**Studies into clubroot (*Plasmodiophora brassicae)* epidemiology and resistance**

by

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# Abstract

Clubroot, caused by *Plasmodiophora brassicae*, is an important soilborne disease of canola (oilseed rape; *Brassica napus*). The effective and sustainable management of clubroot requires a deeper understanding of clubroot epidemiology and improved durability of host resistance. This research aimed to (i) develop a clubroot yield loss model for *B. napus*, (ii) characterize the spatial patterns associated with *P. brassicae* inoculum density and its relationship with soil pH and boron, calcium, and magnesium, and (iii) analyze the polygenic resistance harbored by a doubled haploid population obtained from a cross between 'Aviso' and 'Montego' (*B. napus*).

The effect of clubroot on *B. napus* yield and yield-related parameters was evaluated under field and greenhouse conditions using the canola cultivars '45H31' (susceptible), '45H29' (1st generation resistance) and 'CS2000' (2nd generation resistance), following inoculation with different pathotypes of *P. brassicae*. The field experiment was conducted over 2 years in Edmonton, Alberta, in biosecure nurseries inoculated with low ($5 \times 10^4$ resting spores per plant), intermediate ($5 \times 10^6$ resting spores per plant) or high ($5 \times 10^8$ resting spores per plant) concentrations of pathotype 5X or a mixture of pathotypes 5X and 3H. In the greenhouse experiment, the same cultivars were inoculated with pathotypes 5X, 3H or a 5X + 3H mixture at inoculum concentrations of $1 \times 10^3$, $1 \times 10^4$ and $1 \times 10^6$ resting spores per plant. In both the field and greenhouse, clubroot incidence and disease severity index (DSI) increased with increasing inoculum density; the highest levels of disease were observed in the susceptible cultivar, while the lowest were found in '45H29.' Yield, pods per plant and 1000-grain weight decreased as the DSI increased in all cultivars. Yield was affected by DSI and canola cultivar, but not by pathotype. Regression analysis indicated that under greenhouse conditions, an increment of 1% in the DSI

resulted in a decrease of 0.49% in yield; under field conditions, this percentage was reduced to 0.26%. While the rate of yield reduction was similar among cultivars, overall yield losses were lower in the clubroot resistant hosts, since clubroot was less severe.

To study the spatial patterns of *P. brassicae* inoculum density and their relationship to different soil properties, four clubroot-infested fields in central Alberta were sampled in 2017 and 2019, and *P. brassicae* inoculum density, soil pH, and boron, calcium, and magnesium concentrations were quantified. Spatial autocorrelation of the inoculum density was estimated with the Moran's *I* and semivariograms. A Bayesian hierarchical spatial approach was used to model the relationship between *P. brassicae* inoculum density and the soil parameters. Patchiness of the pathogen was detected, with most patches located at the field edges and adjacent to the entrance. Infested patches grew in size from 2017 to 2019, with an average increase in diameter of 221.3 m and with this growth determined by the maximum inoculum density and active dispersal methods such as movement by machinery and wind. Soil pH, boron, calcium, and magnesium concentrations were not found to have an important effect on the inoculum density of *P. brassicae.*

Finally, the genetic control of DSI and resting spores per plant (RSP) was examined in a doubled haploid population consisting of 114 lines of winter oilseed rape, obtained from the cross 'Aviso' × 'Montego' inoculated with *P. brassicae* isolate 'eH'. Linkage analysis allowed the identification of three quantitative trait loci (QTLs) controlling DSI (pbBn_di_A02, PbBn_di_A04 and PbBn_di_C03). A significant decrease in DSI was observed when combining effects of the three resistance alleles at these QTLs. Only one QTL, PbBn_rsp_C03, was found to control RSP, reducing resting spore production by 40%. PbBn_di_C03 and PbBN_rsp_C03 partially overlapped, allowing the identification of a single region that controls both traits. Alignment of the genetic map with the reference genome of *B. napus* 'Darmor-*bzh*' indicated the presence of

three genes related to disease resistance and defense in the overlapping region of PbBn_di_C03 and PbBN_rsp_C03. Consideration of both DSI and RSP in breeding for clubroot resistance is recommended for the long-term management of this disease.

# Preface

Andrea Botero-Ramírez submitted this dissertation in partial fulfilment of the requirements for the degree of Doctor of Philosophy. She conducted all of the experiments and analyses presented in Chapters 3 and 4. Ms. Botero conducted some of the laboratory experiments, and performed all of the statistical and linkage analyses, in Chapter 5. She prepared the first draft of all chapters. The chapters were then extensively reviewed and edited by her supervisors, Dr. Stephen Strelkov and Dr. Sheau-Fang Hwang, who made suggestions and editorial changes for each chapter. Ms. Botero then addressed these suggestions as appropriate.

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Andrea Botero Ramírez, Stephen E. Strelkov, and Sheau-Fang Hwang contributed to development of the research concept. Stephen E. Strelkov and Sheau-Fang Hwang secured funding support, and supervised and administered the project. Andrea Botero Ramírez designed and directed sample collection and processing, performed statistical analyses, and wrote the first draft of the manuscript. Stephen E. Strelkov provided project guidance and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Andrea Botero Ramírez conducted some of the laboratory analyses, performed the statistical and linkage analyses, and wrote the manuscript. Anne Laperche, Antoine Gravot, and María J. Manzanares-Dauleux contributed to development of the research concept and design of the study and reviewed multiple versions of the manuscript. Anne Laperche and Mélanie Jubault designed and directed the execution of the experiments. Solenn Guichard and Mélanie Jubault helped to carry out the clubroot tests. Stephen E. Strelkov provided project guidance and extensively edited the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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# Chapter 1 Introduction

## 1.1 Canola and clubroot

Canola (oilseed rape; *Brassica napus* L.) is the second most important oilseed crop worldwide, surpassed only by soybean, with 67.72 million tons produced in 2019 (USDA, 2020). The largest canola producer is Canada, with 19 million tons harvested in 2019, followed by the European Union and China with 17 and 13.1 million tons, respectively (USDA, 2020). The term 'oilseed rape' refers to all cultivated species of the family Brassicaceae whose main product is the seed oil, while 'canola' is defined as any *Brassica* species whose seed oil contains < 2% erucic acid, and the solid component of the seed contains < 30 μmol per gram of air-dry, oil-free solid of one or a mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate (Hickling et al., 2011).

Canola is one of the most important field crops in Canada and is grown on more than 8.4 million ha (Statistics Canada, 2020), contributing C$26.5 billion per year to the national economy (LMC international, 2016). Production is concentrated in the Prairie provinces, including Saskatchewan (4.7 million ha), Alberta (2.4 million ha) and Manitoba (1.3 million ha) (Statistics Canada, 2020). One of the major constraints to canola production, however, is clubroot disease caused by *Plasmodiophora brassicae* Wor., a soilborne pathogen causing average losses between 10% to 15% worldwide (Dixon, 2009b). In highly infested fields, yield reductions can be as high as 30% to 100% (Tewari et al., 2005; Pageau et al., 2006). Yearly losses caused by *P. brassicae* in Alberta have been estimated to be around C$2.1 billion (Fredua-Agyeman et al., 2018).

