Factors Influencing Verticillium Stripe in Canola: Blackleg Interaction, Host Resistance, and pH

by

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Abstract

Verticillium stripe, caused by *Verticillium longisporum*, is emerging as an important disease of canola (*Brassica napus*) in Canada. This thesis aimed to enhance understanding of potential interactions between Verticillium stripe and blackleg (*Leptosphaeria maculans*), assess the influence of pH on the growth of *V. longisporum* and the severity of Verticillium stripe, and identify sources of resistance to this disease. The impact of *V. longisporum*/*L. maculans* interactions on yield was evaluated under both field and greenhouse conditions. Co-inoculation resulted in increased blackleg severity and yield losses. In some cases, Verticillium stripe caused greater yield losses than blackleg. The influence of pH on the growth of *V. longisporum* was assessed by measuring colony diameter after 14 and 21 days of incubation on potato dextrose agar at varying pH levels (4.7, 5.5, 6.5, 7.4, and 8.6). Colonies of *V. longisporum* exhibited approximately 16% greater diameter at pH 7.4 and 8.6 compared to pH 5.5. The impact of pH on disease development at the seedling stage was examined using a semi-hydroponic system with half-strength Hoagland's solution at different pH levels (4.4, 5.4, 6.3, 7.5, and 8.4). Disease severity was most pronounced at pH 7.5 and 8.4. In a follow-up experiment, canola seedlings previously inoculated with the fungus were transplanted into potting mix with pH levels of 5.6, 6.4, 7.2, and 7.8. Verticillium stripe was most severe at pH 7.8, indicating a substantial risk of increased disease and yield losses in neutral to slightly alkaline soils. Finally, 211 *Brassica* genotypes were screened for reactions to *V. longisporum* under greenhouse conditions and subjected to a genome-wide association study to identify single nucleotide polymorphism (SNP) markers for resistance. Eleven non-commercial *Brassica* accessions and nine out of 35 commercial canola cultivars displayed a low normalized area under the disease progress curve, suggesting their potential as sources of resistance against *V. longisporum*. Additionally, 45 significant SNP markers were identified, with promising hotspots

located on chromosomes A03 and A10. Collectively, the results highlight the need for proactive strategies to manage Verticillium stripe in canola.

Preface

This thesis is an original work by me, Yixiao Wang. I conducted all of the experiments and wrote the first draft of all chapters. Dr. Stephen Strelkov, my Supervisor, reviewed and provided editorial revisions and suggestions for improvement of each chapter. My Co-Supervisor, Dr. Sheau-Fang Hwang, approved the final version of each chapter prior to inclusion in my thesis. In addition, Dr. Rudolph Fredua-Agyeman, a member of my Supervisory Committee, also contributed to the revision of Chapter 4. I incorporated or addressed all of the revisions as needed. Once the draft thesis was complete, my Supervisory Committee approved the dissertation to go to defense.

The canola and rutabaga collections included in this thesis were obtained from the collections maintained by the Plant Pathology and Applied Plant Pathology Labs of the University of Alberta. The Chinese vegetable varieties included in Chapter 3 were obtained from Dr. Hui Zhang, Chinese Academy of Agricultural Sciences, Beijing, China. Mr. George Turnbull (University of Alberta) and Mr. Zhiyu Yu (University of Alberta) helped to seed, harvest and maintain the field experiments presented in Chapters 2. Dr. Fredua-Agyeman provided guidance and suggestions on the experimental design employed, while Mr. Zhiyu Yu helped with the data analysis, in Chapter 4. Many undergraduate and graduate students from the University of Alberta Plant Pathology Lab assisted with field plot preparation and weeding.

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List of Abbreviations

Abbreviations	Definitions
AUDPC	Area under the disease progress curve
BLINK	Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway
CC	Canadian canola
CFIA	Canadian Food Inspection Agency
DH	Doubled haploid
ECD	European Clubroot Differential
FarmCPU	Fixed and random model Circulating Probability Unification
GLM	General linear model
GWAS	Genome-wide association study
K	Kinship
LD	Linkage disequilibrium
LOD	Logarithm-of-odds
MLM	Mixed linear model
MLMM	Multiple Locus Mixed Linear Model
MR	Moderately resistant
MS	Moderately susceptible
NJ	Neighbor joining
PCA	Principal Coordinate Analysis
PDA	Potato dextrose agar
Q	Population structure
QTL	Quantitative trait loci
Q-Q plot	Quantile-Quantile plot
R	Resistant
S	Susceptible
SNP	Single nucleotide polymorphism
UPGMA	Unweighted pair group method with arithmetic mean

Chapter 1 Introduction and Literature Review

1.1 Canola

1.1.1 Introduction to canola

Canola or oilseed rape (*Brassica napus* L.) is a member of the Brassicaceae family, which includes many agriculturally important crops grown worldwide. The name 'canola' is a registered trademark for *B. napus* and *B. rapa* varieties with low erucic acid and low glucosinolate content, but it is now also used generically as a synonym of oilseed rape in Canada, Australia, and the United States. Canada is the largest producer and exporter of canola (Canola Council of Canada 2023b). The western provinces of Alberta, Saskatchewan and Manitoba account for 99% of Canada's canola production, with the remaining 1% is grown in British Columbia, Ontario, and Quebec (Canola Council of Canada 2023b). Canola contributes $29.9 billion CAD to the Canadian economy annually and is the most profitable commodity for growers (Canola Council of Canada 2023b).

1.1.2 Main diseases of canola in western Canada

Due to the high economic returns from canola production, growers often cultivate this crop over larger areas and in short rotations. Consequently, various diseases affect canola production in Canada and worldwide. While Verticillium stripe is the focus of this dissertation, three other canola important diseases also warrant mention: clubroot, sclerotinia stem rot, and blackleg.

Clubroot

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is a serious soilborne disease of canola and other *Brassica* species. Infection is associated with the abnormal growth and enlargement of host root tissue, resulting in the formation of 'clubs' or galls. These

root galls reduce a plant's ability to obtain water and nutrients from the soil, resulting in wilting, stunting, and premature and uneven ripening of the crop, which can significant yield losses when symptoms are severe (Botero-Ramírez et al. 2022). The disease was first identified on canola in western Canada in 2003, when 12 clubroot-infested crops were found in central Alberta (Tewari et al. 2005). In the past two decades, clubroot has spread quickly, with nearly 4,000 confirmed field infestations recorded across Alberta by 2022 (Strelkov et al., 2023). Clubroot has also spread to Saskatchewan and Manitoba, and while the number of cases is still much lower than in Alberta, the presence of *P. brassicae* DNA has been detected in hundreds of fields in each province (Government of Manitoba, 2023). Since 2013, clubroot has also been found on canola in North Dakota (Chittem et al. 2014).

Disease management strategies for clubroot include cultural practices, chemical controls, soil amendments and genetic resistance. Since clubroot is a soilborne disease, sanitizing field equipment and machinery can effectively reduce pathogen spread (Cao et al. 2009). Additionally, it is recommended to implement long crop rotations without susceptible plants and to practice effective weed management. This includes removing cruciferous weeds and volunteer canola to reduce pathogen inoculum levels (Hennig et al. 2022). While no fungicides are currently registered for clubroot control on canola, recent studies showed that liquid and granular formulations of amisulbrom reduced disease severity in both clubroot-susceptible and moderately resistant cultivars (Yu et al. 2023). Given that clubroot development is favored in acidic soils, the application of lime treatments to increase the soil pH has often been recommended for managing this disease (Donald and Porter 2009). Hennig et al. (2022) reported a significant reduction in clubroot severity and an increase in seed yield by using hydrated lime to raise soil pH from around 5 to 7. Moreover, numerous clubroot resistance gene loci have been identified (Kato et al. 2013;

Piao et al. 2009; Wei et al. 2024) and utilized in resistance breeding. The deployment of clubroot-resistant canola cultivars is the most widely used and economical method for clubroot management in Canada (Hasan et al. 2021; Hwang et al. 2014).

Sclerotinia stem rot

Sclerotinia stem rot, also known as white mold, is caused by *Sclerotinia sclerotiorum* (Lib) deBary, a soilborne disease of not only canola but many other dicot crops. Yield losses due to *S. sclerotiorum* infection can range from 10% to 70% (Del Río et al. 2007). Water-soaked lesions or areas of very light brown discoloration are first observed on leaves, stems, and branches. Eventually, the plant starts wilting, bleaching, and shredding (Hossain et al. 2023). Hard, melanized, black sclerotia with white moldy growth can be observed in the cortex of affected plants. The most effective tool for stem rot management is the application of fungicides, which can decrease disease incidence and minimize yield reductions (Bradley et al. 2006).

Blackleg

Blackleg or phoma stem canker of canola, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. and De Not., is a ubiquitous and economically important disease worldwide. The pathogen is stubble-borne, surviving on canola residues for 1 to 5 years (Hall 1992). On infected stubble, *L. maculans* forms pseudothecia, from which sexual ascospores are released after rainfall; the ascospores serve as primary inoculum and can be wind-dispersed for hundreds of meters (Ash 2000). The fungus also produces asexual pycnidia on both residues and following infection of the current crop. These pycnidia produce pycnidiospores that lead to secondary cycles of infection, spread primarily by rain splash (Rouxel and Balesdent 2005).

Infection of canola by *L. maculans* can occur from the seedling stage onwards, leading to the development of foliar lesions, stem cankers, and root rot (West et al. 2001). Infected tissues may exhibit small, dark-colored pycnidia. Additionally, upon examination in cross-section, infected stems show varying degrees of vascular staining. These symptoms and signs may resemble those caused by other diseases, most notably Verticillium stripe, which can lead to misdiagnosis by growers or agronomists. Severe blackleg infections can result in seed yield losses of up to 100%, whereas mild infections do not reduce yields (Wang et al. 2020). There have been some anecdotal reports of interactions between blackleg and Verticillium stripe potentially leading to increased yield losses. However, these interactions have not yet been scientifically investigated. Management strategies for blackleg include 4-year rotations out of canola, the planting blackleg-resistant canola cultivars, and fungicide treatments such as seed treatments and foliar sprays (Dolatabadian et al. 2022).

Verticillium stripe

Verticillium stripe, caused by the fungus *Verticillium longisporum* (C. Stark) Karapapa, Bainbridge, and Heale, is an emerging disease of canola in Canada. The Canadian Food Inspection Agency (CFIA) first identified *V. longisporum* in 2014 in Manitoba. Since then, the pathogen has been detected in various other Canadian provinces, including British Columbia, Alberta, Saskatchewan, Ontario, and Quebec (Canadian Food Inspection Agency 2018). According to disease surveys conducted in 2022, symptoms of Verticillium stripe were observed in about 2% and 38% of canola crops surveyed in Alberta (Harding et al. 2023) and Manitoba (Kim et al. 2023), respectively. Similarly, by 2022, Verticillium stripe had become a common disease of canola in the eastern part of Saskatchewan, and by the fall of 2023, it had been identified throughout many regions of the province (Arnason 2023). Given its increasing prevalence, Verticillium stripe has garnered

significant attention from growers and researchers alike, making it the focal point of this dissertation.

1.2 *Verticillium longisporum*

1.2.1 Taxonomy

Verticillium is a small genus of soilborne plant pathogenic fungi in the family Plectosphaerellaceae, class Sordariomycetes, of the phylum Ascomycota (Zare 2007). The genus includes 10 species, most of which have a broad range of hosts (Inderbitzin et al., 2011). *Verticillium* is divided into two groups: Clade Flavexudans, characterized by yellow pigmented hyphae, which includes species like *V. albo-atrum*, *V. tricorpus*, *V. zaregamsianum*, *V. isaacii*, and *V. klebahnii*, and Clade Flavnonexudans, lacking yellow hyphal pigmentation, which encompasses species such as *V. nubilum*, *V. dahliae*, *V. longisporum*, *V. alfalfae*, and *V. nonalfalfae* (Inderbitzin et al., 2011).

Among all *Verticillium* species, *V. dahliae* is the most economically important pathogen, causing wilt on many crops, including vegetables, legumes, woody ornamentals, and over 200 other plant species (Inderbitzin et al., 2011a). *Verticillium longisporum* was first identified by Stark (1961) as a variety of *V. dahliae*, primarily attacking hosts in the Brassicaceae family. However, *V. longisporum* is an amphidiploid hybrid with elongated and irregularly shaped resting structures (microsclerotia) (50 µm to 70 µm in length), long asexual spores (conidia) (7.1 µm to 8.8 µm in length), and three phialides per node on the conidiophores. In contrast, *V. dahliae* is haploid and has more spherical and compact microsclerotia (15 µm to 50 µm in length), shorter conidia (3.5 µm to 5.5 µm in length), and four to five phialides per node on the conidiophores (Karapapa et al. 1997). Molecular studies have indicated that a large intron of 830 bp in the SSU-rRNA gene is present in *V. longisporum* but not in *V. dahliae* (Karapapa et al. 1997). Based on this finding, coupled with the variations in fungal morphology, *V. longisporum* was reclassified as a distinct

species separate from *V. dahliae* (Karapapa et al. 1997). Moreover, Inderbitzin et al. (2011b) reported that *V. longisporum* is a diploid consisting of three different lineages (A1/D1, A1/D2, and A1/D3), each resulting from separate hybridization events. The ancestral species A1 and D1 are unknown, while D2 and D3 represented different lineages of *V. dahliae* (Inderbitzin et al., 2011a). Among the three lineages, A1/D1 is the most virulent on oilseed rape (Eynck et al. 2007).

1.2.2 Epidemiology, symptoms, and life cycle

Verticillium stripe is a monocyclic disease, with *V. longisporum* overwintering as microsclerotia. These structures consist of melanized, thick-walled cells that remain dormant and inactive in soil and crop residues in the absence of a host. They can persist in the soil for more than 10 years (Depotter et al. 2016). Microsclerotia germinate and produce hyphae when stimulated by root exudates, growing towards the root surface (Mol 1995). Subsequently, the germinating hyphae penetrate root hairs and root tips, spreading across the cortex both inter- and intracellularly towards the vascular system. In the xylem, the fungus produces conidia (asexual spores) (Eynck et al. 2007). As *V. longisporum* further colonizes the host, it inhibits vascular flow and alters the host membrane permeability, leading to blockage of the xylem and disruption of nutrient flow (Eynck et al. 2007). At later stages of disease development, as the plants begin to senesce, the fungus colonizes the stem parenchyma (Schnathorst 1981). Subsequently, microsclerotia are formed in the pith and beneath the stem epidermis (Schnathorst 1981). At harvest, microsclerotia either remain in the infected stubble or are deposited into the soil, severing as inoculum for future years.

Kemmochi and Sakai (2004) found that in cabbage (*Brassica oleracea* var. *capitata* L.), *V. longisporum* induces stunting, wilting, chlorosis, necrosis, and defoliation of lower leaves, referring to the disease it causes as Verticillium wilt. However, in canola (oilseed rape), the most distinct symptoms and signs of infection include dark unilateral striping on the stem, shredding of

the stem tissues, and the appearance of black microsclerotia during ripening of the crop. Consequently, Verticillium stripe was adopted as the common name to describe the disease as it occurs in canola or oilseed rape (Depotter et al. 2016). In contrast to the symptoms of Verticillium stripe under field conditions, greenhouse symptoms include chlorosis of the leaves or lateral branches, stunting, and early senescence, which can be observed from the seedling stage to flowering (Eynck et al. 2007).

The occurrence of *V. longisporum* has been confirmed in Europe, North America, and Asia. Given that *V. longisporum* and *V. dahliae* were considered a single species before the 1990s, Verticillium wilt caused by the latter was first described in Brussels sprouts (*Brassica oleracea* var. *gemmifera* DC.) in the UK in the 1950s (Isaac 1957), and the disease was detected on horseradish (*Armoracia rusticana* G.Gaertn., B.Mey. & Scherb.) in Germany in 1960 (Stark 1961). However, the first report of Verticillium disease in oilseed rape (*Brassica napus* L.) was in Sweden in 1969, and since the 1970s, it has become economically important (Dixelius et al. 2005). Subsequently, outbreaks of Verticillium stripe have been observed in the main oilseed rape-producing regions of Germany since the 1980s (Gunzelmann and Paul 1990). While the first reports of *V. dahliae*/*V. longisporum* infection of crucifers came from Europe (Inderbitzin et al., 2011a; Johansson et al., 2006), economic losses had been confirmed in 10 countries including Japan and the USA by the mid-2010s (Inderbitzin and Subbarao 2014). As noted earlier, the first reported case of Verticillium stripe case in canola in Canada was confirmed Manitoba in 2014 (Canadian Food Inspection Agency 2018), although how the pathogen was introduced into this country remains unknown.

The main hosts of *V. longisporum* are in the Brassicaceae family, including Brussels sprouts, turnip (*Brassica rapa* subsp. *rapa* Metzg.), and oilseed rape (Zeise and Von Tiedemann 2002). Disease incidences of up to 80% have been reported in oilseed rape in Europe (Dixelius et al.

2005). Studies conducted in Sweden indicated that yield losses associated with Verticillium stripe ranged from 10% to 50% on this crop (Eastburn and Paul 2007). In Canada, yield losses related to Verticillium stripe was reported by producers in 2020 in Saskatchewan (Briere 2020), and preliminary studies showed that yield loss per plant could exceed 60% when the disease was severe (Cui 2024).

1.3 Management strategies

Cultural management

Because *V. longisporum* is a soilborne fungus with microsclerotia that can survive in the soil more than 10 years, preventing the dissemination of the pathogen to uninfested fields is a critical first line of defense against Verticillium stripe in canola. To achieve this, minimizing soil movement, sanitizing farm equipment and tools, reducing tillage, and monitoring the source of seed and fertilizer are recommended as basic biosecurity practices (Canola Council of Canada 2023c). However, if *V. longisporum* is already present in a field, additional management strategies will be required.

Crop rotation stands out as one of the most common and fundamental methods for managing soilborne pathogens. The narrower host range of *V. longisporum* theoretically facilitates its control by crop rotation (Depotter et al. 2016). However, many growers in western Canada opt for short rotations of canola due to its superior economic returns compared with other available crop options. Furthermore, the longevity of *V. longisporum* microsclerotia (Dixelius et al. 2005) suggests that very long rotations may be necessary for effective management of this pathogen. A study conducted with cauliflower (*Brassica oleracea* var. *botrytis* L.) grown in two fields that were naturally infested by *V. longisporum* indicated that the inoculum density of microsclerotia-forming *Verticillium* species in the soil remained unaffected by continuous cropping, fallow periods, or the

removal of cauliflower debris (França et al. 2013). Given its recent emergence as a pathogen in Canada, few crop rotation studies (França et al. 2013; Subbarao et al. 1999) have been conducted to evaluate the potential of this strategy in managing *V. longisporum* in canola cropping systems. Further research on long-term rotations out of host species will be important to examine their efficacy in reducing inoculum of this pathogen in the soil.

Control of Brassicaceous weeds may help reduce *V. longisporum* inoculum levels. The pathogen has been isolated from numerous weedy species, including shepherd's purse (*Capsella bursa-pastoris* (L.) Medik.), annual wall-rocket (*Diplotaxis muralis* DC.), clasping pepperweed (*Lepidium perfoliatum* L.), tumble mustard (*Sisymbrium altissimum* L.), field pennycress (*Thlaspi arvense* L.), and charlock (*Sinapis arvensis* O.F.Müll.). These plants might serve as reservoirs of inoculum (Johansson et al. 2006a). Controlling *Brassica* reservoir plants and volunteer canola can further decrease the density of microsclerotia (Johansson et al. 2006a). Therefore, combining crop rotation with effective weed management may be effective in reducing the inoculum density of *V. longisporum*.

Chemical management

Chemical management, including seed treatments, foliar sprays, and soil treatments, can be an effective approach for the management of some soilborne diseases. Unfortunately, no registered fungicides or fumigants are currently available for managing *V. longisporum*. Most research on the use of chemicals to control Verticillium diseases has focused on *V. dahliae* and its infection of vegetable crops. For example, methyl bromide, a broad-spectrum fumigant commonly applied before planting (Jarvis 1989), has been proven to reduce the inoculum density of *V. dahliae* in the soil (Subbarao 2002). However, due to its destructive effects on stratospheric ozone, it is no longer available for agricultural use. Soil fumigation with metam sodium, either alone or in combination

with 1,3-dichloropropene, has been used to control potato early dying in the United States, as these treatments can effectively reduce soil populations of *V. dahliae* (Rowe and Powelson 2002). Another fumigant, chloropicrin, applied via shank injection, was evaluated for protection of tomato against *V. dahliae* in heavily infested fields. The treatments resulted in a 7% to 10% reduction in the incidence of Verticillium wilt (Gullino et al. 2002). However, no effective fungicides have been identified for controlling *V. longisporum* (Depotter et al., 2016), and there are no published reports on the use of fumigants to manage this pathogen. Therefore, more research on chemical strategies for the management of *V. longisporum*, particularly in canola or oilseed rape, is necessary.

Biological management

The use of biocontrol control agents, such as bacteria and fungi, shows promise in managing diseases caused by *Verticillium* species. For instance, França et al. (2013) demonstrated that a non-pathogenic isolate *V. isaacii*, sourced from a Verticillium wilt-suppressive cauliflower field, effectively mitigated symptoms caused by *V. longisporum* infection and reduced host colonization under controlled conditions. Although the precise mechanism(s) underlying these effects remain unclear, it is possible that competition for infection sites and induction of host resistance responses contributed to the observed outcomes (França et al., 2013). Similarly, the ascomycete fungus *Microsphaeropsis ochracea* has shown efficacy in controlling *V. longisporum in vitro* and under sterile soil conditions (Stadler and Von Tiedemann 2014). However, while this biocontrol agent caused high rates of microsclerotial mortality in controlled environments, its efficacy in the field was limited, likely due to an insufficient capacity to compete with other soil microbes (Stadler and Von Tiedemann 2014).

Narisawa et al. (2004) tested 349 fungal endophytes obtained from various crops to assess their effectiveness in reducing root colonization of Chinese cabbage by *V. longisporum*. Two

isolates of *Phialocephala fortinii* and one isolate of a dark septate endophytic (DSE) fungus from barley roots almost completely suppressed *V. longisporum* infection in Petri dish-grown plants (Narisawa et al. 2004). Furthermore, evaluation of *Heteroconium chaetospira*- and DSE fungus-treated plants under field conditions suggested that both biocontrol agents could inhibit the development of Verticillium wilt caused by *V. longisporum* in Chinese cabbage (Narisawa et al. 2004). Given these promising results, it may be worthwhile evaluating *H. chaetospira* and DSE fungi as potential agents for biocontrol of Verticillium stripe in canola.

In addition to fungal biological control agents, the plant-beneficial bacterium *Serratia plymuthica* HRO-C48 was evaluated for its effectiveness in controlling *V. longisporum* in oilseed rape, as it had previously been used to protect strawberry against *V. dahliae* (Müller and Berg 2008). Three different techniques, pelleting, film coating and bio-priming, were used to apply the biocontrol agent to seeds for evaluation (Müller and Berg 2008). The results indicated that bio-priming resulted in a more stable reduction of disease severity compared to the other two application techniques, at least under greenhouse conditions (Müller and Berg 2008). Additionally, two *Bacillus amyloliquefaciens* strains, UCMB-5036 and UCMB-511, provided significant protection against *V. longisporum* infection *in vitro* and exhibited growth-promoting effects (Danielsson et al. 2007). However, while biological management strategies show promise in controlling *V. longisporum*, their efficacy is often limited to controlled environments rather than field conditions.

Genetic resistance

Given the limited availability of effective fungicides and biological control agents, there is a critical need to explore genetic resistance to Verticillium stripe in canola or oilseed rape. While no major resistance (R)-genes have been identified against *V. longisporum*, Fradin et al. (2009)

reported the effectiveness of the R-gene *Ve1* against *V. dahliae* and *V. albo-atrum*. Originally identified in tomato (*Solanum lycopersicum* L.) (Kawchuk et al. 2001), functional *Ve1* homologues have been found in lettuce (*Lactuca sativa var. crispa* L.), tobacco (*Nicotiana tabacum* L.), and other plant species (Hayes et al. 2011; Zhang et al. 2013). The effector protein, Ave1, is known to activate *Ve1*-mediated resistance against race 1 strains of *V. dahliae* and *V. albo-atrum* (De Jonge et al. 2012). However, the *Ve1* gene was not effective against isolates of *V. longisporum*, suggesting their lack of the Ave1 effector (Fradin et al., 2011). A more recent study indicated that *Ve1* may function as a "resilience" gene rather than a classical R-gene (Nazar et al. 2020). This is because the *Ve*-locus in tomato governs not only resistance to Verticillium wilt but also enhances root growth when the *Ve1* protein is induced, thereby increasing the plant's ability to withstand infection (Robb and Nazar 2021).

At present, most cultivars of canola or oilseed rape exhibit a low level of resistance to *V. longisporum* (Cowling 2007). In contrast, lines derived from *B. carinata*, *B. rapa*, and *B. oleracea*, have shown reduced susceptibility to this fungus (Happstadius et al. 2003). Rygulla et al. (2007) successfully enhanced resistance by resynthesizing *B. napus* from its progenitor species, *B. oleracea* and *B. rapa*, using an embryo rescue-assisted interspecific technique. Test lines generated from this approach exhibited significantly higher levels of resistance to *V. longisporum*. Furthermore, Rygulla et al. (2008) developed 163 doubled haploid (DH) lines using the *B. napus* parental lines 307-406-1 and 307-230-2. They identified two major quantitative trait loci (QTL) located on linkage groups N14 and N15 on the C-genome, which consistently conferred resistance to multiple isolates of *V. longisporum* under greenhouse conditions (Rygulla et al. 2008).

To identify *V. longsiporum* resistance across different genetic backgrounds of *B. napus*, Obermeier et al. (2013) examined 214 DH lines derived from the partially resistant oilseed rape

cv. ‘Express’ and a resistant, resynthesized rapeseed line R53. The study aimed to identify QTL associated with resistance to the fungus under greenhouse conditions. Two major QTL on chromosome C5 and one minor QTL on chromosome C1 were identified (Obermeier et al. 2013). All the detected QTL were on the C-genome, indicating that these sources of quantitative resistance to *V. longisporum* originated from oilseed rape parental cabbage lines (Depotter et al. 2016). While the mechanisms of this resistance are unknown, some of the QTL regions coincided with loci linked to the synthesis of two soluble phenylpropanoid compounds (Obermeier et al. 2013); these compounds have been reported to exhibit a negative correlation with disease severity. Therefore, genes related to the phenylpropanoid pathway may be important in conferring resistance to *V. longisporum*, and could serve as potential candidates for resistance breeding in canola or oilseed rape.

Efforts to understand resistance to *V. longisporum* continue. For instance, Gabur et al. (2020) utilized single nucleotide polymorphism (SNP) markers and single nucleotide absence polymorphism (SNaP) markers to screen a partially resistant population from crosses of a common elite oilseed rape parent with five synthetic *B. napus* parents. Their results identified four QTL regions on chromosomes A03, A05, C05, and C08 (Gabur et al. 2020). More recently, Su et al. (2023) reported that a MYB transcription promoter, *BrMYB108*, in *B. rapa* regulates the generation of reactive oxygen species, thereby enhancing resistance to *V. longisporum*. Despite these advancements, it is evident that additional research on the identification and genetic control of resistance to this fungus is necessary, especially in the context of Canadian canola production.

1.4 Hypotheses and Objectives

Given the recent emergence of *V. longisporum* as a pathogen of canola in Canada, it is crucial to develop effective management strategies. Controlling Verticillium stripe will require a

comprehensive understanding of the pathogen's biology, including its response to soil conditions such as pH, potential interactions with other significant canola pathogens like *L. maculans*, and an enhanced understanding of host resistance and its sources.

The research in this thesis was conducted to test three main hypotheses:

1. Mild blackleg infections do not cause yield losses; however, when co-inoculated with *V. longisporum*, the severities of both blackleg and Verticillium stripe increase, leading to greater yield losses.
2. Increased pH results in greater *V. longisporum* growth *in vitro* and increases Verticillium stripe severity on canola both at the seedling stage and at maturity.
3. Phenotypic differences in *V. longisporum* resistance will be observed in a collection of *Brassica* genotypes, and significant single nucleotide polymorphism (SNP) markers will be identified for resistance.

These hypotheses unpin the following objectives:

1. To establish the relationship between blackleg severity and yield in blackleg-resistant canola hybrids, and to determine whether the co-occurrence of blackleg and Verticillium stripe together can cause greater yield losses. As a secondary objective, specific symptoms were compared to provide guidelines to help non-expert personnel to more easily distinguish between blackleg and Verticillium stripe.
2. To examine the effects of pH on (i) radial growth of *V. longisporum in vitro*, (ii) Verticillium stripe development at the seedling stage under semi-hydroponic conditions, and (iii) Verticillium stripe severity and canola yields at maturity under greenhouse conditions.

