved selective medium for the isolation of *Verticillium dahliae*. Phytoparasitica **3**:133-137.

Bagal, U.R., Leebens-Mack, J.H., Lorenz, W.W., and Dean, J.F. 2012. The phenylalanine ammonia lyase (PAL) gene family shows a gymnosperm-specific lineage. BMC Genomics **13**(3):S1. doi:10.1186/1471-2164-13-S3-S1.

Banno, S., Saito, H., Sakai, H., Urushibara T., Ikeda, K., Kabe, T., Kemmochi, I., and Fujimura, M. 2011. Quantitative nested real-time PCR detection of *V. longisporum* and *V. dahliae* in the soil of cabbage fields. Journal of General Plant Pathology **77**:282-291.

Barton, N.H., 2001. The role of hybridization in evolution. Molecular Ecology **10**:551-568.

Bell, A.A., Puhalla, J.E., Tolmsoff, W.J., and Stipanovic, R.D. 1976. Use of mutants to establish (+)-scytalone as an intermediate in melanin biosynthesis by *Verticillium dahliae*. Canadian Journal of Microbiology **22**:787-799.

Berg, G., Frankowski, J., and Bahl, H. 1999. Biocontrol of Verticillium wilt in oilseed rape by chitinolytic Serratia plymuthica. In Proceedings of the Tenth International Rapeseed Congress, International Rapeseed Congress, Canberra, Australia.

Berg, G., Knaape, C., Ballin, G., and Seidel, D. 1994. Biological control of *Verticillium dahliae* kleb. by natural occurring rhizosphere bacteria. Archives of Phytopathology and Plant Protection **29**:249-262.

Bermingham, N., and Luettich, K. 2003. Polymerase chain reaction and its applications. Current Diagnostic Pathology **9**:159-164.

Burlacu, M.C., Leonte, C., Lipsa, F., Simioniuc, D.P., and Lazarescu, E. 2012. Identification of some cultivars of *Brassica napus* with resistance at *Verticillium longisporum*. Research Journal of Agricultural Science **44**:14-18.

Bustin, S. 2005. Real-time PCR. Encyclopedia of Diagnostic Genomics and Proteomics **10**:1117-1125.

Bustin, S.S., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry **55**:611-622.

Butler, M.J., and Day, A.W. 1998. Fungal melanins: a review. Canadian Journal of Microbiology **44**:1115-1136.

Canadian Food Inspection Agency. 2017. Pest Risk Management Document- *Verticillium longisporum* (Verticillium stripe). Canadian Food Inspection Agency, Winnipeg, Manitoba.

Canola Council of Canada. 2017. *Verticillium*. Canola Council of Canada, Winnipeg, Manitoba.

Choudhary, D.K., Prakash, A., and Johri, B.N. 2007. Induced systemic resistance (ISR) in plants: mechanism of action. Indian Journal of Microbiology **47**:289-297.

Clewes, E., Edwards, S.G., and Barbara, D.J. 2008. Direct molecular evidence supports long-spored microsclerotial isolates of *Verticillium* from crucifers being interspecific hybrids. Plant Pathology **57**:1047-1057.

Cohen, Y.R. 2002. β-aminobutyric acid-induced resistance against plant pathogens. Plant Disease **86**:448-457.

Collins, A., Okoli, C.A., Morton, A., Parry, D., and Edwards, S.G. 2003. Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. Phytopathology **93**:364-376.

Collado-Romero, M., Jiménez-Díaz, R.M., and Mercado-Blanco, J. 2010. DNA sequence analysis of conserved genes reveals hybridization events that increase genetic diversity in *Verticillium dahliae.* Fungal Biology **114**:209-218.

Conn, K.L., Tenuta, M., and Lazarovits, G. 2005. Liquid swine manure can kill *Verticillium dahliae* microsclerotia in soil by volatile fatty acid, nitrous acid, and ammonia toxicity. Phytopathology **95**:28-35.

Cooper, R.M., and Williams, J.S. 2004. Elemental sulphur as an antifungal substance in plant defence. Journal of Experimental Botany **55**:1947-1953.

Debode, J., Clewes, E., DeBacker, G. and 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**:301-309.

Debode, J., Van Poucke, K., França, S.C., Maes, M., Höfte, Monica, and Heungens, K. 2011. Detection of multiple Verticillium species in soil using density flotation and real-time polymerase chain reaction. Plant Disease **95**:1571-1580.

Depotter, J.R., Deketelaere, S., Inderbitzin, P., Tiedemann, A.V., Höfte, M., Subbarao, K.V., Wood, T.A., and Thomma, B.P. 2016. *Verticillium longisporum*, the invisible threat to oilseed rape and other brassicaceous plant hosts. Molecular Plant Pathology **17**:1004-1016.

Dessimoz, M., Enkerli, J., Michel, V. and Widmer, F. 2012. Ribosomal intergenic spacer (rIGS) sequences and MALDI-TOF MS for rapid typing of Verticillium taxa. Molecular Ecology. Submitted for publication.

Dixelius, C., Happstadius, I., and Berg, G. 2005. Verticillium wilt on Brassica oilseed crops – a Swedish perspective. Journal of Swedish Seed Association **115**:36-48.

Duke, K.A., Becker, M.G., Girard, I.J., Millar, J.L., Fernando, W.G.D., Belmonte, M.F., and de Kievit, T.R. 2017. The biocontrol agent *Pseudomonas chlororaphis* PA23 primes *Brassica napus* defenses through distinct gene networks. BMC Genomics **18**:467.

Duressa, D., Rauscher, G., Koike, S.T., Mou, B., Hayes, R.J., Maruthachalam, K., Subbarao, K.V., and Klosterman, S.J. 2011. A Real-Time PCR Assay for Detection and Quantification of *Verticillium dahliae* in Spinach Seed. Phytopathology **102**:443-451.

Eastburn, D.M. and Paul, V.H. 2007. Verticillium wilt. Pages 47-50. in S. R. Rimmer, V. I. Shattuck, and L. Buchwaldt (Eds.). Compendium of Brassica Diseases. APS Press, St Paul, MN.

Erlich, H.A. 1989. Polymerase chain reaction. Journal of Clinical Immunology **9**:437-447.

Eynck, C., Koopmann, B., Grunewaldt-Stöcker, G., Karlovsky, P., and von Tiedemann, A. 2007. Differential interactions of *Verticillium longisporum* and *Verticillium dahliae* with *Brassica napus* detected with molecular and histological techniques. European Journal of Plant Pathology **118**:259-274.

Eynck, C., Koopmann, B., Karlovsky, P., and von Tiedemann, A. 2009. Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. Phytopathology **99**:803-811.

Fähling, M., Graf, H., and Siemens, J. 2003. Pathotype separation of *Plasmodiophora brassicae* by the host plant. Journal of Phytopathology **151**:425-430.

Fan, R., Klosterman, S.J., Wang, C., Subbarao, C.V., Xu, X., Shang, W., and Xiaoping, H. 2017. *Vayg1* is required for microsclerotium formation and melanin production in *Verticillium dahliae.* Fungal Genetics and Biology **98**:1-11.

Fradin, E.F., and Thomma, B.P.H.J. 2006. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology **7**:71-86.

Fravel, D.R. 1998. Role of antibiosis in the control of plant diseases. Annual Review of Phytopathology **26**:75-91.

Goud, J.K. , Termorshuizen, A.J., and Gams, W. 2003. Morphology of *Verticillium dahliae* and *V. tricorpus* on semi-selective media used for the detection of *V. dahliae* in soil. Mycological Research **107**: 822-830.

Greig, D., Louis, E.J., Bortis, R.H., and Travisano, M. 2002. Hybrid speciation in experimental populations of yeast. Science **298**:1733-1775.

Hall, R., and Ly, H. 1972. Development and quantitative measurement of microsclerotia of *Verticillium dahliae*. Canadian Journal of Botany **50**:2097-2102.

Hamiduzzaman, M., Jakab, G., Barnavon, L., Neuhaus, J., and Mauch-Mani, B. 2005. Baminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. Molecular Plant–Microbe Interactions **18**:819-829.

Happstadius, I., Ljungberg, A., Kristiansson, B., and Dixelius, C. 2003. Identification of *Brassica oleracea* germplasm with improved resistance to Verticillium wilt. Plant Breed **122**:30-34.

Heale, J.B. 2000. Diversification and speciation in *Verticillium* - an overview. Pages 1-14. in E. C. Tjamos, R.C. Rowe, J.B. Heale, and D.R. Fravel (Eds.). Advances in Verticillium research and disease management. APS Press, St. Paul, MN.

Heale, J.B. and Karapapa, V.K. 1999. The *Verticillium* threat to Canada's major oilseed crop: canola. Canadian Journal of Plant Pathology **21**:1-7.

Hein. T. 2017. Verticillium stripe: measuring the threat. Canola Digest. Canola Council of Canada, Winnipeg, Manitoba.

Higuchi, R., and Dollinger, G. 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnology **10**:413-417.

Hongbao, M. 2005. Development Application of Polymerase Chain Reaction (PCR). The Journal of American Science **1**:1-47.

Horiuchi, S., Hagiwara, H., and Takeuchi, S. 1990. Host specificity of isolates of *Verticillium dahliae* towards cruciferous and solanaceous plants. Hornby, D. (Ed.). CAB International, Wallingford, Oxon, UK. Publ. 285-298 pp. ISBN : 0851986374

Hornig, H. 1987. Weitere Untersuchungen und Erkenntnisse zur Krankhaften Abreife. Raps **1**:27-30.

Huisman, O.C. 1982. Interrealtions of root growth dynamics to epidemiology of root-invading fungi. Annual Review of Phytopathology **20**:303-327.

Hwang, S.F., Strelkov, S.E., Ahmed, H.A., Zhou, Q., Fu, H., Fredua-Agyeman, R., and Turnbull, G.D. 2017. First report of *Verticillium dahliae* Kleb. Causing wilt symptoms in canola (*Brassica napus* L.) in North America. Canadian Journal of Plant Pathology **39:**514-526.

Inderbitzin, P., and Subbarao, K.V. 2014. Verticillium systematics and evolution: How confusion impedes Verticillium wilt management and how to resolve it. Phytopathology **104**:564-574.

Inderbitzin, P., Bostock, R.M., Davis, R.M., Usami, T., Platt, H.W., and Subbarao, K.V. 2011a. Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. PLoS ONE **6**(12):e28341. doi:10.1371/journal.pone.0028341

Inderbitzin, P., Davis, R.M., Bostock, R.M., and Subbarao, K.V. 2011b. The ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. PLoS ONE **6**(3):e18260. doi:10.1371/journal.pone.0018260

Inderbitzin P., Davis R.M., Bostock R.M., and Subbarao K.V. 2013. Identification and Differentiation of *Verticillium* Species and *V. longisporum* Lineages by Simplex and Multiplex PCR Assays. PLoS ONE **8**(6):e65990. doi:10.1371/journal.pone.0065990

Ingram, R. 1968. *Verticillium dahliae* var. *longisporum*, a stable diploid. Transactions of the British Mycological Society **51**:339-341.

Isaac, I. 1949. A comparative study of pathogenic isolates of *Verticillium*. Transactions of the British Mycological Society **32**:137-157.

Isaac, I. 1953. A further comparative study of pathogenic isolates of *Verticillium*: *V. nubilum* Pethybr. and *V. tricorpus* sp. nov. Transactions of the British Mycological Society **36**:180-195.

Jiménez-Díaz, R.M., Cirulli, M., Bubici, G., Jiménez-Gasco, M.M., Antoniou, P.P., and Tjamos, E.C. 2012. Verticillium wilt, a major threat to olive production: Current status and future prospects for its management. Plant Disease **96**:304-329.

Johansson, A. 2006. *Verticillium longisporum,* infection, host range, prevalence and plant defence responses. Licentiate thesis. Swedish University of Agricultural Sciences. Uppsala: ISBN 91-576-7153-2.

Johansson, A., Goud, J.K.C., and Dixelius, C. 2006. Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. European Journal of Plant Pathology **114**:139-149.

Kambleab, A., Koopmanna, B., and von Tiedemanna, A. 2013. Induced resistance to *Verticillium longisporum* in *Brassica napus* by β-aminobutyric acid. Plant Pathology **62**:552-561.

Kang, Y., Lee, S.H., and Lee, J. 2014. Development of a selective medium for the fungal pathogen *Cylindrocarpon destructans* using radicicol. The Plant Pathology Journal **30**:432-436.

Karapapa, V.K., and Typas, M.A. 2001. Molecular characterization of the host-adapted pathogen *Verticillium longisporum* on the basis of a group-I intron found in the nuclear SSU-rRNA gene. Current Microbiology **42**:217-224.

Karapapa, V.K., Brainbridge, B.W., and Heale, J.B. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb, nov., pathogenic to oilseed rape. Mycological Research **101**:1281-1294.

Katan, J., Greenberger, A., Alon, H., and Grinstein, A. 1976. Solar heating by polyethylene mulching for the control of diseases caused by soil-borne pathogens. Phytopathology **66**:683-688.

Kehr, J., Buhtz, A., and Giavalisco, P. 2005. Analysis of xylem sap proteins from *Brassica napus*. BMC Plant Biology **5**:11.

Klosterman, S.J., Atallah, Z.K., Vallad, G.E., and Subbarao, K.V. 2009. Diversity, pathogenicity, and management of *Verticillium* species. Annual Review of Phytopathology **47**:39-62.

Knüfer. J. 2011. Improvement of winter oilseed rape resistance to *Verticillium longisporum* – assessment of field resistance and characterization of ultrastructural plant responses. Thesis zur Erlangung des Doktorgrades der Fakultät für Agrarwissenschaften der Georg-August-Universität Göttingen.

Kondrashov, A.S., Sunyaev, S., and Kondrashov, F.A. 2002. Dobzhanksy-Muller incompatibilities in protein evolution. Proceedings of the National Academy of Sciences of the United States of America **99**:14878-14883.

Lipp, M., Shillito, R., Giroux, R., Spiegelhalter, F., Charlton, S., Pinero, D., and Song, P. 2005. Polymerase chain reaction technology as analytical tool in agricultural biotechnology. Journal of AOAC international **88**:1.

López-Millán, A.F., Morales, F., Andaluz, S., Gogorcena, Y., Abadía, A., De Las Rivas, J. 2000. Responses of sugar beet roots to iron deficiency. Changes in carbon assimilation and oxygen use. American Society of Plant Physiologists **124**:885-898.

Lopisso, D.T., Knüfer, J., Koopmann, B., and von Tiedemann, A. 2017. Growth of *Verticillium longisporum* in xylem sap of *Brassica napus* is independent from cultivar resistance but promoted by plant aging. Phytopathology **107**:1047-1054.

Lorenz, T.C. 2012. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. Journal of Visualized Experiments: JoVE **63**:3998.

Mathre, D. 1989. Pathogenicity of an isolate of *Verticillium dahliae* from barley. Plant Disease **73**:164-167.

Martin, R.R., James, D., Lévesque, C.A. 2000. Impacts of molecular diagnostic technologies on plant disease management. Annual Review of Phytopathology **38**:207-239.

McDonald, B.A. and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review of Phytopathology **40**:349-379.

Mol, L., and van Riessen, H.W. 1995. Effect of plant roots on the germination of microsclerotia of*Verticillum dahliae.* European Journal of Plant Pathology **101**:673-678.

Mol, L. and Scholte, K. 1995. Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. Potato Research **38**:143-150.

Morales, L., and Dujon, B. 2012. Evolutionary role of interspecies hybridization and genetic exchanges in yeasts. Microbiology and Molecular Biology Reviews: MMBR **76**:721-739.

Nagtzaam, M.P.M., Bollen, G.J., and Termorshuizen, A.J. 1998. Efficacy of *Talaromyces flavus* alone or in combination with other antagonists in controlling *Verticillium dahlia* in growth chamber experiments. Journal of Phytopathology **146**:165-173.

Nelson, R.R. 1963. Interspecific hybridization in the fungi. Annual Review of Microbiology **17**:31-48.

Newcombe, G., Stirling, B., McDonald, S., and Bradshaw, H.D. 2000. *Melampsora x columbiana*, a natural hybrid of *M. medusae* and *M. occidentalis*. Mycological Research **104**:261-274.

Nobel Prize Facts. 2017. Nobelprize.org. Nobel Prize Facts. Nobel Media AB 2017.

Osborn, T.C., Pires, J.C., Birchler, J.A., Auger, D.L., Chen, Z.J., Lee, H.S., Comai, L., Madlung, A., Doerge, R.W., Colot, V., and Martienssen, R.A. 2003. Understanding mechanisms of novel gene expression in polyploids. Trends in Genetics: TIG **19**:141-147.

Parkin, I.A.P., Sharpe, A.G., Keith, D.J., and Lydiate, D.J. 1995. Identification of the A and C genomes of amphidiploid *Brassica napus* (oilseed rape). Genome **38**:1122-1131.

Pegg, G.F., and Brady, B.L. 2002. *Verticillium* wilts. CABI Publishing. CABI International. Cromwell Press, Trowbridge, UK. Publ. ISBN 0851995292

Petit, A.N., Fontaine, F., Vatsa, P., Clément, C., and Vaillant-Gaveau. N. 2012. Fungicide impacts on photosynthesis in crop plants. Photosynthesis Research **111**:315-326.

Pray, L. 2008. The biotechnology revolution: PCR and the use of reverse transcriptase to clone expressed genes. Nature Education **1**:94

Ririe, K.M., Rasmussen, R.P., and Wittwer, C.T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical Biochemistry **245**:154-160.

Rygulla, W., Friedt, F., Seyis, W., Lühs, C., Eynck, A., Von Tiedemann, A., and Snowdon, R.J. 2007a. Combination of resistance to *Verticillium longisporum* from zero erucic acid *Brassica oleracea* and oilseed *Brassica rapa* genotypes in resynthesized rapeseed (*Brassica napus*) lines. Plant Breeding **126**:596-602.

Rygulla, W., Snowdon, R.J., Eynck, C., Koopmann, B., von Tiedemann, A., Lühs, W., and Friedt, W. 2007b. Broadening the genetic basis of *Verticillium longisporum* resistance in *Brassica napus* by interspecific hybridization. Phytopathology **97**:1391-1396.

Sahasrabudhe, M. 1977. Crismer values and erucic acid contents of rapeseed oils. Journal of the American Oil Chemists' Society **54**:323-324.

Schnathorst, W.C. 1981. Life cycle and epidemiology in Verticillium. Pages 81-112. in Mace, M.C., Bell, A.A., and Beckman, C.H. Fungal wilt diseases in plants. Academic Press, New York.

Short, D.P., Sandoya, G., Vallad, G.E., Koike, S.T., Xiao, C.L., Wu, B.M., Gurung, S., Hayes, R.J., and Subbarao, K.V. 2015. Dynamics of *Verticillium* species microsclerotia in field soils in response to fumigation, cropping patterns, and flooding. Phytopathology **105**:638-645.

Sinclair, W.A., and Lyon, H.H. 2005. Diseases of trces and shrubs (2'"1 cdition). Comstock Publishing, Cornell Univcrsity Prcss. lthaca, NY. 660 pp.

Singh, S., Braus-Stromeyer, S.A., Timpner, C., Valerius, O., von Tiedemann, A., Karlovsky, P., Druebert, C., Polle, A., and Braus, G.H. 2012. The plant host *Brassica napus* induces in the pathogen *Verticillium longisporum* the expression of functional catalase peroxidase which is required for the late phase of disease. Molecular Plant-Microbe Interactions: MPMI **25**:569-581.