The clubroot pathogen produces resilient resting spores, which have a half-life of about 4 years (Wallenhammar, 1996; Hwang et al., 2013) but can persist in the soil for up to 17 years

(Wallenhammar, 1996). Recent studies indicate that, despite this apparent longevity, resting spore levels can decline by up to 90% after a 2-year break from canola, later stabilizing (Peng et al., 2015; Ernst et al., 2019) and resulting in a Type III survivorship curve (Rauschert, 2010). The long viability of *P. brassicae* resting spores represents a major challenge for clubroot management, since it is very difficult to eradicate the pathogen from a field once it has become established (Strelkov and Hwang, 2014).

Typically, *P. brassicae* infection results in the formation of root galls that impede water and nutrient uptake, ultimately reducing crop quality and yield (Dixon, 2009b). The direct economic impacts of the disease can include a decline in seed number and weight, grain mass, straw yield, and oil quality (Pageau et al., 2006). The uniformity of crop maturity may also be affected, as infected plants often senesce prematurely (Dixon, 2009b; Strelkov and Hwang, 2014). Beyond these direct economic impacts, clubroot makes crop management more difficult and expensive, and may reduce the value of infested land (Dixon, 2009b).

In Alberta, clubroot was first observed around 1977 on broccoli (*Brassica oleracea* L. var. *italica* Plenk) and cabbage (*B. oleracea* L. var. *capitata* L.) in home gardens in Edmonton, and on cauliflower (*B. oleracea* L. var. *botrytis* L.) in Ohaton (I. Evans, unpublished data). The disease was not identified on canola until 2003, when crops in 12 fields in central Alberta were found to be infected (Tewari et al., 2005). Clubroot has since spread throughout much of the province, reaching a total of 3353 infested fields in 2019 (Strelkov et al., 2020). While disease levels in most canola crops range from mild to moderate, between 10% and 15% of crops are severely infected (Strelkov and Hwang, 2014).

Multiple strategies have been proposed for the management of clubroot on canola, including crop rotation, biological control, chemical control, soil liming, manipulation of the sowing date and planting of clubroot resistant (CR) varieties (Hwang et al., 2014b). The most effective tactic for clubroot management to date has been the deployment of cultivars with monogenic resistance (Diederichsen et al., 2006, 2009). While genetic resistance is the most effective and convenient method for disease management, the selection pressure those cultivars impose on the pathogen can cause rapid shifts in its population structure (Holtz et al., 2018). These shifts can result in a loss of resistance, as has already been observed in Canada and Europe (Kuginuki et al., 1999; Diederichsen et al., 2014; Orgeur et al., 2016; Strelkov et al., 2016; Hollman et al., 2021).

The sustainable and effective management of clubroot requires the implementation of integrated strategies, including already proven successful practices and new and better-suited approaches, to reduce the impact on canola production. The development of novel management practices, however, requires a deeper understanding of clubroot epidemiology, including an accurate assessment of disease impact on yield, and secondly, an analysis of its spatial behaviour.

Accurate estimates of yield losses caused by clubroot are necessary to define economic thresholds for disease risk assessments, and ultimately to select appropriate management strategies to maintain crop profitability (Shtienberg, 2000). Additionally, epidemiological studies on pathogen spatial patterns and spread can help to improve understanding of the pathogen biology and ecology. Such knowledge is essential for the development of improved and more effective sampling methods and disease management programs (Nicot et al., 1984; Campbell and Noe, 1985; Chellemi, 1988; Rekah et al., 1999; Kranz, 2012). Currently, only a few studies have conducted any spatial analysis of clubroot disease at the field level (Cao et al., 2009; Wallenhammar et al., 2012; Vojtěch et al., 2017).

Given the efficacy and ease of use of CR varieties, genetic resistance should remain as an option for clubroot management. Ideally, however, the durability of the resistance could be enhanced via strategies such as the deployment of polygenic resistance, gene pyramiding, and the planting of multilines and cultivar mixtures (Parlevliet and Zadoks, 1977; Pink and Puddephat, 1999). These approaches require a deeper understanding of the genomic regions involved in the resistance harbored by specific host genotypes.

### 1.2 Hypotheses and objectives

The general aim of this research was to improve understanding of clubroot epidemiology and resistance, helping to enable integrated and knowledge-based management of this disease. The work had three specific objectives: i) to develop a yield loss model to estimate the impact of *P. brassicae* infection on canola; ii) to characterize the spatial patterns associated with pathogen inoculum density and its relationship with soil pH and boron, calcium, and magnesium content in the soil; and iii) to analyze the polygenic resistance harbored by a doubled haploid population obtained from a cross between 'Aviso' and 'Montego' (*B. napus*).

The corresponding hypotheses were that: i) yield losses caused by *P. brassicae* in incompatible interactions (resistant cultivars) can be explained by linear models with a slope close to zero, while in compatible interactions (susceptible and partially resistant cultivars), losses can be explained by third order polynomial models; ii) *P. brassicae* inoculum density is negatively affected by soil pH and boron, calcium and magnesium content in the soil, and there is no significant patch growth at the field level in the short term; and iii) there are common quantitative trait loci modulating the disease severity index (DSI) and resting spore production in some host genotypes.

# Chapter 2 Literature Review

## 2.1 Clubroot biology and epidemiology

### 2.1.1 *Plasmodiophora brassicae* life cycle

The lifecycle of *P. brassicae* consists of three main stages: i) survival in the soil as resting spores, ii) primary infection, and iii) secondary infection. The pathogen resting spores can remain viable for up to 17.3 years (Wallenhammar, 1996), thanks in part to thick cell walls composed of five layers: proteinaceous fibres, proteinaceous fibres with lipid granules, a chitinous wall with an inner phospholipid region, and an inner spore membrane (Buczacki and Moxham, 1983). Resting spores are spherical with spines, have a single nucleus (Ingram and Tommerup, 1972) and an average size of 3.2 μm (Buczacki and Cadd, 1976). The primary infection process begins with germination of the resting spores to release primary biflagellate zoospores, with germination rates enhanced by the presence of host and non-host root exudates (Macfarlane, 1970; Friberg et al., 2005; Rashid et al., 2013). Following germination, the zoospores swim through water films toward the root hairs. Upon encountering the cell wall of a root hair, the zoospores retract their flagella and encyst, with the pathogen cytoplasm injected into the host via formation of the Rohr (a long tubular cavity in the cyst) and Stachel (a sharp-pointed rod inside the Rohr), the latter of which penetrates the host wall (Aist and Williams, 1971). Once in the root hair, the pathogen forms a primary plasmodium that divides to produce a zoosporangium that gives rise to new (secondary) zoospores. The secondary zoospores can either be released into the rhizosphere and then infect the host cortical tissue, or move to neighboring cortical cells to infect them; at this point, the third stage of the pathogen life cycle starts (Mithen and Magrath, 1992).