3. To screen a large collection of rutabaga (*B. napus* ssp. *napobrassica*) accessions and commercial canola cultivars from Canada, as well as *B. rapa* and *B. oleracea* genotypes from China, for resistance to *V. longisporum* using a genome-wide association study.

Chapter 2 Blackleg yield losses and interactions with Verticillium stripe in canola (*Brassica napus*) in Canada

2.1 Introduction

Blackleg, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. & De Not. (anamorph: *Phoma lingam* (Tode) Desm.), is an important disease of canola (oilseed rape; *Brassica napus* L.) in Europe, Australia, and Canada (Gugel & Petrie 1992; Salisbury et al. 1995; West et al. 2001). The fungus can attack the stems, leaves and pods of *B. napus*, but the formation of basal stem cankers is most damaging (Howlett et al. 2001). These cankers appear dry and sunken, and may be dotted with black pycnidia, the asexual fruiting bodies of *L. maculans*. In cross-section, a dark discolouration of the vascular tissues is visible in infected canola stems, which may help to diagnose the disease. In recent surveys of western Canadian canola crops, blackleg was found to occur at an average incidence of 12.5% and average prevalence of 80% (Akhavan et al. 2022; Harding et al. 2022; Kim et al. 2022). The deployment of blackleg-resistant cultivars is the most effective strategy for blackleg management (Kutcher et al. 2013). However, the erosion or loss of resistance has been reported in many canola cultivars, reflecting the emergence of virulent isolates of *L. maculans* (Rashid et al. 2021; Zhang et al. 2016).

Severe epidemics of blackleg can result in yield losses of 30-50% (Barbetti & Khangura 1999; Hall et al. 1993; Zhou et al. 1999). In experiments conducted in western Canada, Hwang et al. (Hwang et al. 2016) found a negative linear relationship between blackleg severity and yield loss in a susceptible canola cultivar, 'Westar', with a 17.2% reduction in yield for each unit increase in disease severity, as assessed on a 0-5 scale. More recently, Wang et al. (2020) examined blackleg severity-yield loss relationships in moderately resistant canola hybrids and reported quadratic relationships between blackleg severity and yield losses. A small increase in

yield was found under very mild infection in plants rated as '1' on the 0-5 scale, which was followed by large yield decreases at blackleg severities of ≥ 2. At a severity rating of '5', the percentage yield loss was as high as 99% (Wang et al. 2020). In canola hybrids classified as blackleg resistant, which represent most of the cultivars available in the Canadian market (Canola Council of Canada 2022), the relationship between blackleg severity and yield loss has not been explored.

Over the last few years, another disease, Verticillium stripe caused by *Verticillium longisporum* (C. Stark) Karapapa, Bainbridge and Heale, has also emerged as a potential threat to canola in western Canada. Initially identified in Manitoba in 2014, the presence of *V. longisporum* was soon confirmed across much of the country, including British Columbia, Alberta, Saskatchewan, Ontario, and Quebec (Canadian Food Inspection Agency 2018). By 2021, symptoms of Verticillium stripe were observed in nearly 3% and 30% of canola crops surveyed in Alberta (Harding et al. 2022) and Manitoba (Kim et al. 2022), respectively. Three samples collected in Saskatchewan also tested positive for the presence of *V. longisporum* (Akhavan et al. 2022). Symptoms and signs of Verticillium stripe include necrosis and shredding of the stem tissue, along with the presence of pathogen microsclerotia (Depotter et al. 2016). Infection by *V. longisporum* also results in staining of the vascular tissues, visible in cross-sections of the stem, which can be confused with the symptoms caused by *L. maculans* described above. Yield losses associated with Verticillium stripe were between 10 to 50% in oilseed rape in Sweden (Rimmer et al. 2007).

Considering that both blackleg and Verticillium stripe now occur on canola in western Canada and given the generally greater familiarity of farmers and agronomists with the former (Akhavan et al. 2022; Harding et al. 2022; Kim et al. 2022), it is possible that some *V. longisporum*

infections are being misdiagnosed as *L. maculans*. It is also likely that some canola plants are infected by both pathogens. In this context, it is important to assess if and how co-infection of canola affects yield losses caused by *V. longisporum* and *L. maculans*. Moreover, given the linear vs. quadratic relationships reported (Hwang et al. 2016; Wang et al. 2020) in susceptible vs. moderately resistant canola cultivars, respectively, it is also important to clarify this relationship in hosts classified as resistant. The main objectives of this study were: (1) to establish the relationship between blackleg severity and the yield of blackleg resistant canola hybrids under experimental field conditions and in commercial crops in western Canada, and (2) to examine possible interactions between blackleg and Verticillium stripe with respect to yields. In addition, specific symptoms were compared that could help non-expert personnel to distinguish between blackleg and Verticillium stripe more easily.

2.2 Materials and methods

Inoculum preparation

The isolates of *L. maculans* (11C78103, 11C78301, 11S54041, 11P125232, and 11L999014) used as inoculum in the field plot and greenhouse experiments were originally characterized by Rong et al. (2015), while the isolate of *V. longisporum* (Vl43) was recovered from diseased *B. napus* tissues collected near Edmonton, Alberta. Grain inoculum of both fungi was prepared based on Hwang et al. (2014). Briefly, cultures of *L. maculans* were grown in Petri dishes on V8 medium (composition per litre: 850 mL distilled water, 150 mL V8® Original Vegetable Juice (Campbell Soup Company, Camden, NJ), 1.5 g CaCO3, 15.0 g agar), and then incubated for 21 d at room temperature under fluorescent lighting to encourage pycnidiospore production. Cultures of *V. longisporum* were grown in Petri dishes on potato dextrose agar (PDA; 26 g PDA powder, 850 mL distilled water), and then incubated in darkness at room temperature for 21 d for conidial

production. The colonies of *L. maculans* and *V. longisporum* were cut into small pieces and mixed with sterilized, water-soaked wheat grain (900 mL grain per one culture of *L. maculans* or *V. longisporum*) in separate autoclave bags. The inoculated grain was incubated at room temperature for 28 d, and then dried at 35°C for 2 d. After drying, the inoculated grain was ground in a grain mill and passed through a 2 mm-diam. mesh sieve.

Field experiments for blackleg yield losses

Field experiments to evaluate the relationship between blackleg severity and yield were conducted over 2 years (2019 and 2020) at the Crop Diversification Centre-North, Edmonton, AB, Canada (53°39′N, 113°22′W). Two blackleg-resistant canola hybrids, '45H31' and 'CS2000', were included in the experiments. Treatments were arranged in a split-plot design with four replicates. Each plot consisted of four rows, 6 m in length and 1.5 m in width, with 0.25 m spacing between the rows. Adjacent plots were separated by a 1 m buffer zone, with 2 m between replicates. Each row was seeded with 0.7 g of canola using a push seeder. The grain inoculum was applied (200 mL/row) at seeding by placing it in the seeder together with the seeds. No grain inoculum was included in the control treatments. The plots were seeded on 27 May 2019 and 17 May 2020.

To assess the impact of blackleg on canola yield, all plants within a 1 m^2 area in the center of each plot were carefully excavated from the soil with a shovel at maturity (8 Oct. 2019 and 1 Oct. 2020) and placed in paper bags. The remainder of each plot was harvested with a small plot combine using a straight cut header, and the seed was weighed to determine overall yield. The average blackleg severity per 1 m^2 plot area was assessed on a 0–5 scale as described below, and stem cross-sections were examined by cutting the plant at the soil line. The number of pods and seed yield were recorded for each plant individually. The pods from each plant were threshed manually, and the seeds were cleaned and weighed.

Blackleg yield losses in commercial crops

To evaluate yield losses due to blackleg in commercial fields, nine canola crops in the County of Wetaskiwin and three crops in the County of Lacombe, located in central Alberta, were sampled at maturity on 12 Sep. 2019. These crops were selected for sampling based on the occurrence of symptoms of blackleg. Briefly, entire plants were dug out from the soil within a 1 m^2 area at each of five locations along the arms of a 'W' sampling pattern, with 100 canola plants collected per field (Madden & Hughes 1999). The plants were placed in paper bags for subsequent assessment in the laboratory. Each plant was rated for blackleg severity on the 0-5 scale described below, and pod number and seed yield were recorded. Pods were threshed manually, and the seeds were cleaned and weighed. Six of the fields sampled in Lacombe County were sown to the canola hybrid 'DKTF 94CR', and three were sown to '75-42CR'; both hybrids are rated as blackleg resistant. The three fields in the County of Lacombe were all sown to the hybrid 'DKTF 94CR'.

Field experiments for blackleg and Verticillium stripe interactions

Field trials to evaluate the effect of blackleg/Verticillium stripe interactions on canola yield were conducted over 2 years (2020 and 2021) at two sites located at the Crop Diversification Centre-North. The canola hybrids '45H31'and 'CS2000'were included in the experiments, which were arranged in a split-plot design with four replicates. Each plot consisted of four rows, 6 m in length and 1.5 m in width with 0.25-m spacing between the rows. Adjacent plots were separated by a 1 m buffer zone, with 2 m between replicates. Each row was seeded with 0.7 g of canola as described above. Grain inoculum of *L. maculans* and *V. longisporum* was applied at various ratios in the different treatments: *L. maculans* grain inoculum applied at 200 mL/row; *V. longisporum* grain inoculum at 200 mL/row; a 3:1 mix of *L. maculans* (150 mL/row) and *V. longisporum* (50 mL/row); a 1:1 mix of *L. maculans* (100 mL/row) and *V. longisporum* (100 mL/row); and a 1:3

mix of *L. maculans* (50 mL/row) and *V. longisporum* (150 mL/row) inoculum. Control treatments did not receive any inoculum. The grain inoculum was applied at seeding by placing it in the seeder as above. Experiments were seeded on 17 May 2020 and 18 May 2021.

To assess the impact of blackleg and Verticillium stripe interactions, 15 plants from each plot were carefully collected with a shovel and placed in paper bags. The remainder of each plot was harvested with a small plot combine using a straight cut header, and the seed was weighed to determine overall yield. The percentage yield reduction was calculated relative to non-inoculated controls. Experiments were harvested at maturity on 13 Oct. 2020 and 22 Sept. 2021. The plants were rated for blackleg severity on a 0–5 scale and for Verticillium stripe severity on a 0-4 rating scale as described below. Plant samples were visually examined for the presence of pycnidia of *L. maculans* and microsclerotia of *V. longisporum*. Horizontal and vertical sections of the stems were made with a bypass pruner for comparison of blackleg and Verticillium stripe symptoms.

Greenhouse experiments for blackleg and Verticillium stripe interactions

Greenhouse experiments were conducted with the canola cultivars '45H31' and 'CS2000'. The experiments were arranged in a split-plot design with four replicates using plastic containers (40.9 × 28.2 × 15.0 cm) filled with Sunshine® mix #4 potting medium. Two rows were seeded per container, at a rate of 20 seeds per row, and grain inoculum was placed along with the seeds. Inoculation treatments included: *L. maculans* grain inoculum applied at 20 mL/row; *V. longisporum* grain inoculum applied at 20 mL/row; a 3:1 mix of *L. maculans* (15 mL/row) and *V. longisporum* (5 mL/row); a 1:1 mix of *L. maculans* (10 mL/row) and *V. longisporum* (10 mL/row); and a 1:3 mix of *L. maculans* (5 mL/row) and *V. longisporum* (15 mL/row). Control treatments were not inoculated. The experiment was repeated. All the plants in each container were rated for blackleg severity on a 0–5 scale and Verticillium stripe severity on a 0-4 scale as described below.

Horizontal and vertical sections were made for identification of blackleg and Verticillium stripe. Emergence counts were taken 14 days after seeding. Seed yields were weighed and recorded.

Disease assessments

Plants were rated for blackleg severity on a 0-5 scale, where: 0 = no infection; 1 = lesion area < 25% of the cross-section area of the crown; 2 = lesion area 25-50% of the cross-section area of the crown; 3 = lesion area 51-75% of the cross-section area crown; 4 = lesion area 76-100% of the cross-section area of the crown; and 5 = plant dead [42]. Verticillium stripe severity was assessed on a 0-4 scale based on the amount of fungal microsclerotia on the entire plant, where: 0 = healthy plants with no microsclerotia visible; 1 = slight colonization by microsclerotia < 25%; 2 = moderate colonization by microsclerotia < 75%; 3 = extensive colonization by microsclerotia > 75%; 4 = severe colonization by microsclerotia and peeling of the stem epidermis.

Statistical analysis

Statistical analysis was conducted using R v 4.2.0: (R Core Team, R Foundation for Statistical Computing, Vienna, Australia, 2013). To establish the relationship between blackleg severity and pod number and seed yield, regression analysis was performed. The Akaike information and Bayesian information criteria were used for selection of the best model for the data. Adjusted R^2 values and the F test were used to examine compatibility of the regression. Residual data were tested for normality with the Shapiro–Wilk test in R shapiro.test stats. Regression equations were generated to evaluate the losses in pod number and seed yield with increasing disease severity. The yield of plants with no blackleg symptoms was used as a point estimate, with different yield data points at each disease severity transformed into yield percentages relative to canola yield with no disease. Regression analysis was performed to estimate yield loss percentage per unit increase in disease severity. To examine blackleg and Verticillium stripe interactions, canola hybrid was

considered as a fixed effect, and replication and site-year and their interaction as random effects. Analysis of variance was performed. Least significant difference comparisons were used to determine whether disease severity and seed yield differed among concentrations.

2.3 Results

Field experiments for blackleg yield losses

Mean blackleg disease severity on the non-inoculated canola hybrids '45H31' and 'CS2000' was 0.1 and 0.2, respectively, in 2019, and 0.0 for both hybrids in 2020 (Figure 2.1). On the inoculated treatments, mean blackleg disease severity was 2.4 on '45H31' and 1.4 on 'CS2000' in 2019, and 1.3 and 0.8, respectively, in 2020 (Figure 2.1). A paired t-test indicated that the mean disease severities among the inoculated cultivars were different at $P < 0.01$. In 2019, the seed yield of the non-inoculated canola '45H31' and 'CS2000' was 3.47 and 2.72 t ha^{-1}, respectively, while in the inoculated plots, the yield was 2.18 and 1.87 t ha^{-1}, respectively (Table 2.1). In 2020, the seed yield of the non-inoculated canola '45H31' and 'CS2000' was 1.05 and 0.88 t ha^{-1}, respectively, while in the inoculated plots, yield was 1.00 and 0.82 t ha^{-1}, respectively (Table 2.1).

Regression analysis indicated that pod number and seed yield declined with increasing blackleg severity. When averaged across two years for the canola hybrid '45H31', the average seed yield and pod number (+/- SE) ranged from 5.30 g ± 1.28 g to 41.18 g ± 3.55 g seed per plant and from 44 ± 12 to 433 ± 79 pods per plant. The regression models were $y = -1.767x^2 - 65.456x + 419.093$ ($P < 0.01$, $R^2 = 0.97$) for pod number vs. disease severity (Figure 2a), and $y = -0.2871x^2 - 5.9911x + 42.24$ ($P < 0.01$, $R^2 = 0.97$) for seed yield vs. disease severity (Figure 2b). Average seed yield and pod number for the canola hybrid 'CS2000' ranged from 2.74 g ± 1.05 g to 27.77 g ± 6.37 g seed per plant and from 91 ± 35 to 493 ± 137 pods per plant. The regression models were $y = 2.721x^2 - 92.035x + 454.402$ ($P = 0.08$, $R^2 = 0.69$) for pod number vs. disease severity

(Figure 2.2a), and $y = -0.0652x^2 - 4.4641x + 24.836$ ($P = 0.08$, $R^2 = 0.69$) for seed yield vs. disease severity (Figure 2.2b).

The regression models for percentage yield losses vs. disease severity were $y = 0.698x^2 + 14.5456x - 2.5786$ ($P < 0.01$, $R^2 = 0.97$) for '45H31', and $y = 0.3114x^2 + 21.4063x - 19.0623$ ($P = 0.08$, $R^2 = 0.69$) for 'CS2000' (Figure 2.3). In 'CS2000', plants with a blackleg severity of 0 had a slightly lower yield than plants with a severity of 1. When disease severity was rated as 1, seed yield increased by 6.91 g. However, as disease severity increased further from 2 to 5, yields began to decrease. The percentage yield loss increased by 26.86%–86.87% in plants with disease severities of 2-5, relative to plants with disease severities of 0-1. In contrast, on '45H31', seed yield begun to decrease at a disease severity rating of 1 and continued to decrease as severity increased to a rating of 5. However, a high-adjusted R^2 value indicated the relationship between yield loss and blackleg severity still fit a second-degree quadratic equation better than a linear regression.

Blackleg yield losses in commercial crops

Symptoms of blackleg were identified in all nine canola crops sampled in central Alberta. Mean disease severities on the hybrids 'DKTF 94CR' and '75-42CR' were 2.9 and 2.7, respectively, across all the crops. On 'DKTF 94CR', regression analysis indicated that the average seed yield ranged from 1.80 g ± 0.55 g to 17.69 g ± 3.71 g per plant, while the average pod number ranged from 76 ± 18 to 271 ± 61 pods per plant. The regression model was $y = -8.7969 x^2 + 20.969x + 194.61$ ($P = 0.22$, $R^2 = 0.39$) for pod number vs. disease severity (Figure 2.4a), and $y = -0.2664x^2 - 1.297x + 14.138$ ($P = 0.11$, $R^2 = 0.61$) for seed yield vs. disease severity (Figure 2.4b). In the case of '75-42CR', the average seed yield ranged from 0.80 g ± 0.27 g to 15.79 g ± 3.42 g per plant, while the average pod number ranged from 45 ± 18 to 276 ± 88 pods per plant. The

regression model was y = –32.367x + 233.3 (P = 0.15, R^2 = 0.31) for pod number vs. disease severity (Figure 2.4a), and y = − 3.1563x + 15.911 (P < 0.01, R^2 = 0.96) for seed yield vs. disease severity (Figure 2.4b).

The regression models for percent yield loss vs. disease severity were y = $2.2678x^2$ + 11.04x – 20.339 (P = 0.11, R^2 = 0.61) for 'DKTF 94CR' and y = 19.992x – 0.7785 (P < 0.01, R^2 = 0.96) for '75-42CR' (Figure 2.5). In the case of the hybrid 'DKTF 94CR', plants with a blackleg severity of 1 had a greater seed yield relative to plants with a rating of 0, but as severities increased from 2 to 5, yields began to decrease and the percentage yield loss increased from 26.4-84.7% relative to plants with disease severities of 0 or 1. In contrast, a linear relationship between disease severity and percentage yield loss was observed in '75-42CR'. For each unit increase in blackleg severity, estimated yield losses were about 20%.

Field experiments for blackleg and Verticillium stripe interactions

Mean blackleg disease severity ranged from 0.1 to 1.6 on '45H31', and from 0.0 to 1.3 on 'CS2000', at the two sites over two years (Table 2.2). On '45H31' in 2020, the most severe blackleg (1.3-1.6) at site 1 was observed in treatments inoculated with *V. longisporum* alone or with a 3:1 or 1:1 mix of *L. maculans* and *V. longisporum*; in the *L. maculans* alone treatment, the blackleg severity (1.0) was significantly lower than in the 1:1 mix of pathogens. At site 2 in 2020, the most severe blackleg (1.2-1.5) on '45H31' developed following inoculation with the 3:1 and 1:1 mixes of *L. maculans* and *V. longisporum*, while the lowest disease (0.1) was observed on the control. The *L. maculans* alone and 1:3 mix of *L. maculans* and *V. longisporum*, and *V. longisporum* alone treatments developed intermediate blackleg severities (0.7-1.0). On 'CS2000' in 2020, all inoculated treatments developed blackleg severities ranging from 0.8 to 1.3 at the two sites, which was significantly greater than the severity (0.1) on the non-inoculated control (Table

2). In 2021 at site 1, the most severe blackleg (1.5) on '45H31' developed on the *L. maculans* alone treatment, and the mildest blackleg was observed on the control (0.5) and 1:3 mix of *L. maculans* and *V. longisporum* (0.3) and *V. longisporum* alone (0.7) treatment; the disease severity on the other treatments was intermediate (Table 2.2). Similar trends were observed for '45H31' at site 2 and 'CS2000' at sites 1 and 2 in 2021; the most severe blackleg developed on the *L. maculans* alone treatment, on which disease was generally higher than most other treatments, although the mildest symptoms did not always occur on the non-inoculated control (Table 2.2).

The mean Verticillium stripe severity ranged from 0.0 to 2.2 on the hybrid '45H31' and from 0.0 to 2.0 on 'CS2000' at the two sites over two years (Table 2.3). At site 1 in 2020, the numerically most severe Verticillium stripe (0.5) on '45H31' was observed in the *V. longisporum* alone treatment, although this was not significantly greater than the disease (0.4) that developed following inoculation with the 3:1 and 1:1 mixes of the pathogens. However, Verticillium stripe on the *V. longisporum* alone treatment was significantly more severe than on the control (0.0), 1:3 mix of *L. maculans* and *V. longisporum* (0.2), and *L. maculans* alone (0.3) treatments for '45H31' at site 1 in 2020. At site 2 in 2020, there were no significant differences in Verticillium stripe severity on this hybrid (Table 2.3). In the case of 'CS2000' at both sites in 2020, the most severe Verticillium stripe (0.6-0.7) was observed in treatments inoculated with *V. longisporum*, while the mildest disease was found on the control (0.1-0.2) and *L. maculans* alone (0.2-0.3) treatments (Table 2.3). In 2021 on '45H31' at site 1, the most severe Verticillium stripe (1.0-1.2) was observed on treatments inoculated with 3:1, 1:1, 1:3 mixes of *L. maculans* and *V. longisporum*, as well as on the *V. longisporum* alone treatment at site 1. The no inoculum control (0.5) and *L. maculans* alone (1.1) treatments were significant different from other treatments. While the *L. maculans* alone treatment had a high numerical value (1.1) relative to all other inoculated

treatments, the low standard deviation resulted in significant differences. At site 2 in 2021, the most severe Verticillium stripe was observed on the 1:3 mix of *L. maculans* and *V. longisporum* (1.8) and *V. longisporum* alone (1.9) treatments. The mildest Verticillium stripe was observed on the control (0.6) and *L. maculans* alone (1.1) treatments (Table 2.3). In the case of 'CS2000', the most severe Verticillium stripe was observed on the 1:3 mix of *L. maculans* and *V. longisporum* (1.2) and the *V. longisporum* alone (1.6) treatments. The mildest Verticillium stripe was observed on the control (0.2) and *L. maculans* alone (0.4) treatments at site 1 in 2021. At site 2, the control (0.0) and *L. maculans* alone (0.2) treatments had the lowest Verticillium stripe and significant different from other treatments (1.5-2.0). (Table 2.3).

The mean seed yield was similar in the two hybrids, ranging from 0.9 to 2.5 t/ha on '45H31' and from 0.9 to 2.8 t/ha on 'CS2000', and was significantly greater in 2021 than in 2020 (Figure 2.6a and 2.6b). However, the mean seed yield was not significantly different among treatments for either hybrid in either year.

Comparison of symptoms and signs on canola

While the symptoms and signs of blackleg and Verticillium stripe were superficially similar, they could readily be distinguished with careful examination, even when they occurred together. The microsclerotia of *V. longisporum* were much smaller than the pycnidia produced by *L. maculans*, and were greyer in color (Figure 2.7a). Due to their larger size, individual pycnidia could be discerned more easily, and were generally more darkly pigmented than the microsclerotia. Moreover, while both *V. longisporum* and *L. maculans* caused a vascular discoloration visible in cross-sections of the crown or base of the stem, the staining associated with blackleg was darker (black) and more discrete than the grey, more diffuse staining resulting from Verticillium stripe (Figure 2.7b). Longitudinal sections of the stem further served to distinguish the two diseases. In

the case of blackleg, the vascular discoloration was restricted to the lower stem, affecting the cortex and epidermis (Figure 2.8a); in the case of Verticillium stripe, symptoms extended up the stem, with a hollow, darker centre (Figure 2.8b). In cases where the two pathogens occurred together, longitudinal sections revealed a hollow and darker centre together with black discoloration of the cortex and epidermis (Figure 2.8c). Infection by *V. longisporum* also was usually associated with some shredding of the stem.

Greenhouse experiments for blackleg and Verticillium stripe interactions

Emergence ranged from 41.6 to 94.7% and from 51.9 to 95% in the canola hybrids '45H31' and 'CS2000', respectively, 14 days after seeding in the greenhouse experiments (Table 2.4). For both hybrids, percent emergence was highest (~95%) in the control (non-inoculated) treatments. In the case of 'CS2000', the emergence in all of the treatments that received any inoculum (regardless of the ratio of *L. maculans* and *V. longisporum*) was similar (51.9-63.8%). In contrast, for '45H31', the lowest emergence was observed in the *L. maculans* only treatment and in the 3:1 mix of *L. maculans* and *V. longisporum* (41.6-42.2%), followed by the 1:1 and 1:3 *L. maculans*/*V. longisporum* mixes and the *V. longisporum* only treatment (54.7-57.2%).

The mean blackleg severity ranged from 0.0 to 1.3 on '45H31', with no blackleg detected (severity of 0.0) in either the no inoculum control or *V. longisporum* only treatment. On this hybrid, the greatest blackleg severity (1.3) was obtained with the 3:1 mix of *L. maculans* and *V. longisporum*, followed by the *L. maculans* only (0.9) and 1:3 *L. maculans*/*V. longisporum* (0.6) treatments (Table 2.4). On 'CS2000', blackleg severity ranged from 0.0 to 1.0, with no symptoms of the disease detected on the non-inoculum control or *V. longisporum* only treatment. The most severe blackleg on 'CS2000' was obtained with the 3:1 and 1:1 mixes of *L. maculans* and *V. longisporum*, as well as with the *L. maculans* only treatment (severities of 0.8 to 1.0) (Table 4).

The mean Verticillium stripe severity ranged from 0.0 to 1.9 on both '45H31' and 'CS2000', with no Verticillium stripe detected in the no inoculum control or *L. maculans* only treatment for either hybrid (Table 2.4). On '45H31', the highest Verticillium stripe severity was observed in the *V. longisporum* only treatment, followed by intermediate severities (1.0 to 1.3) in the 3:1, 1:1 and 1:3 *L. maculans*/*V. longisporum* treatments. On 'CS2000', the most severe (1.6-1.9) Verticillium stripe was observed in any treatment that included *V. longisporum*, regardless of the ratio or whether or not *L. maculans* was included (although there seemed to be a numerical increase in severity as the proportion of *V. longisporum* increased) (Table 2.4).

Mean seed yield ranged from 1.5 g to 3.9 g per plant on '45H31' and from 1.2 g to 2.3 g per plant on 'CS2000' (Table 2.4). For '45H31', the lowest yields were observed in the non-inoculated control and *V. longisporum* only treatments, followed by the 1:3 mix of *L. maculans*/*V. longisporum*. The highest yields were obtained in the *L. maculans* only and 3:1 *L. maculans*/*V. longisporum* treatments; yield in the 1:1 *L. maculans*/*V. longisporum* treatment was intermediate. Similar trends were observed for 'CS2000' (Table 2.4). The lowest yields were observed in the non-inoculated control and *V. longisporum* only treatment, and the highest was recorded in the *L. maculans* only treatment; yields in the various mixes of *L. maculans* and *V. longisporum* were intermediate (Table 2.4). Symptoms and signs of Verticillium stripe and blackleg in the greenhouse resembled those described above for the field experiments.

2.4 Discussion

Blackleg is an established disease of canola in Canada, while Verticillium stripe has only recently emerged as a concern on this crop (Canadian Food Inspection Agency 2018; Gugel & Petrie 1992). An improved understanding of the impact of blackleg on canola yields, particularly on blackleg-resistant hybrids, as well as of the potential effects of interactions between *L. maculans* and *V.*

longisporum on infected crops, is important to determine the need for and effectiveness of different disease management strategies. To our knowledge, this is the first report examining blackleg/Verticillium stripe interactions in canola.