Soltis, D.E., and Soltis, P.S. 1999. Polyploidy: recurrent formation and genome evolution. Trends in Ecology and Evolution **14**:348-352.

Stark, C. 1961. Das Auftreten der Verticillium – Tracheomykosen in Hamburger Gartenbaukulturen. Gartenbauwissenschaft **26**:493-528.

Thomas, R. 1993. The denaturation of DNA. Gene **135**:77-79.

Tjamos, E.C., Tsitsigiannis, D.I., Tjamos, S.E., Antoniou, P.P., and Katinakis, P. 2004. Selection and Screening of Endorhizosphere Bacteria from Solarized Soils as Biocontrol Agents Against *Verticillium dahliae* of Solanaceous Hosts. European Journal of Plant Pathology **110**:35-44.

Tjamos, S.E., Flemetakis, E., Paplomatas, E.J., and Katinakis, P. 2005. Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and

pathogenesis-related proteins gene expression. Molecular Plant-Microbe Interactions: MPMI **18**:555-561.

Ton, J., and Mauch-Mani, B. 2004. β-aminobutyric acid-induced resistance against necrotrophic pathogen is based on ABA-dependent priming for callose. The Plant Journal **38**:119-130.

Tsao, P.H. 1970. Selective media for isolation of pathogenic fungi. Annual Review of Phytopathology **8**:157-186.

Tyagi, S., and Kramer, F.R. 1996. Molecular beacons: probes that fluoresce upon hybridization. Nature Biotechnology **14**:303-308.

Typas, M.A., and Heale, J.B. 1980. DNA content of germinating spores, individual hyphal cells and resting structure cells of *Verticillium* spp. measured by microdensitometry. Journal of General Microbiology **121**:231-242.

Tzelepis, G., Bejai, S., Sattar, M.N., Schwelm, A., Ilbäck, J., Fogelqvist, J., and Dixelius, C. 2017. Detection of *Verticillium* species in Swedish soils using real-time PCR. Archives of Microbiology. **199**:1383–1389.

Valasek, M.A. and Repa, J.J. 2005. The power of real-time PCR. Advances in Physiology Education **29**:151-159.

Walker, N.J. 2002. A technique whose time has come. Science **296**:557-559.

Watson, J.D., and Crick, F.H.C. 1953. A structure for deoxyribose nucleic acid. Nature **171**:737-738.

Wysong, D.R., Christin, L., Sugar, A.M., Robbins, P.W., and Diamond, R.D. 1998. Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. Infection and Immunity **66**:1953-1961.

Yu, J.M., Cafarov, I.H., and Babadoost, M. 2016. Morphology, molecular identity, and pathogenicity of *Verticillium dahliae* and *V. longisporum* associated with internally discoloured horseradish roots. Plant Disease **100**:749-757.

Zeise, K., and von Tiedemann, A. 2002. Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. Journal of Phytopathology **150**:112-119.

Zeise, K. 1990. Erste Untersuchungen zu einer durch *Verticillium dahliae* Kleb. verursachten Fruchtfolgekrankheit an Winterraps (*Brassica napus* L. var. *oleifera* Metzger). PhD Thesis, Rostock, Germany, University Rostock.

Zheng, G.G., Chen, R.H., Wang, Y.C., Wang, K.R., and Zheng, X.B. 2005. Molecular detection of *Verticillium albo-atrum* by PCR based on its sequences. Agricultural Sciences in China **4**:760-766.

Zhou, L., Hu, Q., Johansson, A., and Dixelius, C. 2006. *Verticillium longisporum* and *V. dahliae*: infection and disease in *Brassica napus*. Plant Pathology **55**: 137-144.

Zimmerli, L., Metraux, J., and Mauch-Mani, B. 2001. β-aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. Plant Physiology **126**:517-523.

Chapter 2

2. Investigation of Spatial Distribution and Hybrid Lineage of *V. longisporum* of Canola at the First Reported Farm in North America

2.1 Abstract

The soil-borne fungus, *Verticillium longisporum* (Stark) Karapapa et al. (1997) is a common pathogen of Brassica crops in Europe and causes Verticillium stripe in oilseed rape crops. *Verticillium longisporum* was first identified in North America on canola at a research farm in Manitoba, Canada in 2014. Past research has established that *V. longisporum* is a diploid hybrid either of two species of *V. dahliae* (parental lines D2 or D3) or two other unknown species (A1 or D1). Since this is the first ever-documented case of this pathogen of canola in North America, very little is known about its spatial distribution, ability to establish in soil, virulence, and hybrid lineage. To help address these uncertainties, this research study was set out with the objectives to investigate the spatial distribution of *V. longisporum* at the research farm, and to determine the hybrid origin of *V. longisporum* isolates in Manitoba. Fifty nine areas with management differences from 2002 to 2015 were identified at the research farm and a total of 194 soil samples were collected from these areas in fall 2015. A soil extraction real-time PCR assay confirmed 76 of 194 samples positive for the pathogen. The highest pathogen density reported was 69.65 pg *V. longisporum* genomic DNA per g of soil with a mean of 3.99 pg/g soil across all 194 samples. A triplex PCR-based hybrid lineage determination of isolates from the farm indicated the pathogen A1/D1 type, the most virulent hybrid lineage of canola. Additionally, the pathogen seemed fairly established in the farm soil, as it was detected at areas where a suitable host (canola) was not grown in the last 12 years.

2.2 Introduction

The soil-borne phytopathogen *Verticillium longisporum* is responsible for an economically important disease in oilseed rape known as Verticillium stripe (Knüfer 2011). Over the past few years there has been a rapid spread of the pathogen, reported in 10 countries in 2013 (Inderbitzin and Subbarao 2014) to 16 countries in 2017 (Canadian Food Inspection Agency, 2016). This brisk spread and a broad range of infected hosts can be due to long-term resting structures known as microsclerotia and the diploid nature of the pathogen, respectively (Butler and Day 1998; Heale and Karapapa 1999). Microsclerotia are black coloured, highly compacted masses of melanized hyphae that in *V. dahliae* (parent of *V. longisporum*) are known to survive in soil for up to 14 years (Short et al. 2015). The disease cycle of *V. longisporum* (Fig. 2.1) is initiated by microsclerotia on stimulation by host root exudates rich in carbon and nitrogen (Mol and van Riessen, 1995; Huisman 1982; Inderbitzin et al. 2013). Upon stimulation, microsclerotia germinate, producing hyphae that are responsible for penetrating lateral roots and root hairs of the host (Eynck et al. 2007). Once inside its host, the pathogen colonizes and blocks the xylem vessel and produces conidia (asexual spores) to enhance colonization through the host transpiration system (Johansson, 2006; Eynck et al. 2007; Eastburn and Paul 2007). Following the blocking of xylem vessels, the host begins to display symptoms of the disease such as stunting, senescence (Johansson et al. 2006), black striped stem, and appearance of black specs (microsclerotia) in the epidermis of the stem (Heale and Karapapa, 1999; Dixelius et al. 2005; Hein 2017). Later in the disease cycle, microsclerotia are produced in/on the stem pith and upon decay of the host are returned to the soil to serve as resting structures until a new host arrives (Dixelius et al. 2005). Early detection of the pathogen can therefore be extremely vital in disease forecasting (Kang et al. 2014). Detection methods for *V. longisporum* in soil, based on selective media, wet sieving

method (Andersson 2003) and real-time PCR (Debode et al. 2011) have been successfully employed.

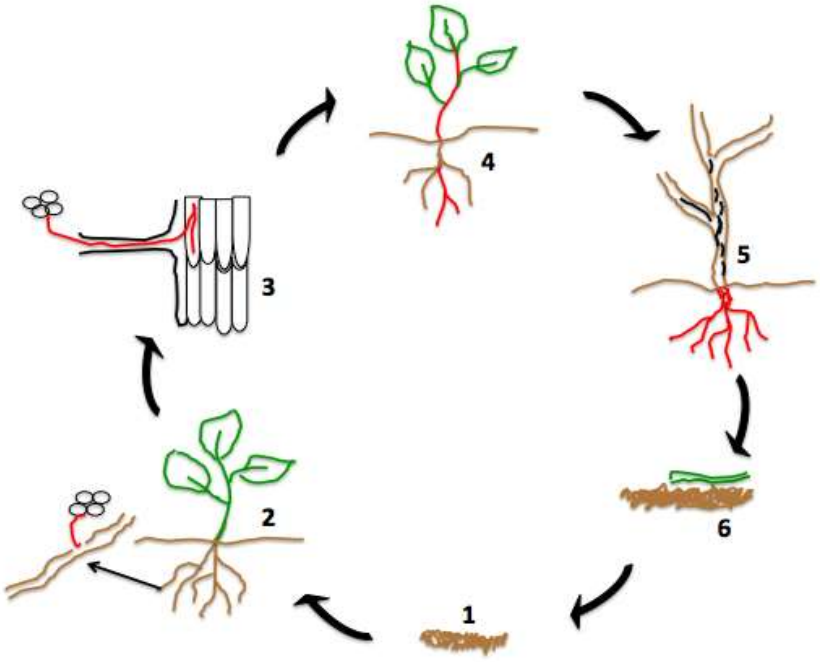


Figure 2.1. Illustration of the Verticillium stripe disease cycle caused by *V. longisporum*. 1. Pathogen resting structure-microsclerotia in soil. 2. Microsclerotia stimulated by host's root exudates and penetration of root by hyphae. 3. Colonization of xylem vessels and production of conidia. 4. Spread of conidia to other parts of the plants. 5. Stunting and senescence in the host and production of microsclerotia in the stem. 6. Decay of the infected host and dispersion of microsclerotia back to the soil, modified from Depotter et al. (2016).

All species of the fungal class Ascomycota are haploid, one exception being *V. longisporum*, which is a non-parasexual recombinant stable diploid (Inderbitzin et al. 2011b) that has a genome twice the size of its parent *V. dahliae* (Typas and Heale 1980). *Verticillium longisporum* acquired a diploid state via hybridization events occurring between four parental lineages across three different species (Fig. 2.2). These lineages include *V. dahliae* D2, *V. dahliae* D3, species A1 and species D1 (Heale 2000). Species A1 and D1, yet to be isolated are unidentified species and have been arbitrarily named so (Heale 2000). Species A1 and D1 were

previously incorrectly identified as *V. albo-atrum* (Clewes et al. 2008) but later the identification was corrected. Hybridization events in the polyphyletic pathogen (Heale 2000) occurred at least three different times (Collins et al. 2003) resulting in three *V. longisporum* hybrid types. Species A1 being involved in all hybridization events, independently hybridizing with species D1, *V. dahliae* lineage D2 and *V. dahliae* lineage D3, resulted in the following *V. longisporum* hybrid types: A1/D1, A1/D2 and A1/D3 (Inderbitzin et al. 2011b).

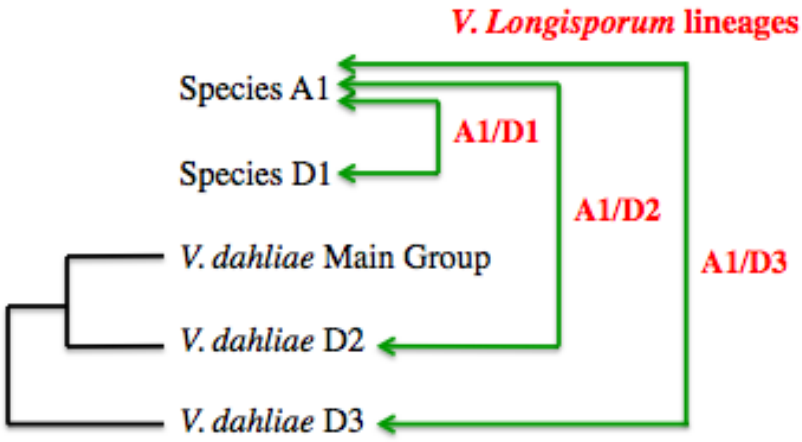


Figure 2.2. Illustration of hybridization events leading to the formation of three *V. longisporum* hybrids A1/D1, A1/D2 and A1/D3, modified from Inderbitzin et al. (2013).

The three resultant hybrids differ from one another and their parental lineage in terms of pathogenicity and virulence. In comparison to the parent *V. dahliae, V. longisporum* is more virulent on Brassicaceae hosts, where the virulence and choice of host depends on the hybrid type (Heale and Karapapa 1999; Inderbitzin et al. 2011b). *Verticillium longisporum* hybrid A1/D1 for example is most virulent on canola and cauliflower, whereas hybrid A1/D2 is most virulent on horseradish and cabbage. *Verticillium longisporum* hybrid A1/D3 infects canola and cauliflower but virulence is not as severe as hybrid A1/D1. In a study conducted by Novakazi et al. (2015), it

was discovered that hybridization has also enabled *V. longisporum* hybrids to surpass or equal the virulence of parent *V. dahliae* on some non-Brassicaceae hosts like tomato, eggplant, lettuce and watermelon. Hybridization involving four different parental lineages has therefore enabled *V. longisporum* to acquire higher virulence and a broader range of susceptible hosts as compared to the parent *V. dahliae* (Novakazi et al. 2015). Some of the most common crops infected by the pathogen include wheat, pea, oats (Johansson et al. 2006), broccoli, cabbage, cauliflower, horseradish, field mustard, turnip and oilseed rape (Horiuchi et al. 1990).

The oil rich crops oilseed rape (*Brassica napus)* and canola (developed from *B. napus*, *B. rapa* or *B. juncea* seeds), are often described interchangeably but technically they do not refer to the same crop (McInnis 2004). Oilseed rape is the predecessor of canola and was developed by Agriculture and Agri Food Canada and the University of Manitoba in the 1970's (Canola Council of Canada 2017d). Canola was developed by crossbreeding of oilseed rape to reduce glucosinolate, erucic acid and eicosenoic acid content in the product (McInnis 2004). Glucosinolate (3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate) (Canola Council of Canada 2017d) are growth inhibitory compounds found in the protein meal fraction of the crushed crop residual that can be harmful to livestock. On the other hand, erucic acid and eicosenoic acid are considered inedible and toxic to humans if consumed in high doses. Unlike oilseed rape, canola was engineered to have a fatty acid profile with less than two percent erucic acid and the non-oil component with less than 30 μmol/g of glucosinolates (McInnis 2004). This made canola an ideal product for commercial use (Canola Council of Canada 2017d). Canola seeds are 44% oil and 56% high protein meal. The oil is rich in essential omega-3 and omega-6 fatty acids and compared to other commercial oils, canola oil has the lowest level of saturated fats (Canola

Council of Canada 2017b). The high protein meal produced from the seeds serves as animal feed for cattle, poultry, swine and fish, and has shown to increase milk production by one liter per day in dairy cows (Canola Council of Canada 2017c).

In just four decades, canola has established a billion dollar economy and has become an extremely profitable crop for its farmers. An analysis conducted by the Canola Council of Canada suggested that the average economic impact of canola on the Canadian economy was C$26 billion per year with a wage impact of C$12.51 billion. The analysis reported a 202% upward increase in the economic impact of canola from 2004 to 2017 (Canola Council of Canada 2017a).

In the last four years there have been two reports of canola infection by two *Verticillium* species in North America. The more recent report was in 2016 from an Alberta farm, where canola infection by *V. dahliae* isolate A1-SS05 was reported for the first time (Hwang et al. 2017). The earlier report is from the current study where in October 2014, canola infection by *V. longisporum* was detected at a research farm just south of the city of Winnipeg by Manitoba Agriculture and the Department of Plant Science at the University of Manitoba. *Verticillium longisporum* infestation in cabbage, cauliflower (California) and horseradish (Illinois) has been recorded in North America in the past (Novakazi et al. 2015). However, this was the first occurrence of canola infestation by the pathogen in the continent. Manitoba Agriculture then sent the suspect canola residue to Agriculture and Agri-Food Canada (AAFC) for further identification. AAFC confirmed *V. longisporum* genotype A1/D1 and shared their findings with the Canadian Food Inspection Agency (CFIA). In November 2014, the CFIA resampled the farm and confirmed the presence of *V. longisporum* A1/D1 in Manitoba. In February 2015, the infested farm was placed under quarantine restrictions to reduce the risk of the pathogen spreading to new areas. These restrictions were lifted in July 2015 when the CFIA discovered the

pathogen was dispersed in the neighboring fields of the infested farm. From August 2015 to January 2016 the CFIA conducted a comprehensive soil survey to evaluate the extent of distribution of *V. longisporum* across Canada. The survey was conducted in nine provinces and a total of 1074 canola fields were tested. Analysis conducted by Plant Pathology Laboratory, ON revealed six of nine provinces tested positive for the pathogen, with the highest number of *V. longisporum* fields interestingly found in Manitoba.

Initial field trials of oilseed rape in Europe resulted in up to 80% disease incidence and up to 50% yield loss in single field crops infested by *V. longisporum* (Novakazi et al. 2015). However, the overall impact of this pathogen on oilseed rape yield in North America is still unknown. The severity of pathogen infestation can vary with environmental conditions like soil pH, temperature and moisture which differ between the two continents. This study of *V. longisporum* of infection of canola in North America is the first of its kind and there is very little information available about its concentration and ability to establish in soil, and virulence on canola. Therefore, the objectives of this study were to investigate *V. longisporum* density, its spatial distribution, and hybrid lineage at the research farm found positive for the pathogen.

2.3 Materials and Methods

2.3.1 Soil Collection from the Farm

The current study commenced in fall 2015 with sampling of the research farm soil. Several varieties of crops like canola, wheat, oat, barley, soybean, corn and alfalfa are grown on the farm with a four-year rotation at any given area. The research farm also allows various agricultural companies like JRI, Bayer, Univar, Advanta, Dupont, BASF, Pioneer, Monsanto, and DOW Agrosciences, as well as the University of Manitoba, to use several areas of the farm as

areas to run field trials. Prior to sampling, cropping history of the farm and Google Earth images from 2002 to 2015 were used to identify areas that were used for field trials and thus deviated from the four-year rotation practice. Using PowerPoint software, polygons were drawn around these unique areas to delineate them from the surrounding areas (Appendix I). All polygons from 2002 to 2015 were plotted as one map so that areas with a unique cropping history could be identified on one image (Fig. 2.3). GIS (Geographic Information System) mapping was used to delineate these areas from each other and a unique number was assigned to each area (Fig. 2.4). The GIS mapping for the 500-acre farm assigned a total of 59 areas. Google Earth Imagery for some years from 2002 to 2015 was not produced (Appendix I) and polygons were not generated for those years. This was overcome by drawing polygons on all areas of the 2015 Google Earth image, such that the entire farm could be sampled.