After penetration of the cortex, binucleate secondary plasmodia develop and proliferate intracellularly, causing hypertrophy and hyperplasia of the infected tissues. This results in unorganized growth and disintegration of the vascular and root tissue structure; at this stage, galls are formed and the typical clubroot symptoms become visible (Ingram and Tommerup, 1972; Mithen and Magrath, 1992). Further divisions of the secondary plasmodia and deposition of cell wall leads to the formation of new resting spores, which are released into the soil as survival structures as the galls decompose (Bulman and Braselton, 2014).

### 2.1.2 Factors affecting clubroot development

The disease triangle is a classical concept in plant pathology, providing a framework for the study of the conditions needed for disease to occur. It reflects the fact that disease development results from the interaction of three factors: host genetics, pathogen genetics and inoculum potential, and the environmental conditions during infection (Scholthof, 2007). Given the importance of the disease triangle for the prediction and control of plant disease, each of the elements affecting clubroot development are reviewed in the following section.

#### 2.1.2.1 Host

The occurrence of disease can be determined by the level of host resistance. Resistance can be defined as "traits that prevent infection or limit its extent" (Schneider and Ayres, 2008), and can be complete or qualitative, or incomplete or quantitative. Qualitative resistance is usually conditioned by a single, major gene, while quantitative resistance is controlled by multiple, minor genes (Poland et al., 2009). Genes conferring qualitative resistance are commonly referred as '*R*-genes' (resistance genes), while genes controlling quantitative resistance are known as 'QTLs'

(quantitative trait loci) (Poland et al., 2009). Compared with qualitative resistance, quantitative resistance confers partial and more durable race-nonspecific resistance (Kou and Wang, 2010).

*Brassica napus* originated approximately 7500 years ago (Chalhoub et al., 2014) by hybridization and later diploidization between *B. rapa* (AA, 2n=20) and *B. oleracea* (CC, 2n=18) (U, 1935); it has 19 chromosomes, A1-A10 from *B. rapa* and C1-C9 from *B. oleracea* (Diederichsen et al., 2009). A better understanding of the resistance harbored by *B. napus* requires consideration of the phylogenetic relationships among *Brassica* species, since *B. rapa* and *B. oleracea* are still closely related to *B. napus,* and the interspecific transfer of clubroot resistance genes has played a major role in resistance breeding and possibly also in spontaneous gene flow (Diederichsen et al., 2009).

In *B. rapa*, multiple major genes controlling resistance to clubroot have been identified, including *Crr1a, Crr1b* (*Crr1a* and *Crr1b* were initially identified as a single locus, *Crr1*), *Crr2, Crr3, CRa, CRb, CRs, Rcr1, Rcr2* and *Rcr4* (Matsumoto et al., 1998; Suwabe et al., 2003; Hirai et al., 2004; Saito et al., 2006; Hatakeyama et al., 2013, 2017; Chu et al., 2014; Huang et al., 2017; Yu et al., 2017; Laila et al., 2019). Two QTLs were also detected that controlled resistance to a Korean *P. brassicae* isolate belonging to pathotype 2 (as classified on the differential set of Williams (1966)) (Choi et al., 2020), three QTLs (*Crr4, CRc,* and *CRk,*) were found to control resistance to several non-pathotyped isolates (Suwabe et al., 2006; Sakamoto et al., 2008), and two QTLs (*Rcr8* and *Rcr9)* controlled the host reaction to a Canadian isolate classified as pathotype 5X on the Canadian Clubroot Differential set (Yu et al., 2017; Strelkov et al., 2018).

In *B. oleracea*, resistance is mostly incomplete and controlled by QTLs with both major and minor effects. Around 40 QTLs have already been identified in multiple *B. oleracea* populations

inoculated with isolates belonging to diverse pathotypes (Table 2-1). QTLs with major effects have been observed mostly on chromosomes C01, C02, and C03. Eight QTLs conferring resistance to pathotypes 2 and 7 (as classified on the differentials of Williams (1966)), $P_1$, $P_2$, $P_4$, $P_7$ (as classified on the differentials of Some et al., (1996), and ECD 16/3/36 (as classified on the European Clubroot Differential (ECD) (Buczacki et al., 1975)) have been identified on chromosome C01, explaining between 6% and 88% of the disease variance (Landry et al., 1992; Figdore et al., 1993; Voorrips et al., 1997; Rocherieux et al., 2004). Four additional QTLs effective against pathotypes 4, 9, $P_1$ and $P_2$ have been found on chromosome C02, explaining between 4% and 47% of the disease variance (Rocherieux et al., 2004; Nagaoka et al., 2010; Lee et al., 2016). On chromosome C03, seven QTLs have been reported to reduce disease levels in plants inoculated with pathotypes 1, 2, 3, 4, 9, $P_7$, and 5X (as classified in the Canadian Clubroot Differential (CCD) set (Strelkov et al., 2018)); those QTLs controlled between 8.9% and 54% of the disease variance (Voorrips et al., 1997; Moriguchi et al., 1999; Rocherieux et al., 2004; Nagaoka et al., 2010; Lee et al., 2016; Farid et al., 2019). About 14 minor QTLs controlling resistance to pathotypes 2, 4, 7, $P_1$, $P_2$, $P_4$, and 3A have been identified on chromosomes C04, C05, C06, C07, C08, and C09 (Landry et al., 1992; Figdore et al., 1993; Rocherieux et al., 2004; Nagaoka et al., 2010; Farid et al., 2019).

Genetic analyses of the control of clubroot resistance in *B. napus* have detected the presence of one major resistance gene (*Cra*) (Zhang et al., 2016), and more than 30 QTLs in various plant populations inoculated with multiple isolates of *P. brassicae* (Table 2-2). The doubled haploid (DH) population Darmor-*bhz* × Yudal (DY) is one of the most widely studied for the presence of QTLs controlling clubroot symptoms, pathogen DNA content in infected roots, and resting spore production. When the DY population was tested against pathotype $P_7$ (as classified on the differentials of Somé et al. (1996)), two QTLs were found controlling 98.3% of the variance in

disease severity index (DSI) (Manzanares-Dauleux et al., 2000). Further research detected the presence of five minor QTLs located on chromosomes A03, A04, A09, C02 and C05, controlling the DSI in this population after inoculation with the pathotype $P_4$ (Manzanares-Dauleux et al., 2000; Laperche et al., 2017). Additionally, when the population was tested against pathotype $P_1$, the DSI was controlled by one major QTL on chromosome C09 that accounted for more than 66% of the variance in the DSI. Likewise, six minor QTLs on chromosomes A01, A05, A07, A10, C02, and C03 were found to control the DSI to some extent (Laperche et al., 2017; Wagner et al., 2019). Further analysis using the same pathotype also found that genetic control of resting spore production by the pathogen is controlled by three QTLs, one on chromosome C09, which accounts for between 30.8% and 78.9% of the variance, one on chromosome C02, which accounts for between 9.6% and 52% variance, and one minor QTL on chromosome A07. Finally, one major QTL on chromosome C09 and two minor QTL*s* on chromosomes C03 and C07 were found to control *P. brassicae* DNA content in infected roots (Wagner et al., 2019).