In general, blackleg severity was low in the field experiments to evaluate yield losses caused by this disease, and never exceeded a mean rating of 2.4 for either canola hybrid in either year of the study. This likely reflected the classification of both '45H31' and 'CS2000' as blackleg-resistant, although no information regarding the specific resistance genes in these hybrids is available. The *L. maculans* isolates used as inoculum in the field plot experiments were classified as Pathogenicity Group (PG)-2, *sensu* Mengistu (Mengistu et al. 1991; Rong et al. 2015). The PG classification system is based on the virulence of the blackleg fungus on the differential canola genotypes 'Westar', 'Quinta' and 'Glacier', where PG-2 isolates are avirulent on 'Quinta' (carrying the *Rlm1* resistance gene) and 'Glacier' (carrying *Rlm2* and *Rlm3*). More recently, *L. maculans* isolates are often classified based on the avirulence (*Avr*) genes they carry, which interact with specific major resistance genes in the host (Balesdent et al. 2005). The isolates used in this experiment were confirmed to carry the *AvrLm4 –7* and *AvrLm6* avirulence genes, while *AvrLm1* was absent (Rong et al. 2015). Although the isolates were not tested for the presence of *AvrLm2* or *AvrLm3*, based on their virulence phenotypes, PG-2 isolates are expected to carry *AvrLm2* (which interacts with *Rlm2*) and *AvrLm3* (which interacts with *Rlm3*) (Kutcher et al. 2010). Most commercial canola hybrids possess the *Rlm3* gene, and PG-2 was predominant in western Canadian *L. maculans* populations for a long time (Chen & Fernando 2006; Zhang & Fernando 2018). However, additional *Avr* genes beyond *AvrLm2* and *AvrLm3* have been identified in PG-2 (Balesdent et al. 2005).

Blackleg development is also influenced by environmental conditions, with humid weather and warm temperatures favouring the disease (Hall 1992). This may explain why the mean blackleg severity ratings on both canola hybrids at both sites were higher in 2019 than in 2020. More rainfall in June and July 2019 (Total precipitation - Quarterly data seasonal for Edmonton. 2022) likely resulted in more severe *L. maculans* infection in the inoculated treatments, leading to more severe disease. In addition, heavy rainfall in May 2020 (Total precipitation - Quarterly data seasonal for Edmonton. 2022) shortly after inoculation may have flushed the inoculum through the soil, resulting in less disease that year.

In the case of '45H31' and 'CS2000', quadratic equations best explained the relationship between blackleg severity and yield. Very mild symptoms (disease severity rating of 1) were associated with an increase in yield relative to plants with no symptoms at all (rating of 0), but as disease severity increased to ≥ 2, yields decreased dramatically. These results are similar to those reported by Wang et al. (2020) in an analysis of moderately resistant canola hybrids. As such, the current study and the earlier report by Wang et al. (2020) suggest that quadratic relationships between blackleg severity and yield loss are common in blackleg-resistant and moderately resistant canola. Hwang et al. (2016), in contrast, found a linear relationship between disease severity and yield loss in a susceptible canola cultivar, 'Westar'. It is difficult, however, to draw any conclusions as to whether the level of resistance influences the nature of the relationship between disease severity and yield loss. In the evaluation of 12 commercial canola crops conducted in the current study, the two hybrids ('DKTF 94CR' and '75-42CR') exhibited different blackleg severity/yield loss relationships despite both being rated as blackleg-resistant. In 'DKTF 94CR', a quadratic relationship was observed, while in '75-42C', the relationship was linear. Despite their rating as resistant, moderate levels (2.9 and 2.7) of blackleg were found on both hybrids, indicating

that even if there was a similar erosion of resistance in both hosts, the relationship between yield loss and disease severity remained distinct. Nonetheless, at higher blackleg severities (2-5), either model (quadratic or linear) would provide a fairly accurate estimate of yield losses.

In the field experiments examining the interaction between *L. maculans* and *V. longisporum*, symptoms of blackleg were detected in both the control (non-inoculated) and *V. longisporum* inoculum only treatments. These symptoms may have reflected the presence of natural inoculum at the field sites, the spread of asexual pycnidiospores via rain-splash, and/or the fact that wind-borne ascospores of *L. maculans* can travel 5-8 km from the source (Bokor et al. 1975; Howlett 2004). This was particularly evident in 2020, a year with higher precipitation that was favorable for blackleg, when no strong relationships were found between the amounts of *L. maculans* applied and the severity of blackleg across the treatments. In contrast, 2021 was a very dry year, and despite the occurrence of mild symptoms on the control and *V. longisporum* only treatments, blackleg tended to be more severe when more *L. maculans* inoculum was applied. In the case of *V. longisporum*, the situation was reversed, with Verticillium stripe being more severe in 2021 vs. 2020. Verticillium stripe is generally favored by hot and dry conditions, with excess moisture making the disease less problematic (Eastburn & Paul 2007). The presence of symptoms of Verticillium stripe on the control and *L. maculans* only treatments suggests the movement of *V. longisporum* microsclerotia via wind-dispersal, rain splash or infested soil (Rouxel & Balesdent 2005) from treatments inoculated with the pathogen. Given the increasing prevalence of this fungus on the Prairies (Oosterhuis 2022), it is also possible that there was natural *V. longisporum* inoculum at the field sites, although there was not history of the disease there.

There were no significant differences in seed yields among any of the treatments in each year of the study examining the interaction between *L. maculans* and *V. longisporum*, although

yields overall were much greater in 2021 than in 2020. Some of the plots were flooded in 2020 due to heavy rainfall, resulting in yield reductions. In 2021, while there were no statistically significant differences among treatments, numerical differences were observed in the yields between the control and inoculated treatments, with the former being slightly higher in both hybrids at both sites.

Under greenhouse conditions, the emergence of both 'CS2000' and '45H31' decreased significantly, relative to the non-inoculated controls, when inoculum of *L. maculans* and/or *V. longisporum* was applied. Barbetti & Khangura (2000) reported that blackleg can reduce stand establishment. Similarly, Cui et al. (2022) found that percentage emergence was reduced under both low and high concentrations of *V. longisporum* inoculum under greenhouse conditions. Further research is needed to explore more fully the impact of these pathogens on stand establishment, particularly under field conditions.

The non-inoculated control and *V. longisporum* alone treatments had the lowest seed yield for both canola hybrids under greenhouse conditions. The control plants were smaller than the plants in the other treatments, as they had higher emergence and hence experienced greater competition. Therefore, single plant seed yield on the non-inoculated control plants was lower than in many of the inoculated treatments. In the treatments receiving *L. maculans* inoculum, blackleg severities ranged from 0.6 to 1.3 across the two hybrids, at which no yield losses are expected according to the empirically derived models from the field experiments. Indeed, yields were highest in these treatments, in comparison not only with the non-inoculated control, but also with the *V. longsiporum* only treatments. The results suggest mild Verticillium stripe infection might cause greater yield losses compared with mild blackleg infection, but this may not be the case at higher disease severities.

Blackleg is generally diagnosed on canola based on the discoloration of cross-sections of the lower stem, but Verticillium stripe also causes cross-section discoloration. Growers and agronomists who are not familiar with Verticillium stripe might have difficulties identifying this disease, given its relative novel emergence in Canada (Canola Council of Canada 2020). This study demonstrated that longitudinal sections could help to more readily differentiate blackleg and Verticillium stripe on canola, enabling more accurate diagnoses and disease monitoring in the field.

Plant pathogens can be affected by the quantity and/or quality of shared resources, leading to resource-mediated interactions between different pathogens (Dutt et al. 2022). While, to our knowledge, there have been no previous studies on the interaction between *L. maculans* and *V. longsiporum*, Toscano-Underwood et al. (2003) examined the co-existence of two closely related species, *L. maculans* and *L. biglobosa*. Epidemiological differences between these pathogens resulted in stable co-existence (Toscano-Underwood et al. 2003). Another study examined two foliar wheat pathogens *Puccinia triticina* (leaf rust) and *Pyrenophora tritici-repentis* (tan spot) under greenhouse conditions and found that infection by *P. graminis* facilitated the development of tan spot (Al-Naimi et al. 2005). In this study, under both field and greenhouse conditions and on both canola hybrids, blackleg severities were generally higher when *L. maculans* was co-inoculated with *V. longisporum* than when *L. maculans* was applied alone. Microsclerotia of *V. longisporum* germinate and the fungus enters the plant vascular system via formation of hyphae (Depotter et al. 2016), while *L. maculans* colonizes the intercellular spaces and also reaches the vascular tissues (Salisbury et al. 1995). Invasion of the vascular tissues by both fungi simultaneously could facilitate nutrient release from degraded host tissues and promote the growth of *L. maculans* even on blackleg resistant or moderately resistant hybrids. The destruction of the

stem cortex caused by *L. maculans* could also facilitate microsclerotium formation by *V. longisporum* at the later plant stages. Regardless of the exact mechanism, the results suggest a synergistic effect, with the presence of both pathogens resulting in more severe disease overall.

2.5 Conclusions

In this study, the relationship between blackleg severity and yield components was explained best by second-degree quadratic equations in most canola hybrids examined, although a linear relationship was observed for one variety sampled in commercial fields. Under natural field conditions, however, multiple plant pathogens may occur together. The recent identification of Verticillium stripe in Canada is of particular concern for canola growers, and the disease may be found in conjunction with blackleg in some fields. When *L. maculans* and *V. longisporum* were inoculated together in field and greenhouse experiments, blackleg severity and yield losses increased relative to when *L. maculans* was applied on its own. The severity of Verticillium stripe also tended to increase, suggesting a synergistic effect between the pathogens. Under low inoculum pressure, *V. longisporum* caused more severe yield losses than blackleg. The two diseases could be readily distinguished by longitudinal sections of the lower stems, which will facilitate surveillance activities and identification by non-expert personnel. The co-occurrence of blackleg and Verticillium stripe on canola represents another challenge to Canadian canola production and will require the development of proactive disease management strategies.

2.6 Tables

Table 2.1 Yield (t ha^{-1}) of canola hybrids '45H31' and 'CS2000' in *Leptosphaeria maculans*-inoculated and non-inoculated treatments in field trials near Edmonton, AB, Canada, in 2019 and 2020.

Year	Treatment	'45H31'	'CS2000'
2019	Non-inoculated	3.47a [1]	2.72a
	Inoculated	2.18b	1.87b
2020	Non-inoculated	1.05A	0.88A
	Inoculated	1.00A	0.82A

[1] Data are the means of four replications; means in a column followed by the same letter are not significantly ($P \leq 0.05$) different for each year according to the Tukey-Kramer test.

Table 2.2 Blackleg severity (0-5) on the canola hybrids '45H31' and 'CS2000' following inoculation with *Leptosphaeria maculans* (*Lm*) and/or *Verticillium longisporum* (*Vl*) alone and in various combinations under field conditions.

Treatment[1]	2020				2021			
	Site 1		Site 2		Site 1		Site 2	
	'45H31'	'CS2000'	'45H31'	'CS2000'	'45H31'	'CS2000'	'45H31'	'CS2000'
Control	0.4d [2]	0.2B	0.1b	0.1B	0.5c	0.2C	0.6bc	0.0C
Lm alone	1.0bcd	0.9A	0.7ab	0.8A	1.5a	1.1A	1.0a	1.2A
3 *Lm*: 1 *Vl*	1.3abc	0.9A	1.2a	1.0A	1.0ab	0.6B	0.4bc	0.4BC
1 *Lm*: 1 *Vl*	1.6a	1.0A	1.5a	0.8A	0.6ab	0.6BC	0.3b	0.6AB
1 *Lm*: 3 *Vl*	1.1cd	0.9A	0.9ab	0.9A	0.3bc	0.5B	0.4bc	0.3BC
Vl alone	1.5ab	1.3A	1.0ab	1.2A	0.7c	0.3BC	0.6c	0.4BC

[1] *Lm* alone = *Lm* applied at 200 mL inoculum/row; 3 *Lm*: 1 *Vl* = 3:1 mix of *Lm* (150 mL/row) and *Vl* (50 mL/row); 1 *Lm*: 1 *Vl* = 1:1 mix of *Lm* (100 mL/row) and *Vl* (100 mL/row); 1 *Lm*: 3 *Vl* = 1:3 mix of *Lm* (50 mL/row) and *Vl* (150 mL/row); *Vl* alone = *Vl* applied at 200 mL/row inoculum.

[2] Data were collected over four site-years in Edmonton, AB, Canada, and are the means of four replications. Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to the Tukey-Kramer test.

Table 2.3 Verticillium stripe severity (0-4) on the canola hybrids '45H31' and 'CS2000' following inoculation with *Leptosphaeria maculans* (*Lm*) and/or *Verticillium longisporum* (*Vl*) alone and in various combinations under field conditions.

Treatment[1]	2020				2021			
	Site 1		Site 2		Site 1		Site 2	
	'45H31'	'CS2000'	'45H31'	'CS2000'	'45H31'	'CS2000'	'45H31'	'CS2000'
Control	0.0c [2]	0.1C	0.0a	0.2B	0.5b	0.2C	0.6c	0.0B
Lm alone	0.3b	0.3BC	0.0a	0.2B	1.1b	0.4C	1.1c	0.2B
3 *Lm*: 1 *Vl*	0.4ab	0.3ABC	0.4a	0.3AB	1.0a	0.5BC	2.2b	1.5A
1 *Lm*: 1 *Vl*	0.4ab	0.6AB	0.2a	0.2AB	1.0a	0.8ABC	1.7b	1.8A
1 *Lm*: 3 *Vl*	0.2bc	0.5ABC	0.2a	0.2AB	1.1a	1.2AB	1.8a	1.6A
Vl alone	0.5a	0.7A	0.3a	0.6A	1.2a	1.6A	1.9a	2.0A

[1] *Lm* alone = *Lm* applied at 200 mL inoculum/row; 3 *Lm*: 1 *Vl* = 3:1 mix of *Lm* (150 mL/row) and *Vl* (50 mL/row); 1 *Lm*: 1 *Vl* = 1:1 mix of *Lm* (100 mL/row) and *Vl* (100 mL/row); 1 *Lm*: 3 *Vl* = 1:3 mix of *Lm* (50 mL/row) and *Vl* (150 mL/row); *Vl* alone = *Vl* applied at 200 mL/row inoculum.

[2] Data were collected over four site-years in Edmonton, AB, Canada, and are the means of four replications. Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to the Tukey-Kramer test.

Table 2.4 Seedling emergence, blackleg severity, Verticillium stripe severity and seed yield of the canola hybrids '45H31' and 'CS2000' following inoculation with *Leptosphaeria maculans* (*Lm*) and/or *Verticillium longisporum* (*Vl*) alone and in various combinations under greenhouse conditions.

Treatment[1]	Emergence (%) [2]		Blackleg severity (0-5)		Verticillium stripe severity (0-4)		Yield (g plant-1)	
	'45H31'	'CS2000'	'45H31'	'CS2000'	'45H31'	'CS2000'	'45H31'	'CS2000'
Control	94.7a	95.0A	0.0d	0.0C	0.0c	0.0B	1.5c	1.2B
Lm alone	41.6c	52.5B	0.9b	0.8AB	0.0c	0.0B	3.9a	2.3A
3 *Lm*: 1 *Vl*	42.2c	52.5B	1.3a	1.0A	1.0b	1.6A	3.8a	2.0AB
1 *Lm*: 1 *Vl*	54.7b	62.2B	0.9bc	0.8AB	1.0b	1.7A	3.4ab	1.8AB
1 *Lm*: 3 *Vl*	57.2b	63.8B	0.6c	0.6B	1.3b	1.8A	2.7b	1.7AB
Vl alone	55.6b	51.9B	0.0d	0.0C	1.9a	1.9A	1.6c	1.4B

[1] *Lm* alone = *Lm* applied at 20 mL inoculum/row; 3 *Lm*: 1 *Vl* = 3:1 mix of *Lm* (15 mL/row) and *Vl* (5 mL/row); 1 *Lm*: 1 *Vl* = 1:1 mix of *Lm* (10 mL/row) and *Vl* (10 mL/row); 1 *Lm*: 3 *Vl* = 1:3 mix of *Lm* (5 mL/row) and *Vl* (15 mL/row); *Vl* alone = *Vl* applied at 20 mL/row inoculum.

[2] Data are the means of four replications in each of two repeats of the experiment, which were combined as they were not significantly different ($P > 0.05$); means in a column followed by the same letter are not significantly different according to the Tukey-Kramer test ($P \leq 0.05$).

2.7 Figures

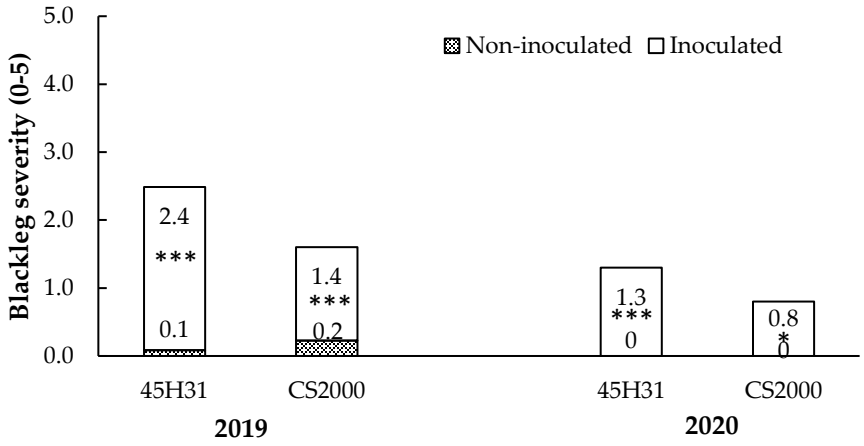

Figure 2.1 Mean blackleg disease severity on the canola hybrids '45H31' and 'CS2000' under field conditions in Leptosphaeria maculans-inoculated and non-inoculated treatments. Data were collected over two years (2019 and 2020) at two sites in Edmonton, AB, Canada. Blackleg severity was assessed on a 0–5 scale, where 0 = no disease and 5 = death of the plant. Significant differences between inoculated and non-inoculated plots of each hybrid in each year are indicated with asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and n.s., not significant.

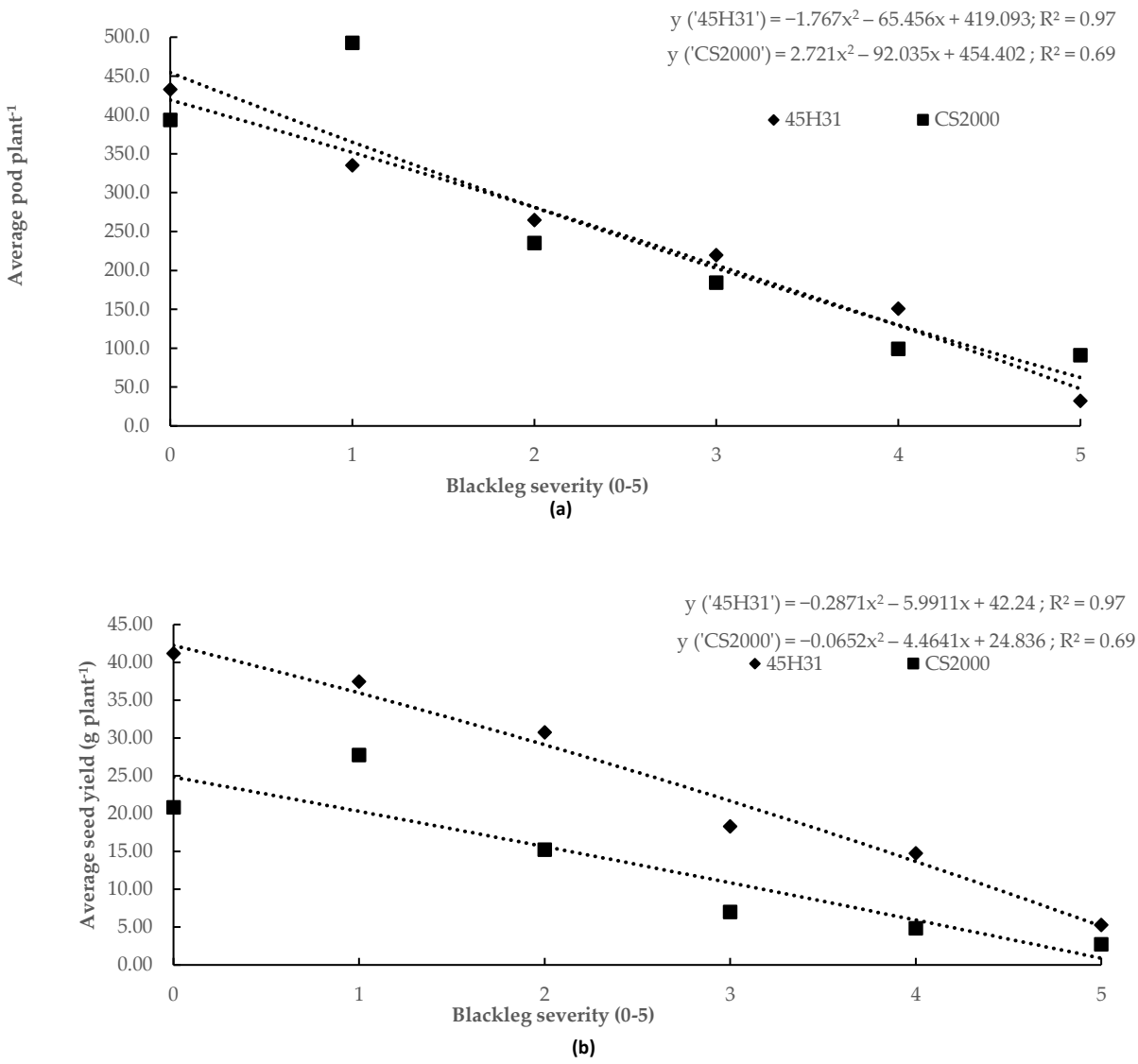

Figure 2.2 Relationship between blackleg severity and pods per plant (a) or seed yield per plant (b) in the canola hybrids '45H31' and 'CS2000' under field conditions. Data were collected over two years (2019 and 2020) at two sites in Edmonton, AB, Canada. Each point represents the mean of four replications × two site-years. Blackleg severity was assessed on a 0–5 scale, where 0 = no disease and 5 = death of the plant.

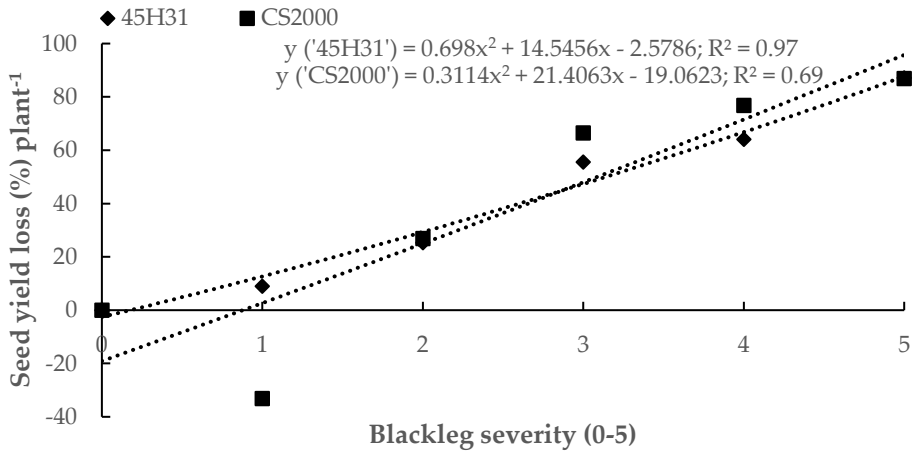

Figure 2.3 Relationship between blackleg severity and yield loss in the canola hybrids '45H31' and 'CS2000' under field conditions. Data were collected over two years (2019 and 2020) at two sites in Edmonton, AB, Canada. The yield loss data were estimated using the y-intercept in the equation averaged over four site-years. The data points were transformed into the percentage of the maximum yield.

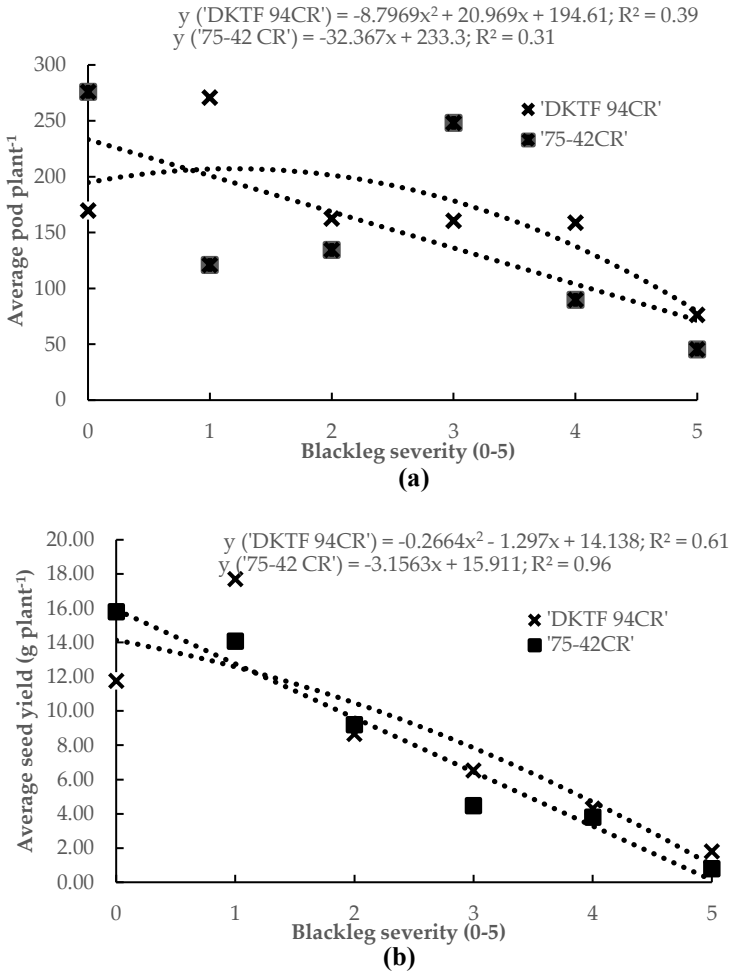

Figure 2.4 Relationship between blackleg severity and pods per plant (a) and seed yield per plant (b) in the canola hybrids 'DKTF 94CR' and '75-42CR' sampled in 12 commercial fields around Lacombe and Wetaskiwin, AB, Canada, in 2019. Each point represents the mean of all fields planted to the same canola hybrid. Blackleg severity was assessed on a 0–5 scale, where 0 = no disease and 5 = death of the plant.

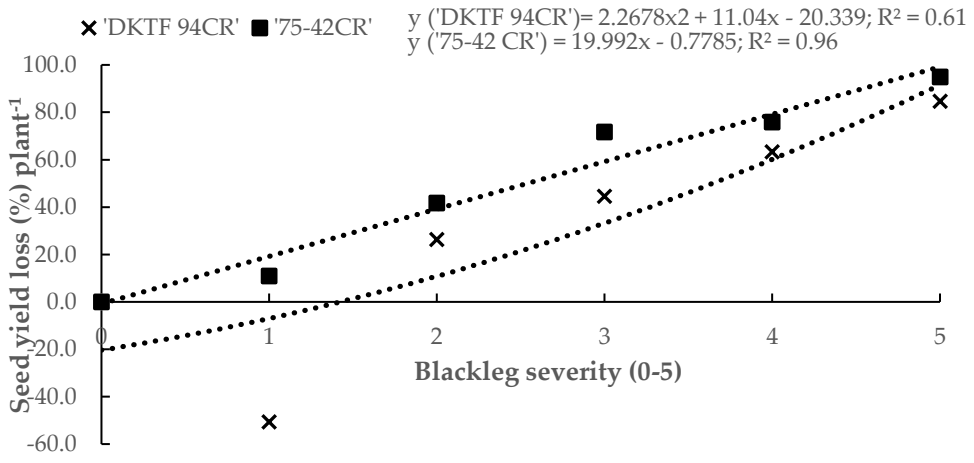

Figure 2.5 Relationship between blackleg severity and yield loss in the canola hybrids 'DKTF 94C' and '75-42CR' sampled in 12 commercial fields around Lacombe and Wetaskiwin, AB, Canada, in 2019. The yield loss data were estimated using the y-intercept in the equation averaged over 12 commercial fields. The data points were transformed into the percentage of the maximum yield.