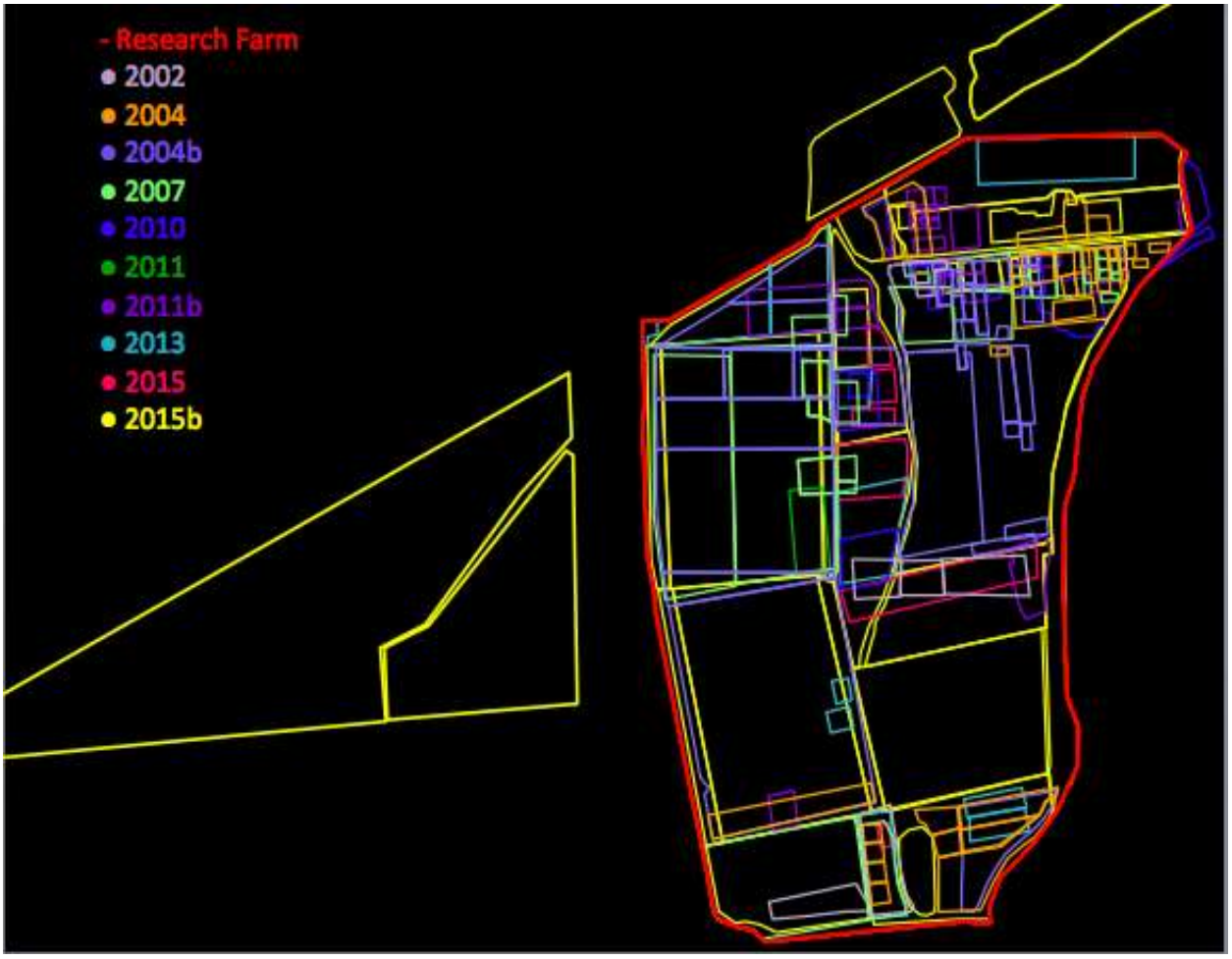


Figure 2.3. Areas with different cropping history (2002-2015) at the research farm delineated by year-wise colour-coded polygons.

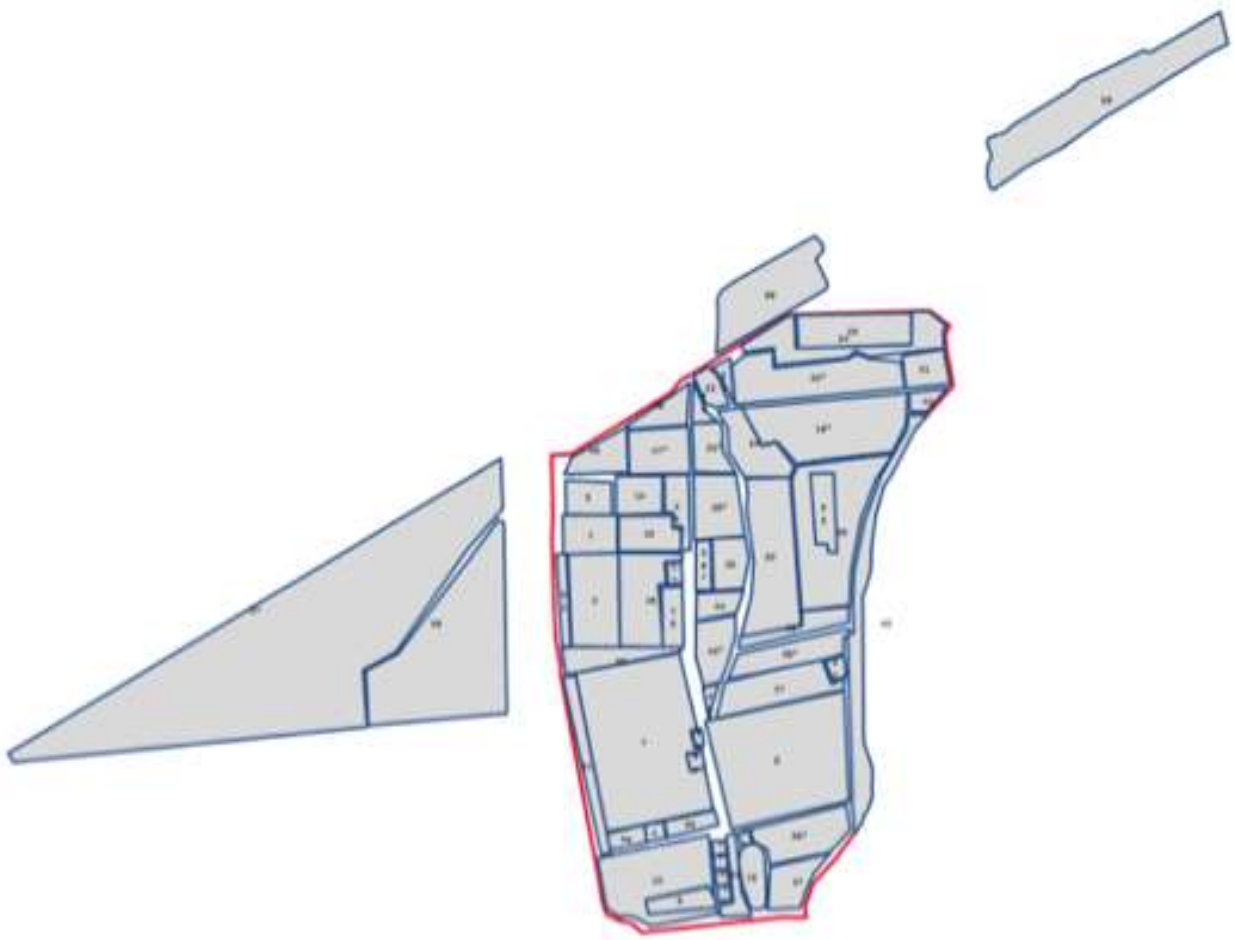


Figure 2.4. GIS image of the 500-acre research farm delineated into 59 areas that had a unique cropping history (2002-2015).

From each area, using 3.5 cm wide standard soil probes (dutch auger), a total of 10 soil sub-samples were collected to a 30 cm depth. In order to achieve this, a central point was established on each area. A three-meter rope was then used to set a radius around the central point and nine flags were placed at equidistance on the circumference of the circle (Fig. 2.5). Sampling was achieved by collecting one sub-sample from the central point together with sub-samples from each of the nine flags. All sub-samples (1+9) were placed into Ziploc bags and a GPS coordinate of the central point at each area was recorded.

Figure 2.5. Illustration of the 2015 soil sampling from the research farm found positive for *V. longisporum*.

To avoid cross-contamination, soil probes were cleaned with virkon solution between collections from different areas. Virkon (active ingredient: potassium peroxymonosulfate) was used as a disinfectant to sanitize the surface removing bacteria and fungi. All of the samples were transported to the University of Manitoba and stored at 4°C. While drawing the polygons on the Google Earth image, some areas were identified with a more complicated history than the rest (Appendix, I). These areas were sampled extensively such that the main area was divided into 6 to 24 sub-areas and 10 sub-samples were collected from each sub-area. The total number of soil samples collected for analysis from the 59 different areas at the farm was 194.

2.3.2 Sample Processing

Sample processing of collected soil commenced in the fall of 2016. The soil was removed from 4°C storage and dried. To facilitate drying, the 10 sub-samples from every area were manually broken into smaller pieces (1 cm^3). The broken pieces were placed on a large metal tray (sterilized with 70% ethanol) and dried at room temperature for seven days. The segregation was such that all sub-samples (10) from each area (194) were placed together on one tray.

After seven days of drying, soil samples on trays were thoroughly mixed by hand (with sterilized gloves to avoid cross contamination) and divided into quarters. Following the seven-day drying, it was found that the soil had become extremely hard which made the downstream pulverization step extremely difficult. To avoid this issue, gradual breakdown of soil every day during the seven-day drying period was found beneficial. One quarter of the dried soil weighing approximately 300 g was saved and the remaining soil was discarded. Weighing equipment was sterilized with 70% ethanol in between samples, to minimize cross contamination. The 300 g quarter was then placed into a plastic bag and labeled with appropriate area number and collection date and stored at room temperature. Using a sterilized mortar and pestle (soaked in 10% bleach overnight and autoclaved at gravity cycle, 121°C, 25 psi for 30 minutes), the 300 g quarter was then pulverized. The pulverized soil was passed through a no. 45 (425 μm) sieve to collect at least 100 g of fine powdered soil. The remaining soil was discarded and the 100 g pulverized soil was placed into plastic specimen cups and stored at 4°C. The sieve used above was chosen to be no. 45 (425 μm) because the microsclerotia of *V. longisporum* is known to range between 30-300 μm in diameter (Yu et al. 2016) and thus a sieve > 300 μm mesh was required for the propagules to pass through. To avoid cross contamination the sieves were cleaned under high air pressure hose to remove any soil residual grains.

2.3.3 DNA Extraction from the Processed Soil

A PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Solana Beach, CA) was used to extract genomic DNA from the processed soil. From the pulverized soil, 0.25 g was weighed and transferred to power-bead tubes (provided in the kit). The downstream steps were found to erase the labeling from the cap therefore the tube itself was labeled too. Using sterile tips and micropipette, 60 µl of Solution C1 was added to the power-bead tubes and vortexed for 2 seconds. Solution C1 is a Sodium dodecyl sulphate (SDS) based detergent that was used to disrupt cell membrane lipids and proteins. SDS is an anionic detergent that solubilizes proteins by donating positive charge and breaks open the cell membrane, releasing the nuclear component: DNA. SDS is also responsible for denaturing histones, the DNA binding proteins (Tan and Yiap 2009). The power-bead tubes mixed with Solution C1 were horizontally secured and mixed in a mini-beadbeater16 (BioSpec Products, Bartlesville, OK) at 3450 strokes/min for 2 minutes. This step made sure there was thorough chemical and mechanical homogenization of cell components. The power-bead tubes from the bead-beater were then centrifuged at 12,000 x g for 2 minutes. Approximately 200-400 µl supernatant was transferred into new sterile 2 ml collection tubes (provided in the kit). A 250-µl volume of Solution C2 was added to the supernatant, tubes were vortexed for 5 seconds and incubated at 4°C for 5 minutes. Solution C2 is a precipitate reagent that removed any non-DNA inhibitors like acids and proteins, and yielded those as a pellet after centrifugation. After 5 minutes incubation, the tubes were centrifuged at 12,000 x g for 2 minutes. Avoiding the pellet, up to 600 µl of supernatant was transferred into fresh sterile collection tubes. A 200-µl volume of Solution C3 was added to these tubes, vortexed for 2 seconds and incubated at 4°C for 5 minutes. Solution C3 is used as a secondary wash to remove any proteins and DNA inhibitors not removed by Solution C2. After 5 minutes incubation, the tubes were centrifuged at 12,000 x g for 2 minutes and up to 750 µl of supernatant

was transferred into fresh sterile collection tubes. Solution C4 was gradually added to the tubes until the mixture reached the upper ring of the collection tube. Solution C4 is a high salt solution that causes DNA to precipitate and bind to the Spin Filter tube's silica membrane in the downstream step. Following the addition of Solution C4, the tubes were vortexed for 5 seconds. A 650-µl volume solution from the collection tube was loaded onto clean the Spin Filter tubes (provided in the kit) and centrifuged at 12,000 x g for 2 minutes. Flow through was discarded and another 650-µl volume of solution from the collection tube was loaded onto the Spin Filter tubes. Tubes were centrifuged at 12,000 x g for 2 minutes and the flow through was again discarded. The remaining solution from the collection tube was loaded on to the Spin Filter tube and centrifuged at 12,000 x g for 2 minutes. Flow through was discarded once again. A 500-µl volume of Solution C5 was added to the Spin Filter tubes, centrifuged at 12,000 x g for 35 sec and the flow through collected was discarded. Solution C5 is an ethanol-based solution used to wash the DNA and release it from the bound silica. The tubes were again centrifuged at 12,000 x g for 1 minute but this time the spin filter (detachable) was removed from Spin Filter tubes and carefully placed into new collection tubes. The tubes were centrifuged again to remove any residual ethanol from Solution C5. A 50-µl volume of Solution C6 was added right in the centre of the white filter membrane of the spin filter and the tubes were allowed to stand for 2 minutes. Solution C6 is a Tris buffer based (10 µM) reagent that maintains a pH of 7.5 to 9 and prevents nucleic acid degradation from inhibitory enzymes. At the end of 2 minutes the tubes were centrifuged at 12,000 x g for 40 sec, the spin filters were discarded as the DNA had eluted down into the collection tubes. Using Solution C6 as a blank, the extracted DNA was quantified on the NanoDrop Spectophotometer (Thermo Scientific, Wilmington, DE) to determine the concentration (ng/µl) and its purity (260/280 ratio). The NanoDrop Spec determined the purity of the extracted DNA by measuring UV light absorbance of the sample at 260 nm (absorbed by

DNA) and 280 nm (absorbed by proteins and phenols). The Spec calculated and reported the 260/280 ratio of the sample. 260/280 values close to 1.8 are considered pure for DNA and values considerably lower indicate protein contamination (Desjardins and Conklin 2010). Following purity measurements, DNA samples were stored at -20°C until the next real-time PCR quantification step. All DNA tubes were carefully labeled with the corresponding area number from which the soil sample was collected.

2.3.3.1 Protocol Optimization

The above-described protocol was an optimized version of the original. The original protocol suggested only 1 minute of centrifugation followed by the bead-beater step. This was changed to 2 minutes centrifugation after a series of optimization steps testing from 1 minute through 5 minutes, which increased the yield of supernatant. At the Spin Filter steps where centrifugation occurs thrice, the original protocol of 11,000 x g for 1 minute was optimized to 12,000 x g for 2 minutes. Also the 675 µl volume loaded onto the Spin Filter tubes was reduced to 650 µl to prevent splashing of solution from the overfilled tubes onto the insides of the centrifuge. These steps improved the 260/280 ratio of the extract and therefore yielded purer DNA.

2.3.4 Identification and Quantification of *V. longisporum* genomic DNA in Extracts

A SYBR®Green based real-time PCR assay for quantification of *V. longisporum* in the above extracted DNA samples was performed using a protocol designed by Banno et al. (2011). The real-time PCR assay was tested by Molina (2016) and found to be specific for *V. longisporum* detection with no cross detection for any other Verticillium species.

All tubes and pipette tips used in the PCR assay were sterilized before use by autoclaving. The collection tubes containing extracted DNA samples were removed from the -20°C freezer and placed in the fridge to thaw at 4°C. The DNA samples once thawed were diluted 10 fold using autoclaved distilled water. For every sample, a PCR reaction mixture of total volume of 20 µl was prepared in Bio-Rad optical 0.2 ml 8-cap flat tubes and strips (Bio-Rad Laboratories, Hercules, CA). The reaction mixture contained; 2 µl of 1:10 diluted DNA, 10 µl Ssofast Evagreen Supermix (Bio-Rad Laboratories), 0.6 µl of each primer (10 µM) and 6.8 µl distilled water. SYBR®Green primers specific to *V. longisporum* (Vlsp- F1: AGCCTGAGTCACGAGAGATATGGG, Vlsp- R4: CAAACCACGCCACTGCATTCTCGT; Banno et al. 2011) were obtained from IDT (Integrated DNA Technologies, Coralville, IA). The primers Vlsp F-1 and Vlsp R-4 are complimentary to the group-I intron within the 18s rDNA of *V. longisporum* species, making them specific for detection of the pathogen (Banno et al. 2011). The primers were delivered at a concentration of 100 µM, and were diluted to 10 µM using autoclaved distilled water. The reaction mixtures were prepared on a 96 well plate and to reduce variability, the assay was performed in technical replicates of four for every sample. The subsequent four Cq values obtained for every sample were then averaged to obtain one Cq value per sample. The PCR assay was conducted using a CFX96 detection system (Bio-Rad Laboratories), programmed for 2 min at 98°C and 40 cycles of 5 seconds at 98°C and 5 seconds at 60°C. To distinguish potential primer dimer and nonspecific amplification products, amplification was followed by melt curve analysis at temperatures ranging from 65 to 95 °C, with temperature increased by 0.5 °C every 5 sec. The assay was performed with four sets of control and one set of non-template control (no DNA control, reaction volume of 18 µl). The control setup for the assay was as follows, positive DNA control (DNA from *V. longisporum* isolate 243378e (1ng/µl)), negative DNA control (DNA from non-*Verticillium* species *Gibellulopsis*

nigrescens isolate MBGn80 (1ng/µl)), positive soil control (1:10 diluted DNA from *V. longisporum* positive soil), and negative soil control (1:10 diluted DNA from *V. longisporum* negative soil). The 1:10 dilution between the control soil extracts and sample extracts was kept consistent in the assay. The DNA controls served as checks for the real-time PCR assay whereas the soil controls served as checks for the DNA extraction assay.

To determine the concentration of *V. longisporum* in every farm soil extract, standard concentrations of *V. longisporum* isolate PD624 (isolated by University of California, maintained by University of Manitoba) genomic DNA were obtained by preparing eight serial dilutions (ten fold each) ranging from 10 ng/µl to 10^{-6} ng/µl. In replicates of four, all eight standard dilutions were analyzed on the real-time PCR assay using the above-described protocol. Using the Bio-Rad CFX Manager 3.0.1215.0601 program, a standard curve was generated by plotting the Cq values of the standards versus the log of concentrations of DNA standards (Appendix II). Baseline threshold, y-intercept and slope values from the standard curve were recorded. The baseline threshold obtained from the standard curve was used to calibrate Cq values of all DNA extracts from the farm soil. Once calibrated to the standard curve baseline threshold, Cq values of all samples and standards were converted to *V. longisporum* pg/g soil (genomic DNA concentration in soil) using the following equations:

Mean Cq value for each sample was calculated using equation1.

$$\text{Mean Cq value} = \frac{Cq1 + Cq2 + Cq3 + Cq4}{4} \quad (1)$$

where, Cq1 through Cq4 are Cq values of the four replicates of every sample,

Mean Cq value was converted to ng genomic DNA per reaction by using equation 2.

$$\text{ng } \textit{Verticillium}\text{/reaction} = 10^{\frac{(\text{mean Cq value} - \text{y intercept})}{\text{slope}}} \quad (2)$$

where, slope and y-intercept were obtained from the standard curve of isolate PD624,

Reaction concentration of genomic DNA converted to density in soil using equation 3.

$$\text{ng } \textit{Verticillium}\text{/mg soil} = \frac{\text{ng } \textit{Verticillium}\text{/reaction}}{\text{mass of soil (mg)}} \quad (3)$$

where, mass of soil used in extraction was 250 mg (0.25 g),

And finally, density unit conversion by using equation 4.

$$\text{pg } \textit{Verticillium}\text{/g soil} = \text{ng } \textit{Verticillium}\text{/mg soil x 1000 x 1000 x dilution factor} \quad (4)$$

where ng is converted to pg by multiplying by 1000, mg converted to g by multiplying the equation by 1000, and dilution factor of 10 multiplied to the equation to factor in the ten-fold dilution of the DNA extracts prepared before the real-time PCR assay.