In a study in which another *B. napus* DH population was inoculated with *P. brassicae* isolates of unknown pathotype, four major QTL*s* were identified on chromosome A03, which accounted for between 15.8% and 67.5% of the variance in disease severity (Werner et al., 2007). Another two QTL*s* were found on chromosome A08, which accounted for between 18.7% and 22.2% of the variance, and three QTL*s* were identified on chromosome C09, which accounted for between 10.3% and 42.3% of the variance. Finally, one QTL on chromosome C08 accounted for 43.8% of the variance in disease severity, while an additional eight minor QTLs were found controlling the disease reaction on chromosomes A09, C06, A02 and C03 (Werner et al., 2007).

Although multiple genetic regions quantitatively control the clubroot response in *B. napus*, the resistance in most commercially available CR canola or oilseed rape varieties is based mostly on

one major resistance gene (*Cra)* (Diederichsen et al., 2009). This gene is derived from the European winter oilseed rape 'Mendel' (Diederichsen et al., 2014; Fredua-Agyeman et al., 2018), which was one of the first CR winter oilseed rape hybrids with acceptable agronomic performance released in Europe (Diederichsen et al., 2003, 2009). The resistance in 'Mendel' is conferred by one dominant and two recessive genes (Diederichsen et al., 2006), and was effective against the major pathotypes of *P. brassicae* identified in Canada prior to 2013 (Rahman et al., 2011; Deora et al., 2012, 2013). Most canola varieties available in Canada appear to carry resistance derived from 'Mendel' (Fredua-Agyeman et al., 2018). As of 2019, 28 CR canola varieties were available in western Canada, of which only five were reported to carry novel (i.e., different from 'Mendel') clubroot-resistance (Canola Council of Canada, 2019).

While CR *B. napus* cultivars can be very useful for clubroot management, the emergence of 'new' *P. brassicae* pathotypes in Canada and Europe that are able to overcome major gene resistance (Kuginuki et al., 1999; Diederichsen et al., 2014; Orgeur et al., 2016; Strelkov et al., 2016; Hollman et al., 2021) indicates the need for efforts to develop more durable CR cultivars via the inclusion of multiple resistance genes and/or QTLs in a single cultivar. Such efforts can be hampered, however, by the narrow genetic background of *B. napus*, which limits the use of the primary gene pool (*B. napus*), and by the complexities associated with interspecific hybridization when transferring resistance genes from the secondary gene pool (*B. rapa* and *B. oleracea*) (Rahman et al., 2015).

#### 2.1.2.2 Pathogen

One of the main factors affecting the occurrence and severity of soilborne diseases is the inoculum potential, which is a function of inoculum density and the effects of the environment

upon it (Baker, 1971). In clubroot, different authors have observed that disease incidence and severity increase with increasing pathogen inoculum, following a sigmoidal dose-response curve (Voorrips, 1996; Murakami et al., 2002). Voorrips (1996) reported that an inoculum density between $1 \times 10^3$ and $1 \times 10^5$ resting spores plant$^{-1}$ was required to cause a disease incidence of 50% in susceptible cabbage. Moreover, generalized one-hit models could successfully explain the relationship between *P. brassicae* inoculum density and clubroot incidence, suggesting that interactions between pathogen individuals do not have great influence on the probability of infection (Voorrips, 1996). Murakami et al. (2002) also found that inoculum densities $> 1 \times 10^3$ resting spores gram$^{-1}$ of soil were required to cause disease in Chinese cabbage, cabbage, and broccoli, and that disease severity increased at higher inoculum levels. Moreover, Murakami et al. (2002) noted that Chinese cabbage was more susceptible than cabbage or broccoli. While Chinese cabbage developed a DSI close to 100% at inoculum densities of $1 \times 10^5$ resting spores gram$^{-1}$ of soil, the same inoculum density resulted in a DSI of only 30% and 50% on susceptible cabbage and broccoli, respectively. Partially resistant broccoli varieties presented a DSI < 50% even at $1 \times 10^6$ resting spores gram$^{-1}$ of soil. Both Murakami et al. (2002) and Voorrips (1996) concluded that changes in clubroot incidence and severity observed at certain inoculum densities are dependent on environmental conditions, host susceptibility, and differences in the virulence of the *P. brassicae* inoculum.

On clubroot-susceptible canola under greenhouse conditions, Hwang et al. (2011a) found a DSI > 60% at $1 \times 10^5$ resting spores mL$^{-1}$ soil, which increased to 100% at $1 \times 10^8$ resting spores mL$^{-1}$ soil. This led to the suggestion that disease response curves in susceptible canola varies with the type of inoculum used and the plant age (Hwang et al., 2011a). In a later study, in which nine canola genotypes were tested against six populations of *P. brassicae*

at inoculum densities ranging from $1 \times 10^3$ resting spores $mL^{-1}$ of soil to $2 \times 10^6$ resting spores $mL^{-1}$ of soil, Hwang et al. (2017) concluded that there was a specific relationship between pathogen virulence, host resistance and inoculum density. Some cultivars developed clubroot symptoms at inoculum densities as low as $1 \times 10^3$ resting spores $mL^{-1}$ when these represented highly virulent populations of *P. brassicae*. Moreover, infection rates in resistant canola increased with exposure to higher inoculum concentrations, and at very high inoculum densities, clubroot severity in some resistant cultivars was similar to that observed in susceptible cultivars (Hwang et al., 2017). In commercial crops, clubroot symptoms begun to appear at an inoculum density of $1 \times 10^5$ resting spores $g^{-1}$ of soil, depending on moisture level (Hwang et al., 2017).

Pathogen fitness is another important factor influencing disease development, since fitness affects inoculum production, which in turn drives inoculum potential for future crop cycles. Pathogen fitness can be defined as the combined ability of a pathogen to survive and reproduce, and can be measured by traits such as reproductive rate, infection efficiency or aggressiveness (Leach et al., 2001). Murakami et al. (2004) conducted one of the first assessments of the reproductive capacity of *P. brassicae* in susceptible hosts. They estimated that on Chinese cabbage, cabbage and broccoli, about $1 \times 10^8$ resting spores $plant^{-1}$ were produced on plants with mild clubroot, about $1 \times 10^9$ resting spores $plant^{-1}$ were produced on plants with intermediate levels of disease, and $1 \times 10^{10}$ resting spores $plant^{-1}$ were produced on severely infected plants. On canola, it was estimated that one cycle of a susceptible cultivar contributed approximately $2 \times 10^8$ resting spores $g^{-1}$ soil, while a cycle of a resistant cultivar contributed $1.2 \times 10^7$ resting spores $g^{-1}$ soil (Hwang et al., 2011b). Susceptible canola cultivars were found to produce an average of 2.9 g of galled root tissue per plant, representing about $2.9 \times 10^{10}$ resting spores $plant^{-1}$, while resistant cultivars produced only 0.19 g of galled root tissue, or $1.9 \times 10^8$ resting spores $plant^{-1}$ (Hwang et

al., 2013). A recent study with a doubled haploid population of winter oilseed rape found that between $1 \times 10^7$ and $1 \times 10^9$ resting spores were produced on a single plant (Aigu et al., 2018). Interestingly, clubroot severity and the amount of inoculum produced were not always directly correlated, and some genotypes with mild symptoms produced high numbers of resting spores (Aigu et al., 2018).