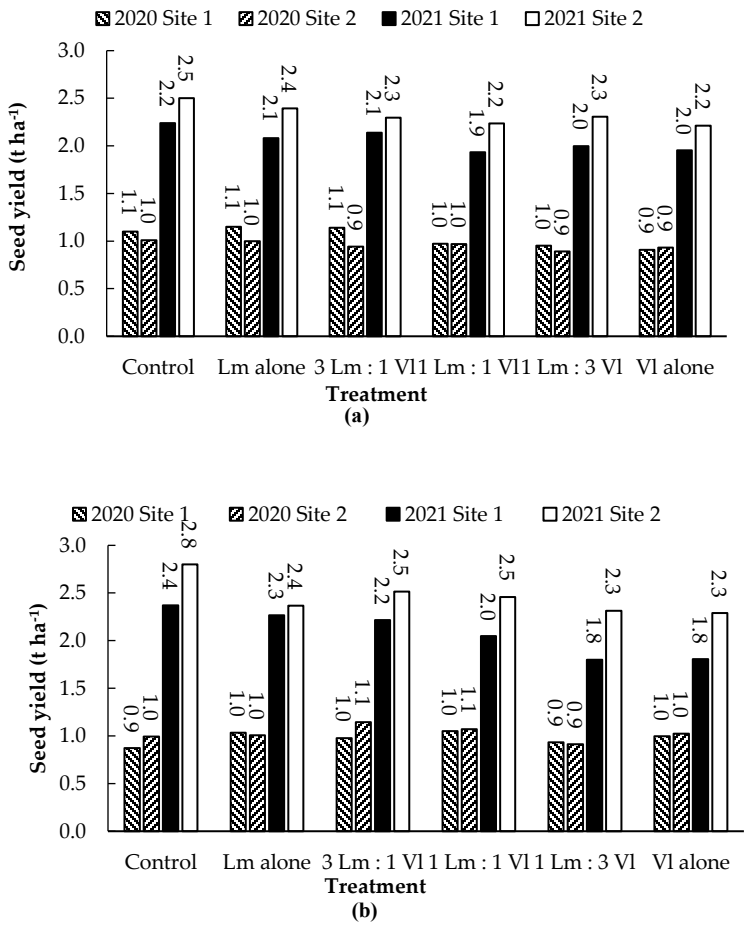

Figure 2.6 Mean seed yield of the canola hybrids '45H31' (a) and 'CS2000'(b) under field conditions. Data were collected over two years (2020 and 2021) in Edmonton, AB, Canada, following inoculation with *Leptosphaeria maculans* (*Lm*) and *Verticillium longisporum* (*Vl*) alone or in various combinations (3:1, 1:1, 1:3). Values represent the mean of four replications for each year. Mean seed yields were not significantly different according to the Tukey-Kramer test ($P > 0.05$) among any of the treatments.

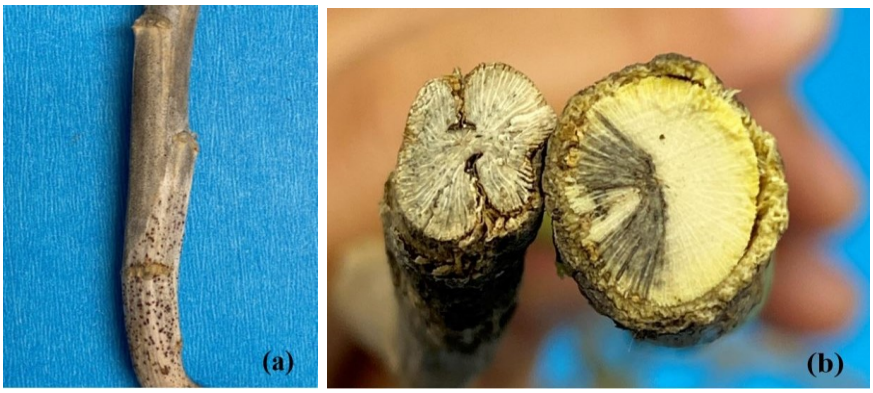

Figure 2.7 Pycnidia of *Leptosphaeria maculans* (lower portion of stem) and microsclerotia of *Verticillium longisporum* (upper portion) occurring on the same canola stem (a). Cross-sections of canola stems showing the discoloration caused by Verticillium stripe (left) and blackleg (right) (b).

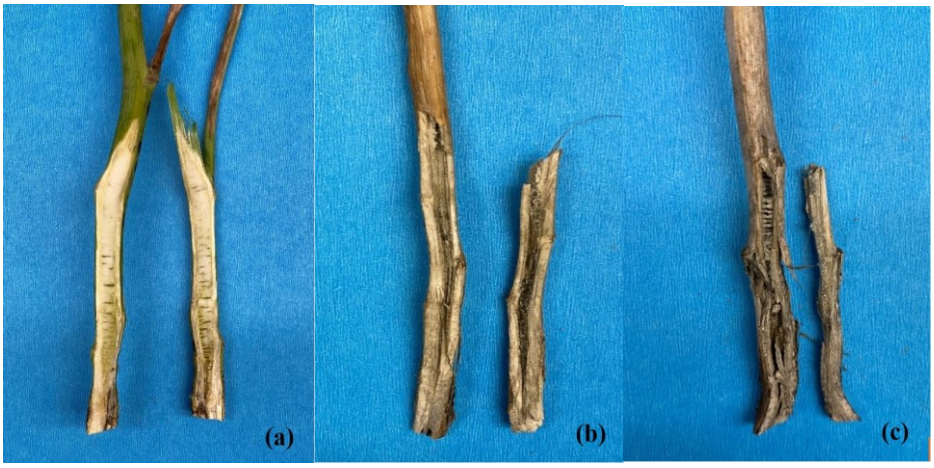

Figure 2.8 Longitudinal sections of canola stems infected by *Leptosphaeria maculans* (a), *Verticillium longisporum* (b), and both pathogens (c).

Chapter 3 Influence of pH on the Growth of *Verticillium longisporum* and Verticillium Stripe Severity in Canola (*Brassica napus*)

3.1 Introduction

Verticillium longisporum (C. Stark) Karapapa, Bainbridge and Heale is the causal agent of Verticillium stripe, an emerging disease of canola (*Brassica napus* L.) in Canada. It was first identified in Manitoba in 2014 (Canadian Food Inspection Agency 2018). A national survey conducted by the Canadian Food Inspection Agency (CFIA) confirmed its presence in other provinces, including British Columbia, Alberta, Saskatchewan, Ontario, and Quebec (Canadian Food Inspection Agency 2018). Mature plants infected with *V. longisporum* typically exhibit unilateral streaking, peeling of the stem tissues, and the appearance of microsclerotia (Heale & Karapapa 1999). Yield losses attributed to *V. longisporum* have been reported to range from 10% to 50% (Dunker et al. 2008). The growing prevalence and incidence of Verticillium stripe across western Canada (Kamchen 2023) have generated increasing concern in the canola sector. Given that the survival structures (microsclerotia) of *V. longisporum* can persist in soil for over 10 years (Heale & Karapapa 1999), effective disease management strategies include minimizing soil movement, sanitizing field equipment, and implementing longer rotations without host crops. Such practices are not always practical, however, especially in the large acreage canola cropping systems of western Canada. Moreover, since no chemical controls or resistant canola cultivars are available (Dunker et al. 2008), managing Verticillium stripe presents significant challenges.

Phenotypic diversity in the *V. longisporum* fungus is influenced by various parameters, including ambient temperature, relative humidity, and substrate or growing medium pH. Variations in these factors can affect the fungal growth rate, the pathogenicity, and, potentially, the disease incidence (Cruz et al. 2019). The pH of the soil can play an important role in the development of

soilborne pathogens, influencing the severity of disease. For example, Sclerotinia stem rot (*Sclerotinia sclerotiorum* (Lib.) de Bary) is affected by pH in liquid media, with acidic conditions favoring sclerotium development (Rollins & Dickman 2001). Similarly, studies on *Rhizoctonia solani* J.G. Kühn have shown that its optimal growth pH is 6.0 (Goswami et al. 2011). The clubroot of canola, caused by the rhizarian parasite *Plasmodiophora brassicae* Wor., is favored in acidic soils (Gossen et al. 2013). While an increased temperature generally promotes mycelial growth in some *Verticillium* species (Sbeiti et al. 2023), the effects of pH appear to be more variable (Baard and Pauer 1981; Fayzalla et al. 2008; Rampersad 2010). For example, *Verticillium dahliae* Kleb. was reported to cause accelerated wilt symptoms on cotton at higher pH levels (Hu et al. 2013), while another isolate from pumpkins showed larger colony diameters at pH 5.2 (Rampersad 2010). The growth of *V. alfalfae* Inderb., H.W. Platt, Bostock, R.M. Davis & Subbarao, the cause of Verticillium wilt of alfalfa, was greatest at pH 6.0 (Li et al. 2021). While the effects of pH on other *Verticillium* species have been studied, little information is available regarding the pH sensitivity of *V. longisporum* and its impact on disease development in canola. An improved understanding of how pH influences this fungus and its pathogenicity could assist growers in implementing effective management strategies for Verticillium stripe. Therefore, the objectives of the current study were to: (i) evaluate the effects of pH on the radial growth of *V. longisporum* in vitro, (ii) assess the effects of pH on Verticillium stripe development at the seedling stage under semi-hydroponic conditions, and (iii) determine the effects of pH on Verticillium stripe severity and yield in canola at maturity under controlled environmental conditions.

3.2 Materials and methods

Effects of pH on fungal growth

A single-spore isolate VL43 of *V. longisporum* (Cui et al. 2022) was grown in Petri dishes (9 cm diam.) filled with potato dextrose agar (PDA). The cultures were incubated under darkness at room temperature (23 °C) for 28 days. A 5 mm diam. plug of a developing colony was transferred to the center of a 9 cm diam. Petri dish containing PDA amended to pH 4.7, 5.5, 6.5, 7.4, or 8.6. The pH value of the full-strength PDA medium was 5.5 ± 0.10. The medium at different pH values was prepared by adding 0.072 M HCl solution (pH 4.7 ± 0.01) or 0.1 M NaOH solution (pH 6.5 ± 0.02, 7.4 ± 0.01, and 8.6 ± 0.03), as necessary. The Petri dishes were incubated in darkness at room temperature. At day 14 and day 21, two measurements of colony diameter (growth) were taken at right angles to each other using a digital caliper. The average of the two measurements, minus the 5 mm diam. of the original agar plug, was then calculated. The experiment was arranged in a completely randomized design with five replicates (Petri dishes) per treatment and was repeated independently.

Effects of pH on Verticillium stripe severity under semi-hydroponic conditions

Fungal cultures grown as described above were utilized to harvest conidial suspensions. Briefly, 10 mL of sterile distilled water was added to each Petri dish, and spores were gently dislodged using a sterile inoculating loop (Cui et al. 2022). The spore suspension was then filtered through four layers of sterile cheesecloth to remove larger mycelial fragments. The concentration of conidia was estimated with a haemocytometer (Hausser Scientific, Horsham, PA, USA) and adjusted to 1×10^6 spores mL^{-1} with sterile distilled water.

Seeds of the *V. longisporum*-susceptible canola cultivar 'Westar' were placed on moistened filter paper in Petri dishes for 10 days to allow for germination. Roots of 10-day-old seedlings were soaked in a conidial suspension for 2 h. Non-inoculated controls were soaked in sterile water instead. After inoculation, 10 seedlings were placed on germination paper that had been moistened with half-strength Hoagland's solution (pH 5.4 ± 0.15) or half-strength Hogland's solution, the pH of which had been adjusted to 4.4 ± 0.14, 6.3 ± 0.12, 7.5 ± 0.12, or 8.4 ± 0.22 using 0.072 M HCl or 0.1 M NaOH. The paper with the seedlings was rolled up and tied with an elastic band in the middle of each roll (Yang et al. 2024). Four rolls were placed in a 2 L glass beaker containing 1.5 L of the respective half-strength Hoagland's solution. Inoculated and non-inoculated seedling rolls were placed in separate beakers. To prevent disease escapes, an additional 5 mL of the conidial suspension was added to the Hogland's solution in each beaker for the inoculated treatments. The beakers were then incubated for 10 days in a growth cabinet at 28 °C with a 16 h photoperiod. The experiment was arranged in a split-plot design and repeated independently.

After 10 days of growth in the semi-hydroponic system, the seedlings were evaluated for disease severity using a 0 to 4 scale, where 0 = no symptoms and a normal root system; 1 = slight brown discoloration between the stem and root and reduced root size; 2 = a damaged stem, brown discoloration between the stem and root, and a reduced root size; 3 = a severely stunted seedling and minimal root development; and 4 = a dead seedling (Figure 3.1). In addition, the plant height was measured using a ruler, and the total plant biomass was determined by weighing on a balance.

Effects of pH on Verticillium Stripe Severity and Yields at Maturity

The effect of pH on Verticillium stripe severity and canola yields at maturity was assessed in a greenhouse using Sunshine Mix #4 growing mix (Sun Gro Horticulture, Vilna, AB, Canada) at various pH levels. Initially, the growing mix had a pH of 6.5 ± 0.24, which was adjusted by either

adding 0.1 M HCl with a watering can to reduce it to 5.6 ± 0.27 or by incorporating hydrated lime (Graymont, Richmond, BC, Canada) to raise it to pH 7.2 ± 0.21 and 7.8 ± 0.16. The pH adjustments were performed on 40 L aliquots of the growing mix at a time, followed by thorough mixing in 53 L plastic tubs. Afterward, the original and pH-amended growing mixtures were stored for 7 days to ensure pH stability and then used to fill 0.38 L plastic pots for use in the experiments. Ten-day-old canola 'Westar' seedlings were inoculated with *V. longisporum* by dipping the roots in a conidial suspension (1×10^6 spores mL^{-1}) for 2 h, as described above, and planted into the different pH potting mixtures at a density of one seedling per pot. The experiment was arranged in a split-plot design. Each treatment included four replicates consisting of 10 plants (pots) per replicate. The layout for the non-inoculated controls mirrored this arrangement. The greenhouse study was repeated independently.

Verticillium stripe severity was evaluated at plant maturity on a 0 to 4 scale, based on the amount of fungal microsclerotia on the entire plant, as described by Wang et al. (Wang et al. 2023). Briefly, a rating of 0 = healthy plants with no microsclerotia visible; 1 = slight colonization by microsclerotia < 25%; 2 = moderate colonization by microsclerotia ≥ 25% to < 75%; 3 = extensive colonization by microsclerotia ≥ 75%; 4 = severe colonization by microsclerotia and peeling of the stem epidermis. The plant height was measured using a ruler, as above. The seeds were harvested manually and weighed on a scale, with yields calculated for each replicate (10 plants).

Statistical analysis

The data were analyzed with R v. 4.2.3: A Language and Environment for Statistical Computing (R Core Team 2013, R Foundation for Statistical Computing, Vienna, Austria). The pH values were considered as fixed effects, and replicates were random effects. Inoculated and non-inoculated plants, the pH levels, and their interactions were considered as fixed effects. Replications within

inoculated and non-inoculated plants (whole-plots), replications within pH levels (split-plots), and inoculated and non-inoculated plant interactions were considered as random effects.

3.3 Results

Effects of pH on *in vitro* fungal growth

The results of the two independent repeats of the experiment were pooled, since they were not significantly different. At both time-points examined, the pH showed significant effects ($p < 0.001$). The mean colony diameter ranged from 35.9 mm to 42.2 mm across the different pH conditions after 14 days of incubation (Figure 3.2). The greatest average diameter was 42.2 mm and 41.9 mm, observed at pH 7.4 and 8.6, respectively. This was followed by an average diameter of 38.1 mm and 37.9 mm at pH 4.7 and 6.5, respectively. The smallest colony diameter, 35.9 mm, was obtained at pH 5.5 (Figure 3.2). Similar trends were observed after 21 days of incubation. At this time, the mean fungal colony diameter ranged from 54.7 mm to 62.0 mm across all pH conditions (Figure 3.2). The greatest colony diameter was again observed at pH 7.4 (60.4 mm) and 8.6 (62.0 mm), while the lowest was found at pH 5.5 (53.0 mm) and 4.7 (54.7 mm) (Figure 3.2).

Effects of pH on Verticillium stripe severity under semi-hydroponic conditions

The results of the two independent repeats of the experiment were combined, as they were not significantly different. The mean Verticillium stripe severity ranged from 0.72 to 2.10 at the seedling stage across the various pH treatments in the inoculated plants. As expected, no disease symptoms were observed on the non-inoculated controls (Table 3.1). The most severe disease symptoms, with severities of 2.10 and 1.91, respectively, were observed on seedlings grown in Hoagland's solution at pH 7.5 and 8.4. At pH 6.3, an intermediate Verticillium stripe severity of 1.59 was obtained, whereas the mildest disease, with severities of 0.72 and 1.19, respectively, was

observed at pH 5.4 and 4.4 (Table 3.1). The mean plant height for the non-inoculated controls ranged from 77 mm to 84 mm, with no significant differences detected. In contrast, the inoculated plants had heights ranging from 56 mm to 66 mm (Table 3.1). The mean biomass for the non-inoculated controls ranged from 0.22 g to 0.27 g, while for the inoculated plants, it ranged from 0.10 g to 0.15 g (Table 3.1).

Effects of pH on Verticillium stripe severity and yields at maturity

The results of the two independent repeats of this experiment were pooled, as they were not significantly different. The mean disease severity for the inoculated plants across all pH treatments at maturity ranged from 0.33 to 1.58, with no symptoms of Verticillium stripe observed on the non-inoculated controls (Table 3.2). The most severe symptoms, with a disease severity of 1.58, were obtained at pH 7.8, followed by intermediate severity (1.24) at pH 6.5 and 7.2. The lowest disease severity, with a mean of 0.33 on the 0–4 rating scale, was observed at pH 5.6. In the non-inoculated controls, the plant height ranged from 58.4 cm to 113 cm, with the tallest and shortest plants obtained at pH 7.8 and pH 5.6, respectively (Table 3.2). At pH 6.5 and 7.2, the plant height was intermediate (79.6 cm to 87.5 cm) (Table 3.2). In the inoculated treatments, the plant height ranged from 54.2 cm to 93.7 cm, with the shortest plants observed at pH 5.6. No significant differences were detected for height at pH 6.5, 7.2, or 7.8, with values ranging from 85.4 cm to 91.4 cm (Table 3.2). The mean seed yield for the non-inoculated plants ranged from 0.88 g to 1.66 g, with the highest seed yield observed at pH 7.8 and the lowest observed at pH 5.6 (Table 3.2). The mean seed yield for the inoculated plants ranged from 0.61 g to 0.79 g, but there were no statistically significant differences among the pH treatments. The seed yield in the inoculated treatments was significantly lower than that in the non-inoculated treatments at pH 6.5, 7.2, and 7.8 (Table 3.2).

3.4 Discussion

Fungi generally demonstrate a wide tolerance to pH variations, with their optimal growth pH typically ranging between 5.0 and 6.0 (Dix and Webster 1995; Deacon 1985). For instance, previous research indicated that *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen performed best at pH 6.3 (Cruz et al. 2019), while *R. solani* exhibited optimal mycelial growth at pH 5.6 across various media (Chaudhary et al. 2018). This study represents the first attempt to assess the effects of pH on *V. longisporum* under diverse conditions. The findings provide evidence that pH influences the growth of this fungus and its capacity to cause disease on canola. Notably, *V. longisporum* displayed significantly smaller colony diameters when the pH was < 6.5, suggesting poorer growth under acidic conditions. Conversely, the fungus showed the fastest in vitro radial growth at pH 7.4 and 8.6, indicating a preference for neutral to alkaline conditions. This preference was consistent, at least when assessed after 14 and 21 days of incubation. Furthermore, the influence of pH was reflected in the disease severity observed on canola, both at the seedling and adult plant stages. The symptoms of infection generally became more pronounced as the pH increased.

Similar pH effects have been documented with *V. dahliae*. In a study involving antirrhinum plants, disease symptoms progressed from mild to severe as the pH increased from 3.5 to 9.5 following inoculation with this fungus (Dutta 1981). Additionally, other studies using *V. dahliae* isolated from cotton plants demonstrated comparable results, showing increased fungal growth, microsclerotia production, and severity of disease symptoms in alkaline conditions (Baard & Pauer 1981; Liu et al. 2021; Hu et al. 2013). Furthermore, the growth of *V. dahliae* on tomato was favored at pH 8 (Fayzalla et al. 2008). In contrast, *V. dahliae* isolates recovered from artichokes, pumpkins, and other hosts were reported to show an optimal pH for fungal growth of around pH 5 (Kabir et

al. 2004; Rampersad 2010). Given the broad host range of *V. dahliae*, which infects over 300 plant species (Bautista-Jalón et al. 2021), the varying effects of pH on the growth, microsclerotia formation, and pathogenicity of different isolates may be due to their diverse host origins. *Verticillium longisporum* has a narrower host range, with an apparent preference for species in the Brassicaceae family (Depotter et al. 2016). As such, isolates of this fungus may exhibit reactions to pH that are more consistent. Unfortunately, at the time that this study was conducted, very few *V. longisporum* isolates that had been recovered from canola in western Canada were available, and only one was included in this study. As the pathogen becomes more widespread, it may be informative to evaluate a large collection of isolates from different regions of western Canada to test this hypothesis. Other *Verticillium* species, such as *V. alfalfae* (Li et al. 2021) and *V. albo-atrum* (Malca et al. 1966), showed optimal growth and sporulation at pH 6.

The severe disease development observed on the canola seedlings across various treatments under semi-hydroponic conditions suggests the potential of this system for studying Verticillium stripe. This system, based on a recently published hydroponic assay designed for investigating root architectural traits (Yang et al. 2024), appears effective for *V. longisporum* research. Additionally, similar systems may prove suitable. For instance, a hydroponics-based method was employed to screen for Phytophthora root rot resistance in chickpea, allowing for the more accurate observation of early host responses to infection (Amalraj et al. 2019). Considering the consistent results observed for in vitro fungal growth and disease development on canola under greenhouse and semi-hydroponic conditions in this study, the semi-hydroponic system may be a valuable tool for the high-throughput screening of multiple isolates and/or host genotypes. It has the advantage of requiring less time and space compared to pot-based methods in the greenhouse.

In the semi-hydroponic conditions, the seedling height did not vary significantly across different pH levels in the non-inoculated treatments, and no clear trends emerged in the height of inoculated seedlings. However, inoculated seedlings were consistently shorter than non-inoculated ones across all pH levels tested. Conversely, in the greenhouse study at maturity, the plant height was the lowest at pH 5.6, irrespective of the inoculation status. There was also no significant difference in height between the inoculated and non-inoculated treatments at this pH level. These observed effects of pH on the height of canola in the absence of inoculum may reflect the pH preferences of canola itself. Baquy et al. (Baquy et al. 2017) found that the plant height decreased as the soil pH declined from pH 7 to 4. Indeed, in this study, the seed yield at maturity was not significantly different between the inoculated and control plants when grown at pH 5.6, while at pH 6.5–7.8, the inoculated treatments had significantly lower yields. This likely further reflects the interactions between the pH optimum of the crop itself and the effects of *V. longisporum* infection. The growth of plants in acidic soils might be reduced due to pH-related toxicities and/or nutrient deficiencies [28]. While the plant biomass was recorded only in the semi-hydroponic study at the seedling stage, it was significantly lower in the inoculated vs. non-inoculated treatments; Cui et al. (2022) also reported that dry weight decreased when plants were infected with this fungus.

The collective findings of this study suggest that *V. longisporum* exhibits faster growth and induces more severe disease symptoms under neutral to alkaline conditions. However, the mechanism(s) by which pH influences the growth or pathogenicity of this fungus remain(s) unclear and requires further investigation. Van Wyk and Baard (Van Wyk and Baard 1971) reported an increase in the conidial germination of *V. dahliae*, from 0% to over 60%, as the soil pH rose from 3.8 to 7.9. A similar phenomenon may occur in *V. longisporum*, wherein heightened germination at higher pH levels contributes to heightened disease severity. Nonetheless, this hypothesis does

not account for the greater mycelial growth with increasing pH levels observed in the in vitro experiments.

Regardless of the exact mechanism(s), the preference of *V. longisporum* for higher pH conditions observed in this study may help explain the higher prevalence of Verticillium stripe in Manitoba (Kim et al. 2023) relative to Saskatchewan and Alberta (Akhavan et al. 2023; Harding et al. 2023). The soil in most regions of Manitoba is neutral to alkaline (Manitoba Agriculture 2017). Meanwhile, the majority of the more than 3 million ha of cultivated soils in the Prairies with a pH < 6.0 are found in the eastern and southwestern regions of Saskatchewan, as well as in central and northern Alberta (Canola Council of Canada 2023a; Les 2020). Conversely, the widespread distribution of clubroot, caused by *P. brassicae*, in Alberta has been attributed to the prevalence of lower-pH soils in many regions of the province. This pathogen is known to favor acidic soils (Gossen et al. 2013). These observations underscore potential conflicting best management practices for these diseases; treatments for increasing the soil pH for clubroot mitigation (Donald and Porter 2009) may inadvertently exacerbate the Verticillium wilt severity due to the preferred pH range of the respective pathogens. Similarly, several other important soilborne diseases of canola, including Sclerotinia stem rot (Rollins and Dickman 2001) and Fusarium wilt (*F. oxysporum*) (Cruz et al. 2019), are also favored by acidic soils. These factors should be taken into consideration when developing an integrated crop protection plan for canola.

Studying the pH effects on *V. longisporum* could offer valuable insights for growers and agronomists in devising integrated management strategies not just for Verticillium stripe but potentially for other diseases as well. To date, Verticillium stripe has been identified in Europe (Gladders et al. 2011; Tzelepis et al. 2017), North America (Heale and Karapapa 1999; Chapara et al. 2023), and Asia (Si et al. 2024), where another significant disease of crucifers, clubroot, is also

frequently reported (Struck et al. 2022). Since clubroot development is favored in acidic soils, liming to increase soil pH is often recommended as a method for controlling this disease (Faggian et al. 2017; Struck et al. 2022; Niwa et al. 2007; Chai et al. 2014). However, liming should be reconsidered in fields or regions where Verticillium stripe or other diseases favored by alkaline environments are also an issue.

This study investigated the impact of pH on *V. longisporum* and Verticillium stripe development in canola under various controlled environmental conditions. While some studies have explored the effect of pH on *V. dahliae*, to our knowledge, this is the first report on the influence of pH on *V. longisporum* in canola. The findings of this study suggest that neutral to alkaline environments promote pathogen growth, resulting in more severe disease symptoms. The observation that pH can profoundly affect the growth and virulence of *V. longisporum* may be valuable for growers, highlighting the potential importance of this parameter in Verticillium stripe development. Future research, particularly under field conditions, could further enhance our understanding of the pH effects on *V. longisporum*, thus facilitating knowledge-based disease management strategies.

3.5 Tables

Table 3.1 Effect of *Verticillium longisporum* inoculation on canola seedling height, disease severity, and biomass at various pH levels under semi-hydroponic conditions.

	Plant Height (mm) ***		Disease Severity (0–4 Scale) ***		Biomass (g 10^{-1} Plants) ***	
pH	**Control**	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
4.4 ± 0.01	81 ± 3.8 A	59 ± 4.3 ab	0 A	1.19 ab	0.27 ± 0.03 A	0.11 ± 0.01 ab
5.4 ± 0.10	84 ± 4.4 A	66 ± 2.5 a	0 A	0.72 a	0.27 ± 0.01 AB	0.15 ± 0.02 a
6.3 ± 0.02	82 ± 3.7 A	56 ± 1.3 b	0 A	1.59 bc	0.26 ± 0.01 AB	0.12 ± 0.01 ab
7.5 ± 0.01	79 ± 5.1 A	56 ± 8.5 b	0 A	2.10 d	0.24 ± 0.02 AB	0.10 ± 0.03 b
8.4 ± 0.03	77 ± 2.2 A	59 ± 1.9 ab	0 A	1.91 cd	0.22 ± 0.01 B	0.11 ± 0.01 ab

Note: 'Control' refers to non-inoculated plants, while 'Inoculated' refers to seedlings inoculated with a *Verticillium longisporum* conidial suspension (1×10^6 spores mL^{-1}). Plant height, disease severity, and biomass were assessed at 10 days after inoculation. Means in a column and category followed by the same uppercase or lowercase letter do not differ based on Tukey's method at $P = 0.05$. Data are the least square means of four replications. Significant differences in height, disease severity, and biomass between inoculated and non-inoculated plants are indicated with three asterisks: ***, $P < 0.001$.

Table 3.2 Effect of *Verticillium longisporum* inoculation on the plant height, disease severity, and seed yield of canola at maturity under greenhouse conditions.