To examine the confidence range of the real-time PCR assay, another dilution series using isolate PD624 was established. Similar to the standard curve, eight serial dilutions ranging from 10 ng/µl to 10^{-6} ng/µl were prepared along with an additional dilution of concentration $5x10^{-7}$ ng/µl (half of the most diluted concentration in the former series). At the farm, the Red River

flows east of the commercial fields and passes around a non-commercial oak forest. Wild vegetation growing in the oak forest was observed. These included plants such as *Symphoricarpos albus*, *Bromus* species, *Cirsium arvense*, *Asclepias syriaca, Poa pratensis,* and *Rosa arkansana*, none of which are known to be host to *V. longisporum*. Hence, the soil from the forest was expected to be negative for *V. longisporum* genomic DNA. Therefore, to assign a minimum reportable pathogen concentration (pg/g soil) and the corresponding Cq value to the farm samples, soil samples from three areas in the oak forest were sampled in a V-shaped pattern and processed. The new dilution series (10 ng/µl to $5x10^{-7}$ ng/µl) and the oak forest samples were analyzed similar to the above-explained method on the real-time PCR assay.

2.3.5 GIS Mapping *of V. longisporum* Concentration and Host Cropping History

Since this was the first episode of *V. longisporum* infection on canola in North America, the ease of establishment in soil was unknown. A high concentration of the pathogen at non-canola grown areas would suggest that the pathogen was not restricted to canola and thus was easily established in the farm soil. Therefore, a correlation between pathogen DNA concentrations in farm soil and number of years of canola grown at the farm was investigated using GIS mapping, linear regression and Spearman Rank Analyses (explained later) as visual and statistical tools, respectively.

Two GIS multi-layer maps of the research farm were created using aerial Google Earth imagery with *V. longisporum* concentration (pg/g soil) determined at each area, and Google Earth imagery with number of years canola was grown at each area. The maps were generated using ArcView GIS 3.3 (ESRI, Redlands, CA) software. The first GIS map was prepared by plotting the pathogen concentration on the corresponding GPS coordinate where the soil sample was

originally collected. In contrast the second GIS map was prepared by plotting the number of times canola was grown at each of the 59 areas from 2003 to 2015. Since the Google Earth imagery used for delineating the farm dated back to 2002, the farm owner provided cropping history from the same year and onwards from 2003. Therefore, canola years from 2003 to 2015 were plotted. Both GIS maps were compared to investigate association, if any, between the pathogen concentration and cropping history of canola at the farm.

2.3.6 Hybrid Lineage Determination of *V. longisporum* Isolates in Manitoba

The second objective of this study was to identify the hybrid lineage of the pathogen at the research farm under investigation. The three *V. longisporum* hybrids differ in virulence based on the host under infestation. Identifying the hybrid lineage at the farm can help forecasting susceptible hosts and disease virulence. In order to identify the hybrid lineage of *V. longisporum* at the research farm, six isolates of the pathogen, originally isolated from the research farm, were obtained for the analysis. Isolates CDC600 and CDC602, isolated by Manitoba Agriculture, and isolates 243377d, 243377e, 243377e and 243378e, isolated by the CFIA during the National Soil Survey, were obtained (Appendix III). Two other *V. longisporum* isolates, PD348 and PD624 of known hybrid lineage (A1/D1) isolated by the University of California, were also obtained for the analysis and served as positive controls. Along with *V. tricorpus* isolate MBVt40 and *V. dahliae* isolate MBVd12 that served as a negative control and *V. longisporum* parental lineage control, respectively. Hybrid lineage determination was performed on all ten isolates in a two-step assay: DNA extraction followed by triplex PCR and gel electrophoresis.

2.3.6.1 DNA Extraction from *V. longisporum* Isolates

In a sterile laminar flow equipped with HEPA filters, individual PDA (Potato Dextrose Agar) plates were inoculated with the isolates, preferably in replicates of two. After 2-3 weeks following mycelial growth on the plates, DNA was extracted using a protocol designed by Dr. Maria Henriquez, AAFC (unpublished). All tubes and pipette tips used in the following assay were sterilized by autoclaving before use. About half of the mycelia growing on the PDA plates were aseptically transferred into sterile 2 ml tubes containing 1.066 g of 1 mm glass beads (BioSpec Products, Bartlesville, OK). Transfer of some agar along with the mycelia was inevitable but did not pose problems in the downstream steps. A 1000-µl volume of TES extraction buffer was added to each tube and vortexed for 5 seconds. A 100-µl volume of 1% SDS was added to the tubes and vortexed for 5 seconds. The tubes were then horizontally secured and mixed in the mini-beadbeater16 (BioSpec Products, Bartlesville, OK) at 3450 strokes/min for 3 minutes. The tubes were then centrifuged at 12,000 x g for 3 minutes and all of the supernatant and pellet (excluding the glass beads) was transferred into new 1.5 ml sterile tubes. A 2-µl volume of proteinase K was added to the tubes and vortexed for 5 seconds. The tubes were then placed in a water bath at 65°C for 1 hour. The following steps were performed under a fume hood. The tubes were removed from the water bath and 400 µl of 7.5 M ammonium acetate was added to each tube and vortexed for 5 seconds and incubated at room temperature for 10 minutes. The tubes were then centrifuged at 12,000 x g for 10 minutes and up to 800 µl supernatant from each tube was transferred into new 1.5 ml tubes. A 300-µl volume of chloroform-isoamyl alcohol was added to each tube, vortexed for 5 seconds and centrifuged at 13,000 x g for 10 minutes. A 700-µl volume of the top layer (without disturbing the middle layer) was transferred into new 1.5 ml sterile tubes. Again 300 µl chloroform-isoamyl alcohol was added to each tube, vortexed for 5 seconds and centrifuged at 13,000 x g for 10 minutes. A 600-µl volume of top layer of the

supernatant was transferred into new sterile tubes. Ice-cold isopropanol was added to each tube until the solution reached the top ring marked in the tube. The tubes were flicked by hand to mix the solution, labelled and stored at -20°C overnight in an anti-flammable freezer.

The next day all the tubes were centrifuged at 12,000 x g for 10 minutes, the supernatant was discarded. An 800-µl volume of cold 70% ethanol was added to each tube and using a 200 µl sterile pipette tip the pellet was manually broken. The tubes were then centrifuged at 12,000 x g for 5 minutes and the supernatant was discarded. The tubes were centrifuged briefly and the remaining supernatant was removed. On a large Kim wipe under the laminar flow, the tubes were allowed to air dry for 15 minutes to evaporate any remaining ethanol. A 50-µl volume of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA) was then added to each tube and the pelleted DNA was mixed thoroughly in the buffer. A 5-µl volume of RNase (1mg/ml) was added to each tube, vortexed for 5 seconds and incubated in the water bath for 1 hour at 37°C. Using TE buffer as a calibration blank, the extracted DNA was quantified on the NanoDrop Spectophotometer to analyse the concentration (ng/µl) and its purity (260/280 ratio). Following purity measurements, DNA samples were stored at -20°C until the next step in the hybrid lineage determination.

2.3.6.2 Triplex PCR and Gel Electrophoresis of *V. longisporum* Isolate DNA

Triplex PCR-based identification of *V. longisporum* hybrid lineage of isolates was performed using a protocol reported by Inderbitzin et al. (2013). All tubes and pipette tips used in the PCR assay were sterilized by autoclave. The tubes containing extracted DNA samples from the *V. longisporum* isolates were removed from the -20°C freezer and allowed to thaw. The DNA samples once thawed were diluted to a 10 ng/µl concentration using autoclaved distilled water as diluent. For every DNA sample, a PCR reaction mixture of total volume of 25 µl was

prepared in Bio-Rad optical 0.2 ml 8-cap flat tubes and strips. The reaction mixture contained 10 µl of template dilution (10 ng/µl), 12.5 µl GoTaq Colourless Master Mix (Promega Corp., Madison, WI), and 2.5 µl of triplex primer stock. The triplex primer stock was prepared by mixing three primer pairs in the following volumes; 6.25 µl each of primer pair D1f and AlfD1r, 6.25 µl each of primer pair A1f and A1r, 3.125 µl each of primer pair Df and Dr and 93.75 µl of distilled water. The initial concentration of each primer added in the preparation of triplex primer stock was 100 µM. Primers were obtained from IDT (Integrated DNA Technologies, Coralville, IA) with sequence as follows: D1f: CCCCGGCCTTGGTCTGAT and AlfD1r: TGCCGGCATCGACCTTGG, A1f: AAGTGGAGCCCCGTATCTTGAAT and A1r: CAACTGGCAACAGGGCTTGAAT and, Df: CCGGTCCATCAGTCTCTCTG and Dr: CTGTTGCCGCTTCACTCG. The PCR assay was conducted using vapo.protect detection system (Eppendorf AG Laboratories, VWR International Inc., Mississauga, ON), programmed for 2 min denaturation step at 94°C and 35 cycles of 10 seconds at 94°C, 20 seconds annealing step at 64°C and 1 minute extension at 72°C followed by final extension of 7 minutes at 72°C. At the end of cycle, PCR products were stored at -20°C until the next step.

Gel electrophoresis was performed in a large gel box with 16-sample comb. The gel was prepared with 1.35 g agarose (Invitrogen, Carlsbad, CA) and 2 µl GelRed DNA staining dye (Biotium, Fremont, CA) in 100 ml 0.5X TAE buffer. Agarose in concentrated TAE buffer, 1.35% w/v. 50X TAE buffer (242g tris base, 57.1 ml glacial acetic acid and 100ml of 500mM EDTA) topped up to 1 liter with distilled water and diluted 1:100 folds to form the 0.5X concentration. A 4-µl volume of each PCR product mixed with 2 µl of 6X (30% glycerol, 0.2% bromophenol blue, 10mM Tris-HCl pH 8.0, 1mM EDTA) loading dye (Thermo Scientific, Wilmington, DE) was loaded into the wells along with 1 µl of 1Kb DNA reference ladder (Thermo Scientific,

Wilmington, DE) loaded in the end wells on either side of the gel. The gel was run at 95 V for 45 minutes in TAE buffer and the amplicons were visualized under UV light in G:Box F3 (Syngene, Frederick, MD) using GeneSys program at exposure setting of 360 ms, lighting setting at T-LUM and filter setting at UV032.

Primer pair A1f/A1r are complementary to the EF locus of Species A1, primers pair D1f/AlfD1r are complementary to the GPD locus of Species D1 and primer pair Df/Dr are complementary to the ITS region of *V. dahliae* lineage D3 (Table 2.1). Therefore identification of the A1/D1 hybrid lineage is indicated by amplicons for EF and GPD alleles of 310 bp and 1020 bp. The A1/D2 hybrid is indicated by an amplicon solely for the EF allele of 310 bp and hybrid A1/D3 is identified by amplicons of the EF allele and ITS region of 310 bp and 490 bp.

Table 2.1. Details of primers used in the triplex PCR based identification of *V. longisporum* hybrid lineage. Includes complementary locus in the targeted species and expected PCR product band size**.**

Target Species	Target loci†	Primer pair	Expected PCR amplicon (bp)‡
Species A1	EF	A1f/A1r	310
Species D1	GPD	D1f/AlfD1r	1020
V. dahliae lineage D3	ITS	Df/Dr	490

†EF: elongation factor 1 alpha, GPD: glyceraldehyde-3-phosphate dehydrogenase and ITS: internal transcribed spacer.
‡bp: nucleotide base pairs.

2.3.7 Statistical Analysis

The Cq values, the coefficient of determination (R^2 efficiency) and efficiency of every real-time PCR assay were calculated automatically by the Bio-Rad CFX manager software 3.0.1215.0601 (Bio-Rad Laboratories). *Verticillium longisporum* concentration (pg/g soil) for all 194 samples was calculated from their respective Cq values using equations one through four. Finally, a histogram was generated to graphically report pathogen genomic DNA concentration in all samples.

Association between *V. longisporum* density and canola grown years at the farm was examined statistically. For this purpose, correlation between the two variables was determined using linear regression and Spearman Rank Correlation analyses. The linear regression was used to associate the proportional change in pathogen density (dependent variable) to the change in number of canola years (independent variable) at the farm. On the contrary, the Spearman Rank Correlation analysis was used to associate change in both the variables in a monotonic relationship. To avoid redundancy, Spearman Correlation was chosen over Pearson Correlation, since the latter also tests for linear relationship (similar to linear regression) between the two variables (Hauke and Kossowski 2011).

The data generated from both the GIS maps was summarized. *Verticillium longisporum* concentration of all areas at the research farm with the same number of canola years (2003-2015) were averaged and tabulated (Appendix IV). Using Microsoft® Excel®, Version 14.7.7 (Microsoft, Redmond, WA), these average concentrations were analyzed on linear regression with respect to the corresponding number of canola year(s). The data was then analyzed for correlation (R^2 value) and significance at $P < 0.05$.

For Spearman Rank Analysis the following hypotheses were examined. Null hypothesis: there is no correlation between pathogen concentration and number of canola grown years at the farm; alternate hypothesis: there is a positive correlation between pathogen concentration and number of canola grown years at the farm. For the analysis, pathogen concentration at areas with the same canola grown years were averaged and tabulated (Appendix IV). As a second step, ranks in ascending order were assigned to every canola year (R1) and to the corresponding average pathogen concentration (R2). From 2003 to 2015, canola years at the farm for any given area ranged from 0 to 7, thus the first set of ranks was assigned from 7 to 1 respectively. The second set of ranks was assigned to the corresponding average pathogen concentration. Lastly, the corresponding ranks between canola years and pathogen concentration were subtracted and squared. A rank coefficient (R) to determine the correlation between canola years and pathogen concentration was calculated manually using equation 5. The rank coefficient ranges between the value of +1 (positive correlation) and -1 (negative correlation). R-value close to zero represents no correlation (Rebekić et al. 2015).

$$(R) = 1 - \frac{6\sum d^2}{n^3 - n} \tag{5}$$

where, n is the sample size and d^2 is the square of the difference between the two ranks.

Significance of interaction in the Spearman Rank analysis was determined by evaluating the P-value, which was achieved by plotting the rank coefficient (R) and degrees of freedom on the Spearman Rank significance graph (Appendix V). The alternate hypothesis and correlation was declared significant at $P < 0.05$.

2.4 Results

2.4.1 *V. longisporum* Genomic DNA Concentration in Soil

The average reaction concentration of DNA extracted from the 194 soil samples was 4.96 ng/µl with approximate 260/280 purity of < 1.65, as reported by the Nanodrop Spectrophotometer. The relationship between the Cq values and log of DNA concentration on the standard curve generated for isolate PD624 was linear ($y = -3.06x + 20.597$, $R^2 = 0.99$, amplification efficiency = 1.12) with baseline threshold of 386.6, slope value of -3.06 and y-intercept value of 20.597 (Appendix II). The mean Cq values of the PD624 isolate on the standard curve ranged from 16.94 for the 10 ng/µl DNA standard to 33.38 for the 10^{-6} ng/µl DNA standard (Appendix VI). The mean Cq value of the *V. longisporum* positive DNA control (isolate 243378e) was 18.47 whereas the mean Cq value of the *V. longisporum* negative DNA control (isolate MBGn80) was 36.35. The mean Cq value of DNA from *V. longisporum* positive soil was 28.6, whereas the mean Cq value of DNA from the *V. longisporum* negative soil was 34. The melt curves revealed species-specificity of the assay by generating melt temperatures of 83°C for DNA extracts positive for *V. longisporum*. All extracts negative for the pathogen generated melt temperatures of 79°C (Appendix VII).

The mean Cq values of the samples from the oak forest were 34, 33.86 and 33.42, which corresponded with pathogen DNA concentrations of 1.67 pg/g soil, 1.85 pg/g soil and 2.58 pg/g soil, respectively. Moreover, the additional dilution ($5x10^{-7}$ ng/µl) on the second standard curve was undetected (no Cq value generated) by the Bio-Rad CFX Manager. Therefore, based on the results from forest samples and secondary standard curve, the most diluted standard (10^{-6} ng/µl) of isolate PD624 on the primary standard curve with Cq value of 33.38 and pathogen genomic

DNA concentration of 2.65 pg/g soil was assigned as the lowest threshold concentration for any sample to be considered positive for the pathogen. Any farm sample concentration < 2.65 pg/g soil was considered outside the range of confidence for this real-time PCR assay and unreportable for the pathogen.

For all of the 194 soil samples collected from the farm, a histogram was generated depicting the frequency of the pathogen concentration in samples (Fig. 2.6). The results were asymmetrically distributed in a highly right-handed skewed fashion (Doane and Seward 2011) with a mean *V. longisporum* DNA concentration across all 194 samples of 3.99 pg/g soil and sample median of 2.01 pg/g soil. Standard deviation and coefficient of variation across all 194 samples were 6.9 and 172%, respectively. Based on the lowest reportable pathogen concentration (> 2.65 pg/g soil), 76 of 194 samples (39.1%) were found positive for *V. longisporum* of canola from the first reported farm in North America. The mean pathogen concentration across positive samples was 8.19 pg/g soil. The results were normally distributed with a median of 5.16 pg/g soil, standard deviation of 9.5 and coefficient of variation as 116%, across the positive samples. The highest pathogen concentration found was 69.65 pg/g soil.

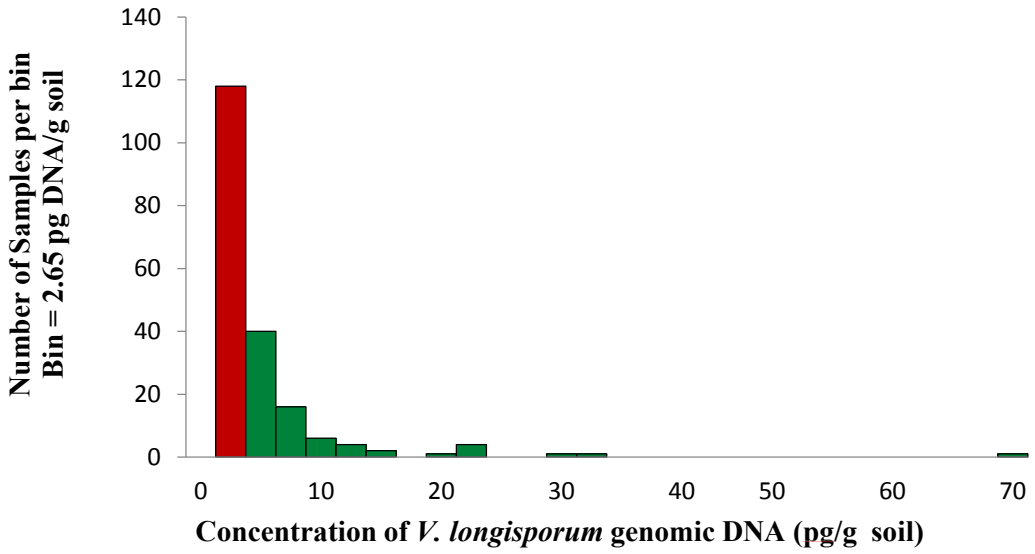


Figure 2.6. Histogram representation of *V. longisporum* genomic DNA concentration in soil samples collected from the Research Farm. In red, all the samples that tested negative for the pathogen (< 2.65 pg/g soil) and in green, all samples that tested positive for the pathogen (> 2.65 pg/g soil).