#### 2.1.2.2.1 Physiological specialization

Physiological specialization in *P. brassicae* was first demonstrated by Honing (1931), who proposed the existence of at least three different physiological races classified based on the host species attacked. Scheijgrond and Vos (1954) also suggested the existence of physiological strains of *P. brassicae* after observing different disease responses in 14 turnip cultivars grown across four locations in the Netherlands. Many studies have since confirmed the occurrence of physiological specialization in the clubroot pathogen, and various host differential systems have been proposed to identify *P. brassicae* races or pathotypes.

The most widely used systems to classify *P. brassicae* include the differentials of Williams (1966), Somé et al. (1996) and the European Clubroot Differential (ECD) set (Buczacki et al., 1975). The differentials of Williams (1966) consist of four host genotypes (two *B. napus* and two *B. oleracea*), allowing for a theoretical maximum of 16 pathotypes, which were originally proposed from studies conducted in the USA in the 1960s. The differential set of Somé et al. (1996) was developed to characterize *P. brassicae* collections from France, and includes three genotypes of *B. napus*. The ECD set, which, as the name suggests, was developed as a pan-European initiative, consists of 15 host genotypes representing fives genotypes of each of *B. rapa, B. napus* and *B. oleracea* (Buczacki et al., 1975). One of the main issues associated with the use of

differential sets for the classification of *P. brassicae* pathotypes is that while a host line may distinguish populations from one region, it may also be uniformly resistant or susceptible to populations from another region (Some et al., 1996). Limited information regarding the number and identity of resistance genes in the differential hosts may also sometimes result in a lack of reproducibility, particularly with respect to the ECD set (Diederichsen et al., 2009).

Some of the early evaluations of *P. brassicae* pathotype composition in Canada were carried out using the differentials of Williams (1966) with pathogen collections from cruciferous vegetables. Pathotype 2 was identified as the most common variant in pathogen collections from Quebec, the Maritimes (Nova Scotia, Prince Edward and New Brunswick), and Ontario. Pathotypes 1 and 3 were also recorded from the Maritimes (Williams, 1966; Ayers, 1972; Reyes et al., 1974; Hildebrand and Delbriidge, 1995), while pathotype 6 was detected in isolates from British Columbia and Quebec (Williams, 1966; Ayers, 1972; Reyes et al., 1974). Pathotype 2 appeared to be predominant on rutabaga, while pathotype 6 was more common on broccoli, cabbage, cauliflower and pak choi (Reyes et al., 1974). With the identification of clubroot on canola in western Canada (Tewari et al., 2005), more extensive pathotyping began of *P. brassicae* collections, with testing not only on the differentials of Williams, but also on the hosts of Somé et al. and the ECD set. This testing indicated that pathotype 3, $P_2$ or ECD 16/15/12, as identified on each of these systems, was predominant in central Alberta (Strelkov et al., 2006, 2007; Xue et al., 2008; Cao et al., 2009). In contrast, pathotype 5, $P_3$ or $P_2$, and ECD 16/15/0, was more common in southern Alberta, Manitoba and Quebec (Strelkov et al., 2007; Cao et al., 2009). Testing of *P. brassicae* isolates from Ontario confirmed the earlier identification of pathotype 6, which were classified as pathotype $P_4$ (1996) or ECD _/0/12 on the differentials of Somé et al. and the ECD set, respectively. Various other less common pathotypes, including Williams' pathotype 8, were

also detected in pathogen collections before 2010 (Strelkov et al., 2006; Xue et al., 2008; Cao et al., 2009).

While the differentials of Williams, Somé et al. and the ECD set have been useful for the characterization of *P. brassicae* in Canada, new virulence phenotypes of the pathogen were identified, beginning in 2013, that could not be distinguished based on the existing pathotype classification systems (Strelkov et al., 2016). These 'new' strains of *P. brassicae* were capable of overcoming the resistance in the recently released CR canola cultivars, and their identification led to the development of the Canadian Clubroot Differential (CCD) set (Strelkov et al., 2018). The CCD set consists of 13 host genotypes, including the differentials of Williams and Somé et al., selected of the hosts of the ECD set, as well as the winter oilseed rape 'Mendel', the open pollinated spring canola 'Westar', and the CR hybrid canola '45H29'. In the CCD system, isolates are assigned a pathotype denomination that includes a number based on their Williams' classification, followed by a letter denoting their CCD designation (Strelkov et al., 2018; Askarian et al., 2021). This provides continuity with earlier pathotype designations based solely on Williams' hosts. Since the CCD set also includes the hosts of Somé et al. (1996), pathotype designations based on this system are also generated, but do not form part of the formal CCD pathotype nomenclature. Recent studies have identified 36 CCD pathotypes across Canada, over half of which can overcome the resistance in most CR canola cultivars (Strelkov et al., 2018, 2021; Askarian et al., 2021; Hollman et al., 2021). Among the resistance-breaking pathotypes, the CCD pathotype 3A is predominant (Strelkov et al., 2018, 2021; Hollman et al., 2021).

#### 2.1.2.2.2 The spread of *P. brassicae*

Disease spread results from inoculum dispersal, which can be defined as the movement of infectious units of a pathogen from one place to another (Madden et al., 2007). Active dispersal of soil microorganisms through the soil matrix is generally very limited (depending on the taxon, in the order of a few millimetres to centimeters per day), although passive dispersal over larger distances is possible via accidental or specialized animal vectors, water and wind (Ettema and Wardle, 2002). In the case of *P. brassicae,* active spread is restricted because zoospore motility is limited (Hwang et al., 2012b), but various mechanisms of passive dispersal have been identified.

The main mechanism of passive field-to-field spread is through the movement of *P. brassicae*-infested soil and plant debris on farm machinery, with clubroot most often found at field approaches where contaminated equipment enters a field (Cao et al., 2009; Donald and Porter, 2014). Other dispersal mechanisms also have been identified, including the movement of inoculum in water, wind, and as an external seed contaminant (Datnoff et al., 1984; Rennie et al., 2011, 2015), although these appear to play minor roles relative to the spread of infested soil on machinery (Rennie et al., 2011; Strelkov and Hwang, 2014). Nonetheless, the identification of *P. brassicae* resting spores in windblown dust from infested fields in Alberta suggested that wind-mediated dispersal of the pathogen is possible and may contribute to its local spread (Rennie et al., 2015). Movement of the pathogen via water or water-mediated soil erosion was observed by Datnoff et al. (1984), who identified clubroot on cabbage in seedbeds with no history of cabbage production, but which had been irrigated using run-off water from clubroot-infested soils. The presence of resting spores was also confirmed in soil-tag and dust associated with the seeds of various crops and tubers produced in *P. brassicae*-infested fields, although commercial seed cleaning appeared sufficient to reduce this risk to negligible levels (Rennie et al., 2011). Finally, livestock also has

been identified as a possible clubroot vector, since *P. brassicae* resting spores have been detected in manure from chicken and pigs, suggesting that pathogen spread via contaminated manure is likely more effective than spread through seedborne transmission (Chai et al., 2016).