	Plant Height (cm)		**Disease Severity (0–4 Scale) *****		**Seed Yield (g 10^{-1} Plants)**	
pH	**Control**	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
5.6 ± 0.27	58.4 ± 3.9 C	54.2 ± 1.7b (n.s.)	0 A	0.33 c	0.88 ± 0.13 C	0. 79 ± 0.14 a (n.s.)
6.5 ± 0.24	87.5 ± 5.8 B	85.4 ± 5.8a (n.s.)	0 A	1.24 b	1.35 ± 0.07 B	0.76 ± 0.02 a (***)
7.2 ± 0.21	79.8 ± 9.4 B	93.7 ± 6.8a (*)	0 A	1.24 b	1.50 ± 0.11 AB	0.71 ± 0.03 a (***)
7.8 ± 0.16	113 ± 5.5 A	91.4 ± 3.5a (***)	0 A	1.58 a	1.66 ± 0.05 A	0.61 ± 0.03 a (***)

Note: 'Control' refers to non-inoculated plants, while 'Inoculated' refers to seedlings inoculated with a *Verticillium longisporum* conidial suspension (1×10^6 spores mL^{-1}). Plant height, disease severity, and biomass were assessed at plant maturity. Means in a column and category followed by the same uppercase letter or lowercase letter do not differ based on Tukey's method at $p = 0.05$. Data are the least square means of four replications. Significant differences in height, disease severity, and biomass between inoculated and non-inoculated plants are indicated with asterisks: n.s., non-significant; *, $P \leq 0.05$; ***, $P < 0.001$.

3.6 Figures

Figure 3.1 Scale for evaluating the disease severity of canola seedlings inoculated with *Verticillium longisporum*. The seedlings were evaluated on a 0 to 4 scale, where 0 = no symptoms and a normal root system; 1 = slight brown discoloration between the stem and root, a reduced root size; 2 = a damaged stem, brown discoloration between the stem and root and a reduced root size; 3 = a severely stunted seedling and minimal root development; and 4 = a dead seedling.

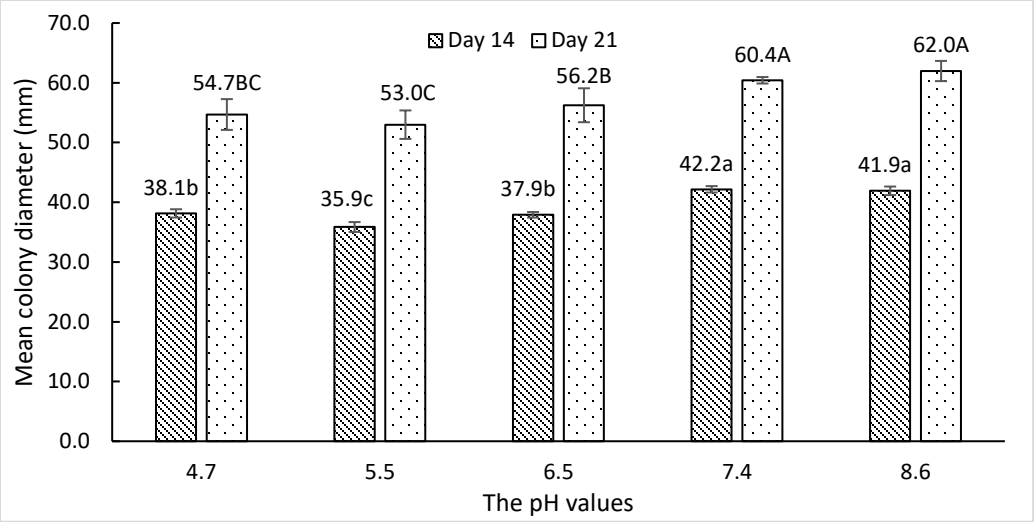

Figure 3.2 Mean diameter of *Verticillium longisporum* colonies after 14 or 21 days of growth on potato dextrose agar (PDA) at different pH levels. Means with the same uppercase letter or lowercase letter are not significantly different based on Tukey's method at $p = 0.05$. The error bars indicate the standard deviation of the mean across replicates.

Chapter 4 Genome-wide association study of *Verticillium longisporum* resistance in *Brassica* genotypes

4.1 Introduction

Verticillium stripe, caused by the fungal pathogen *Verticillium longisporum* (C. Stark) Karapapa, Bainbridge and Heale, is an important soilborne disease of canola (*Brassica napus* L.) in Canada. The first case of Verticillium stripe in this country was identified in 2014 (Canadian Food Inspection Agency 2018). Subsequently, *V. longisporum* has been detected in other Canadian provinces, including British Columbia, Alberta, Saskatchewan, Ontario, and Quebec (Canadian Food Inspection Agency 2018). Yield losses due to *V. longisporum* infection were reported to range from approximately 10% to 50% on canola, although they could exceed 80% on a single plant (Dunker et al. 2008). Since the survival structures (microsclerotia) of *V. longisporum* can persist in the soil for up to 10 years (Schnathorst 1981), strategies such as minimizing soil movement, implementing longer rotations out of host crops, and good weed management can potentially reduce *V. longisporum* inoculum levels (Johansson et al. 2006b). However, these strategies may not be practical for growers due to economic concerns. Moreover, there are currently no registered fungicides available for controlling this disease (Dunker et al. 2008). Therefore, genetic resistance stands out as the most effective and environmentally friendly approach for managing Verticillium stripe. Unfortunately, no commercial canola varieties in Canada have been registered as resistant to *V. longisporum* (Norman 2023).

Verticillium longisporum mainly attacks hosts in the *Brassicaceae* family, such as *B. napus* (canola/oilseed rape and rutabaga), *Brassica rapa* L. (including pak choy, Chinese cabbage, and turnip), *Brassica oleracea* L. (including broccoli, cauliflower, cabbage, and kale), and *Brassica juncea* L. (including brown and leaf mustard) (Depotter et al. 2016; Zeise and Von Tiedemann

2002). Some progenitor species of *B. napus* (AACC, *n* = 19), including *B. rapa* (AA, *n* = 10) and *B. oleracea* (CC, *n* = 9) (Nagaharu 1935), have been reported to exhibit higher levels of resistance to several significant canola diseases such as blackleg (Zou and Fernando 2024), clubroot (Fredua-Agyeman et al., 2019), and Sclerotinia stem rot (Khan et al. 2023). Consequently, the screening of various *Brassica* species for genetic resistance to *V. longisporum* is an important breeding objective. Rygulla et al. (2008) identified two major quantitative trait loci (QTL) for *V. longisporum* resistance on chromosomes C04 and C05. Additionally, Obermeier et al. (2013) found a major QTL on chromosome C05 and a minor QTL on C01, both correlated with *V. longisporum* resistance, whereas Gabur et al. (2020) reported a QTL for resistance on chromosome C09. In a recent study, Su et al. (2023) demonstrated that the MYB transcription factor BrMYB108 in *B. rapa* directly targets genes encoding respiratory burst oxidase homologues, leading to resistance against *V. longisporum* through the regulation of reactive oxygen species (ROS) generation (Su et al. 2023).

However, to the best of our knowledge, no screening or resistance gene/QTL detection studies have been conducted in Canada for the identification of *Brassica* germplasm suitable for breeding *V. longisporum* resistance in commercial canola cultivars. Therefore, the objective of this study was to screen a large collection of rutabaga (*B. napus* ssp. *napobrassica*) accessions and commercial canola cultivars from Canada, as well as *B. rapa* and *B. oleracea* genotypes from China, for resistance to this fungus. Additionally, a genome-wide association study (GWAS) was utilized to identify accessions and genomic regions associated with *V. longisporum* resistance.

4.2 Materials and methods

Plant materials

Two-hundred eleven *Brassica* accessions, commercial cultivars, and differential hosts were evaluated for their reaction to *V. longisporum*. Among these were 110 rutabaga (*B. napus* spp. *napobrassica*) accessions previously screened for clubroot resistance by Fredua-Agyeman et al. (2020), and utilized in genetic diversity studies by Yu et al. (2021). In addition, the evaluation included 35 Canadian canola cultivars, 40 *B. rapa* vegetable cultivars, and 15 *B. oleracea* vegetable cultivars from China. Furthermore, 11 hosts of the European Clubroot Differential set (ECD; Buczacki et al., 1975) were tested, including ECD 06, ECD 08, ECD 09, ECD 10 (*B. napus*), ECD 01, ECD 02, ECD 03, ECD 04, ECD 05 ('Granaat') (*B. rapa*), ECD 11, and ECD 13 (*B. oleracea*). Among these 211 *Brassica* genotypes, the Canadian canola cultivar 'Westar' was included as a susceptible check, while *B. rapa* var. *pekinensis* 'Granaat' (ECD 05) served as a moderately resistant check (Rygulla et al., 2007). The details of the plant materials used are presented in Supplementary Table S1.

Resistance phenotyping

The single-spore isolate VL43 of *V. longisporum*, collected from an infected canola plant sampled near Edmonton, Alberta (Cui et al., 2023), was cultured in Petri dishes (9-cm diameter) filled with potato dextrose agar (PDA). The multiplex PCR method described by Inderbitzin et al. (2013) was employed to identify isolate VL43 as *V. longisporum* lineage A1/D1. Cultures were incubated in darkness at room temperature for 28 days before harvesting the conidia. Briefly, 10 mL of sterile distilled water was added to each Petri dish, and a sterile inoculating loop was used to gently dislodge the spores. The resulting conidial suspension was filtered through four layers of sterile

cheesecloth to remove mycelial fragments. The spore concentration was then estimated using a haemocytometer (Hausser Scientific, Horsham, Pennsylvania, USA), and adjusted to 1×10^6 spores mL^{-1} with sterile distilled water.

Seven-day old seedlings of the 211 *Brassica* genotypes were inoculated using the root-dip method as described by Cui et al. (2022). Non-inoculated controls were dipped in sterile distilled water instead. The experimental setup consisted of 32 L plastic tubs filled with Sunshine Mix #4 growing mix (Sun Gro Horticulture, Vilna, Alberta, Canada). Each tub accommodated five seedlings of the same genotype, with four *Brassica* genotypes per tub, totaling 20 plants (5 plants × 4 genotypes) per tub. The plants were maintained in a greenhouse under an 18-h photoperiod (22°C day/16°C night).

Disease severity assessments were conducted weekly for each plant over a 4-week period. The assessment utilized a 1-9 rating scale as described by Eynck et al. (2009), where a rating of 1 = no symptoms, while 9 = the plant is dead. The experiment was arranged in a randomized completely block design with four replicates, and was independently repeated.

Statistical analysis of the disease data

The area under the disease progress curve (AUDPC) was calculated for each host genotype based on Verticillium stripe severity using the formula described by Campbell & Madden (1990): $AUDPC = \sum_{i=1}^{n}(y_i+y_{i+1}/2) \times (t_{i+1} - t_i)$, where y_i is the disease severity for each observation number i, t_i is the number of days after inoculation at the time of observation number i, and n is the number of observations. Non-inoculated plants were also assessed on the same scale at the same times. A net AUDPC value ($AUDPC_{net}$) was calculated following Eynck et al. (2009): $AUDPC_{net} = AUDPC(X_{inoc.}) – AUDPC(X_{contr.})$, where $X_{inoc.}$ is the inoculated plants and $X_{contr.}$ is non-inoculated controls.

The AUDPC values were normalized for each genotype relative to the susceptible check 'Westar' and moderately resistant check 'Granaat' to account for fluctuating disease severity between trials. The normalized AUDPC ($AUDPC_{norm.}$) was calculated according to Eynck et al., (2009):

$$\text{AUDPCnorm.} = \frac{\text{AUDPCnet (X)}}{(\text{AUDPC net Westar} + \text{AUDPC net Granaat})/2}$$

The phenotype data was analyzed statistically using R: A Language and Environment for Statistical Computing (R Core Team 2013). *Brassica* genotypes with significantly lower $AUDPC_{norm.}$ compared to the moderately resistant cultivar 'Granaat' were considered resistant (Eynck et al. 2009; Rygulla et al. 2007).

SNP genotyping

SNP genotyping was performed on all 211 *Brassica* genotypes using a *Brassica* 19K SNP array from TraitGenetics GmbH (Gatersleben. Germany). This array included 9,966 SNP markers on the A-genome, 7,740 SNP markers on the C-genome, and 1,146 SNP markers on scaffolds. After filtering monomorphic, low-coverage site markers, as well as markers with minor allele frequency (MAF) ≤ 0.05 and those missing data for >10%, 4,972 A-genome markers and 4,621 C-genome markers were retained for the GWAS. The GWAS was conducted separately on the 45 *B. rapa* (AA) and 149 *B. napus* (AACC) accessions using the A-genome markers, and on the 17 *B. oleracea* (CC) and 149 *B. napus* accessions using the C-genome markers. Additionally, the average inter-SNP marker distance was determined for each combination and each chromosome.

Linkage disequilibrium estimation

Intra-chromosomal linkage disequilibrium (LD) between allelic values at two loci was estimated using Pearson's squared correlation coefficient (r^2) statistic with TASSEL 5.0 (Bradbury et al. 2007). To determine the significance of pairwise marker r^2-values, $P < 0.001$ of the Chi-square ($\chi 2$) statistic for each SNP pair was used according to Fredua-Agyeman et al. (2020). The LD decay curves were determined by calculating the Chi-square (χ^2) statistic for each SNP pair in relation to physical map distance (in Mb) using R v. 4.3.2 (R Core Team 2013). The extent of LD was estimated based on the interaction of the fitted LD decay curve and r^2-threshold lines for each chromosome (Bellucci et al. 2015; Breseghello & Sorrells 2006).

Population structure analysis

To determine the population structure (Θ) of the *Brassica* accessions used in this study, a Bayesian clustering approach was employed. Burn-in periods ranged from 5,000 to 100,000 iterations, and Markov Chain Monte Carlo (MCMC) analyses ranged from 5,000 to 100,000 permutations through the population-genetic software *STRUCTURE* v. 2.3.4 (Pritchard et al. 2000). The *Brassica rapa* + *B. napus* genotypes and *B. oleracea* + *B. napus* genotypes were analyzed separately to determine the number of genetically homogeneous clusters (*K*) based on 4,972 and 4,621 SNP markers, respectively. Runs for each *K*=1-10 were replicated 10 times. The number of clusters and average log-likelihood plots were determined according to Evanno et al. (2005) through STRUCTURE HARVESTER (Earl & vonHoldt 2012).

The genetic diversity of *B. rapa* + *B. napus* genotypes and *B. oleracea* + *B. napus* genotypes was determined separately. This analysis was based on the 4,972 A-genome markers and 4,621 C-genome markers. The unweighted pair group method with arithmetic mean (UPGMA)

and the neighbor joining (NJ) method implemented in TASSEL v. 5.0 were used to generate phylogenetic trees.

Genome-wide association analyses

Three general linear models (GLM) and four mixed linear models (MLM) implemented in TASSEL v. 5.0 (Bradbury et al. 2007) were tested for the marker-trait association studies. The GLM tested consisted of the population structure (Q)-only, Kinship (K)-only, and Principal Coordinate Analysis (PCA)-only models. The MLM models comprised Q+K, PCA+K, Q+PCA and PCA+D (Distance matrices) (Fredua-Agyeman et al. 2020). Furthermore, three additional GWAS methods were employed using the GAPIT v. 3 (Wang & Zhang, 2021) package in R. These included the Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway (BLINK) (Huang et al. 2019), the Fixed and random model Circulating Probability Unification (FarmCPU) (Liu et al. 2016), and the Multiple Locus Mixed Linear Model (MLMM) (Segura et al. 2012).

GWAS was conducted for the *B. rapa* + *B. napus* accessions using the 4,972 A-genome SNP marker data. Two independent AUDPC measurements and the average of two sets of AUDPC phenotype data were utilized. This analysis was performed using the seven models and three methods noted above. Similarly, the 4,621 C-genome SNP marker data and two independent measurements of AUDPC, along with the average of two sets of AUDPC data for each genotype, were used for the GWAS of the *B. oleracea* + *B. napus* genotypes. For each model/method and genotype combination, Quantile-Quantile (Q-Q) plots were examined to identify which plot showed the least amount of deviation from the expected $-\log_{10}$ *P*-value. Significant markers associated with Verticillium stripe resistance were identified by examining the best-fitted Q-Q plots and Manhattan plots. These plots were generated using the CMplot package in R. To establish

the significance cut-off ($-\log_{10}$ (0.05/n), n = number of markers), the Bonferroni correction was applied (Benjamini and Hochberg 1995). A slightly lower threshold of $-\log_{10} P = 3.0$ was employed for association. Significant SNP markers associated with Verticillium stripe resistance were identified using the various models and methods.

Candidate gene prediction

The sequences of significant SNP markers were utilized in BlastN searches of the *B. rapa* (AA), *B. oleracea* (CC), *B. napus* (AACC), and *Arabidopsis thaliana* genome assemblies in the EnsemblPlants (plants.ensembl.org) and National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov) databases. Using a threshold of ≥ 90% identity and an E-value ≤ $1e^{-20}$, candidate genes were mapped to the reference genomes. The physical locations of these genes were determined up to 1,000 bp upstream or downstream of the closely related gene.

4.3 Results

Verticillium stripe phenotyping

Excluding the susceptible and moderately resistant checks, 20 (9.6%) of the remaining 209 *Brassica* genotypes tested were classified as resistant (R), 13 (6.2%) as moderately resistant (MR), 89 (42.6%) as moderately susceptible (MS), and 87 (41.6%) as susceptible (S) (Figure 4.1A).

Among the 110 rutabaga accessions screened, three (2.7%) were R, two (1.8%) were MR, 54 (49.1%) were MS, and 51 (46.4%) were S (Figure 4.1B). The $AUDPC_{norm.}$ scores from the first and second rounds of screening ranged from 0.295 ± 0.181 to 2.741 ± 0.717, and from 0.242 ± 0.095 to 4.497 ± 2.051, respectively, while the average $AUDPC_{norm.}$ score of the two rounds ranged from 0.269 ± 0.137 to 3.533 ± 1.690 (Supplementary Figure S1A).

The $AUDPC_{norm.}$ values for 34 of the 35 Canadian commercial canola cultivars, excluding the susceptible check 'Westar', showed that nine (26.5%) were classified as R, seven (14.7%) as MR, 13 (47.1%) as MS, and five (11.8%) as S (Fig 4.1C). The first round of $AUDPC_{norm.}$ scores ranged from 0.016 ± 0.028 to 3.448 ± 0.442, the second round ranged from 0.177 ± 0.037 to 2.541 ± 0.836, and the average $AUDPC_{norm.}$ scores from the two rounds ranged from 0.101 ± 0.188 to 2.994 ± 0.786 (Supplementary Figure S1B).

Among the 40 *B. rapa* vegetable cultivars from China, two (5.0%) were classified as R, one (2.5%) as MR, 15 (37.5%) as MS, and 22 (55.0%) as S (Figure 4.1D). The $AUDPC_{norm.}$ scores from the first and second rounds ranged from 0.120 ± 0.034 to 3.370 ± 0.012, and from 0.138 ± 0.037 to 3.040 ± 0.421, respectively, while the average $AUDPC_{norm.}$ scores from the two rounds ranged from 0.129 ± 0.034 to 3.202 ± 0.084 (Supplementary Figure S1C).

In the case of the 15 *B. oleracea* vegetable cultivars from China, five (33.3%) were classified as R, three (20.0%) as MR, three (20.0%) as MS, and four (26.7%) as S (Figure 4.1E). The first round of $AUDPC_{norm.}$ scores ranged from 0.158 ± 0.026 to 0.918 ± 0.411, the second round ranged from 0.159 ± 0.063 to 1.718 ± 0.928, and the average $AUDPC_{norm.}$ score from the two rounds ranged from 0.168 ± 0.058 to 1.315 ± 0.709 (Supplementary Figure S1D).

Besides the moderately resistant check ECD 05 ('Grannat'), among the other 10 selected hosts of the ECD set, one (10.0%) (ECD 11) was classified as R, two (20.0%) (ECD 08 and ECD13) as MR, one (10.0%) as MS (ECD 09), and six (60.0%) (ECD 01, ECD 02, ECD 03, ECD 04, ECD 06 and ECD10) as susceptible (S) (Figure 4.1F). The $AUDPC_{norm.}$ scores from the first and second rounds of screening ranged from 0.064 ± 0.051 to 3.599 ± 0.232, and from 0.208 ± 0.134 to 2.989 ± 0.338, respectively. The average $AUDPC_{norm.}$ scores from the two rounds ranged from 0.186 ± 0.161 to 3.294 ± 0.473 (Supplementary Figure S1E).

Based on the average $AUDPC_{norm.}$ scores, 20 *Brassica* genotypes showing a strong level of resistance to *V. longisporum* were identified. These included the rutabagas FGRA043, FGRA053, and FGRA063; canola cultivars CC2, CC4, CC5, CC7, CC10 and CC15; *B. rapa* cultivars 'Jingyan Zikuaicai', and 'Jingjian No.70'; *B. oleracea* cultivars 'Zigan2', 'Zhongqing 18', 'Zhongqing 12', 'Zhonggan 11', and '8398'; and ECD 11 (Figure 4.2).

Distribution of polymorphic SNP markers

Table 4.1 presents the number and distribution of SNP markers retained in the GWAS to determine resistance to *V. longisporum*. In the GWAS of the *B. rapa* and *B. napus* accessions, the mean number of filtered SNP markers was 497.2 ± 130.6, ranging from 370 on chromosome A09 to 779 on chromosome A03 (Table 4.1). The filtered set of 4,972 markers covered 291.6 Mb of the A-genome in *B. rapa* and *B. napus* (Table 4.1). The mean inter-SNP marker distance or density for the A-genome was 61.6 ± 21.5 Kb, ranging from 41.3 on chromosome A07 to 115.7 on chromosome A09 (Table 4.1). In the GWAS of the *B. oleracea* and *B. napus* accessions, the mean number of filtered SNP markers was 513.4 ± 207.5, ranging from 231 on chromosome C09 to 829 on chromosome C03 (Table 4.1). The filtered set of 4,621 markers covered 463.4 Mb of the C-genome in *B. oleracea* and *B. napus* (Table 4.1). The mean inter-SNP marker distance or density for the C-genome was 117.8 ± 59.3 Kb, ranging from 65.0 on chromosome C07 to chromosome C09 255.5 (Table 4.1).

Linkage disequilibrium

The average of the squared allele correlation LD (r^2) for all chromosomes is presented in Table 1. The mean r^2 value for the A-genome of *B. rapa* and *B. napus* was calculated to be 0.163, ranging from 0.144 on chromosome A09 to 0.223 on chromosome A08 (Table 4.1). The average extent of LD decay for the 10 A-genome chromosomes (A_1-A_{10}) ranged from 0.42 Mb on chromosome A08

to 1.15 Mb on chromosome A09, with an estimated mean of 0.60 Mb (Table 4.1). The mean r^2 for the C-genome of *B. oleracea* and *B. napus* was 0.277, ranging from 0.243 on chromosome C08 to 0.358 on chromosome C01 (Table 4.1). The estimated mean LD decay for the nine C-genome chromosomes (C_1 to C_9) ranged from 0.68 Mb on chromosome C07 to 1.85 Mb on chromosome C09, with an estimated mean of 0.42 Mb (Table 4.1). Therefore, the extent of LD for the C-genome chromosomes was slightly greater than for the A-genome chromosomes.

Population structure analyses

Two clusters (*K*=2) were determined at all runs (10,000, 50,000 and 100,000 burn-in iterations and MCMC lengths) by the method of Evanno et al. (2005) using *STRUCTURE* for the analyses of both *B. rapa* + *B. napus* (Figure 3A) and *B. napus* + *B. oleracea* (Figure 3B). At a probability of 70%, 77 (39.7%) of the *B. rapa* and *B. napus* genotypes were placed in group 1, 108 (55.7%) were placed in group 2, while 9 (4.6%) were classified as admixtures (Figure 3C). In group 1, there were 45 *B. rapa* genotypes, including 40 vegetable cultivars from China and the ECD lines 01-05, along with 32 Canadian canola cultivars. In Group 2, there were 106 rutabagas, along with ECD 10 and one Canadian canola cultivar. Additionally, there were two Canadian canola cultivars, along with ECD 06-09 and four rutabagas classified as admixtures.

Among the *B. oleracea* and *B. napus* genotypes, 51 (30.7%) were placed in group 1, 110 (66.3%) in group 2, while five (3.0%) were classified as admixtures based on a probability of 70% (Figure 3D). In group 1, there were 17 *B. oleracea* genotypes, including 15 vegetable cultivars, ECD 11, and ECD 13, as well as 34 Canadian canola cultivars. Group 2 consisted of 108 rutabagas, ECD 10, and one Canadian canola cultivar. The admixtures included ECD 06-09 and two rutabagas.

NJ and UPGMA Cluster analyses

The NJ and UPGMA clustering was performed using 4,972 A-genome SNP markers for 194 *B. rapa* (45) and *B. napus* (149) genotypes, and 4,621 C-genome SNP markers for 166 *B. oleracea* (17) and *B. napus* (149) genotypes.

The cluster analyses of *B. rapa* and *B. napus*, using both the NJ and UPGMA methods, grouped them into five major branches. These comprised 4, 40, 34, 6, and 110 accessions in clusters 1, 2, 3, 4, and 5, respectively (Figures 4.4A and 4.4B). Cluster 1 (N1 and U1) included ECD 01-04 (*B. rapa*). Cluster 2 (N2 and U2) included 39 *B. rapa* vegetable cultivars and ECD 05. Cluster 3 (N3 and U3) encompassed ECD 06-09, two rutabagas, and one Canadian canola cultivar. Cluster 4 (N4 and U4) consisted of 34 Canadian canola cultivars and one *B. rapa* vegetable cultivar. The remaining 108 rutabagas, along with ECD 10 and one Canadian canola cultivar, were grouped into cluster 5 (N5 and U5) (Figures 4.4A and 4.4B).

In the *B. oleracea* and *B. napus* cluster analyses, both the NJ and UPGMA methods grouped the genotypes into four major clusters, where clusters 1, 2, 3, and 4 comprised 33, 17, 6, and 110 accessions, respectively (Figures 4.4C and 4.4D). Cluster 1 (N1 and U1) consisted of 33 Canadian canola cultivars. Cluster 2 (N2 and U2) included 15 *B. oleracea* vegetable cultivars, ECD 11, and ECD 13. Cluster 3 (N3 and U3) comprised ECD 06-09, one Canadian canola cultivar, and two rutabagas. The remaining 108 rutabagas, along with ECD 10 and one Canadian canola cultivar, were grouped as cluster 4 (Figures 4.4C and 4.4D).

Association mapping of Verticillium stripe resistance loci

In the two GWAS, the observed $-\log_{10} P$ distribution showed greater deviation from the expected distribution in the Q-Q plots of the three GLMs than in the four MLMs. Among the four MLMs, the observed $-\log_{10} P$ distribution of the PCA + K and Q + K models deviated the least from the

expected distribution compared with the Q + D and PCA + D models (Supplementary Figures S2A-G, K-Q). The observed -$\log_{10}$ *P* distribution of three other GWAS methods, namely BLINK, FarmCPU and MMLM, also exhibited minimal deviation from the expected distribution (Supplementary Figures S2H-J, R-T). Therefore, among the 10 models and methods tested, the PCA + K and Q+K models, along with the BLINK, FarmCPU, and MLMM methods, generated the best Q-Q plots. Consequently, Manhattan plots for these five methods were utilized to identify significant SNPs for Verticillium stripe resistance (Figure 4.5A-E and Figure 4.6A-E). Based on the Manhattan plots, 45 SNP markers were found to be associated with resistance to this disease (Table 4.2). Among these significant markers, 38 SNPs were identified on the A-genome, while seven SNPs were on the C-genome (Table 4.2). The significant SNPs were distributed across all chromosomes except for chromosomes C01, C04, C07, and C09 (Table 4.2).

Functions of proteins encoded by significant sequences

Three of the 45 SNP marker sequences identified in this study were reported to be associated with disease resistance, plant immunity, and stress genes. These genes encoded multisubstrate pseudouridine synthase 7, leucine-rich repeat receptor-like serine, and L-type lectin-domain containing receptor kinase S.5 (Table 4.2). Other genes encoded functional proteins include formin-like protein, NAD-dependent protein, cyclic nucleotide binding, nucleotide-sugar transmembrane transporter, internal metabolism, and biosynthesis, which are associated with cellular and biochemical processes (Table 4.2). Additionally, genes that encoded zinc transporter 12, COP1-interactive protein 1, transcription factor PIF3, kelch repeat-containing F-box family protein, and transcription factor DIVARICATA, play an important role in basic plant biological and physiological processes (Table 4.2). Proteins of unknown molecular function were also detected (Table 4.2).