2.4.2 Spatial Distribution of *V. longisporum* with Respect to the Canola Cropping History at the Farm

Using GIS mapping, two multi-layer maps were generated to plot the pathogen concentrations (Fig. 2.7) on corresponding (geo-referenced) areas from where the soil was originally sampled, and canola cropping history at the farm from 2003 to 2015 (Fig. 2.8).

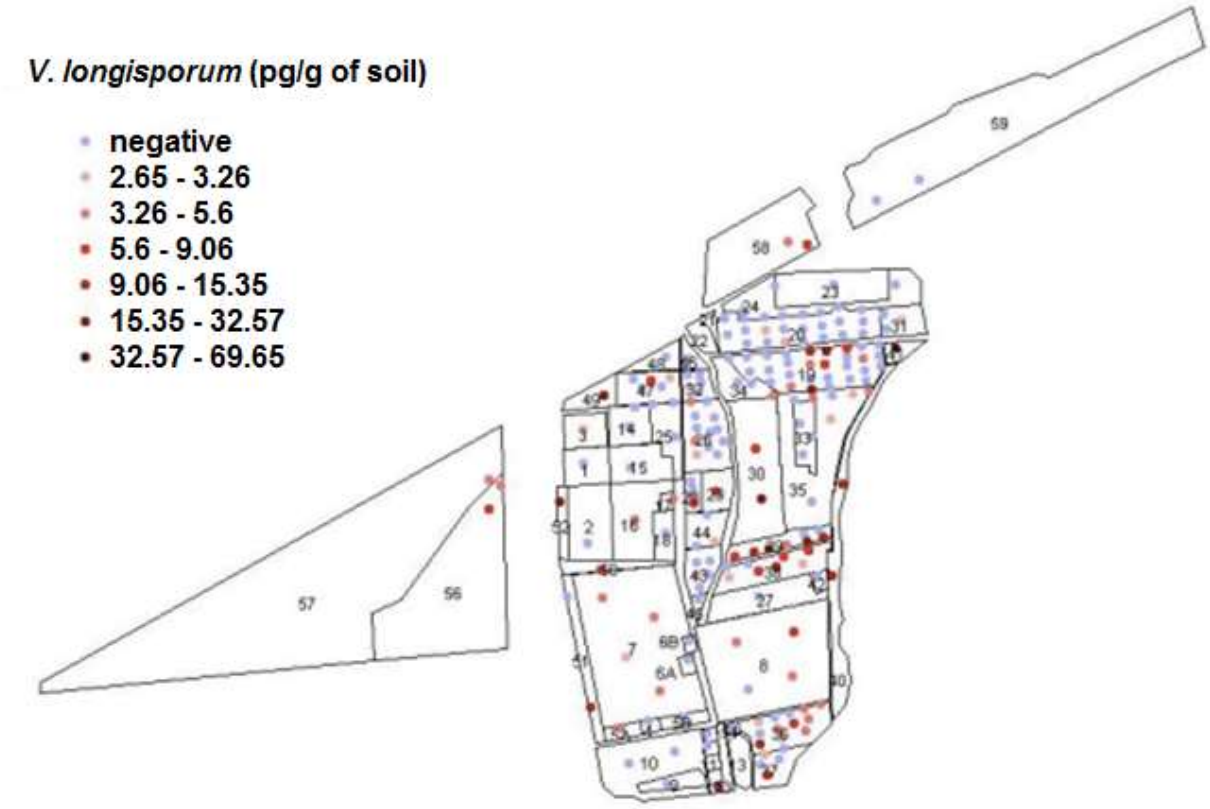


Figure 2.7. GIS multi-layer map of the research farm positive for *V. longisporum*. A visual representation of pathogen genomic DNA concentration plotted on the area from which the soil was originally. Soil samples with pathogen DNA concentrations < 2.65 pg/g soil are considered negative for *V. longisporum.*

Samples from different sub-areas from within the same area were found positive and negative for the pathogen. There seemed to be a high variation in spatial distribution of the pathogen at the farm. For example, area #8 had four sub-areas sampled (Fig. 2.7) and pathogen concentrations ranged from unreportable to 5.51 pg/g soil across the four sub-areas.

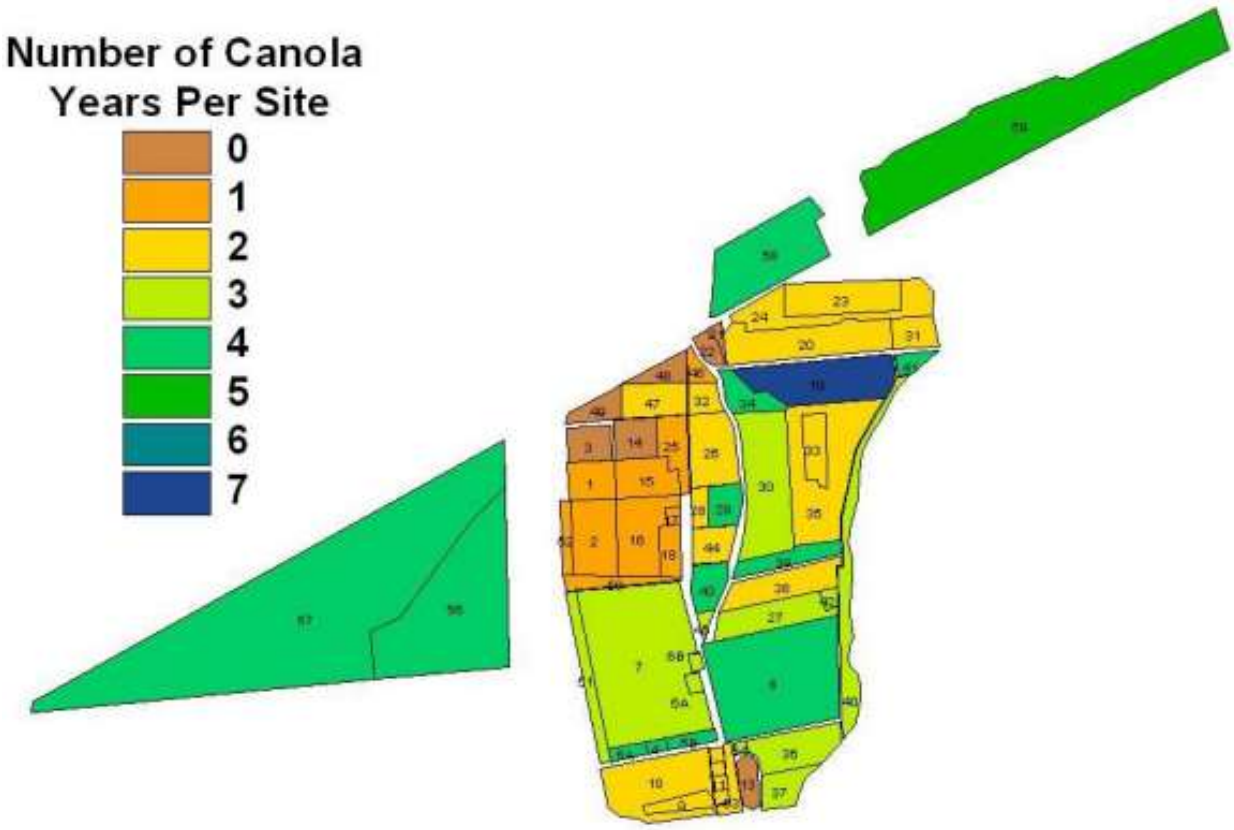


Figure 2.8. GIS multi-layer map of the research farm positive for *V. longisporum*. A visual representation of number of years canola was grown (2003 to 2015) at each of 59 areas sampled at the research farm.

As per the acquired cropping history, from 2003 to 2015 canola was not grown even once at area #3 and #49 (Fig. 2.8) and yet these areas were found positive for the pathogen (Fig. 2.7). In contrast, some areas (#6a, #6b, #11, #23, #24 etc.) with >1 canola year (Fig. 2.8) were found negative for the pathogen (Fig. 2.7). Therefore, based on the two GIS maps generated, there seemed to be no correlation between the pathogen concentration and number of canola grown years at the farm. A weak, non significant association ($R^2 = 0.28$, $P > 0.05$) reported by the linear regression plot (Fig. 2.9) between pathogen concentration and canola years at the farm, supported the results from the two GIS multi-layer maps.

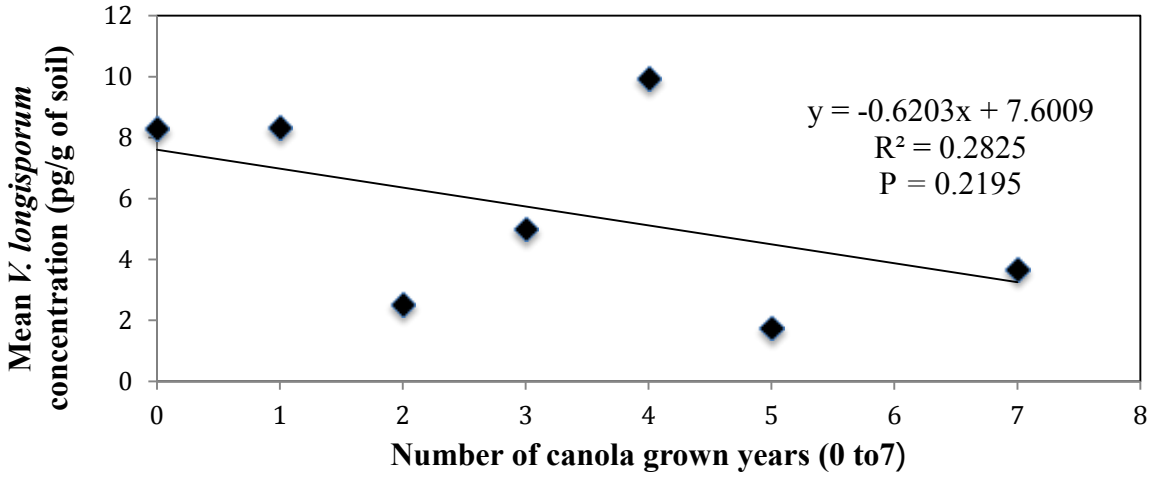


Figure 2.9. Relationship between mean *V. longisporum* concentrations (pg/g soil) at various areas of the research farm and corresponding number of canola grown years at those areas, from 2003 to 2015.

The results provided by the GIS maps and the linear regression plot were further confirmed using Spearman Rank Correlation Analysis. Using the data reported in Table 2.2 and equation 5, the R-value (correlation coefficient) of the Spearman Rank Analysis was found to be 0.39 with $P > 0.05$ (Appendix V). The alternate hypothesis of the Spearman Rank analysis was thus rejected and the null hypothesis was accepted; there was a weak, non-significant correlation ($R = 0.39$, $P > 0.05$) between pathogen concentration and canola grown years at the farm. Since the pathogen was not restricted to the areas where canola was mostly grown, the results suggested that *V. longisporum* was fairly established in the farm soil.

During the acquisition of the cropping history of the farm to investigate correlation between canola grown years and *V. longisporum* concentration, an interesting observation was made between the association of the pathogen to the number of wheat grown years at the farm (Appendix VIII). This will be discussed in Chapter 3 in detail.

Table 2.2. Spearman Rank Correlation Analysis for canola years and pathogen concentration at the research farm positive for the pathogen.

No. of canola grown years at various areas at the research farm from 2003 to 2015	No. of areas with the corresponding canola grown years	Rank 1 for canola years	Mean *V. longisporum* concentration at the corresponding areas (pg/g of soil)	Rank 2 for *V. longisporum* density	d (Rank1-Rank2)	d^2
0	7	7	8.3	3	4	16
1	10	6	8.34	2	4	16
2	16	5	2.54	6	-1	1
3	12	4	5	4	0	0
4	12	3	9.95	1	2	4
5	1	2	1.76	7	-5	25
7	1	1	3.67	5	-4	16

2.4.3 Hybrid Lineage of *V. longisporum* Isolates in Manitoba

DNA was extracted from the mycelia of six *V. longisporum* isolates (CDC600, CDC602, 243377d, 243377e, 243378d, and 243378e) from the farm. Concentration of the extracted DNA was measured on the Nanodrop Spec and DNA purity was reported in terms of the 260/280 ratio (Table 2.3). Analysis also included four controls; two *V. longisporum* isolates, PD624 (251.3 ng/µl) and PD348 (1202.1 ng/µl) of known hybrid lineage (A1/D1), one non-related *V. longisporum* isolate MBVt40 (524.3ng/µl) and one *V. longisporum* parental isolate MBVd12 (818.8 ng/µl) of known parental lineage (D3).

Table 2.3. DNA concentration of six *V. longisporum* isolates obtained from the research farm for hybrid lineage determination.

Isolate name †	DNA concentration (ng/µl)	260/280 ratio
CDC600	31.6	1.55
CDC602	210.3	1.97
243377d	242.2	2.04
243377e	426.6	2.00
243367d	497.3	2.00
243378e	1625.8	2.13

† *Verticillium longisporum* isolates from the farm: CDC600, CDC602, 243377d, 243377e, 243378d, and 243378e.

The multiplex PCR assay with three locus-specific primer pairs was successfully able to amplify the target loci (Table 2.1) on the extracted DNA. Visualization of the amplicon size using gel electrophoresis (Fig. 2.10) enabled determination of the hybrid lineage. The negative control isolate, MBVt40, did not amplify in the multiplex PCR assay and yielded no amplicon(s). Parental control isolate MBVd12 was identified by an amplicon of 480 bp indicating *V. dahliae* lineage D3, one of the parent species of *V. longisporum* A1/D3 lineage. The two *V. longisporum* reference isolates, PD348 and PD624, of known lineage were identified by amplicons of 310 bp and 1020 bp. An amplicon of 310 bp represented the amplified EF locus of species A1 and amplicon of 1020 bp represented the amplified GPD locus of Species D1. Therefore the two reference isolates were confirmed to be of A1/D1 hybrid lineage. All of the six *V. longisporum* isolates of unknown hybrid lineage from the research farm were similarly determined to be of A1/D1 hybrid lineage based on amplicons of 310 bp and 1020 bp. Hence, the hybrid lineage of

V. longisporum of canola at the first reported farm in North America was A1/D1, the most virulent hybrid on oilseed rape.

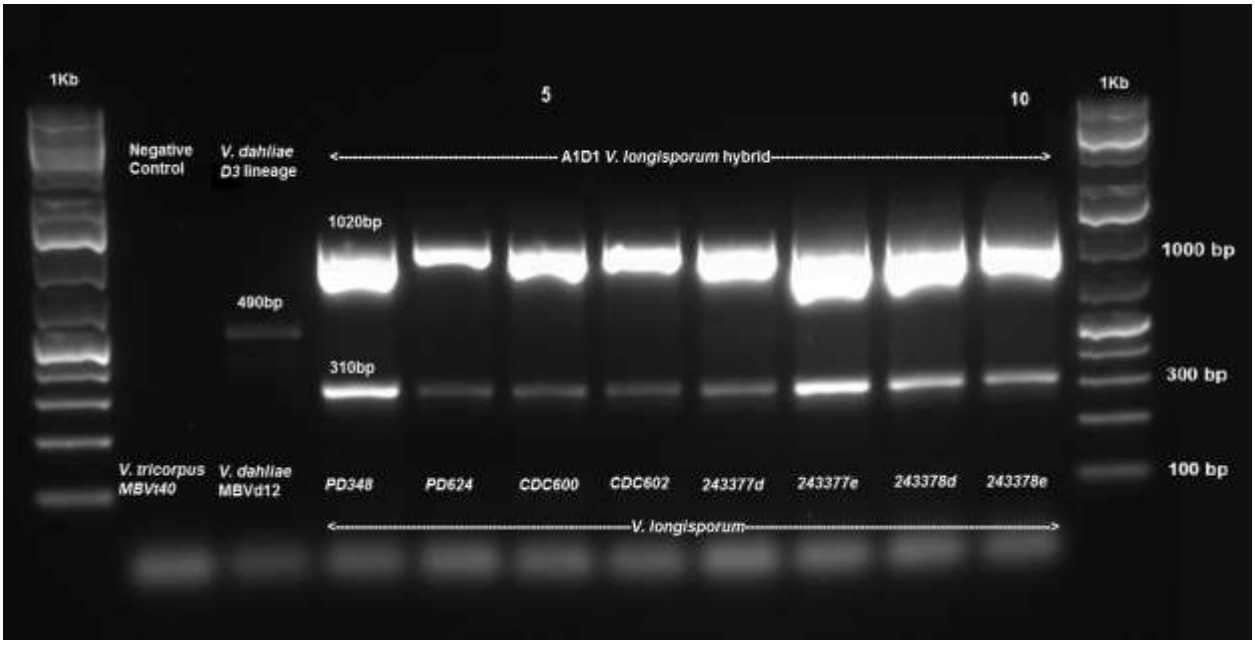


Figure 2.10. Gel electrophoresis of products of multiplex PCR for determination of *V. longisporum* hybrid lineage. The gel is delimited by two 1 Kb ladder on either side with relevant size markers labeled on the ladder on the right. Lanes are numbered from left to right; every fifth lane is numbered. Lane 1: *V. tricorpus* negative control. Lane 2: *V. dahliae* positive control for D3 lineage. Lane 3-4: *V. longisporum* reference isolates PD348 and PD624 of known A1/D1 lineage. Lane 5-10: Six *V. longisporum* isolates (CDC600, CDC602, 243377d, 243377e, 243378d, and 243378e) from the research of A1/D1 hybrid lineage.

2.5 Discussion

In November 2014, a research farm just south of the city of Winnipeg, MB was confirmed positive for *V. longisporum* by the CFIA. In the current study, the 500-acre farm was delineated into 59 areas from which 194 soil samples were quantified for pathogen DNA concentration using real-time PCR analysis. Spatial distribution and the pathogen ability to establish freely in soil with correlation to the host cropping history were also assessed. Finally, hybrid lineage of the pathogen at the research farm was also determined.

Prior to sampling the farm, Google Earth imagery for some years during the 2002 to 2015 timeline were not generated (Appendix I). The farm under investigation is quite dynamic in terms of variation in the types and locations of field trials that are conducted every year. For this reason, the missing Google Earth imagery and subsequent polygons could have revealed additional sub-areal boundaries enclosing locations that deviated from the conventional farming. Therefore, there may have been a possibility of delineating > 59 areas and subsequently sampling > 194 soil samples, hence increasing the sample size of the analysis and improving accuracy, diversity and validity of the current study. Nonetheless, the method involved in soil sampling and processing in the current study was quite comprehensive; therefore, chances of errors are low but still a possibility.

2.5.1 *V. longisporum* of Canola at the First Documented Farm in North America

Introduction of pathogens into new geographic areas can impose selection pressure and lead to spread of new diseases, originally absent in those regions. Comparison of environmental conditions between the original ecosystem inhabited by the pathogen and the new affected region can shed light on disease incidence and impact (Depotter et al. 2017).