#### 2.1.2.3 Environment

##### 2.1.2.3.1 Environmental factors and clubroot severity

Clubroot development and *P. brassicae* resting spore survival are affected by multiple environmental factors, the most widely studied being temperature, soil moisture, and soil properties including pH and nutrient content. Multiple studies have demonstrated that temperatures between 20°C and 25°C are optimal for root hair and cortical infection and favor greater clubroot severity (Ayers, 1944; Thuma et al., 1983; Sharma et al., 2011b, 2011a; Gossen et al., 2012, 2013a; Luo et al., 2014). Conversely, infection and disease severity are reduced at lower or higher temperatures. A delay in the onset of symptoms has been observed at temperatures < 17°C, while at 10°C root hair infection is reduced and cortical infection is not observed (Sharma et al., 2011b, 2011a; Gossen et al., 2012; Luo et al., 2014). The production of inoculum (resting spores) for future infections is also affected by temperature. At 25°C, about $2.32 \times 10^8$ resting spores $g^{-1}$ gall tissue were produced in Shanghai pak choi (*B. rapa* subsp. *chinensis*). This declined to $5.7 \times 10^7$ resting spores $g^{-1}$ of gall at 20°C, or $7.9 \times 10^7$resting spores $g^{-1}$ of gall at 30°C (Sharma et al., 2011a). Resting spore production was lowest at 15°C ($3.4 \times 10^7$ resting spores $g^{-1}$ of gall).

Soil moisture has been regarded as one of the most important factors affecting clubroot development, with disease incidence and severity increasing together with moisture levels (Samuel and Garrett, 1945; Hamilton and Crête, 1978; Dobson et al., 1982). Samuel and Garrett (1945) observed that primary infection was reduced about 300-fold in soils at 30% of the water-holding

capacity (WHC) compared with soils at 80% of WHC. Interestingly, these authors did not observe infection in a loamy soil at 30% WHC, while some root hairs were infected in a sandy soil at the same moisture level; they suggested that this occurred because the water in the sandy soil was more readily available, facilitating resting spore germination and root hair infection. In contrast, Dobson et al. (1982) concluded that root hair infection was possible at a minimum water potential of -800 mbars (< 10% WHC), while cortical infection was not possible below -200 mbar in muck soils or -150 mbar in mineral soils (50% to 70% WHC). Likewise, Hamilton and Crête (1978) observed that in organic soils, clubroot only occurred at soil moisture > 60% WHC, while in a mineral soil, the minimum moisture required was 25% WHC. Colhoun (1953) found that clubroot development was favored in soils at 70% WHC, and that fluctuating moisture levels were equally effective at causing disease as was constant high moisture. More recently, Narisawa et al. (2005) did not observe clubroot when soil moisture was ≤ 40% WHC, while severe disease symptoms were observed at a soil moisture of 80% WHC. Collectively from these studies, it can be inferred that root hair infection requires less moisture than cortical infection, and that while soil moisture is critical for clubroot development, the amount of moisture required is highly dependent on the soil type.

Soil pH can also have an important influence on clubroot development, with the disease generally more severe in acidic soils. Various authors have found that clubroot is favoured at pH values between 5.0 and 6.0, reduced at pH ≥ 7.0, and eliminated at pH > 8.0 (Myers et al., 1983; Webster and Dixon, 1991b; Donald and Porter, 2004). Nevertheless, severe clubroot symptoms can sometimes occur in alkaline soils, particularly under high spore loads and favourable moisture and temperature (Colhoun, 1953; Fletcher et al., 1982; Myers and Campbell, 1985; Gossen et al., 2013a). The suppression of clubroot under neutral and alkaline conditions (pH > 7.2) has been

attributed to the inhibition of resting spore germination in the rhizosphere (Niwa et al., 2008; Rashid et al., 2013), declines in the number of root hair infections, inhibition of the maturation and dehiscence of the sporangia, and abortion of the zoosporangia (Myers and Campbell, 1985; Webster and Dixon, 1991b, 1991a; Rashid et al., 2013).

In addition to pH, soil nutrient composition can significantly affect clubroot development. Calcium and boron are among the most widely studied cations interfering with *P. brassicae* infection in cruciferous plants; magnesium seems to reduce clubroot as well, but its effect has not been examined to the same extent. The influence of calcium has been difficult to separate from the effect of pH, although several studies have shown that calcium itself inhibits clubroot (Hamilton and Crête, 1978; Fletcher et al., 1982). For instance, Hamilton and Crête (1978) reported that plants grown in soils that had been limed to increase the pH to the same level developed a clubroot index of disease that was 27.6% lower following $CaSO_4$ vs. $Na_2CO_3$ treatment. Calcium reduces clubroot mainly by decreasing primary infection by inhibiting the maturation and dehiscence of the primary plasmodia (Webster and Dixon, 1991a). Additionally, the effect of calcium has been ascribed to its involvement in the induction of defense-related compounds and *P. brassicae*-induced cell death in the host (Takahashi et al., 2002, 2006).

Magnesium and calcium seem to affect clubroot development in a similar manner, with high concentrations of either nutrient tending to decrease disease levels (Myers and Campbell, 1985). More specifically, high concentrations of magnesium reduce primary infection by limiting the release of zoospores and cortical infection (Myers and Campbell, 1985). The effect of calcium and magnesium on clubroot development is pH dependent, since the amount of cations required to reduce disease decreases as the pH increases (Myers and Campbell, 1985; Donald and Porter, 2004), an observation explained by their pH-dependent absorption (Myers and Campbell, 1985).

Boron reduces clubroot by supressing and delaying primary infection and cortical colonization, and inhibiting plasmodium development to subsequent stages (Webster and Dixon, 1991a; Deora et al., 2011). Its effects have been reported to be erratic and highly dependent on the soil type and the dosage, with phytotoxicity as a major risk (Deora et al., 2011, 2014). Deora et al. (2011) observed a quadratic decline in root hair infection on canola plants as boron dosage was increased; this decline was associated with an inhibition of the differentiation of plasmodia and zoosporangia, and an inhibition of zoosporangial dehiscence. Beyond its effects on primary infection, boron also reduces cortical infection (Webster and Dixon, 1991a).

#### 2.1.2.3.2 Environmental factors and *P. brassicae* resting spore viability

Few studies have addressed the effects of environmental factors on the survival of *P. brassicae* resting spores, although there is evidence that resting spore viability is greatly affected by soil pH, with spore germination rates reduced at acidic pH in the absence of a host (Macfarlane, 1952; Takahashi, 1994). After incubating infested soils at 25°C for 15 days, Takahashi (1994) observed that the viability of resting spores was greatest (40-60%) in soils with a pH between 6.5 and 8, intermediate at pH 4.5 (about 20%), and lowest at pH 5.5 (< 10%). Similarly, after incubating infested soil for 6 weeks, Macfarlane (1952) found that the number of primary infections in cabbage declined rapidly in wet acid soils (80% of WHC and pH 5.8) and more slowly in dry alkaline soils (30% of WHC and pH 8.0). After 24 weeks of incubation, however, *P. brassicae* activity was similar in both soils. This initial decrease in primary infection was hypothesized to reflect resting spore germination rates in the absence of a host (Macfarlane, 1952).