4.4 Discussion

As an emerging canola disease, Verticillium stripe continues to spread across the Canadian prairies (Oosterhuis, 2022), and has recently been detected in North Dakota (Chapara et al., 2023). However, no *V. longisporum* resistant canola cultivars has been registered in Canada, resulting in increased yield losses (Norman 2023). Therefore, the identification of germplasm for breeding resistant cultivars and identifying molecular makers tightly linked with *V. longisporum* resistance for marker-assisted selection is critical. Association mapping, based on linkage disequilibrium of markers with QTLs, is a powerful tool for marker-assisted selection, enabling the exploitation of variation in plant materials (Jestin et al. 2011). GWAS is one of the most popular approaches for association mapping, offering significant advantages over linkage analysis. It provides higher resolution, incorporates a greater number of alleles, and allows for the simultaneous analysis of various traits of interest (Zhu et al. 2008). Currently, single nucleotide polymorphism (SNP) markers are widely utilized for GWAS (Ben-Ari & Lavi 2012). These markers are co-dominant and suitable for high-throughput genotyping (Ben-Ari & Lavi 2012). Their biallelic and high heritability contribute to increased genotyping accuracy (Zhu et al. 2008). In this study, GWAS was employed to find significant SNP markers associated with *V. longisporum* resistance in a large collection of *B. napus*, *B. rapa*, and *B. oleracea* genotypes.

To minimize missing data, the *B. rapa* and *B. napus* accessions were analyzed separately using only the A-genome markers, while the *B. oleracea* and *B. napus* accessions were analyzed separately using only the C-genome markers. The filtered set of 4,972 SNP markers obtained from the *Brassica* 19K SNP array covered 291.6 Mb of the A-genome of *B. rapa* and *B. napus*. For the GWAS of the *B. oleracea* and *B. napus* accessions, the filtered set of 4,621 SNPs covered 463.4 Mb of the C-genome. On genetic maps, 1 cM of the *B. napus* was reported to correspond to ~500

kb (Eynck et al. 2007; Suwabe et al. 2006), so the two GWAS analyses covered approximately 580 cM and 925 cM of the A- and C-genomes, respectively. The two GWAS conducted in this study had approximately the same A-genome coverage and close to 1.5× more coverage of the C-genome compared to a previous study that utilized a *Brassica* 13.2K SNP array from SGS TraitGenetics (Fredua-Agyeman et al. 2020). In the current study, the mean maker density was 61.6 ± 21.5 Kb (8.57 SNP markers/cM) on the A-genome, and 117.8 ± 59.3 Kb (5.02 SNP markers/cM) on the C-genome. In contrast, Fredua-Agyeman et al. (2020) reported mean marker densities using the *Brassica* 13.2K SNP assay of approximately 63.4 ± 21.9 Kb (8.46 SNP markers/cM) and 15.0 ± 8.4 Kb (44.3 SNP markers/cM) for the A- and C-genomes, respectively. Therefore, the marker density on the A-genome was similar, but on the C-genome, the density was almost 9× lower with the 19K SNP array used in this study. While the 19K SNP array provided increased coverage, more C-genome markers need to be developed.

Linkage disequilibrium refers to the association and linkage of alleles among SNPs within a genomic sequence, which is important in GWAS for identification of genetic markers (Joiret et al. 2022). Wang et al. (2017) observed that the extent of LD decay ranged from 0.15 to 3.3 Mb for the A-genome and from 0.4 to 8.3 Mb for the C-genome. Using a *Brassica* 60K Illumina Infinium SNP array, Xu et al. (2015), reported LD decay for the A-genome ranging from 0.6 to 5.6 Mb and from 1.2 to 8.5 Mb for the C-genome. In another study, Fredua-Agyeman et al. (2020) estimated LD decay ranging from 1.1 to 2.3 Mb for the A-genome and from 0.20 to 1.5 Mb for the C-genome using the *Brassica* 13.2K SNP array. The extent of LD decay found in this study ranged from 0.42 to 1.15 Mb for the A-genome and from 0.68 to 1.85 Mb for the C-genome, which was similar to the values reported in these earlier studies.

In the present study, GWAS was conducted using three GLMs (Q-only, K-only, and PCA-only) and four MLMs (PCA+D, PCA+K, Q+K, and Q+D), in addition to three methods (BLINK, FarmCPU, and MLMM). Mixed linear models are versatile and widely used in GWAS. They offer a balance between complexity and computational efficiency by incorporating population structure and kinship to adjust associations on markers (Yang et al. 2014). However, MLMs can lead to increased false positive rates due to overfitting (Kaler et al. 2020). Therefore, other GWAS algorithms were also employed in this study. To reduce false positive or false negative associations, BLINK considers both main effects and interactions among genetic variants (Huang et al. 2019). FarmCPU integrates fixed and random effects and adjusts for population structure and relatedness using a kinship matrix (Kaler et al. 2020), while MLMM simultaneously considers multiple loci, accommodating polygenic effects (Kaler et al. 2020). By employing multiple GWAS methods, SNP markers could be identified more consistently, thereby increasing the accuracy and efficiency of QTL detection.

Among the 45 significant SNP markers identified in this study, 38 were on the A-genome and only seven were on the C-genome. No significant markers were found on chromosomes C01, C04, C07 and C09. While six SNPs were identified on chromosome A03 in this study, none coincided with a QTL for *V. longisporum* resistance previously reported by Gabur et al. (2020) on chromosome A03 (physical position 7,963,059 to 11,419,476). Interestingly, the SNP marker Bn_A03_p14870270 was found in a genomic region known as a hotspot for clubroot resistance (Fredua-Agyeman et al., 2021). This region contains the clubroot resistance gene(s)/QTLs *Bn.A3P2F*, *Crr3*, *CRk*, and *CRd*. The rutabaga accessions used in this study were also screened for clubroot resistance by Fredua-Agyeman et al. (2020). Unfortunately, accessions previously classified as resistant or moderately resistant to clubroot (FGRA036, FGRA037, FGRA044,

FGRA072, FGRA106, FGRA108, FGRA109 and FGRA112) were found to be susceptible or moderately susceptible to *V. longisporum*. Hirani et al. (2018) reported that ECD 01, ECD 02, ECD 03 and ECD 04 (*B. rapa*), as well as ECD 11 (*B. oleracea*), were resistant to Canadian field isolates of the clubroot pathogen, while ECD 05 (*B. rapa*) was susceptible. In the current study, ECD 01, ECD 02, ECD 03 and ECD 04 were all susceptible to *V. longisporum* isolate VL43. Among all of the ECD hosts screened, only ECD 11 exhibited resistance to this fungus, while ECD 05 was moderately resistant. Similarly, ECD 08 (*B. napus*) and ECD 13 (*B. oleracea*) were moderately resistant to *V. longisporum*, but they were susceptible and segregated in response to clubroot (Hirani et al. 2018). Consequently, the present findings suggest a negative relationship between resistance to Verticillium stripe and clubroot.

Of the 20 Verticillium stripe-resistant *Brassica* genotypes identified in this study, eight were *B. rapa* and *B. oleracea* vegetable types, three were rutabagas, and nine were canola. Gabur et al. (2020), Rygulla et al., (2008), and Su et al. (2023) identified QTLs/genes for Verticillium stripe resistance in vegetable-type *Brassica* germplasm. This indicates that *B. rapa* and *B. oleracea* might be potential donors for resistance breeding programs in canola/oilseed rape. Nine or about a quarter of the Canadian canola cultivars in this study were found to be resistant to *V. longisporum*, suggesting that the deployment of resistant hosts holds promise for the management of Verticillium stripe.

In the current GWAS, two SNP markers were identified that were associated with plant disease resistance and immunity mechanisms. The SNP marker Bn_A03_p21487106, located on chromosome A03, overlapped with a leucine-rich repeat receptor (LRR)-like serine protein. The LRR proteins have been reported to play a significant role in plant defense responses, for example by modulating the development of *Phytophthora sojae* on soybean (Si et al. 2021). The other SNP

marker Bn_A10_p15719803, located on chromosome A10, overlapped with an L-type lectin-domain containing receptor kinase. The L-type lectins were initially identified in the seeds of leguminous plants, and have been found to positively regulate disease resistance against *Phytophthora* in pepper (Woo et al. 2020). It is possible that the genomic regions containing these two significant SNP markers also harbor genes controlling *V. longisporum* resistance. Fourteen of the 45 significant SNP markers identified (including SNP marker Bn_A10_p15719803) were located on chromosome A10, between 15,236,993 to 17,366,723 bp. This region may be a hotspot for *V. longisporum* resistance.

In conclusion, screening 211 *Brassica* genotypes for resistance to *V. longisporum* identified 20 resistant accessions/cultivars, including representatives from *B. rapa*, *B. oleracea*, and *B. napus*. Additionally, significant SNP markers on chromosome A03 may be important for Verticillium stripe resistance breeding. Furthermore, the GWAS indicated that chromosome A10 in both *B. rapa* and *B. napus* may harbor a hotspot for *V. longisporum* resistance.

4.5 Tables

Table 4.1 Single nucleotide polymorphism (SNP) marker density and extent of intrachromosomal linkage disequilibrium (LD) in *Brassica rapa*, *Brassica napus* and *Brassica oleracea* genotypes included in a genome-wide association study of resistance to *Verticillium longisporum*.

Linkage group or Chromosome	Total # of SNP markers	# Filtered SNP markers	Length covered (kb)	Average inter-SNP marker distance (kb)	Pairwise comparisons of all linked SNP markers	Number (%) of SNP pairs in significant LD*	Average r^2 value/chromosome	Estimated LD decay (Mb) $^{\phi}$
A01	865	430	29050.96	67.7	92235	24630 (26.7%)	0.153	0.58
A02	787	427	29805.34	70.0	90951	28445 (31.3%)	0.178	0.91
A03	1553	779	37644.04	48.4	303031	71089 (23.5%)	0.148	0.48
A04	984	493	22049.36	44.8	121278	31756 (26.2%)	0.161	0.58
A05	981	455	29222.52	64.4	103285	29260 (28.3%)	0.167	0.75
A06	1102	530	31805.48	60.1	140185	42146 (30.1%)	0.157	0.61
A07	1395	667	27493.45	41.3	222111	52218 (23.5%)	0.148	0.43
A08	673	382	21796.85	57.2	72771	27800 (38.2%)	0.223	0.42
A09	811	370	42688.79	115.7	68265	17568 (25.7%)	0.144	1.15
A10	815	439	20088.28	45.9	96141	30124 (31.3%)	0.167	0.44
A-genome	9966	4972	291646.1	61.6±21.5	1310253	355036 (27.1%)	0.163	0.60
C01	790	311	43826.6	141.4	48205	20992 (43.5%)	0.358	0.99
C02	813	528	61056.6	115.9	139128	49779 (35.8%)	0.280	1.10
C03	1524	829	61857.1	74.7	343206	119219 (34.7%)	0.282	0.81
C04	1147	798	56008.8	70.3	318003	105924 (33.3%)	0.267	0.65
C05	558	329	46342.5	141.3	53956	21564 (40.0%)	0.277	1.45
C06	816	545	45790.4	84.2	148240	46786 (31.6%)	0.255	0.82
C07	871	587	38104.3	65.0	171991	69196(40.2%)	0.284	0.68
C08	734	463	51664.0	111.8	106953	41807 (39.1%)	0.243	0.80
C09	487	231	58767.0	255.5	26565	10633 (40.0%)	0.265	1.85
C-genome	7740	4621	463417.2	117.8±59.3	1356247	485900 (35.8%)	0.277	0.42

*The number and percentages of SNP pairs in significant LD were determined from Chi-squared tests at p-value < 0.001.$^{\phi}$ The extent of LD decay was estimated from the projection of the intersection between the fitted curve of the data points and the 95th percentile of an unlinked r^2 threshold line (background LD) onto the physical distance axis.

Table 4.2 Single nucleotide polymorphism (SNP) markers identified in two genotype combinations, *Brassica rapa* + *Brassica napus* and *Brassica oleracea* + *B. napus*, including their chromosomal locations and linkage association with resistance to *Verticillium longisporum*.

Model Used[o]	**Genotype combination**	**[α]SNP marker**	**Marker position**		**[β]Linkage group**	**Description**
			Start	End		
BLINK	*B.rapa* + *B. napus*	Bn_A01_p21776155	30012708	30012828	A01	molecular function unknown
FarmCPU	*B.rapa* + *B. napus*	Bn_A01_p3134159	3201000	3201200	A01	lysosomal Pro-X carboxypeptidase
Q+K	*B.rapa* + *B. napus*	Bn_A02_p11087388	10648939	10649139	A02	molecular function unknown
FarmCPU	*B.rapa* + *B. napus*	Bn_A02_p12044265	14034247	14038116	A02	calmodulin binding / cyclic nucleotide binding / ion channel
BLINK	*B.rapa* + *B. napus*	Bn_A02_p26154897	31129887	31130162	A02	zinc transporter 12
FarmCPU	*B.rapa* + *B. napus*	Bn_A02_p9353942	8716238	8716438	A02	kelch repeat-containing F-box family protein
FarmCPU/MLMM/PCA+K/Q+K	*B.rapa* + *B. napus*	Bn_scaff_16269_1_p296261	9618803	9619003	A02	formin-like protein 13
FarmCPU	*B.rapa* + *B. napus*	Bn_A03_p14037892	14458777	14459077	A03	putative cyclic nucleotide-gated ion channel 13
BLINK/FarmCPU	*B.rapa* + *B. napus*	Bn_A03_p14870270	14869760	14869980	A03	multisubstrate pseudouridine synthase 7
FarmCPU/MLMM	*B.rapa* + *B. napus*	Bn_A03_p2130281	2195728	2195848	A03	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_A03_p21487106	22058721	22058841	A03	leucine-rich repeat receptor-like serine/threonine-protein kinase BAM2
BLINK	*B.rapa* + *B. napus*	Bn_A03_p28202050	28195947	28202482	A03	DNA topoisomerase family protein

BLINK	*B.rapa* + *B. napus*	Bn_A03_p6335597	6438602	6438801	A03	dehydrogenase/reductase SDR family member FEY
BLINK	*B.rapa* + *B. napus*	Bn_A04_p1311487	1309052	1312331	A04	nucleotide-sugar transmembrane transporter/ sugar hydrogen symporter
BLINK/FarmCPU/MLMM/PCA+K/Q+K	*B.rapa* + *B. napus*	Bn_A04_p14410667	16921876	16921996	A04	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_A04_p5853514	7982622	7982821	A04	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_A04_p7442886	9487825	9487945	A04	molecular function unknown
FarmCPU	*B.rapa* + *B. napus*	Bn_A05_p14338060	14336950	14337170	A05	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_A05_p7098949	7098783	7098915	A05	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_A05_p817036	817042	818136	A05	late embryogenesis abundant protein, putative / LEA protein, putative
BLINK/PCA+K/Q+K	*B.rapa* + *B. napus*	Bn_A06_p22051862	54212892	54213013	A06	molecular function unknown
FarmCPU	*B.rapa* + *B. napus*	Bn_A06_p24886436	57569198	57569511	A06	MALE DISCOVERER 1, LRR receptor-like serine/threonine-protein kinase
FarmCPU	*B.rapa* + *B. napus*	Bn_A06_p3255819	3676547	3676847	A06	transcription factor PIF3
BLINK	*B.rapa* + *B. napus*	Bn_A02_p771313	16493261	16493561	A07	COP1-interactive protein 1
BLINK	*B.rapa* + *B. napus*	Bn_A02_p808711	16542128	16542248	A07	molecular function unknown
FarmCPU/MLMM	*B.rapa* + *B. napus*	Bn_A07_p10370541	14732056	14732176	A07	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_A07_p3569093	7886469	7886775	A07	molecular function unknown

FarmCPU	*B.rapa* + *B. napus*	Bn_A07_p5030137	5029471	5029603	A07	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_scaff_18505_1_p254578	10671651	10672980	A07	Pentatricopeptide repeat
BLINK	*B.rapa* + *B. napus*	Bn_A08_p6828854	18168606	18168806	A08	TOG array regulator of axonemal microtubules protein 1
BLINK	*B.rapa* + *B. napus*	Bn_A09_p30329663	50495251	50495372	A09	molecular function unknown
PCA+K/Q+K	*B.rapa* + *B. napus*	Bn_A10_p15237975	15236993	15237409	A10	molecular function unknown
BLINK/MLMM	*B.rapa* + *B. napus*	Bn_A10_p15719803	15717774	15719732	A10	L-type lectin-domain containing receptor kinase S.5
BLINK	*B.rapa* + *B. napus*	Bn_A10_p15727608	15727042	15727174	A10	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_A10_p15731773	15731207	15731339	A10	molecular function unknown
BLINK	*B.rapa* + *B. napus*	Bn_A10_p16620627	16620061	16620190	A10	transcription factor DIVARICATA
BLINK/FarmCPU	*B.rapa* + *B. napus*	Bn_A10_p16836688	16835914	16836910	A10	WCRKC2 | WCRKC2 (WCRKC THIOREDOXIN 2)
BLINK	*B.rapa* + *B. napus*	Bn_A10_p17367157	17366591	17366723	A10	molecular function unknown
PCA+K/Q+K	*B.oleracea* + *B. napus*	Bn_scaff_15714_1_p2995346	2021706	2021906	C02	molecular function unknown
MLMM	*B.oleracea* + *B. napus*	Bn_scaff_18482_1_p138097	23803397	23803597	C03	molecular function unknown
BLINK	*B.oleracea* + *B. napus*	Bn_scaff_19310_1_p376747	40810873	40810993	C03	molecular function unknown
BLINK	*B.oleracea* + *B. napus*	Bn_scaff_18181_1_p572911	8090231	8090531	C05	molecular function unknown
MLMM	*B.oleracea* + *B. napus*	Bn_scaff_15892_1_p310757	34598807	34598929	C06	molecular function unknown
MLMM	*B.oleracea* + *B. napus*	Bn_scaff_15892_1_p404259	38035252	38035372	C06	molecular function unknown
Q+K	*B.oleracea* + *B. napus*	Bn_A09_p33459299	46230681	46230802	C08	sister chromatid cohesion protein PDS5 homolog E

[ø]Mixed Linear Model (MLM) designations: PCA, principal component analysis; Q, population structure; K, Kinship. GWAS method designations: BLINK, Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway; FarmCPU, the Fixed and random model Circulating Probability Unification; MLMM, the Multiple Locus Mixed Linear Model. [ɑ]SNP markers denoted with the same superscript letter mapped to multiple chromosomes on the reference genomes. The type of PCR-based markers showing trait association has been specified. [β]Linkage groups A1-A10 = *B. rapa*, *B napus*, and C1-C9 = *B. oleracea*, *B. napus*. Putative functions are based on matching entries in the EnsemblPlants and NCBI GenBank databases.

4.6 Figures

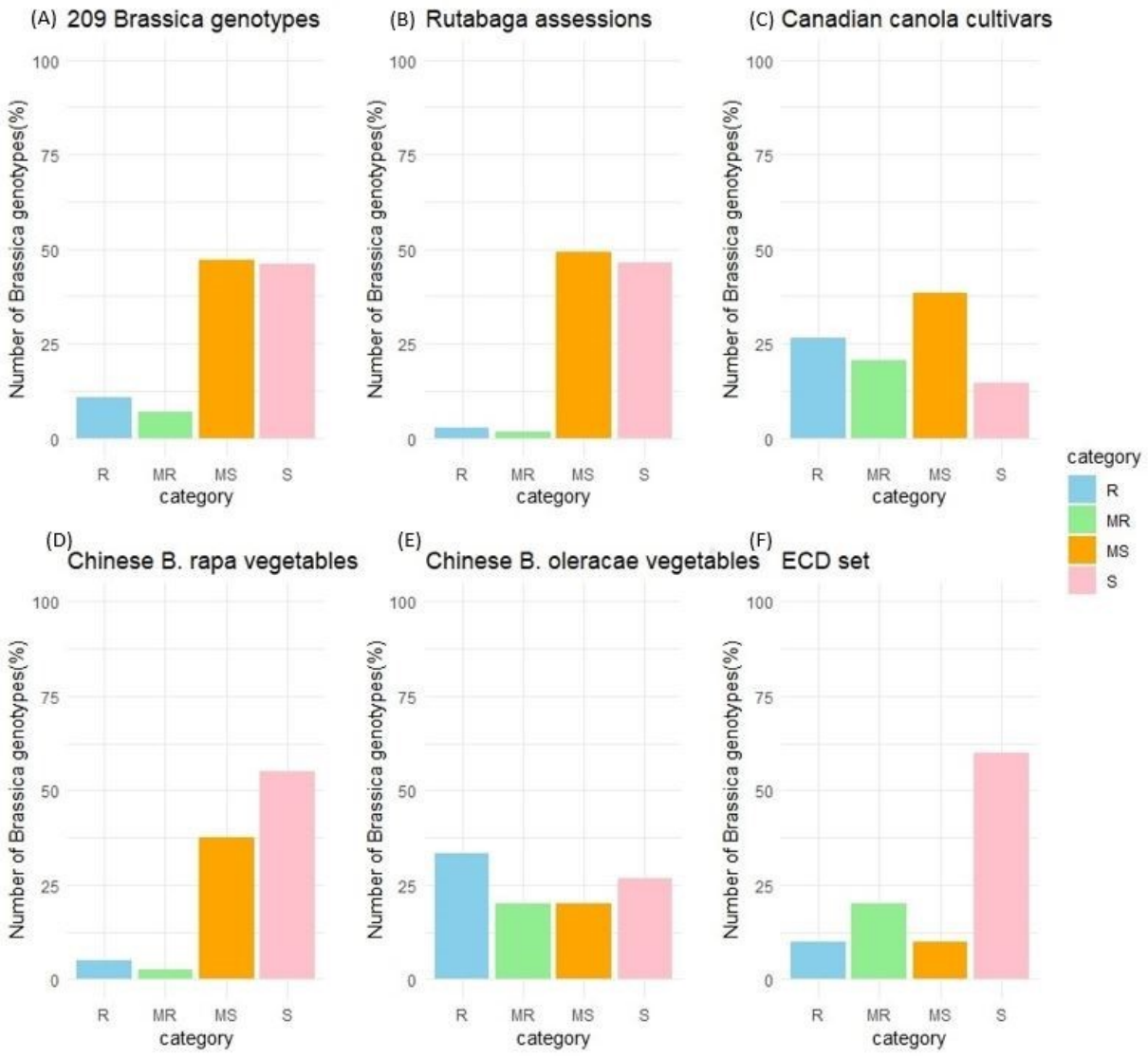

Figure 4.1 Reaction of *Brassica* genotypes to inoculation with *Verticillium longisporum*. Genotypes were rated as susceptible (S), moderately susceptible (MS), moderately resistant (MR), or resistant (R) to the fungus based on disease severity. Frequency distributions for host reactions are shown for (A) the entire collection of 209 genotypes excluding the susceptible and moderately resistant checks; (B) 110 rutabagas (*B. napus* ssp. *napobrassica*); (C) 34 canola (*B. napus*) cultivars; (D) 39 vegetable-type *B. rapa*; (E) 15 vegetable-type *B. oleracea*; and (F) 10 selected hosts of the European Clubroot Differential (ECD) set, excluding the moderately resistant-check ECD 05.

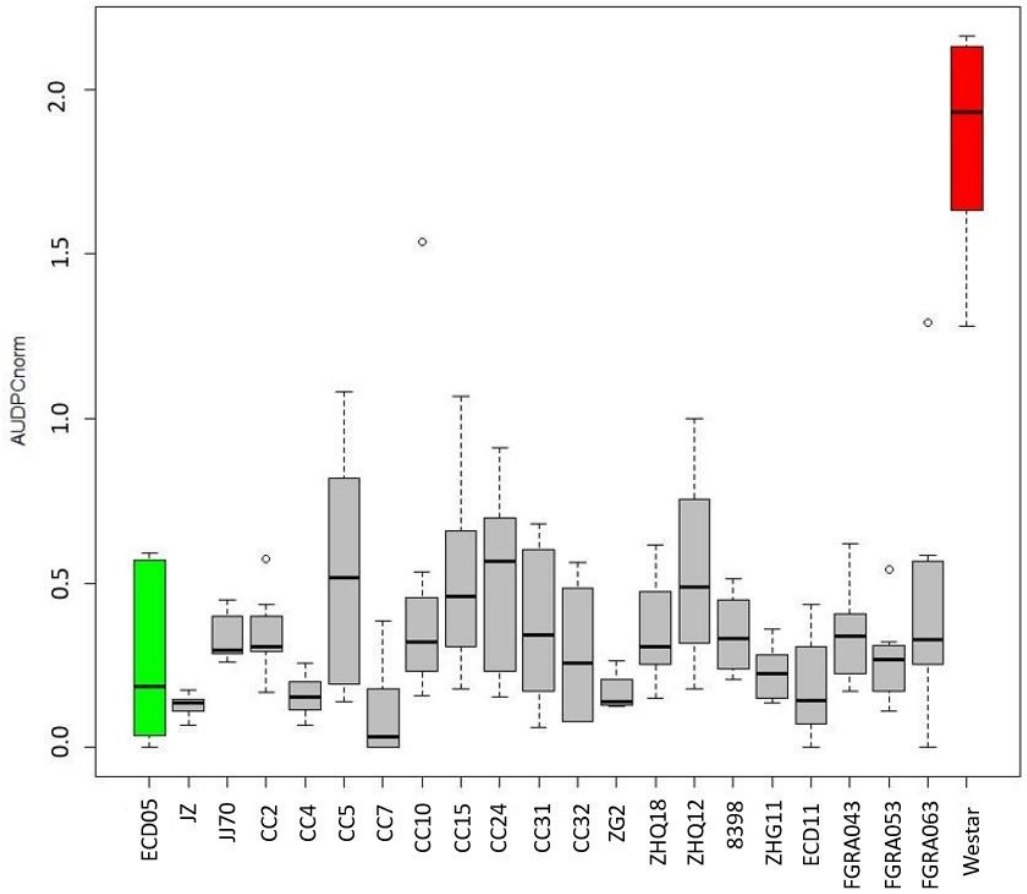

Figure 4.2 Normalized area under the disease progress curve ($AUDPC_{norm.}$) for 20 *Brassica* genotypes showing resistance to *Verticillium longisporum.* The $AUDPC_{norm.}$ was calculated from disease severities rated from 1-9 following Eynck et al. (2009), where 1 = no symptoms and 9 = the plant is dead. The grey bars show the range of the maximum and minimum $AUDPC_{norm.}$, and the black lines in each box indicate the mean of $AUDPC_{norm.}$ values among eight replicates in two independent repeats. The green bar denotes the moderately resistant check *B. rapa* var. *pekinensis* 'Granaat' (ECD 05), while the red bar denotes the susceptible check *B. napus* 'Westar'. The other genotypes shown include the rutabagas (*B. napus* ssp. *napobrassica*) FGRA043, FGRA053, and FGRA063; canola (*B. napus*) cultivars CC2, CC4, CC5, CC7, CC10 and CC15; *B. rapa* cultivars 'Jingyan Zikuaicai' (JZ), and 'Jingjian No.70' (JJ70); *B. oleracea* cultivars 'Zigan2' (ZG2), 'Zhongqing 18' (ZHQ18), 'Zhongqing 12' (ZHQ12), 'Zhonggan 11' (ZHQ11), and '8398'; and ECD 11.