In a DNA extraction study on different types of USA soil conducted by Schneegurt et al. (2003) a low yield of 1.7 ng/µl was reported for clay soil. This was found consistent with the current study where the soil was also rich in clay with a low average DNA concentration of 4.96 ng/µl across all farm samples. This was mainly because DNA in clay soil strongly adheres to the soil colloids, which makes it difficult to extract DNA from clay-rich soil samples (Schneegurt et al. 2003). In another recent study of DNA extraction from soil followed by PCR amplification, Fatima et al. (2014), using a commercial DNA extraction kit, reported DNA concentration of

0.79 ng/µl with 260/280 purity of 1.21, which was in line with the low 260/280 purity of < 1.65 reported in the current study. In a 2015 study using the MoBio DNA extraction kit, Tanasea et al. (2015) also reported low DNA concentration of 1.29 ng/µl from their assay (260/280 purity not determined). These findings were also in line with the low concentration reported from our assay using the same method. Soil, along with being a source to pathogen genomic DNA, is also an abundant source to several organic acids of broad ranges of solubility and charge distribution. This often makes it difficult to deduce an optimum extraction and amplification method using soil as a source to genetic matter (Schneegurt et al. 2003).

In a SYBR®Green based real-time PCR assay study conducted by Bilodeau et al. (2012) on *V. longisporum* parent *V. dahliae* in a strawberry field in the USA, the minimum reportable *V. dahliae* concentration was 1 microsclerotia/g soil, which corresponds to 1 pg *V. dahliae* DNA per g of soil (Tzelepis et al. 2017). These findings were in line with the current study where the minimum reportable *V. longisporum* DNA concentration was 2.65 pg/g soil which interestingly corresponds to 1 microsclerotia/g soil as well (each *V. longisporum* microsclerotium equals 2 pg DNA/g soil) (Tzelepis et al. 2017).

In research conducted by Debode at al. (2011) based on detection of *Verticillium* species in soil using real-time PCR, the lowest concentration reported for *V. tricorpus* was 0.01 pg/g soil, whereas the lowest concentration reported for *V. longisporum* was 0.04 pg/g soil. The assay used in the Debode at al. (2011) study was found to be more sensitive than the current study where the minimum reportable concentration was 2.65 pg/g soil. This suggested that the sensitivity of the assay in the current study could be further optimized. In the Debode at al. (2011) study, instead of extracting DNA directly from soil, a density floatation step with 70% sucrose solution preceded DNA extraction and real-time PCR analysis. The use of 100 g of soil for the density floatation

step (instead of 0.25 g for direct DNA extraction) avoids the use of a large multiplication factor in the *Verticillium* genomic DNA calculations that increases sensitivity and reduces overestimation of DNA concentration in the assay (Debode at al. 2011). Therefore, this is a possible explanation for the lower sensitivity and higher minimum reportable pathogen concentration found in the current study.

Quantification of pathogen DNA in our assay, using the Vlsp primer pairs complementary to the group-I intron in the 18s rRNA gene, has some limitations. The intron is found in the rRNA gene of the small sub-unit of the ribosome; therefore, there is high intra-genomic variability in copy number of the intron at most rDNA repeats among individual isolates. This can therefore lead to inconsistencies in the reported pathogen concentration when quantifying on a real-time PCR assay (Papaioannou et al. 2014). Moreover, the intron is also found absent in the 18s rRNA gene of the hybrid A1/D3 (Tran et al. 2012) and can thus, lead to false negatives using the Vlsp primer pairs. Quantification based on primer pairs complementary to the elongation factor 1-alpha (EF) loci in allele A1 (consistent in all lineages) is a possible alternative to overcome these limitations (Inderbitzin et al. 2013).

Recent research conducted on Swedish soil samples to quantify *V. longisporum* concentrations, revealed fascinating similarities with the current study (Tzelepis et al. 2017). In terms of geography, the location of areas sampled in the Tzelepis et al. (2017) study and those of the current study were both located in the Northern Temperate Region. Therefore the weather conditions at both the locations during growing seasons are expected to be similar. In terms of methodology, both studies involved sampling of areas in sub-samples of 10 and using real-time PCR for *V. longisporum* quantification. In the current study a range in the pathogen concentration ranging from 2.65 pg/g soil to 69.65 pg/g soil was reported, which was in line with a wide range

of concentration from 0.005 pg/g soil to 121.62 pg/g soil in the Tzelepis et al. (2017) study. In the Tzelepis et al. (2017) study, soil was sampled from two different zones: southern (297 samples) and western (132 samples). The southern zone was reported to have more samples positive for *V. longisporum* whereas the western zone was reported to have more samples with *V. dahliae*. This was suspected mainly due to the increase in oilseed rape cultivation in the southern zone from 24,100 ha in 2006 to 44,700 ha in 2016. In the southern zone crops like oilseed rape, winter wheat and barley were grown, which was interestingly similar to the cropping history of the research farm in the current study. In the Tzelepis et al. (2017) study, out of the 297 soil samples analyzed from the southern zone, 120 tested positive for *V. longisporum*. Thus 40% of the samples from the southern zone were reported positive for the pathogen. Fascinatingly, this percentage was extremely close to the 39.1% samples found positive for the pathogen in the current study. Since the current study was the first-documented episode of *V. longisporum* infestation in Canada, the integrity of the results so obtained was confirmed with the Tzelepis et al. (2017) study based on similarities between geographical locations, weather conditions, methodology, cropping history and percentage of positive samples between the two studies.

The mode of arrival of *V. longisporum* from other countries to Canada is still unknown. Perhaps it is similar to the outbreak of Verticillium stripe in the UK from a latent established population of *V. longisporum* (Depotter et al. 2017). Among other possible reasons, the most probable mode of arrival of the pathogen to the Manitoban farm includes; global trade, arrival through infested soil from the USA, and arrival through footwear of personnel harbouring microsclerotia. Until the 2014-biosecurity restrictions, the free flow of traffic may have also contributed to the arrival of the pathogen to the farm. Moreover, the pathogen may have been present on seed or may have originated in the farm through hybridization somewhere in the

Canadian Prairies. Origin through hybridization in Canada, especially in Manitoba, is most likely since the parent *V. dahliae* is found in Manitoban soil in high genomic DNA concentrations of 87 pg/g soil (Molina 2016). However, introduction of pathogens to novel geographical locations is not the only prerequisite for pathogenicity. Several factors like farming practices, environmental conditions (Depotter et al. 2017), host-pathogen interaction in the rhizosphere, pathogen mechanism to avoid host disease response (Targett 1983) and hybrid lineage (Inderbitizin et al. 2013) can play a valuable role in determining the pathogenicity and disease outcome of *V. longisporum* of canola in North America. Currently, presence of the pathogen across six provinces of Canada can also, be attributed to the favourable environmental conditions available to the pathogen, such as ideal soil pH range of 3.5-10.5; (Dutta 1981), soil temperatures of 15-19°C and air temperature of 15-23°C (Canola Council of Canada 2017c), all of which are found during the growing season across Canada (Environmental Canada 2017**).**

2.5.2 *V. longisporum* Ability to Establish at the Research Farm

Soil as a source of detection for pathogen concentrations is quite complex. Several factors like soil texture, pH, and organic matter affect the downstream processes of DNA extraction and real-time PCR quantification. These factors influence the overall quality, quantity and variability in pathogen concentrations across different samples (Tzelepis et al. 2017). This concurs with the high variability in *V. longisporum* concentrations reported in the current study.

The pathogen concentration data plotted on the histogram was asymmetrically distributed and skewed to the right. Typical of right-skewed data, the median (2.01 pg/g soil) of the pathogen concentration was found to be less than the mean (3.99 pg/g soil) of the pathogen concentration (Doane and Seward 2011). This was mainly due to high number of samples with very low

pathogen concentration. The % coefficient of variation (C.V.) of pathogen concentration across all areas of the farm was 172%, in other words, the standard deviation of the data set was 172% of the mean. The unit-free statistical tool C.V. is a measure of relative variability in the data set; therefore, C.V. of 172% suggested extremely high variation in pathogen DNA concentrations when compared across all 194 areas of the farm.

Soil-borne pathogens survive by producing and dispersing inoculum in high amounts, such that even a small residue is sufficient to cause infection in a new host. *Verticillium* species are known to produce 100-300 propagule/g soil, which in terms of pathogen population is over 2 million/cu ft. of top soil. Production of the initial inoculum of *V. longisporum* is extremely important, as the microbe survives in soil solely in the form of microsclerotia produced in the dying host. The pathogen can produce up to 50000 viable propagules (microsclerotia) in the decaying host stem during the end of the disease cycle. Dispersal of propagules occurs in two episodes; the first dispersal episode occurs at the beginning of the growing season when infected plants from the previous season are disced during the preparation of the seedbed. These crops fall on the soil but most of the pathogen propagules are still contained in the stems of these crops. It is during the start of the growing season when the second dispersal episode occurs as the microsclerotia reaches the soil in high numbers upon the decay of the diseased host. From the soil, microsclerotia are distributed unevenly to the neighbouring locations by tillage and combining practices during the growing the seasons (Menzies 1970).

Fungal propagules are dispersed in soil by various other sources that cause movement of the infested soil. These include sources like farm machinery, vehicle wheels, footwear, animals, water and wind. Long distance dispersal of fungal propagules occurs via transport of other non-host infested crops and seeds. Verticillium propagules are concentrated mostly in the topsoil,

corresponding to 30 cm of the soil profile. Dispersal of propagule from the topsoil is advantageous as it eases dispersal via wind (Berlanger and Powelson 2000). *Verticillium longisporum* propagules in particular are dispersed through soil erosion, flooding, farm equipment and wind-borne microsclerotia and crop debris (Canadian Food Inspection Agency 2016).

Average wind speed in the city of Winnipeg from 2002 through 2015 (timeline used for delineation of the farm) was approximately 20 km/hr with maximum speeds recorded up to 75 km/hr (Winnipeg Historical Wind Speed). The Red river flows for a stretch of 1.68 km, east of the sampled farm and possibly brings in strong wind channels due to the natural phenomenon called sea-land breeze. These winds are a result of change in air pressure due to the temperature difference between land and water during day and night (Yan 2005). The 500-acre farm is an open plain with no tall structures like trees or buildings that could obstruct the wind flow. Therefore, wind possibly played a crucial role in dispersal of *V. longisporum* propagules to distant locations at the farm. Also, until 2015, there were no quarantine and biosecurity protocols practiced at the farm. The free movement of farm traffic, machinery, personnel, and seeds may have contributed to the dispersal of *V. longisporum* propagules across the farm.

The *V. longisporum* propagules in the current study were found easily established in the farm soil. These findings were in line with field research conducted by Short el al. (2015) on California soil to evaluate propagule concentration and distribution of *Verticillium* species. The study revealed similar unequal distribution of *V. longisporum* propagules across six delineated areas of 64 quadrants, 1-by1-m each. In the current study, pathogen presence was not found to be restricted to the areas that had higher canola cultivated years. The pathogen population in the soil was not correlated ($R = 0.39$, $P > 0.05$) to the presence of host, therefore the propagules were

found easily established in the farm soil. These findings were in line with the CFIA delimitation survey (July 2015) of adjacent fields to the research farm. *Verticillium longisporum* was found present in adjacent fields and CFIA concluded that the pathogen was not restricted to the original areas of identification and was dispersed across the farm. In the current study, detection of *V. longisporum* at areas where canola was absent can also be attributed to the presence of field mustard (*Brassica rapa* var. *rapa*), a weed at the research farm, which is also a common Brassicaceae host of the pathogen (Horiuchi et al. 1990). Since field mustard grows as a weed at the research farm, unlike canola, there is no documented information about its growth years and area location. Therefore, it can be concluded that there are various factors involved in the settlement of the pathogen at the research farm, including abundance of an alternate host in the form of an unmonitored weed, favourable environmental conditions and ease of establishment in soil via resistant propagules known as microsclerotia.

2.5.3 *V. longisporum* Hybrid Lineage at the Research Farm

The colonies of *V. longisporum* isolates grown on PDA agar during the hybrid lineage determination were 2-3 cm in diameter and mycelia were white to dark grey in colour. These observations were in line with a study conducted by Inderbitzin et al. (2011a) where *V. longisporum* colonies grown on PDA showed similar visual morphological characteristics.

The hybrid lineage of *V. longisporum* isolates from the research farm was reported to be of A1/D1 hybrid lineage. *Verticillium longisporum* diploid hybrid A1/D1 is a result of an independent non-parasexual hybridization (Inderbitzin et al. 2011b) event occurring between Species A1 and Species D1 (Collins et al. 2003; Inderbitzin et al. 2011b). Species A1 (differs from *V. dahliae* sequence by 4.4%) and Species D1 (differs from *V. dahliae* sequence by 1.4%)

are unknown species of non-Verticillium origin that are yet to be independently isolated and only occur as hybrid parents of *V. longisporum* (Heale 2000). These unknown species therefore are most likely to be non-pathogenic to agricultural crops or non-pathogenic saprotrophs in nature (Inderbitzin et al. 2011b). *Verticillium longisporum* can be considered an interspecific hybrid, since the parents involved are of different distinct species (Schardl and Craven 2003).

Determination of the hybrid lineage was based on primer pairs complementary to three intron-rich protein-coding genes: elongation factor 1-alpha (EF), glyceraldehyde-3-phosphate dehydrogenase (GPD) and internal transcribed spacer (ITS). On gel electrophoresis analysis of the multiplex PCR products (Inderbitzin et al. 2013), hybrid lineage A1/D1 was recognized by the 310 bp amplicon of the amplified EF gene of Species A1 along with the 1020 bp amplicon of the amplified GPD gene of Species D1. The negative control, isolate MBVt40 of *V. tricorpus* species, did not amplify in the multiplex PCR assay due to the absence of primer pair Tf/AaTr targeting the ACT locus in the pathogen (Inderbitzin et al. 2013). Absence of false-amplification in the analysis was thus confirmed by the non-amplification of *V. tricorpus* isolate in the assay. Parent species to hybrid A1/D3, *V. dahliae* lineage D3, was confirmed by including isolate MBVd12 that generated a 480 bp amplicon of the amplified ITS region. Also included in the analysis were two reference isolates of *V. longisporum (*PD348 and PD624) of known hybrid lineage. Isolates PD348 and PD624 were confirmed to be of A1/D1 hybrid lineage. These findings were in line with various hybrid-related studies conducted on *V. longisporum*. In the Inderbitzin et al. (2013) and the Novakazi et al. (2015) studies, hybrid lineage of the isolate PD348 was reported as A1/D1. Similarly in the Inderbitzin et al. (2011b) study, hybrid lineage of PD624 was reported A1/D1 as well. The hybrid lineages of the reference isolates in the current

study were found to be consistent with the literature, verifying the hybrid lineage determination (A1/D1) of other isolates from the research farm.

A1/D1 hybrid is the most virulent hybrid on canola and cauliflower. In a study conducted by Novakazi et al. (2015) on determination of virulence of 16 *V. longisporum* isolates based on 11 different hosts, it was discovered that hybrid A1/D1 was most virulent on canola and cauliflower with marginal virulence effects on canola twice that of any other hybrid and thrice that of the parent *V. dahliae.* Interestingly, five of the six *V. longisporum* isolates analysed in the current study were isolated from canola by Manitoba Agriculture and the CFIA (Appendix III).

Verticillium longisporum is different from other Dikaryomycota hybrids in that it does not undergo parasexual recombination (fusion of parent nuclei) following karyogamy, rather it continues to maintain a diploid state. The dikaryotic stage during hybridization provides the hybrid with adaptations like genetic diversity, effective hyphae, defense against mutations and diversified spores (Murphy et al. 2015). Hybridization is a tool for development of new pathogens, which possibly played a crucial role in the evolution of the soil-borne pathogen *V. longisporum.*

The origin of *V. longisporum* and its hybrid lineage is unknown. Interestingly, all three of the hybrid lineages of *V. longisporum* are genetically similar and differ only in MAT1-1 substitution. Allele A1 (involved parent in every *V. longisporum* hybrid) is also the same in all the three lineages without any variation. The lack of genetic variation across the three lineages indicates that *V. longisporum* has originated recently. Hybrid lineage A1/D2 has only been reported from the USA whereas lineage A1/D3 has ben found in Europe and Japan. It is most likely that these locations are the centers of origin for these hybrids. The origin of hybrid A1/D1

on the other hand is difficult, to determine as the pathogen has been reported in Europe, USA, Japan and Russia (Inderbitzin et al. 2011b) and now Canada.

2.6 Conclusions

Verticillium longisporum of canola has been a serious problem in European countries like Sweden, Germany, France, etc. for over 50 years. Previously, the pathogen has been identified in North America but only on cabbage, cauliflower and horseradish (Novakazi et al. 2015). The findings of this study confirm that the first farm documented in detail did in fact harbour canola infected with *V. longisporum*. Up to 39.1% of the 500-acre farm was populated by pathogen propagules where the minimum reportable pathogen genomic DNA concentration was 2.65 pg/g soil. The mean pathogen DNA concentration at the farm (all 194 samples) was 2.99 pg/g of soil, whereas the mean pathogen DNA concentration across positive samples (76) was 8.19 pg/g soil. The findings of this study suggest that the pathogen concentration at a given geo-referenced location at the farm is independent of the number of years of canola grown at the same location. The pathogen seems to be distributed across the farm mainly due to the ability to establish via its propagules known as microsclerotia.

The hybrid lineage of *V. longisporum* of canola at the first reported farm in North America is determined to be A1/D1. The hybrid A1/D1 is most virulent on canola and raises serious concerns for the billion-dollar economy that depends on the crop, especially in Canada. The diploid hybrid nature of the pathogen enables it to acquire a broad host range and enhanced virulence when compared to the parent *V. dahliae* (Novakazi et al. 2015).

The confirmation of *V. longisporum* of canola in North America based on the current study and the National soil survey conducted by the CFIA signifies the need for control methods.

In Europe, canola yield losses of up to 50% and disease incidence of up to 80% caused by *V. longisporum* have already been reported (Novakazi et al. 2015). In the absence of a registered fungicide, two of the most suitable control methods as recommended by the CFIA and Depotter et al. (2017) respectively are; destruction of infected crops before decay commences and microsclerotia is released in the soil, and breeding of resistant oilseed rape lines to prevent the spread of Verticillium stripe disease to new geographical locations.

2.7 Literature Cited

Andersson, C. 2003. Detection methods of *Verticillium longisporum* in soil and in oilseed rape. M.Sc. Dissertation. Swedish University of Agricultural Sciences. Uppsala **45**: ISSN 1651-5196.

Banno, S., Saito, H., Sakai, H., Urushibara T., Ikeda, K., Kabe, T., Kemmochi, I., and Fujimura, M. 2011. Quantitative nested real-time PCR detection of *V. longisporum* and *V. dahliae* in the soil of cabbage fields. Journal of General Plant Pathology **77**:282-291.

Berlanger, I., and Powelson, M.L. 2000. Verticillium wilt. The Plant Health Instructor. The American Phytopathological Society. doi:10.1094/PHI-I-PHI-1-2000-0801-01.

Bilodeau, G. J., Koike, S. T., Uribe, P., and Martin, F. N. 2012. Development of an assay for rapid detection and quantification of Verticillium dahliae in soil. Phytopathology **102:**331-343.