Temperatures > 30°C inactivate *P. brassicae* resting spores, but this depends on the length of exposure, with shorter exposure times required at higher temperatures (White and Buczacki, 1979;

Myers et al., 1983; Fayolle et al., 2006). Resting spores can survive for 28 days at 35°C if soil moisture content is low (10% of WHC), whilst under high moisture (soil at field capacity) at the same temperature, resting spores survive only for 14 days (Porter et al., 1991). Among the cations affecting clubroot development, only the effect of calcium on resting spore survival has been evaluated. Myers et al. (1983) observed that the incubation of resting spores in 1M calcium chloride prior to inoculation reduced spore viability by 10%, possibly due to the fungistatic effects of residual calcium on resting spore germination or zoospore infection.

### 2.2 The effect of clubroot on canola/oilseed rape yield

While clubroot is a cause for concern worldwide, replicated experiments to determine the yield or economic losses associated with infection of canola or oilseed rape have been very limited (Rempel et al., 2014; Strehlow et al., 2014). Instead, most yield loss estimates have been based on anecdotal reports or approximations of grower-expected vs. harvested yields. In Australia, yield losses were estimated to be around 50% (1.6 t $ha^{-1}$) (Donald and Porter, 2003), while in Canada, they were estimated to be around 30% in heavily infested soils where the disease incidence was 94% (Tewari et al., 2005). In at least one canola crop in central Alberta, clubroot was so severe that the crop was not harvested, so the yield loss was effectively 100% (Strelkov and Hwang, 2014). Worldwide, losses across the family Brassicaceae have been estimated to be between 10-15% (Dixon, 2009b). While approximations of yield losses caused by *P. brassicae* are useful to evaluate the general impact of clubroot on canola/oilseed rape production, the relationship between disease incidence and severity, pathogen inoculum density, and the genetics of the host and pathogen have not been studied.

In both winter and spring canola/oilseed rape, it has been observed that as clubroot incidence and severity increase, the yield diminishes (Wallenhammar et al., 2000; Hwang et al., 2011a; Strehlow et al., 2014; McGrann et al., 2016). In China, the yield of two spring oilseed rape crops with a disease incidence of 56.2% or 100% declined by 23.5% and 56.4%, respectively (Ren et al., 2012). These yield losses reflected decreases in the first effective branch number, the effective pod number per plant, the seed number per pod, and 1000-grain weight. In another study where resistant spring oilseed turnip rape (*B. rapa*) were tested, yield losses varied between 5% and 8% when the average clubroot incidence was 17%, while at higher disease levels (>40% infected plants) yield losses increased (Wallenhammar et al., 2000). In Quebec, Canada, Pageau et al. (2006) reported yield reductions between 80% and 90% in susceptible canola, and between 69% and 89% in spring oilseed turnip rape grown in *P. brassicae*-infested fields. Moreover, 1000-grain weight decreased between 13% and 19%, while oil content was reduced between 2% and 6%. The authors also identified two resistant lines whose yield or yield parameters were not affected in infested soils (Pageau et al., 2006).

In Alberta, Hwang et al. (2011a) evaluated the impact of different *P. brassicae* inoculum densities on clubroot severity and yield loss, and found that yield decreased while severity increased with increasing inoculum density. Variance analysis showed that inoculum density accounted for 65% of the variation in canola yield. In addition to seed yield losses, premature ripening was observed in susceptible plants grown in *P. brassicae*-infested fields. While yield decreased as the pathogen inoculum increased, no significant differences were observed among inoculated plants when inoculum densities were between $1 \times 10^5$ and $1 \times 10^8$ resting spores $cm^{-3}$ potting mix (Hwang et al., 2011a). Seed yield was higher in a resistant cultivar, which was not

affected by inoculation with *P. brassicae*, while significant declines were observed in a susceptible cultivar (Hwang et al., 2011b).

In the UK, susceptible and resistant winter oilseed rape genotypes were evaluated in different *P. brassicae*-infested fields, and a highly significant correlation was observed between clubroot severity at harvest and yield across all sites and varieties (McGrann et al., 2016). The predicted yield in the absence of clubroot was 3.85 t $ha^{-1}$, but when the disease severity reached 100%, yield was predicted to be approximately 1 t $ha^{-1}$, for an estimated loss of 0.03 t $ha^{-1}$ per 1% increase in clubroot severity index. When the host varieties were analysed separately, yield losses in the susceptible and resistant cultivars 'Kommando' and 'Mendel' were 0.03 t $ha^{-1}$ and 0.028 t $ha^{-1}$ per 1% increase in clubroot index, respectively, whereas yield was not affected by clubroot in the highly resistant cultivar 'Cracker' (disease severity <5%). Those results, however, were based on a single trial, which could lead to low reproducibility (McGrann et al., 2016). In another study conducted on winter oilseed rape, plant survival and seeds per pod were reduced in the susceptible cultivar 'Visby' when *P. brassicae* was inoculated at rates between $1 \times 10^6$ and $1 \times 10^8$ resting spores $l^{-1}$ soil mix (Strehlow et al., 2014). Yield was reduced by 63%, 93% and 100%, compared with the control, as the inoculum was increased to $1 \times 10^6$, $1 \times 10^7$ and $1 \times 10^8$ resting spores $l^{-1}$, respectively. The number of pods per plant and 1000-grain weight per plant, however, were not different from the non-inoculated control or among the inoculated treatments (Strehlow et al., 2014). Yield of the CR cultivar 'Mendelson' was not significantly affected. Regression analysis indicated that spore concentration and disease severity were significant predictors of seed yield in the susceptible winter oilseed rape (Strehlow et al., 2014).

From the previous reports, it appears that the impact of clubroot on canola or oilseed rape needs to be determined more accurately by conducting experiments of sufficient size under greenhouse

conditions and in naturally infested fields. Such estimates are required since even low levels of *P. brassicae* inoculum in the soil can impair grower profitability, and since accurate estimates of potential yield losses are required to determine action thresholds for clubroot control.

### 2.3 Plant-pathogen interactions

The plant immune system consists of two layers. The first layer involves recognition of molecular patterns generally associated with pathogens (pathogen-associated molecular patterns or PAMPs) and is known as PAMP-triggered immunity (PTI), while the second layer involves recognition of specific pathogen virulence factors (effectors), and is known as effector-triggered immunity (ETI) (Jones and Dangl, 2006). Neither PTI nor ETI are well characterized in the host-*P. brassicae* pathosystem (Pérez-López et al., 2018), although there has been some progress in this area in recent years. It is not clear if chitin is one of the PAMPs of *P. brassicae* that can elicit the initial response of the host, although there is some evidence to suggest that initial recognition of *P. brassicae* may occur by the perception of chitin present in the resting spores (Schwelm et al., 2015; Chen et al., 2016).