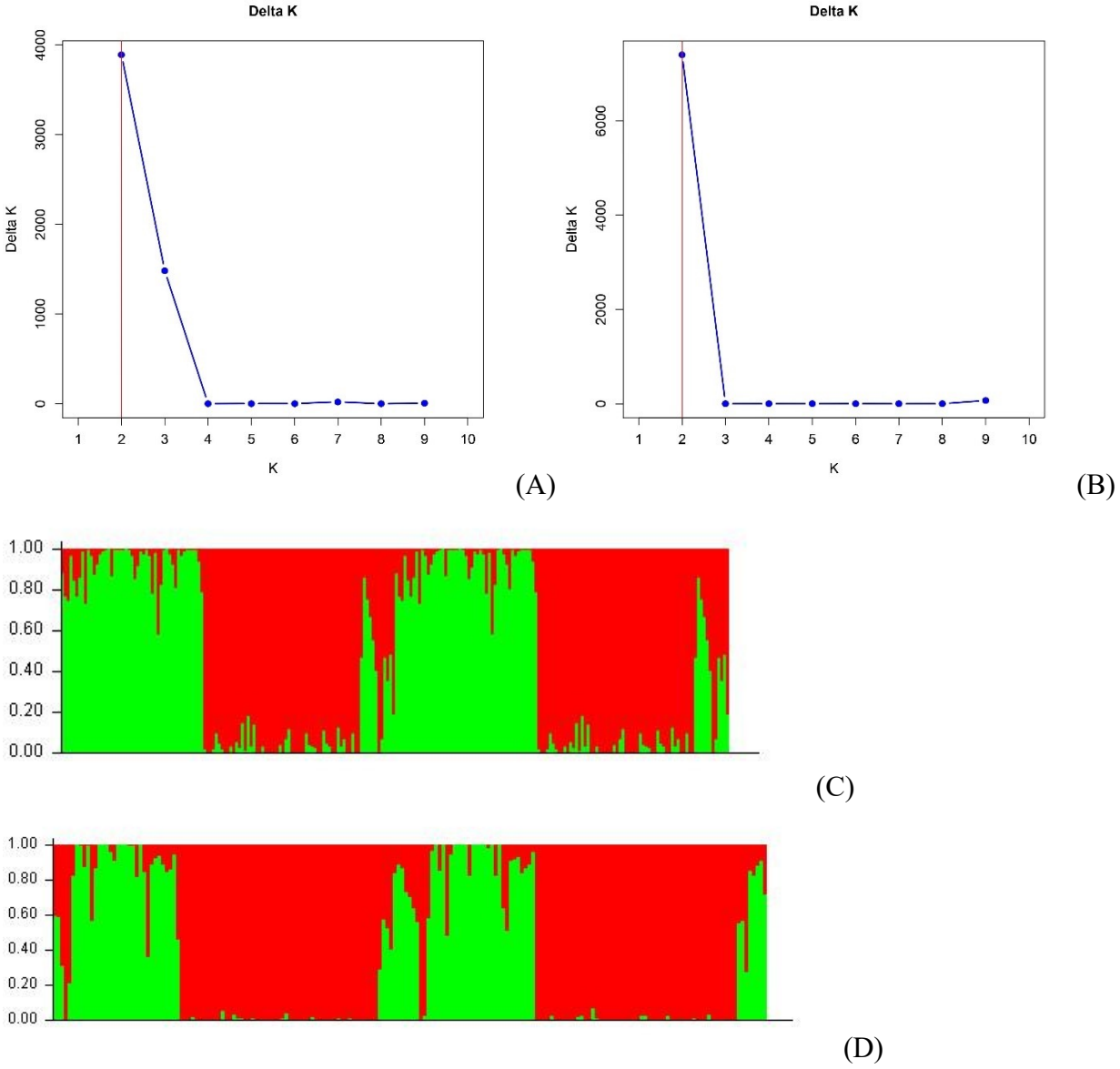

Figure 4.3 Bayesian cluster analysis of 211 *Brassica* accessions including *B. napus*, *B. oleracea* and *B. rapa* estimated with STRUCTURE using 50,000 burn-in iterations and Markov Chain Monte Carlo (MCMC) lengths. The value of K, determined following Evanno et al. (2005), indicated two clusters for the *B. rapa* and *B. napus* genotypes (A), and for the *B. oleracea* and *B. napus* genotypes (B), in all runs. Detailed Bayesian clustering of the *B. rapa* and *B. napus* genotypes (C), and of the *B. oleracea* and *B. napus* genotypes (D), is also shown, with each color representing one ancestry component. The simplified view suggests two ancestral populations.

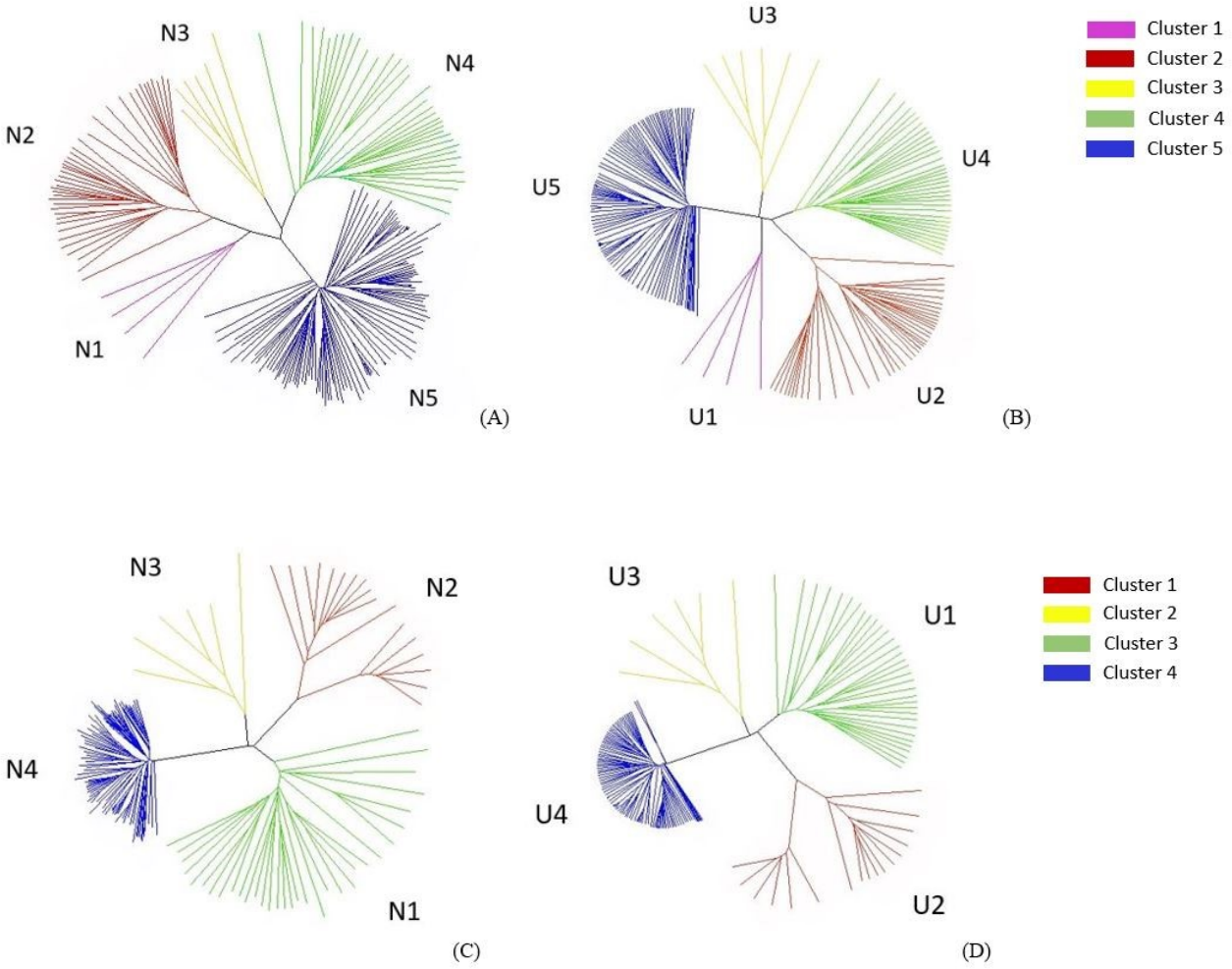

Figure 4.4 Neighbour joining (NJ) (A) and unweighted pair group method with arithmetic mean (UPGMA) (B) analysis with 4,972 A-genome markers grouped 194 *Brassica rapa* and *Brasscia napus* genotypes into five clusters. NJ (C) and UPGMA (D) analysis with 4,621 C-genome markers grouped 166 *Brassica oleracea* and *B. napus* genotypes into four clusters.

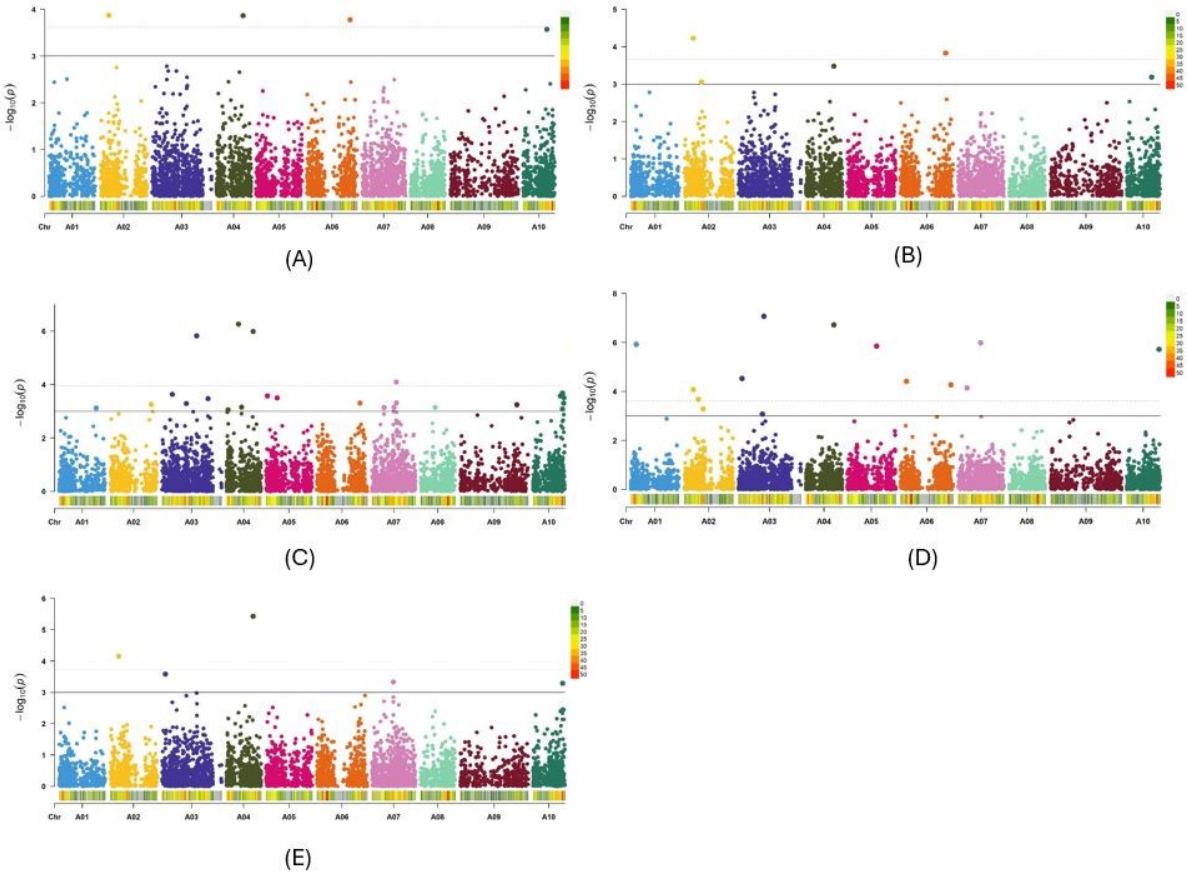

Figure 4.5 Manhattan plots of the PCA+K (A), Q+K (B), BLINK (C), FarmCPU (D) and MLMM (E) models for identifying *Verticillium longisporum* resistance in *Brassica rapa* + *Brassica napus* genotypes. The dashed horizontal lines indicate the Bonferroni-adjusted significance threshold known as "logarithm-of-odds" (LOD score). The solid lines indicate a slightly lower threshold of -log10 P = 3.0. The dots above the significance threshold indicate single-nucleotide polymorphisms (SNPs) associated with resistance to *V. longisporum*.

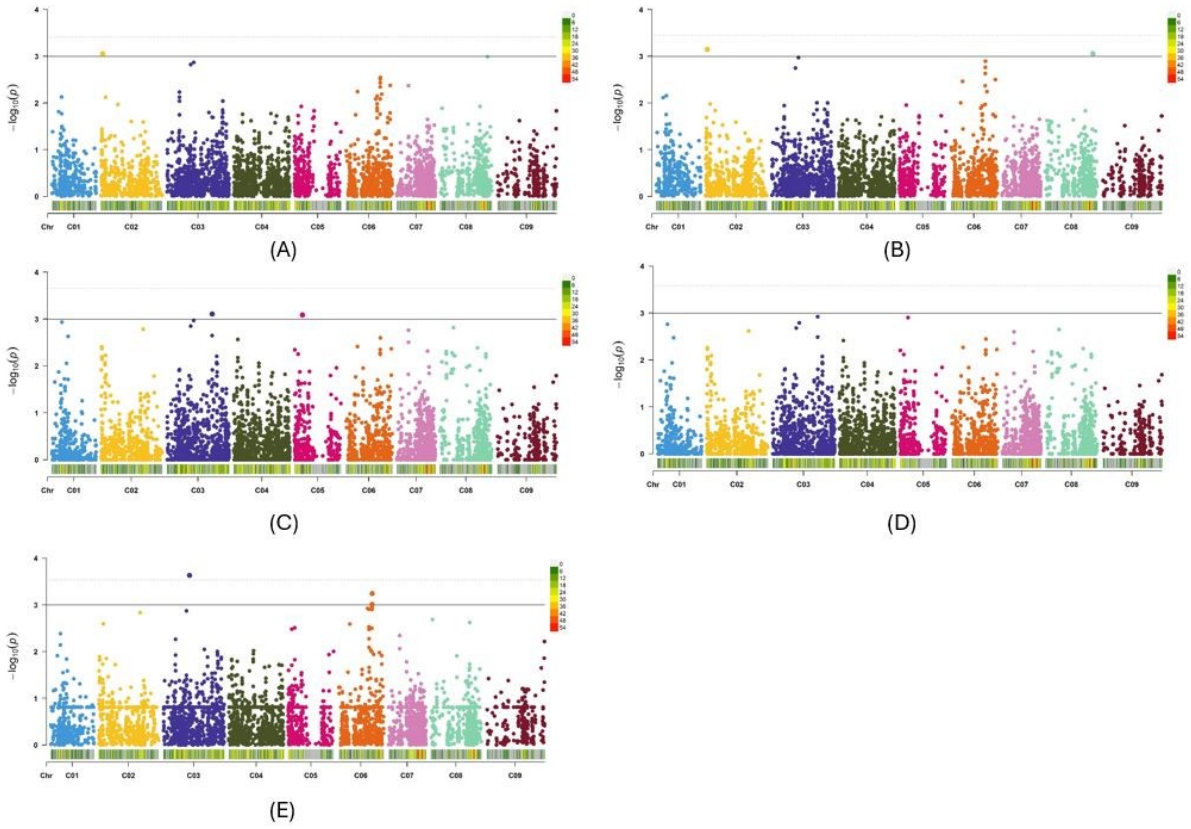

Figure 4.6 Manhattan plots of the PCA+K (A), Q+K (B), BLINK (C), FarmCPU (D) and MLMM (E) models for identifying *Verticillium longisporum* resistance in *Brassica oleracea* + *Brassica napus* genotypes. The dashed horizontal lines indicate the Bonferroni-adjusted significance threshold known as "logarithm-of-odds" (LOD score). The solid lines indicate a slightly lower threshold of -log10 $P = 3.0$. The dots above the significance threshold indicate single-nucleotide polymorphisms (SNPs) associated with resistance to *V. longisporum*.

Chapter 5 Conclusion

The identification of Verticillium stripe, caused by *Verticillium longisporum*, in canola (*Brassica napus* L.) within Canada has raised significant concern, especially considering the limited strategies currently available for managing this disease. With its increasing prevalence in western Canada (Akhavan et al. 2023; Harding et al. 2023; Kim et al. 2023), there is a pressing need for information on the pathogen's biology and host resistance to facilitate knowledge-based control of Verticillium stripe. This research was undertaken to generate such information, serving as the basis for further studies and ultimately forming the foundation for developing sustainable disease management approaches.

In Chapter 2, the impact of interactions between *Leptosphaeria maculans* and *Verticillium longisporum* (C. Stark) Karapapa, Bainbridge, and Heale, was assessed under field and greenhouse conditions. In most canola hybrids, the relationship between blackleg severity and yield components was best explained by second-degree quadratic equations, although a linear relationship was found for one variety sampled in commercial fields. When *L. maculans* and *V. longisporum* were inoculated together, blackleg severity and yield losses increased. In some cases, Verticillium stripe caused greater yield losses than blackleg. The results suggested a synergistic effect, with the presence of both pathogens causing more severe losses overall. This study also demonstrated that longitudinal sections of affected stem tissue could help differentiate blackleg and Verticillium stripe more readily on canola, enabling more accurate diagnoses and disease monitoring in the field.

In Chapter 3, the impact of pH on both the growth of *V. longisporum* and its virulence on canola was investigated. Fungal growth was measured by colony diameter following 14 and 21 days of incubation on potato dextrose agar at varying pH levels (4.7, 5.5, 6.5, 7.4, or 8.6). The

results indicated that colonies of *V. longisporum* were approximately 16% greater in diameter at pH 7.4 and 8.6 compared to pH 5.5. The impact of pH on disease development at the seedling stage was studied using a semi-hydroponic system with different pH levels (4.4, 5.4, 6.3, 7.5, and 8.4) in half-strength Hoagland's solution. Verticillium stripe was most severe at pH 7.5 and 8.4 after a 10-day period in the semi-hydroponic system.

In a second inoculation experiment, canola seedlings previously inoculated with *V. longisporum* were transplanted into potting mix amended to four pH levels (5.6, 6.4, 7.2 and 7.8). The transplants were cultivated under greenhouse conditions and evaluated for Verticillium stripe severity at plant maturity. Disease severity was greatest at pH 7.8. This was the first study on the effects of pH on *V. longisporum* in canola, suggesting a substantial risk of increased disease severity and yield losses in regions with neutral to slightly alkaline soils.

In Chapter 4, a large collection of *Brassica* genotypes was evaluated for their reactions to *V. longisporum*, and a genome-wide association study (GWAS) was carried out to identify single nucleotide polymorphism (SNP) markers associated with resistance. The plant material included 110 rutabaga (*B. napus* ssp. *napobrassica*), 35 canola, 40 *Brassica rapa*, and 15 *Brassica oleracea* accessions or cultivars, alongside 11 hosts from the European Clubroot Differential set. This material was tested for resistance under greenhouse conditions and genotyped using a 19K *Brassica* SNP array. Three general linear models (GLM), four mixed linear models (MLM), and three GWAS methods were employed to evaluate the markers. Eleven non-commercial *Brassica* accessions and nine out of 35 commercial canola cultivars displayed a low normalized area under the disease progress curve ($AUDPC_{norm.}$). Some of these accessions could prove valuable as potential sources of resistance to *V. longisporum*. In addition, 45 significant SNP markers were identified in the GWAS. The genomic regions identified on chromosomes A03 and A10 represent

promising hotspots for marker-assisted selection in the future development of Verticillium stripe-resistant canola.

Several new potential studies could be developed from the current research, which could further contribute to improved management of Verticillium stripe. Some potential avenues for further investigation include:

- Field Trials: Conducting field trials to validate the efficacy of identified resistance sources and markers under more natural growing conditions.
- Pathogen Biology: Further exploring the biology of *V. longisporum*, including its interactions with other pathogens and environmental factors, to develop more targeted control strategies.
- Genetic Studies: Expanding genetic studies to identify additional resistance genes and markers and elucidate the underlying mechanisms of resistance.
- Biocontrol Agents: Investigating the potential of biocontrol agents, such as bacteria and fungi, for managing Verticillium stripe in canola fields under western Canadian conditions.
- Crop Management Practices: Assessing the impact of different crop management practices, such as crop rotation, tillage methods, and soil amendments, on Verticillium stripe severity and canola yield.
- Host-Pathogen Interactions: Studying host-pathogen interactions at the molecular level to improve understanding of the molecular mechanisms of resistance in canola and virulence in *V. longisporum*.

By pursuing these avenues, researchers could further advance our understanding of Verticillium stripe and develop more effective strategies for its management in canola crops.

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Appendices

Supplementary Table 0.1 The *Brassica* genotypes used in this study.

Cultivar name	Common name	Species
FGRA001	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA003	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA004	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA005	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA006	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA007	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA009	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA010	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA011	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA013	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA015	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA016	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA017	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA018	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA020	Rutabaga	*Brassica napus* spp. *napobrassica*
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FGRA022	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA023	Rutabaga	*Brassica napus* spp. *napobrassica*
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FGRA027	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA028	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA029	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA030	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA034	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA036	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA037	Rutabaga	*Brassica napus* spp. *napobrassica*
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FGRA043	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA044	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA045	Rutabaga	*Brassica napus* spp. *napobrassica*

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FGRA050	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA051	Rutabaga	*Brassica napus* spp. *napobrassica*
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FGRA077	Rutabaga	*Brassica napus* spp. *napobrassica*
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FGRA089	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA090	Rutabaga	*Brassica napus* spp. *napobrassica*

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FGRA097	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA098	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA099	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA100	Rutabaga	*Brassica napus* spp. *napobrassica*
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FGRA123	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA124	Rutabaga	*Brassica napus* spp. *napobrassica*
FGRA125	Rutabaga	*Brassica napus* spp. *napobrassica*
Westar	Canadian canola	*Brassica napus*
CC1	Canadian canola	*Brassica napus*
CC2	Canadian canola	*Brassica napus*
CC3	Canadian canola	*Brassica napus*
CC4	Canadian canola	*Brassica napus*
CC5	Canadian canola	*Brassica napus*
CC6	Canadian canola	*Brassica napus*
CC7	Canadian canola	*Brassica napus*

CC8	Canadian canola	*Brassica napus*
CC9	Canadian canola	*Brassica napus*
CC10	Canadian canola	*Brassica napus*
CC11	Canadian canola	*Brassica napus*
CC12	Canadian canola	*Brassica napus*
CC13	Canadian canola	*Brassica napus*
CC14	Canadian canola	*Brassica napus*
CC15	Canadian canola	*Brassica napus*
CC16	Canadian canola	*Brassica napus*
CC17	Canadian canola	*Brassica napus*
CC18	Canadian canola	*Brassica napus*
CC19	Canadian canola	*Brassica napus*
CC20	Canadian canola	*Brassica napus*
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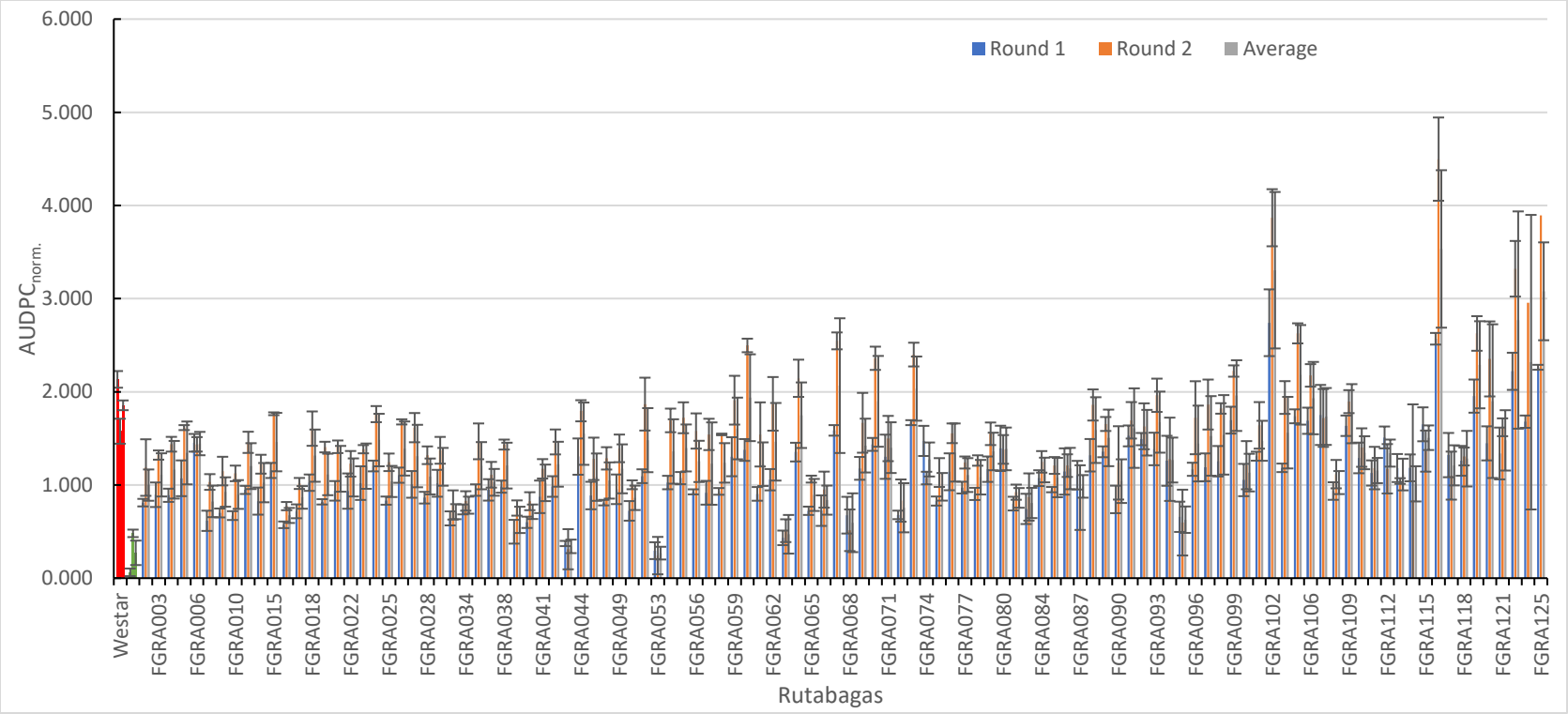
CC24	Canadian canola	*Brassica napus*
CC25	Canadian canola	*Brassica napus*
CC26	Canadian canola	*Brassica napus*
CC27	Canadian canola	*Brassica napus*
CC28	Canadian canola	*Brassica napus*
CC29	Canadian canola	*Brassica napus*
CC30	Canadian canola	*Brassica napus*
CC31	Canadian canola	*Brassica napus*
CC32	Canadian canola	*Brassica napus*
Laurentian	Canadian canola	*Brassica napus*
Mendel	Canadian canola	*Brassica napus*
Jing guan	Chinese vegetable	*Brassica rapa*
Chunyou No5	Chinese vegetable	*Brassica rapa*
Chunyou No1	Chinese vegetable	*Brassica rapa*
Guoxia No3	Chinese vegetable	*Brassica rapa*
Cuiying 256	Chinese vegetable	*Brassica rapa*
Ziguan No1	Chinese vegetable	*Brassica rapa*
New naibai	Chinese vegetable	*Brassica rapa*
Cui bai No3	Chinese vegetable	*Brassica rapa*
Jingcui 60	Chinese vegetable	*Brassica rapa*
Siji Kuaicai No1	Chinese vegetable	*Brassica rapa*
Zhongbai 61	Chinese vegetable	*Brassica rapa*
Jing lv No7	Chinese vegetable	*Brassica rapa*
Lv Zhen	Chinese vegetable	*Brassica rapa*
Xueli Jinhua	Chinese vegetable	*Brassica rapa*

Beijing Autumn 1518	Chinese vegetable	*Brassica rapa*
Beijing Autumn No4	Chinese vegetable	*Brassica rapa*
Beijing Xiaoza No60	Chinese vegetable	*Brassica rapa*
Lvjian No60	Chinese vegetable	*Brassica rapa*
Beijing Orange Heart	Chinese vegetable	*Brassica rapa*
Susheng Lvxiu	Chinese vegetable	*Brassica rapa*
Lvsun No70	Chinese vegetable	*Brassica rapa*
Jingyan Zikuaicai	Chinese vegetable	*Brassica rapa*
Xia lv No2	Chinese vegetable	*Brassica rapa*
Beijing Daniuxin	Chinese vegetable	*Brassica rapa*
Jingjian No70	Chinese vegetable	*Brassica rapa*
Beijing New No3	Chinese vegetable	*Brassica rapa*
Zhongbai 76	Chinese vegetable	*Brassica rapa*
Kuaicai No5	Chinese vegetable	*Brassica rapa*
Jingqiu No3	Chinese vegetable	*Brassica rapa*
Jihong 308	Chinese vegetable	*Brassica rapa*
Li Chun No1	Chinese vegetable	*Brassica rapa*
Spring xiaobao No2	Chinese vegetable	*Brassica rapa*
Zhongbai 81	Chinese vegetable	*Brassica rapa*
Jinglv Wutacai	Chinese vegetable	*Brassica rapa*
qingxiangbiyu	Chinese vegetable	*Brassica rapa*
CR4141	Chinese vegetable	*Brassica rapa*
Beijing Spring Yellow No2	Chinese vegetable	*Brassica rapa*
Jinglv No1	Chinese vegetable	*Brassica rapa*
Spring xiaobao 366	Chinese vegetable	*Brassica rapa*
Bi Yu	Chinese vegetable	*Brassica rapa*
Zhongqing 16	Chinese vegetable	*Brassica olearcea*
Zigan 2	Chinese vegetable	*Brassica olearcea*
Zhonggan 21	Chinese vegetable	*Brassica olearcea*
Zhonggan 301	Chinese vegetable	*Brassica olearcea*
Zhonggan 27	Chinese vegetable	*Brassica olearcea*
Zhonggan 828	Chinese vegetable	*Brassica olearcea*
Zhonggan 628	Chinese vegetable	*Brassica olearcea*
Zhongqing 11	Chinese vegetable	*Brassica olearcea*
Zhongqing 18	Chinese vegetable	*Brassica olearcea*
Zhongqing 9	Chinese vegetable	*Brassica olearcea*
Zhongqing 518	Chinese vegetable	*Brassica olearcea*
Zhongqing 12	Chinese vegetable	*Brassica olearcea*
8398	Chinese vegetable	*Brassica olearcea*
Zhonggan 11	Chinese vegetable	*Brassica olearcea*