Butler, M.J., and Day, A.W. 1998. Fungal melanins: a review. Canadian Journal of Microbiology **44**:1115-1136.

Canadian Food Inspection Agency. 2016. Verticillium longisporum – survey results. Canadian Food Inspection Agency, Winnipeg, MB.

Canola Council of Canada. 2017a. Canola now worth $26.7 billion to Canadian economy. Canola Council of Canada, Winnipeg, Manitoba.

Canola Council of Canada. 2017b. Health benefits of canola oil. Canola Council of Canada, Winnipeg, Manitoba.

Canola Council of Canada. 2017c. Verticillium. Canola Council of Canada, Winnipeg, Manitoba.

Canola Council of Canada. 2017d. What is canola? Canola Council of Canada, Winnipeg, Manitoba.

Clewes, E., Edwards, S.G., and Barbara, D.J. 2008. Direct molecular evidence supports long-spored microsclerotial isolates of *Verticillium* from crucifers being interspecific hybrids. Plant Pathology **57**:1047-1057.

Collins, A., Okoli, C.A., Morton, A., Parry, D., and Edwards, S.G. 2003. Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. Phytopathology **93**:364-376.

Debode, J., Van Poucke, K., França, S.C., Maes, M., Höfte, M., and Heungens, K. 2011. Detection of multiple *Verticillium* species in soil using density flotation and real-time polymerase chain reaction. Plant Disease **95**:1571-1580.

Depotter, J.R., Deketelaere, S., Inderbitzin, P., Tiedemann, A.V., Höfte, M., Subbarao, K.V., Wood, T.A., and Thomma, B.P. 2016. *Verticillium longisporum*, the invisible threat to oilseed rape and other brassicaceous plant hosts. Molecular Plant Pathology **17**:1004-1016.

Depotter, J.R.L., Rodriguez-Moreno, L., Thomma, B.P.H.J., and Wood, T.A. 2017. The emerging British *Verticillium longisporum* population consists of aggressive *Brassica* pathogens. Phytopathology **107**:1399-1405.

Desjardins, P., and Conklin, D. 2010. NanoDrop microvolume quantitation of nucleic acids. Journal of Visualized Experiments: JoVE. (45):2565. doi:10.3791/2565.

Dixelius, C., Happstadius, I., and Berg, G. 2005. Verticillium wilt on Brassica oilseed crops – a Swedish perspective. Journal of Swedish Seed Association **115**:36-48.

Doane, D.P. and Seward, L.E. 2011. Measuring skewness: a forgotten statistic? Journal of Statistics Education **19**:1-18.

Dutta, B.K. 1981. Effect of the chemical and physical condition of the soil on Verticillium wilt of antirrhinum. Plant and Soil **63**:217-225.

Eastburn, D.M. and Paul, V.H. 2007. Verticillium wilt. Pages 47-50. in S. R. Rimmer, V. I. Shattuck, and L. Buchwaldt (Eds.). Compendium of Brassica Diseases. APS Press, St Paul, MN.

Environmental Canada 2017. Historical Climate Data. Government of Canada. Retrieved from http://climate.weather.gc.ca/ on December 21, 2017.

Eynck, C., Koopmann, B., Grunewaldt-Stöcker, G., Karlovsky, P., and von Tiedemann, A. 2007. Differential interactions of *Verticillium longisporum* and *Verticillium dahliae* with *Brassica napus* detected with molecular and histological techniques. European Journal of Plant Pathology **118**:259-274.

Fatima, F., Pathak, N., and Verma, S.R. 2014. An Improved Method for Soil DNA Extraction to Study the Microbial Assortment within Rhizospheric Region. Molecular Biology International 518960:1-6.

Hauke J., and Kossowski T. 2011. Comparison of values of Pearson's and Spearman's correlation coefficient on the same sets of data. Quaestiones Geographicae **30**:87-93.

Heale, J.B. 2000. Diversification and speciation in *Verticillium* - an overview. Pages 1-14. in E. C. Tjamos, R.C. Rowe, J.B. Heale, and D.R. Fravel (Eds.). Advances in Verticillium research and disease management. APS Press, St. Paul, MN.

Heale, J.B., and Karapapa, V.K. 1999. The *Verticillium* threat to Canada's major oilseed crop: canola. Canadian Journal of Plant Pathology **21**:1-7.

Hein. T. 2017. Verticillium stripe: measuring the threat. Canola Digest. Canola Council of Canada, Winnipeg, Manitoba.

Horiuchi, S., Hagiwara, H., and Takeuchi, S. 1990. Host specificity of isolates of *Verticillium dahliae* towards cruciferous and solanaceous plants. Hornby, D. (Ed.). CAB International, Wallingford, Oxon, UK. Publ. 285-298 pp. ISBN : 0851986374

Huisman, O.C. 1982. Interrealtions of root growth dynamics to epidemiology of root-invading fungi. Annual Review of Phytopathology **20**:303-327.

Hwang, S.F., Strelkov, S.E., Ahmed, H.A., Zhou, Q., Fu, H., Fredua-Agyeman, R., and Turnbull, G.D. 2017. First report of *Verticillium dahliae* Kleb. Causing wilt symptoms in canola (*Brassica napus* L.) in North America. Canadian Journal of Plant Pathology **39:**514-526.

Inderbitzin, P., Bostock, R.M., Davis, R.M., Usami, T., Platt, H.W., and Subbarao, K.V. 2011a. Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. PLoS ONE **6**(12):e28341. doi:10.1371/journal.pone.0028341

Inderbitzin, P., Davis, R.M., Bostock, R.M., and Subbarao, K.V. 2011b. The ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. PLoS ONE **6**(3):e18260. doi:10.1371/journal.pone.0018260

Inderbitzin P., Davis R.M., Bostock R.M., and Subbarao K.V. 2013. Identification and Differentiation of *Verticillium* Species and *V. longisporum* Lineages by Simplex and Multiplex PCR Assays. PLoS ONE **8**(6):e65990. doi:10.1371/journal.pone.0065990

Inderbitzin, P., and Subbarao, K.V. 2014. Verticillium systematics and evolution: How confusion impedes Verticillium wilt management and how to resolve it. Phytopathology **104**:564-574.

Johansson, A. 2006. *Verticillium longisporum,* infection, host range, prevalence and plant defence responses. Licentiate thesis. Swedish University of Agricultural Sciences. Uppsala: ISBN 91-576-7153-2.

Johansson, A., Goud, J.K.C., and Dixelius, C. 2006. Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. European Journal of Plant Pathology **114**:139-149.

Kang, Y., Lee, S.H., and Lee, J. 2014. Development of a selective medium for the fungal pathogen *Cylindrocarpon destructans* using radicicol. The Plant Pathology Journal **30**:432-436.

Knüfer. J. 2011. Improvement of winter oilseed rape resistance to *Verticillium longisporum* – assessment of field resistance and characterization of ultrastructural plant responses. Thesis zur Erlangung des Doktorgrades der Fakultät für Agrarwissenschaften der Georg-August-Universität Göttingen.

McInnis, A. 2004. The transformation of rapeseed into canola. Winning the Prairie Gamble. The Saskatchewan Story exhibit, North Battleford, WDM.

Menzies, J.D. 1970. Factors affecting plant pathogen populations in soil. Pages 16-21. in T.A. Tousson, R.V. Bega, and P.E. Nelson (Eds.). Root Diseases and Soil-Borne Pathogens. University of California, Berkeley.

Mol, L., and van Riessen, H.W. 1995. Effect of plant roots on the germination of microsclerotia of*Verticillum dahliae.* European Journal of Plant Pathology **101**:673-678.

Molina, O. (2016). Quantification of *Verticillium* species producing microsclerotia in potato fields from Manitoba. Chapter 3 of PhD Thesis, University of Manitoba, Winnipeg, Manitoba, Canada.

Murphy, T.M., Rost, T.L., and Barbour, M.G. 2015. Plant Biology. Chapter 20: Kingdom Fungi. Pages 1-42. University of California, Davis.

Novakazi, F., Inderbitzin, P., Sandoya, G., Hayes, R.J., von Tiedemann, A., and Subbarao, K.V. 2015. The three lineages of the diploid hybrid *Verticillium longisporum* differ in virulence and pathogenicity. Phytopathology **105**:662-673.

Papaioannou, I.A., Dimopoulou, C.D., and Typas, M.A. 2014. Cryptic group-I intron in the nuclear SSU rRNA gene of *Verticillium dahliae*. Current Genetics **60**:135-148.

Rebekić, A., Lončarić, Z., Petrović, S., and Marić, S. 2015. Pearson's or Spearman's correlation coefficient - Which one to use? Poljoprivreda **21**:47-54. doi:10.18047/poljo.21.2.8

Schardl, C.L., and Craven, K.D. 2003. Interspecific hybridization in plant-associated fungi and oomycetes: a review. Molecular Ecology **12**:2861-2873.

Schneegurt, M.A., Dore, S.Y., and Kulpa Jr., C.F. (2003). Direct extraction of DNA from soil for studies in microbial ecology. Current Issues Molecular Biology **5**:1-8.

Short, D.P., Sandoya, G., Vallad, G.E., Koike, S.T., Xiao, C.L., Wu, B.M., Gurung, S., Hayes, R.J., and Subbarao, K.V. 2015. Dynamics of *Verticillium* species microsclerotia in field soils in response to fumigation, cropping patterns, and flooding. Phytopathology **105**:638-645.

Tanasea, A.M., Mereutab, I., Chiciudeanb, I., Ionescub, R., Mileac, L., Corneaa, C.P., Vassub, T., and Stoica, I. 2015. Comparison of total DNA extraction methods for microbial community form polluted soil. Agriculture and Agricultural Science Procedia **6**:616-622.

Tan, S.C., and Yiap, B.C. 2009. DNA, RNA, and protein extraction: the past and the present. Journal of Biomedicine and Biotechnology 2009:1-10.

Targett, G.A.T. 1983. **What determines the pathogenicity of parasites.** Journal of the Royal Society of Medicine **76**:888-890.

Tran, V.T., Braus-Stromeyer, S.A., Timpner, C., and Braus, G.H. 2012. Molecular diagnosis to discriminate pathogen and pathogen species of the hybrid *Verticillium longisporum* on the oilseed crop *Brassica napus*. Applied Genetics and Molecular Biotechnology **97**:4467-4483.

Typas, M.A., and Heale, J.B. 1980. DNA content of germinating spores, individual hyphal cells and resting structure cells of *Verticillium* spp. measured by microdensitometry. Journal of General Microbiology **121**:231-242.

Tzelepis, G., Bejai, S., Sattar, M.N., Schwelm, A., Ilbäck, J., Fogelqvist, J., and Dixelius, C. 2017. Detection of *Verticillium* species in Swedish soils using real-time PCR. Archives of Microbiology. **199**:1383–1389.

Winnipeg Historical Wind Speed. Retrieved from https://winnipeg.weatherstats.ca/metrics/wind_speed.html on December 29, 2017.

Yan, Y.Y. 2005. Land and Sea Breezes. in: J.E. Oliver (Eds.). Encyclopedia of World Climatology. Encyclopedia of Earth Sciences Series. Springer, Dordrecht

Yu, J.M., Cafarov, I.H., and Babadoost, M. 2016. Morphology, molecular identity, and pathogenicity of *Verticillium dahliae* and *V. longisporum* associated with internally discoloured horseradish roots. Plant Disease **100**:749-757.

Zhou, L., Hu, Q., Johansson, A., and Dixelius, C. 2006. *Verticillium longisporum* and *V. dahliae*: infection and disease in *Brassica napus*. Plant Pathology **55**:137-144.

Chapter 3

3. Overall Synthesis

Soil, a dynamic medium of growth, is a source to water, air, minerals, organic matter, plants and several species of soil-borne pathogens (Byers et al. 1938). The 200-year old genus *Verticillium* (Inderbitzin and Subbarao 2014) is one such soil-borne pathogens that can infect over 200 economically important crops (Klosterman et al. 2009). In the absence of an effective fungicide, the only diploid species of the genus, *V. longisporum* (Johansson 2006) is responsible for an economically devastating disease known as Verticillium wilt. After a series of incorrect identifications, the pathogen was classified as an individual species quite recently (Karapapa and Typas 2001). Interestingly, on oilseed rape crops such as canola, symptoms of wilt are absent and instead black coloured stripes on stems are observed. Hence the name Verticillium stripe was coined to describe *V. longisporum* infection on oilseed rape (Depotter et al. 2016). Verticillium stripe of canola is a common economically important disease in European countries but was observed for the first time in 2014 on a North American research farm documented in the current study. The pathogen, therefore, poses a serious threat to the billion-dollar canola industry in Canada. The research chapter of this thesis (Chapter 2) thus evaluated the following hypotheses: 1) *V. longisporum* pathogen will be widespread throughout the farm due to the inevitability of dispersal of spores via wind, field equipment traffic and other organisms; 2) *V. longisporum* hybrid lineage at the farm will be one of three lineages; A1/D1, A1/D2, or A1/D3. The results of this thesis provide the first information on *V. longisporum* of canola in North America that contributes towards understanding the pathogen concentration and ability to establish in soil and its virulence on canola.

3.1 Importance of Discoveries

The in-situ PCR analysis of the farm soil (described in Chapter 2), confirmed the presence of *V. longisporum*. Our findings provided new information about the pathogen genomic DNA load (pg/g of soil) in the farm soil at up to 59 geo-referenced locations. The results also established a minimum threshold concentration for a soil sample to be considered positive for the pathogen using real-time PCR, which is useful for future research. Moreover, the thesis determined dispersion of the pathogen in the soil and its hybrid lineage to be the most aggressive on canola. This study therefore, documents in detail the episode of *V. longisporum* of canola in North America.

The quantification method described in this thesis (Chapter 2) was extremely specific to *V. longisporum* detection without cross-detection of other Verticillium species. The group-I intron in the 18s rDNA gene of the pathogen, targeted by the Vlsp primer pairs in the real-time assay, allowed us to overcome the non-specificity of other conventional detection methods such as plating on selective media and the wet sieving method (Bilodeau et al. 2012). Therefore, the highly sensitive methodology used in this thesis allows future researchers to successfully detect and quantify *V. longisporum* in any soil sample.

The results of this thesis are extremely important to canola growers in Manitoba (highest number of fields with *V. longisporum*) and all over Canada. Farmers now know that *V. longisporum* of canola is not just a suspected pathogen but rather it has established a population on a North American farm. The threat is real but based on this thesis, now the canola growers have a very specific method to quantify pathogen concentration in their farms. The molecular detection method to quantify the pathogen in soil can thus be used as a means to predict possible Verticillium stripe disease incidence. The implications of this thesis extend further as the results

also provide canola growers a minimum pathogen concentration threshold (2.65 pg/g soil) for farm soil to be considered infested with *V. longisporum.* The study also informs canola growers that the hybrid lineage of the pathogen found at the Manitoba farm is lineage A1/D1 which is known to be most virulent on canola. Moreover, farmers now know that the 4-year crop rotation policy used at most farms is ineffective in controlling *V. longisporum* inoculum in soil, as the pathogen propagules were found easily established in the farm soil due to the inevitability of farm traffic, wind, water, other organisms etc.

The perquisite to disease management is the availability of information on pathogen morphology, density, and virulence. Chapter 1 and Chapter 2 of this thesis describe pathogen genetic and anatomical morphology, and density in soil and virulence, respectively. Unfortunately, an effective registered fungicide to control *V. longisporum* is still unavailable. This is mainly due to the mode of pathogen infection that colonizes in the xylem of its host, where fungicides cannot enact without collaterally killing the host (Schnathorst, W.C. cited by Johansson 2006). Interspecific hybridization of *B. rapa* (gene bank accession 56515) and *B. oleracea* (gene bank accession Kashirka 202) to enhance resistance in *B. napus* has, however, proved successful as the resultant hybrid demonstrated resistance towards *V. longisporum* (Rygulla et al. 2007). Replacing the traditional canola strains in Manitoban farms with this hybrid can prevent possible disease epidemics and high economic losses. Alternatively, use of chemical compounds like elemental sulphur, methyl bromide (Cooper and Williams, 2004), nitrous acid, ammonia and volatile fatty acids (Conn et al. 2005) have been efficient in reducing *Verticillium* microsclerotia in soil. This is another disease management method for traditional canola growers, unwilling to grow canola hybrids on their farms. Additionally, biocontrol through bacterial species, *S. plymuthica* C48 and *P. alvei* K165 have been reported to be effective in controlling *V.*

longisporum populations in soil (Fravel 1988). Inclusion of these bacteria in soil maintains an organic cultivation for canola growers that do not believe in the use of chemical compounds.

3.2 Interesting Observations

3.2.1 Wheat, a Potential Reservoir for *V. longisporum* Inoculum

While studying the cropping history of the research farm to investigate a correlation between canola abundance and *V. longisporum* concentration, an interesting observation was made. Several areas at the farm where canola was not grown during the period of 2003 to 2015, were found positive for *V. longisporum* genomic DNA. Interestingly, at each of these areas, wheat was grown in high numbers during the same timeline and was also the last crop grown before the farm soil was sampled in 2015 for the current study (Appendix VIII).

V. longisporum is most virulent on crops of the Brassicaceae family but its pathogenicity is not restricted to Brassica crops. It can also infect non-Brassica crops such as wheat, pea and oats, although not as aggressively as canola. Microsclerotia formation in these crops is comparatively lesser than in canola, therefore these crops do not display severe disease symptoms but are potential reservoirs for *V. longisporum* inoculum (Johansson et al. 2006). A similar phenomenon is also witnessed for parent *V. dahliae* microsclerotia, which are concealed in the roots of wheat and oats as a reservoir (Mathre 1989).

In a greenhouse study of Swedish soils conducted by Johansson et al. (2006), *V. longisporum* microsclerotia formation was confirmed in non-Brassica crops like wheat, oats and peas. This was in line with the current study, where *V. longisporum* genomic DNA was found in areas where no Brassica host was planted in the last 10 years but instead wheat was grown in abundance (Appendix VIII). There are two possible explanations for these findings: 1) wheat is

acting as a reservoir for *V. longisporum* inoculum keeping the pathogen alive in soil; 2) this is early evidence of the pathogen broadening its host range to non-Brassica crops in Canada. Because pathogen recovery from infested crops was not assessed in the current study, further research including isolation of pathogen DNA from wheat and observation of pathogen microsclerotia in other non-Brassica hosts is required to confirm this hypothesis.

3.3 Recommendations for Future Research

Future research on detection and quantification of *V. longisporum* should be based on the real-time PCR method described in this thesis and should replace other non-sensitive conventional methods like plating on selective media and wet sieving. In order to re-evaluate the sensitivity of the real-time PCR assay used in the current study, it is recommended that the farm soil is re-sampled and re-quantified for pathogen density using the density floatation method from the Debode at al. (2011), as described in Chapter 2. Comparative analysis between the efficiency and sensitivity of the real-time PCR analysis reported in this study and new values from the re-sampled soil can further confirm the integrity and reproducibility of the assay developed in our study.

Based on other studies, it is known that *V. longisporum* hybrid A1/D1 is most virulent on canola (Novakazi et al. 2015; Johansson et al. 2006) but this information was not examined in the current study. Further research based on *V. longisporum* microsclerotia recovery from various crops grown at the farm can thus help evaluate virulence and host range of the reported hybrid A1/D1 and can also confirm the presence of any potential reservoirs of pathogen inoculum in Canada. The protocol described in the Novakazi et al. (2015) study to test pathogenicity and virulence of all *V. longisporum* hybrids can be used for this purpose.

Moreover, the hybrid lineage determined in the current study was based on 6 isolates obtained from various agencies. It is recommended that the hybrid lineage should also be determined directly from the soil at the farm. For this purpose a monosporic culture based on Choi et al. (1999) can to be grown from the soil samples followed by DNA extraction for triplex PCR analysis using the methodology described in Chapter 2. The results can confirm the presence of other possible hybrids across the 500-acre farm, beside the A1/D1 hybrid identified in the current study. Such analysis can be useful for predicting other susceptible hosts like horseradish, cabbage and cauliflower (susceptible to hybrids A1/D2 and A1/D3) in Canada.

Canola growers in Canada would like to maintain sustainable yet profitable canola production. Therefore, tests on the least susceptible oilseed rape cultivars, including Express and RBN 03 (Burlacu et al. 2012) and resistant oilseed rape genotypes SEM 05-500256 and AVISO (Eynck et al. 2009), are also recommended. A study conducted by Eynck et al. (2009) investigated in detail the role of organic compounds like lignin and phenol and plant hormones auxin and ethylene, involved in the resistance mechanisms to Verticillium stripe infection in SEM 05-500256. Future research based on this knowledge can lead to a breakthrough in the control of Verticillium stripe worldwide.

3.4 Literature Cited

Bilodeau, G. J., Koike, S. T., Uribe, P., and Martin, F. N. 2012. Development of an assay for rapid detection and quantification of Verticillium dahliae in soil. Phytopathology **102:**331-343.

Burlacu, M.C., Leonte, C., Lipsa, F., Simioniuc, D.P., and Lazarescu, E. 2012. Identification of some cultivars of *Brassica napus* with resistance at *Verticillium longisporum*. Research Journal of Agricultural Science **44**:14-18.

Byers, H.G., Kellogg, C.E., Anderson, M.S., and Thorp, J. 1938. Formation of soil. Pages 948-978. in Soils and Men. Yearbook of Agriculture, USDA, Washington, DC.

Choi, Y.W., Hyde, K.D. and Ho, W.H. 1999. Single spore isolation of fungi. Fungal Diversity **3:**29-38.

Conn, K.L., Tenuta, M., and Lazarovits, G. 2005. Liquid swine manure can kill *Verticillium dahliae* microsclerotia in soil by volatile fatty acid, nitrous acid, and ammonia toxicity. Phytopathology **95**:28-35.

Cooper, R.M., and Williams, J.S. 2004. Elemental sulphur as an antifungal substance in plant defence. Journal of Experimental Botany **55**:1947-1953.

Debode, J., Van Poucke, K., França, S.C., Maes, M., Höfte, M., and Heungens, K. 2011. Detection of multiple *Verticillium* species in soil using density flotation and real-time polymerase chain reaction. Plant Disease **95**:1571-1580.

Depotter, J.R., Deketelaere, S., Inderbitzin, P., Tiedemann, A.V., Höfte, M., Subbarao, K.V., Wood, T.A., and Thomma, B.P. 2016. *Verticillium longisporum*, the invisible threat to oilseed rape and other brassicaceous plant hosts. Molecular Plant Pathology **17**:1004-1016.

Eynck, C., Koopmann, B., Karlovsky, P., and von Tiedemann, A. 2009. Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. Phytopathology **99**:803-811.

Fravel, D.R. 1998. Role of antibiosis in the control of plant diseases. Annual Review of Phytopathology **26**:75-91.

Inderbitzin, P., and Subbarao, K.V. 2014. Verticillium systematics and evolution: How confusion impedes Verticillium wilt management and how to resolve it. Phytopathology **104**:564-574.

Johansson, A. 2006. *Verticillium longisporum,* infection, host range, prevalence and plant defence responses. Licentiate thesis. Swedish University of Agricultural Sciences. Uppsala: ISBN 91-576-7153-2.

Johansson A., Goud, J.K.C., and Dixelius, C. 2006. Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. European Journal of Plant Pathology **114**:139-149.

Karapapa, V.K., and Typas, M.A. 2001. Molecular characterization of the host-adapted pathogen *Verticillium longisporum* on the basis of a group-I intron found in the nuclear SSU-rRNA gene. Current Microbiology **42**:217-224.

Klosterman, S.J., Atallah, Z.K., Vallad, G.E., and Subbarao, K.V. 2009. Diversity, pathogenicity, and management of Verticillium species. Annual Review of Phytopathology **47**:39-62.

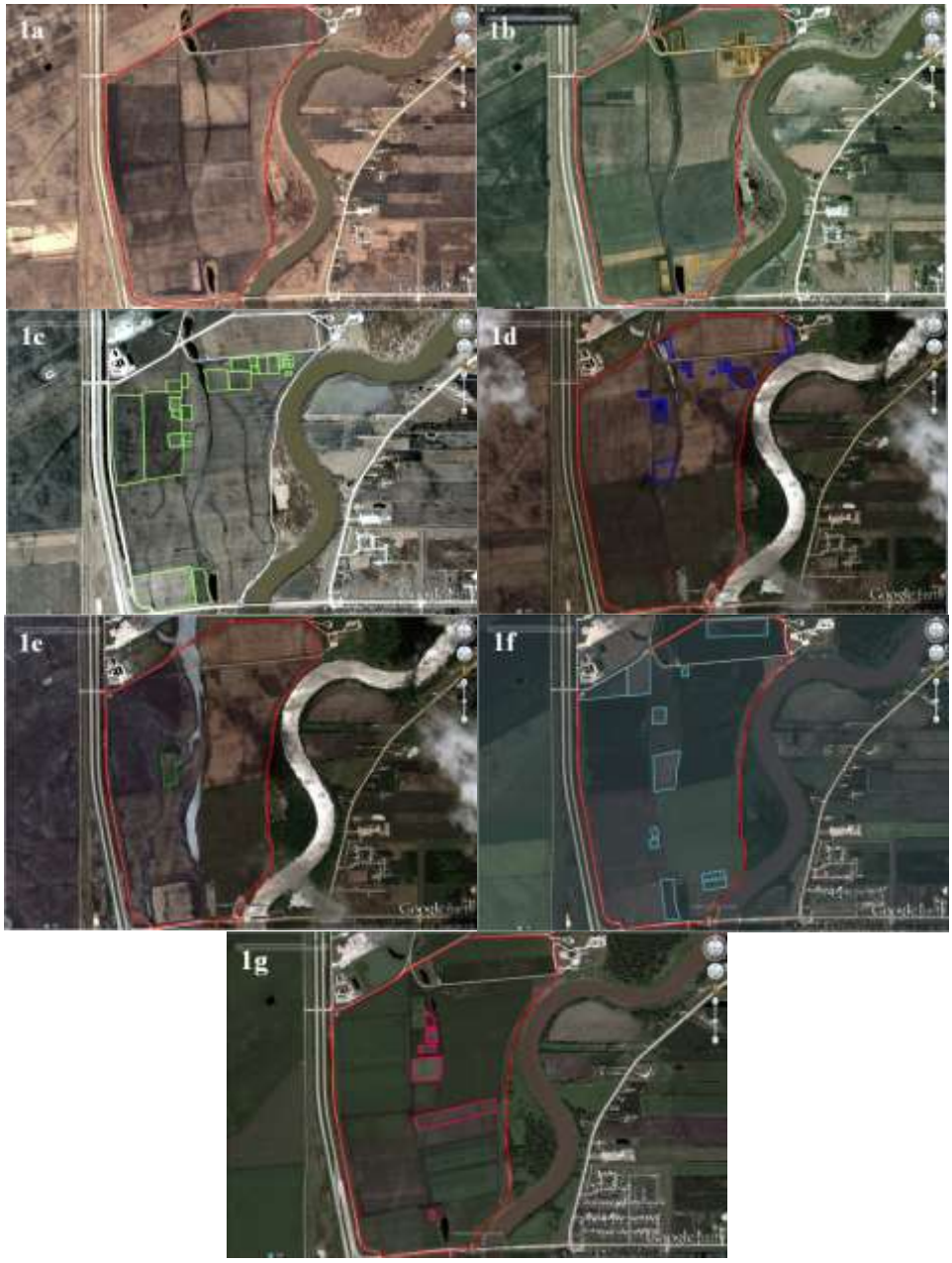
Mathre, D. 1989. Pathogenicity of an isolate of *Verticillium dahliae* from barley. Plant Disease **73**:164-167.

Novakazi, F., Inderbitzin, P., Sandoya, G., Hayes, R.J., von Tiedemann, A., and Subbarao, K.V. 2015. The three lineages of the diploid hybrid *Verticillium longisporum* differ in virulence and pathogenicity. Phytopathology **105**:662-673.

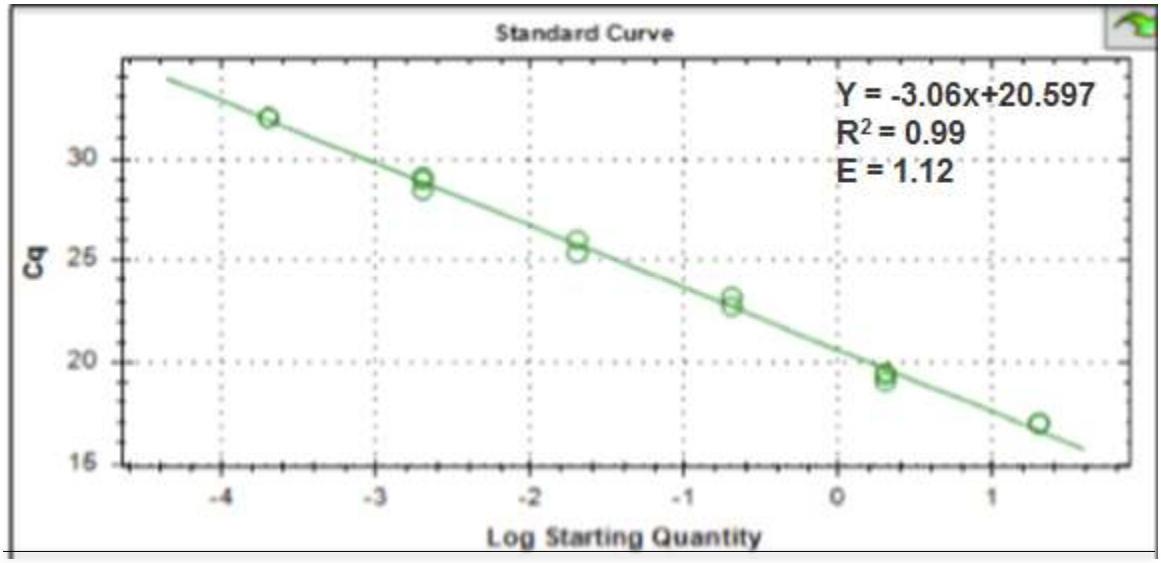
Rygulla, W., Snowdon, R.J., Eynck, C., Koopmann, B., von Tiedemann, A., Lühs, W., and Friedt, W. 2007. Broadening the genetic basis of *Verticillium longisporum* resistance in *Brassica napus* by interspecific hybridization. Phytopathology **97**:1391-1396.

Appendices

Appendix I. Unique areas at the research farm, identified by polygons drawn over 2002 to 2015 Google Earth images of the farm. 1a. Five areas identified on the 2002 Google Earth image of the farm. 1b. 28 areas identified on the 2004 Google Earth image of the farm. 1c. 27 areas identified on the 2007 Google Earth image of the farm. 1d. 12 areas identified on the 2010 Google Earth image of the farm. 1e. One area identified on the 2011 Google Earth image of the farm. 1f. 11 areas identified on the 2013 Google Earth image of the farm. 1g. 6 areas identified on the 2015 Google Earth image of the farm.



Appendix II. Real-time PCR standard curve of 10-fold serially diluted genomic DNA of *V. longisporum* isolate PD624 using Vlsp F-1 and Vlsp R-4 primers.



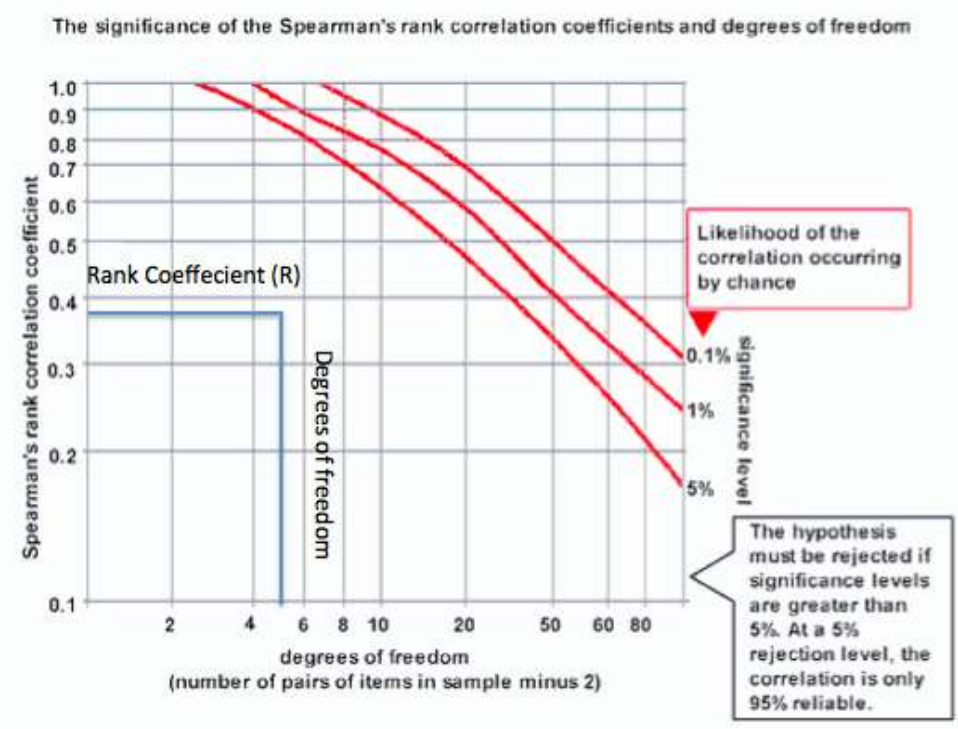
Appendix III. Isolate information of various *Verticillium* species used in the study**.**

Isolate Name	Species	Host	Region	Year isolated
MBGn80	*V. tricorpus*	Potato	Manitoba, CA	2012
CDC600	*V. longisporum*	Canola	Manitoba, CA	2015
CDC602	*V. longisporum*	Canola	Manitoba, CA	2015
243377d	*V. longisporum*	Canola	Manitoba, CA	2015
243377e	*V. longisporum*	Canola	Manitoba, CA	2015
243378d	*V. longisporum*	Canola	Manitoba, CA	2015
243378e	*V. longisporum*	Soil	Manitoba, CA	2015
PD348	*V. longisporum*	Cauliflower	California, USA	Not Available
PD624	*V. longisporum*	Cauliflower	California, USA	Not Available
MBVt40	*V. tricorpus*	Soil	Manitoba, CA	2013
MBvd12	*V. dahliae*	Soil	Manitoba, CA	2012

Appendix IV. Mean *V. longisporum* concentrations of all areas at the research farm with the same number of canola years from 2003 to 2015.

No. canola years at various areas at the research farm from 2003 to 2015	No. of areas with the corresponding canola grown years	Mean *V. longisporum* concentration at the corresponding group of areas (pg/g soil)	% C. V.
0	7	8.30	113.99
1	10	8.34	157.19
2	16	2.54	118.89
3	12	5.00	116.40
4	12	9.95	191.35
5	1	1.76	-
7	1	3.67	-

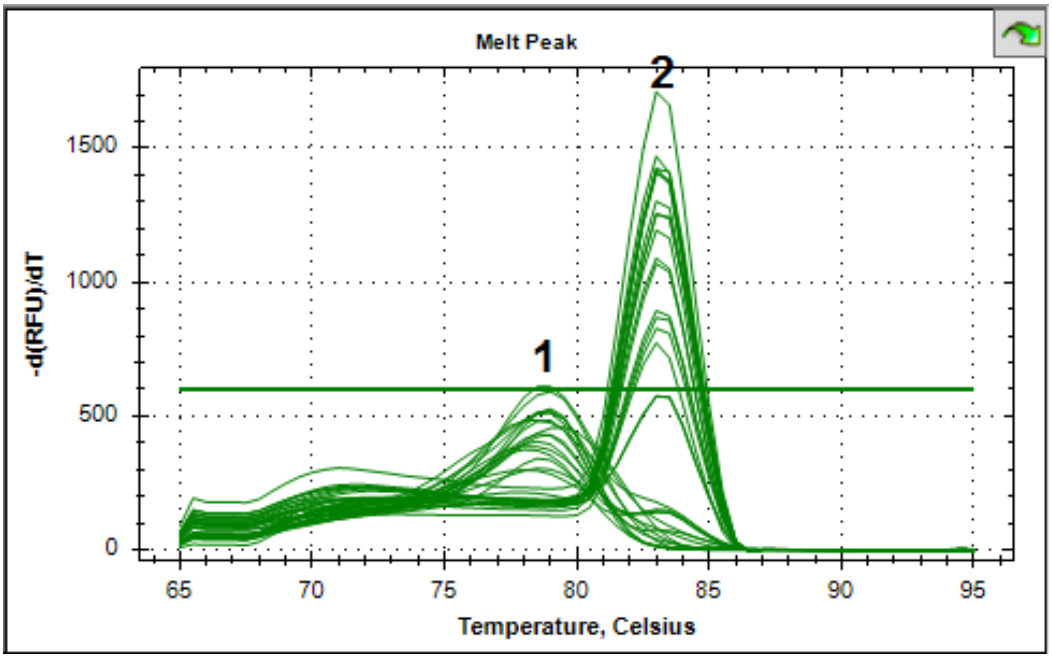
Appendix V. Graphical representation of significance of interaction between Spearman's rank coefficient (R) and degrees of freedom in order to determine the P-value for the interaction between pathogen concentration and canola grown years at the farm.



Appendix VI. Mean Cq values and corresponding concentration of genomic DNA standards of *V. longisporum* isolate PD624 obtained from the standard curve.

Standard	Standard DNA Concentration (ng/ul)	Mean Cq Value	*V. longisporum* concentration (pg/g soil)
Standard 1	10	16.94	626841
Standard 2	1	19.44	95535
Standard 3	10^{-1}	22.06	13303
Standard 4	10^{-2}	25.39	1085.73
Standard 5	10^{-3}	28.46	107.76
Standard 6	10^{-4}	31.71	9.34
Standard 7	10^{-5}	32.3	5.99
Standard 8	10^{-6}	33.38	2.65

Appendix VII. Melt derivative curve, displaying specific peaks of melting temperatures generated from PCR amplicons in the SYBR® Green-based real-time assay using Vlsp primer pair for detection of *V. longisporum.* Melt Peak 1 at 79°C, typical for samples negative for the pathogen and melt peak 2 at 83°C typical for amplicons positive for the pathogen.



Appendix VIII. Comparative analysis of areas found positive for *V. longisporum* that have low number of canola grown years but high number of wheat grown years from 2003 to 2015.

Area #	*V. longisporum* concentration (pg/g soil)	Number of canola grown years (2003 to 2015)	Number of wheat grown years (2003 to 2015)
3	3.26	0	4
49	19.23	0	5
16	4.11	1	9
17	5.12	1	9
52	22.02	1	9
38	5.3	2	6