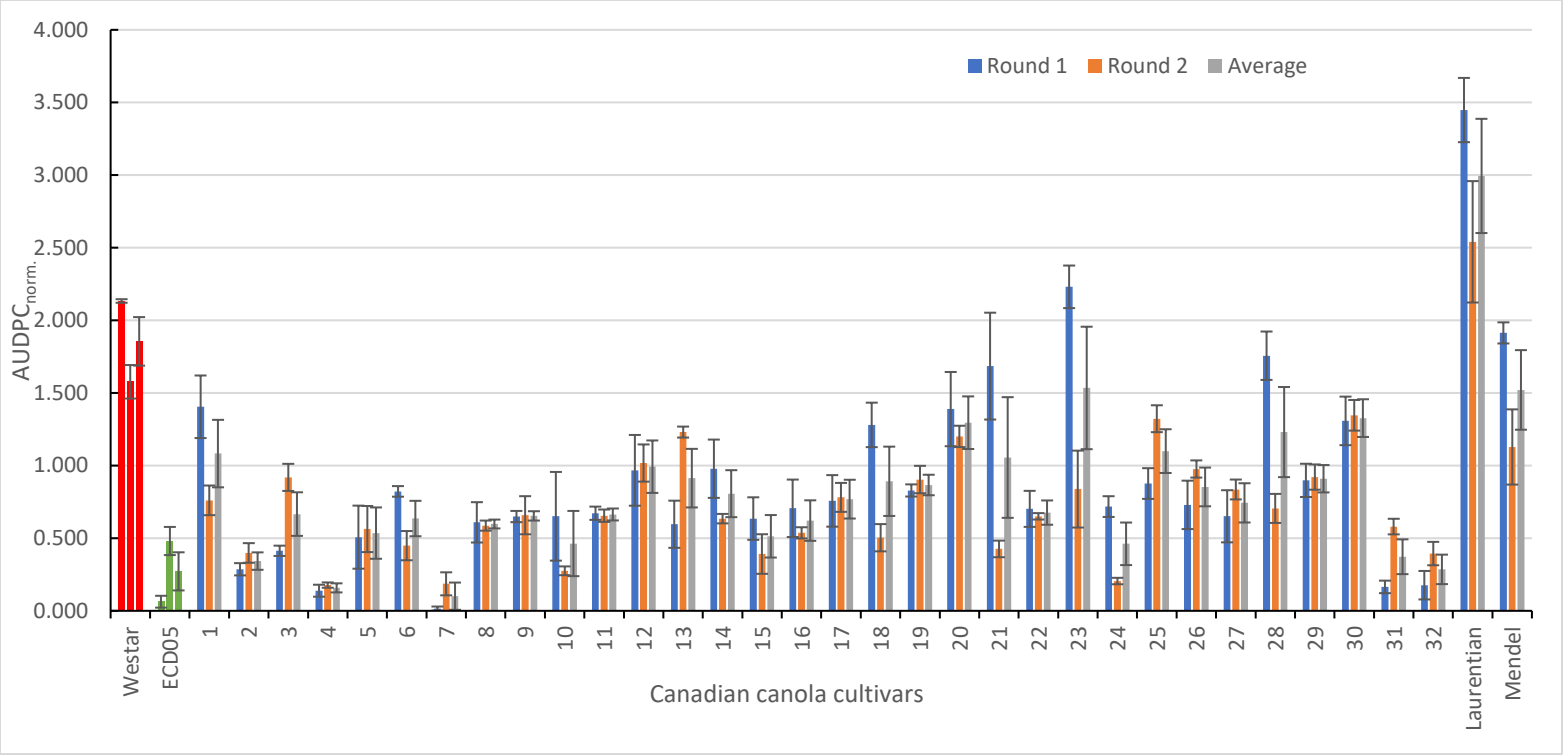
Zhonggan 103	Chinese vegetable	*Brassica olearcea*
ECD 01		*Brassica rapa*
ECD 02		*Brassica rapa*
ECD 03		*Brassica rapa*
ECD 04		*Brassica rapa*
ECD 05	Granaat	*Brassica rapa*
ECD 06	Nevin	*Brassica napus*
ECD 08	Giant Rapa selection	*Brassica napus*
ECD 09	New Zealand resistant rape	*Brassica napus*
ECD 10	Wilmesburger	*Brassica napus*
ECD 12	Bindsachsener	*Brassica olearcea*
ECD 13	Jersey Queen	*Brassica olearcea*

Supplementary Figures



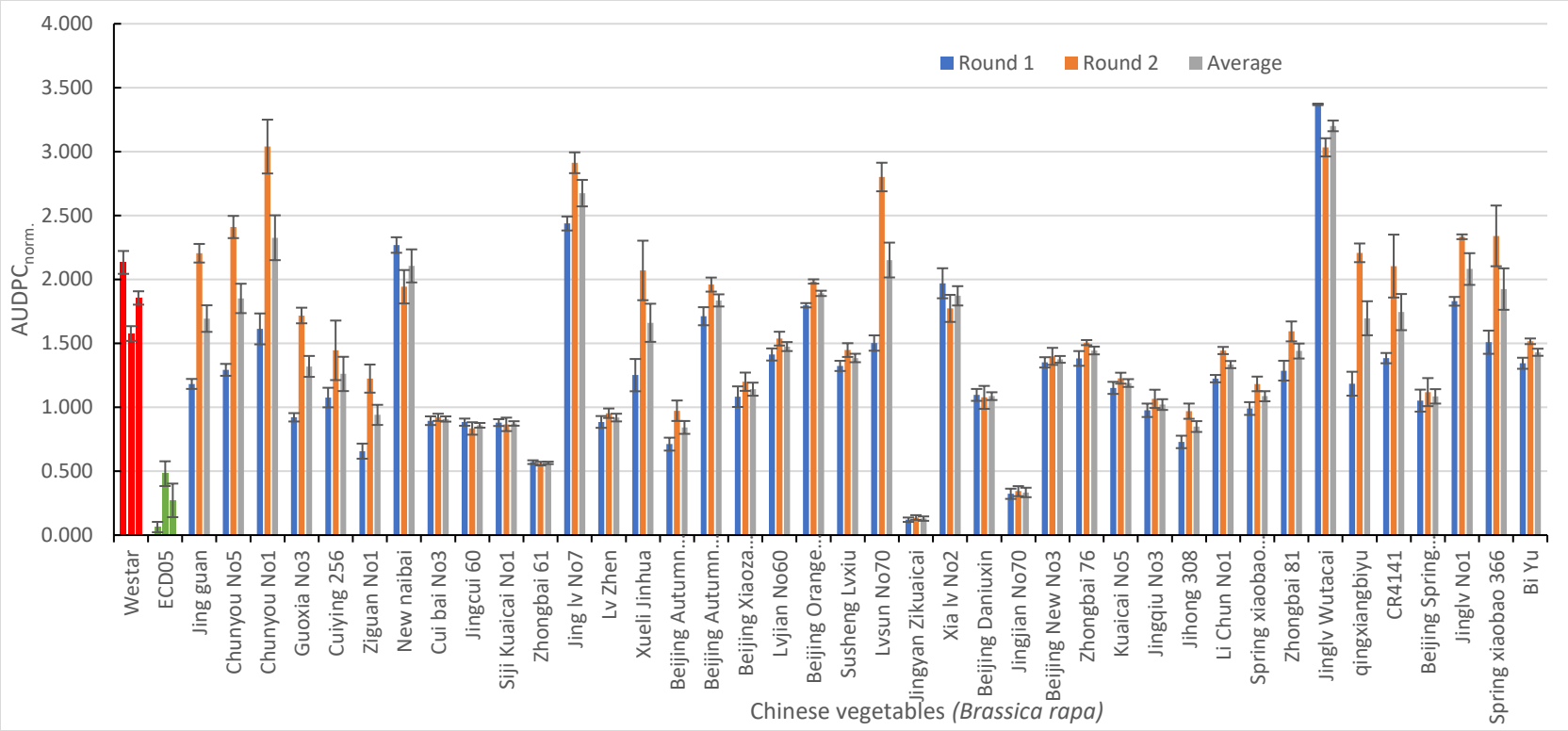
(A)

Supplementary Figure S1A.*Verticillium longisporum* resistance responses measured by area under the disease progress curve ($AUDPC_{norm,}$) for 110 rutabagas (*B. napus*) compared to the susceptible check 'Westar' (red) and moderately resistant check 'Granaat' (ECD 05) (green). Columns and bars represent mean values of four replicates of round 1, round 2 and average of two rounds with standard deviations. Round 1 represents the first independent repeat; round 2 represents the second independent repeat and average represents the mean AUDPC values of two independent repeats.



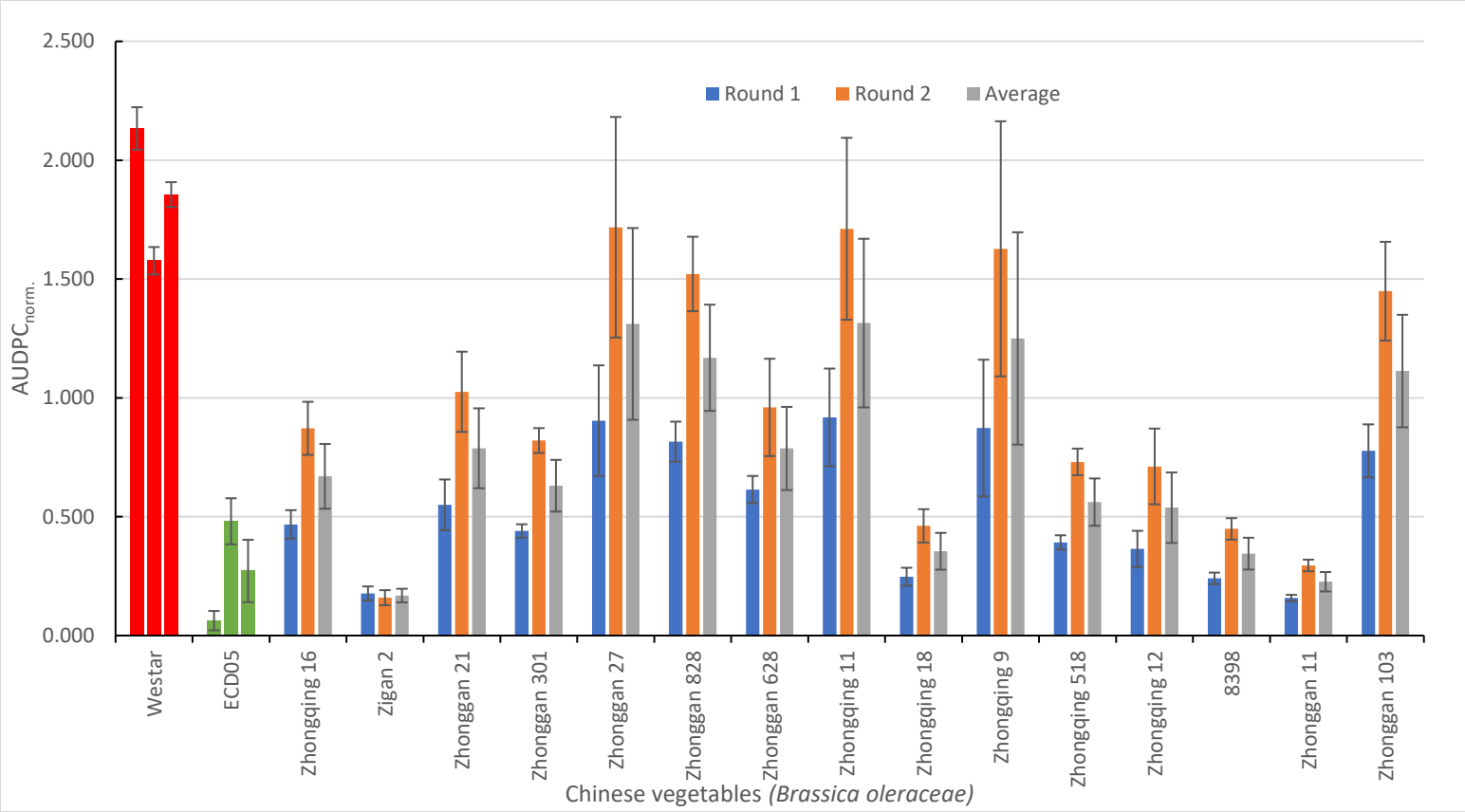
(B)

Supplementary Figure S1B. *Verticillium longisporum* resistance responses measured by area under the disease progress curve ($AUDPC_{norm,}$) for 34 of the 35 Canadian canola cultivars (*B. napus*) compared to the susceptible check 'Westar' (red) and moderately resistant check 'Granaat' (ECD 05) (green). Columns and bars represent mean values of four replicates of round 1, round 2 and average of two rounds with standard deviations. Round 1 represents the first independent repeat; round 2 represents the second independent repeat and average represents the mean AUDPC values of two independent repeats.



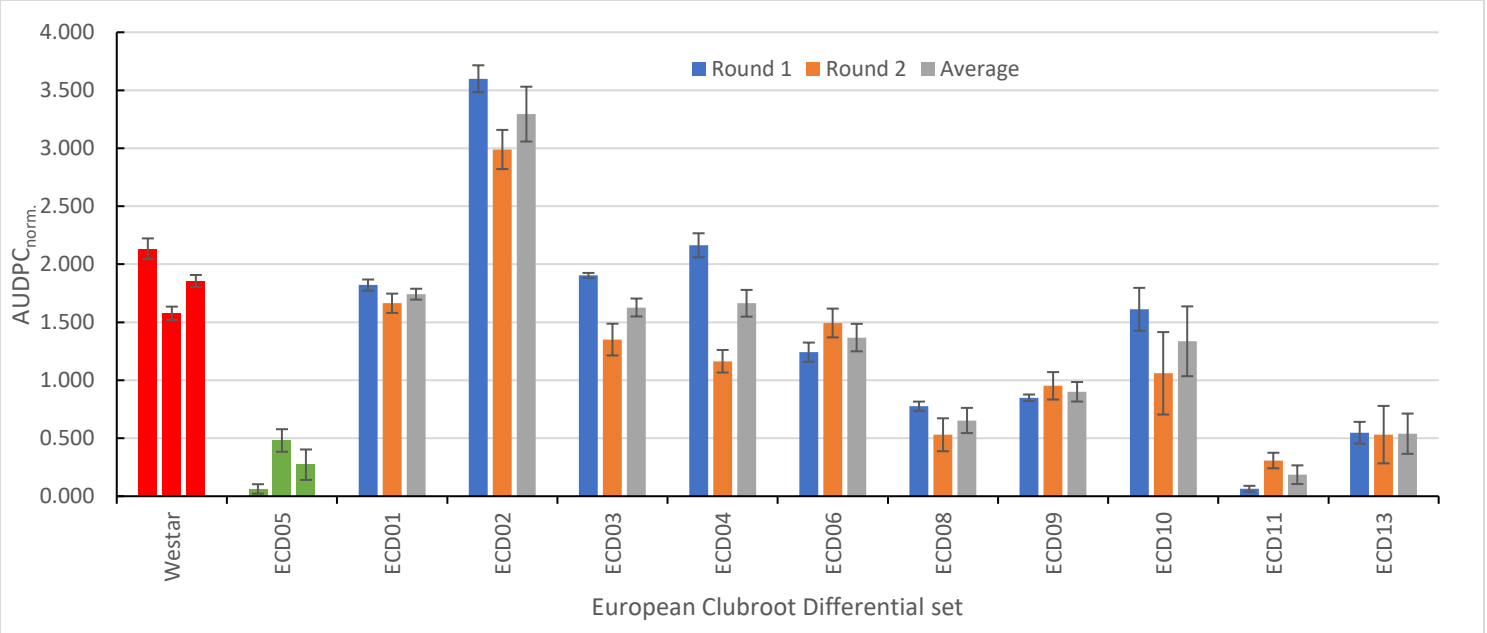
(C)

Supplementary 0. *Verticillium longisporum* resistance responses measured by area under the disease progress curve ($AUDPC_{norm,}$) for 40 *B. rapa* vegetable cultivars from China compared to the susceptible check 'Westar' (red) and moderately resistant check 'Granaat' (ECD 05) (green). Columns and bars represent mean values of four replicates of round 1, round 2 and average of two rounds with standard deviations. Round 1 represents the first independent repeat; round 2 represents the second independent repeat and average represents the mean AUDPC values of two independent repeats.



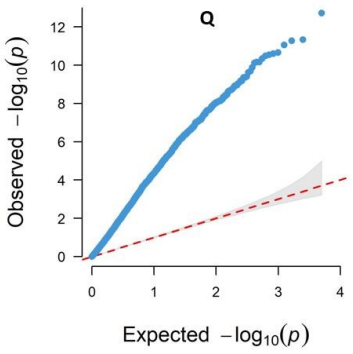
(D)

Supplementary Figure S1D. *Verticillium longisporum* resistance responses measured by area under the disease progress curve ($AUDPC_{norm,}$) for 15 *B. oleracea* vegetable cultivars from China compared to the susceptible check 'Westar' (red) and moderately resistant check 'Granaat' (ECD 05) (green). Columns and bars represent mean values of four replicates of round 1, round 2 and average of two rounds with standard deviations. Round 1 represents the first independent repeat; round 2 represents the second independent repeat and average represents the mean AUDPC values of two independent repeats.

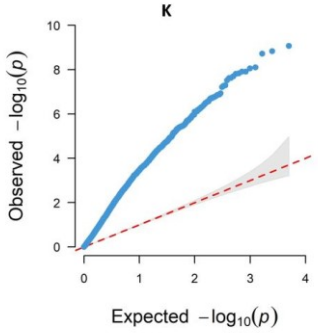


(e)

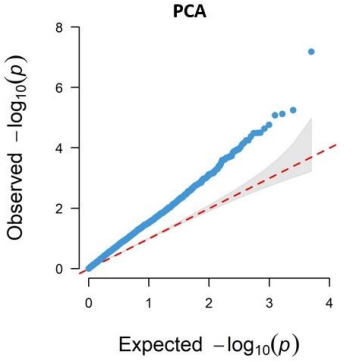
Supplementary Figure S1E. *Verticillium longisporum* resistance responses measured by area under the disease progress curve ($AUDPC_{norm,}$) for 10 selected hosts of the ECD set apart from ECD05 ('Granaat') compared to the susceptible check 'Westar' (red) and moderately resistant check 'Granaat' (ECD 05) (green). Columns and bars represent mean values of four replicates of round 1, round 2 and average of two rounds with standard deviations. Round 1 represents the first independent repeat; round 2 represents the second independent repeat and average represents the mean AUDPC values of two independent repeats.


Q
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

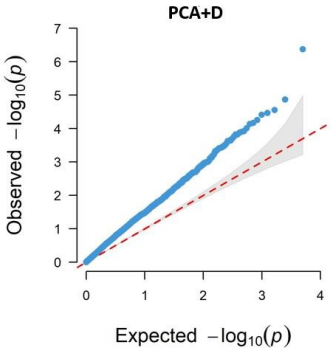
(A)


K
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

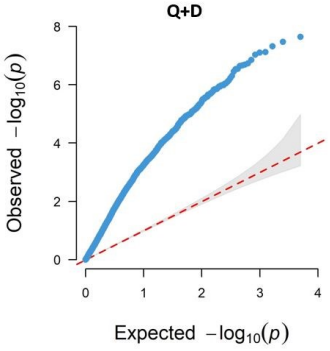
(B)


PCA
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

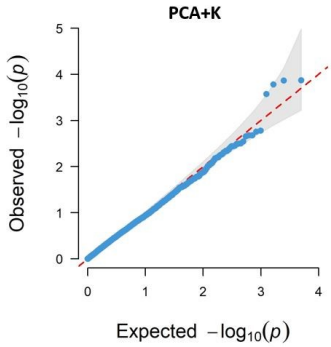
(C)


PCA+D
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

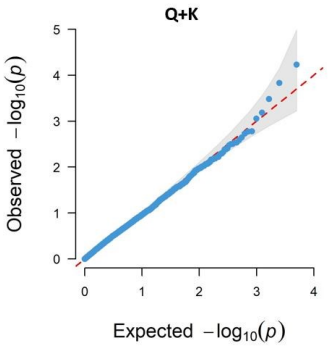
(D)


Q+D
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

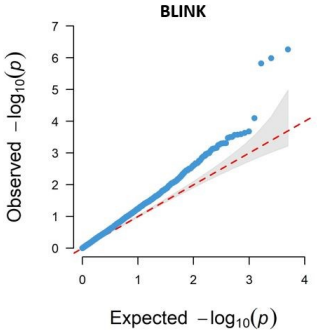
(E)


PCA+K
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

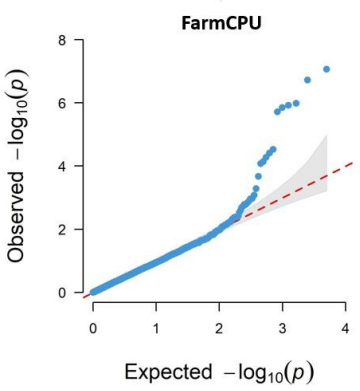
(F)


Q+K
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

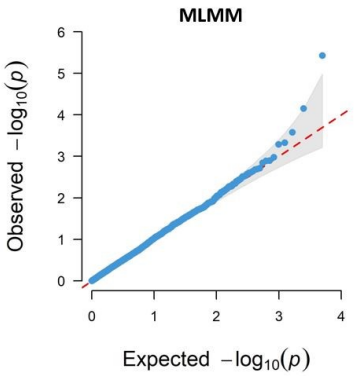
(G)


BLINK
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

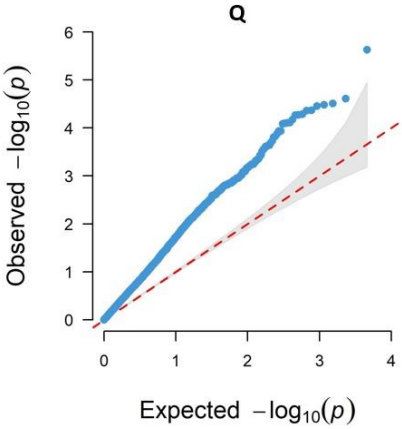
(H)


FarmCPU
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

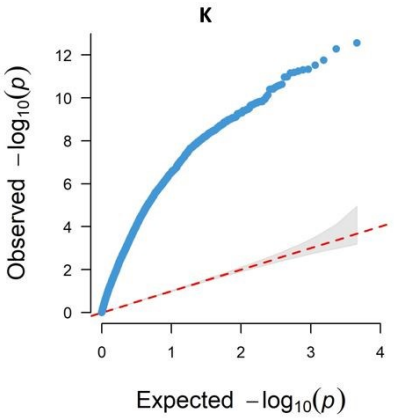
(I)


MLMM
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

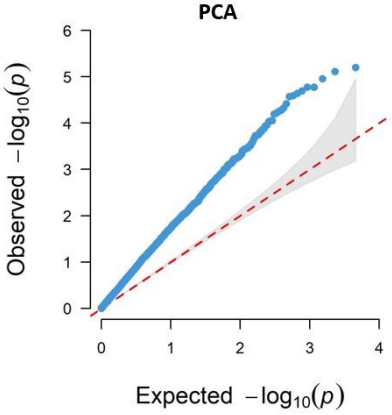
(J)


Q
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

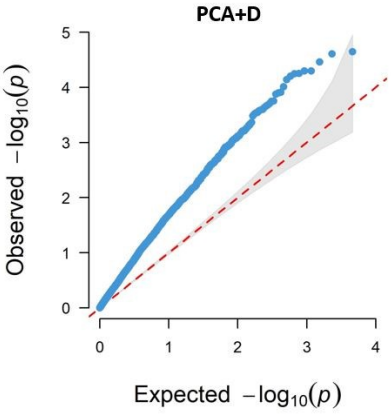
(K)


K
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

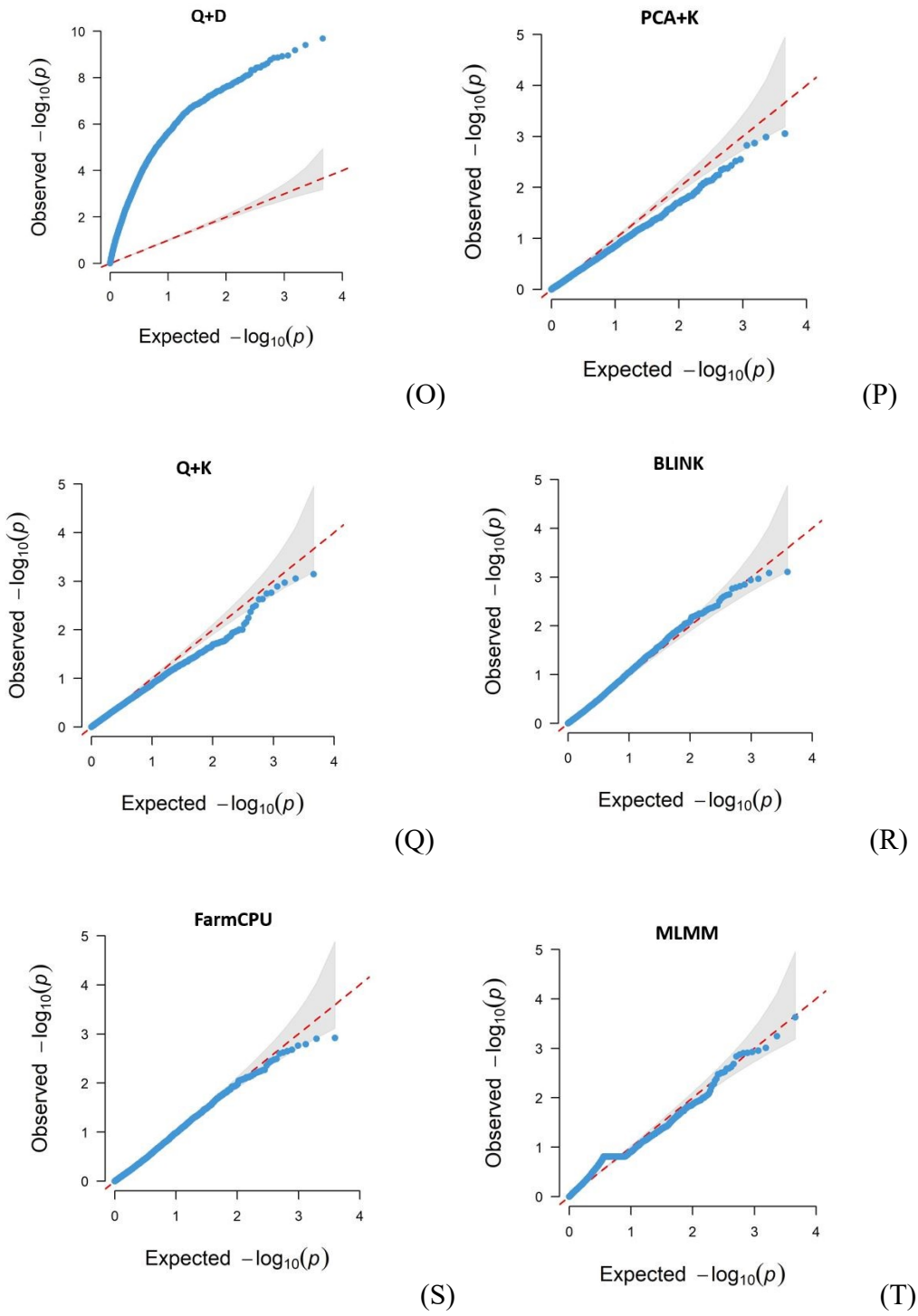
(L)


PCA
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

(M)


PCA+D
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$

(N)



Supplementary Figure 2. Quantile-Quantile comparison of GWAS model Q-only (A), K-only (B), PCA-only (C), PCA+D (D), Q+D (E), PCA+K (F), Q+K (G), BLINK (H), FarmCPU (I) and

MLMM (J) models for *B. rapa* + *B. napus* genotypes. QQ-plot of GWAS model Q-only (K), K-only (L), PCA-only (M), PCA+D (N), Q+D (O), PCA+K (P), Q+K (Q), BLINK (R), FarmCPU (S) and MLMM (T) models for *B. oleracea* + *B. napus* genotypes. The red dash line is the expected -log10 *P* distribution while colored lines are the observed -log10 *P* distribution.