Recognition of *P. brassicae* by the host is completed through host pattern recognition receptor proteins (PRRs). Those proteins are up-regulated in the host after initial contact with the pathogen (Chen et al., 2016) and later during secondary infection (Ning et al., 2019). However, key PRRs, such as brassinosteroid insensitive 1-associated kinase 1 (BAK1), flagellin sensing 2 (FLS2), chitin elicitor receptor kinase (CERK), and chitin elicitor binding protein (CEBiP), have not been found to be expressed differentially in *B. rapa* after *P. brassicae* infection, suggesting that the initial response to the clubroot pathogen differs from other pathosystems (Chen et al., 2016).

While pathogen recognition occurs in both susceptible and resistant hosts, the subsequent response in either host type is insufficient to arrest primary infection by *P. brassicae*. As a consequence, root hair infection can be observed in both compatible (susceptible) and incompatible (resistant) interactions (Takahashi et al., 2006; Deora et al., 2012, 2013). In contrast, cortical infection rarely occurs in incompatible interactions (Takahashi et al., 2006; Deora et al., 2012, 2013). Collectively, these results suggest that the pathogen may use primary infection to overcome the basal resistance of the plant, enabling cortical infection (Feng et al., 2013).

Transcriptomic and proteomic analyses of compatible and incompatible host-*P. brassicae* interactions have shown that host metabolism is highly modified at different points following infection. Compatible interactions between *P. brassicae* and different susceptible host species share a common set of metabolic routes, which are activated upon infection. The most up-regulated pathways include growth and cell cycle, sugar phosphate metabolism, cell rescue and defense, detoxification of reactive oxygen species (ROS), signaling, intracellular homeostasis, and cellular transport (Devos et al., 2006; Siemens et al., 2006; Cao et al., 2008; Rolfe et al., 2016; Jia et al., 2017; Li et al., 2018). In contrast, lignin biosynthesis is down-regulated (Cao et al., 2008). In incompatible interactions, glycolysis is downregulated, likely inhibiting pathogen-induced metabolism (Song et al., 2016).

In contrast, up-regulation of metabolic pathways involved in biosynthesis and lignification of the cell walls, ethylene (ET) response, synthesis and activation of PRRs, pathogenesis-related (PR) proteins and R proteins, salicylic acid (SA) signal transduction, calcium influx for calmodulin activation, the oxidase/respiratory burst, MAP kinase (MAPK) cascades, transcription factors and chitinases, has been observed during secondary infection by *P. brassicae* (Takahashi et al., 2002; Chen et al., 2016; Jia et al., 2017; Ning et al., 2019). Furthermore, most cell division and

expansion-related genes are downregulated in resistant hosts, indicating that these genes are important for the cell multiplication and enlargement required for club formation (Chen et al., 2016).

In resistant hosts, up-regulation of R proteins has been observed consistently following pathogen challenge (Ning et al., 2019). One transcriptomic analysis implicated the involvement of different *MAPK* in the activation of R proteins (Chen et al., 2016). Resistance responses have also been related to strong activation of receptor kinases and G proteins at the start of root hair infection (Mei et al., 2019). Despite the presence of major *R* genes and strong resistance reactions suggesting ETI, evidence for the HR in *P. brassicae*/host interactions has been limited (Kobelt et al., 2000; Takahashi et al., 2006). The R proteins modulating clubroot and their interaction with *P. brassicae* effectors remain to be characterized more fully. Similarly, the pathogen effectors are mostly unknown and uncharacterized as well. An analysis on *P. brassicae* proteins allowed the identification of two proteins putatively involved in pathogenesis, a serine protease *PRO 1* and one MeSa (Feng et al., 2010; Ludwig-Müller et al., 2015).

More recent studies have used multiple approaches to identify additional *P. brassicae* effectors. Using RNA-seq, 53 candidate small secreted proteins (putative effectors) were identified during primary infection (Chen et al., 2019), and 32 were found during cortical infection (Pérez-López et al., 2020). None, however, has been characterized completely or confirmed as an effector. The recent appearance of affordable and effective omics technologies holds great promise not only for improved understanding of *P. brassicae* virulence mechanisms, but also for facilitating the development of CR canola and other hosts (Zhou et al., 2020).

## 2.4 Clubroot management

The prolonged survival of *P. brassicae* resting spores in the soil makes clubroot management difficult. As a result, a number of management strategies have been explored (Hwang et al., 2014b), usually with two main objectives: i) avoiding introduction of the pathogen into previously non-infested fields, and ii) reducing clubroot disease incidence and severity in crops grown in fields where the pathogen is present already.

### 2.4.1 Cultural control

Given the biological characteristics of *P. brassicae*, the best management strategy is exclusion via the sanitization of farm machinery and other equipment (Donald et al., 2009; Hwang et al., 2014b; Diederichsen et al., 2014; Howard et al., 2010). After a field becomes infested, however, the most commonly used management practices to reduce disease incidence and severity include liming of the soil (Murakami et al., 2002; Pei et al., 2015), the deployment of resistant cultivars (Sharma et al., 2012; Cao et al., 2014; McGrann et al., 2016), and the application of fungicides or soil fumigants (Hwang et al., 2014a; Peng et al., 2014). Most of these practices have been developed for use in vegetable brassica crops, and generally, with the exception of genetic resistance, are too expensive or impractical for use in broad acre crops (Donald and Porter, 2003; Strelkov and Dixon, 2014). Longer rotations out of clubroot hosts, while often effective in reducing soil inoculum loads, are not always economically feasible for growers (Strelkov et al., 2011; Peng et al., 2014, 2015; Hwang et al., 2015; Anders et al., 2016; Ernst et al., 2019).

### 2.4.2 Chemical control

The application of fungicides can be an attractive strategy for clubroot management (Peng et al., 2014). The soil fungicides fluazinam, cyazofamid + methiadinil, cyazofamid and flusulfamide

have shown to reduce disease levels and increase yields in broccoli, cauliflower, cabbage, and Chinese cabbage (Donald et al., 2001; Mitani et al., 2003; Wang et al., 2017; Liu et al., 2019). In canola, quintozene also was found to reduce clubroot severity (Hwang et al., 2011c). Seed treatment with the fungicides azoxystrobin, thiamethoxam + difenoconazole + metalaxyl + fludioxonil, flusulfamide, clothianidin + carbathiin + trifloxystrobin + metalaxyl, and carbathiin + thiram also has been evaluated for the control of clubroot on canola in western Canada (Hwang et al., 2012a). While these treatments showed some promise under greenhouse conditions, they were not generally effective in the field, likely because the protection they afforded did not last sufficiently long to protect them from the persistent soil inoculum (Hwang et al., 2012a). Furthermore, while some fungicides have shown efficacy against clubroot, in "the current legislative climate it is unlikely that any soil applied fungicide would gain approval for use particularly on the scale of oilseed rape" (McGrann et al., 2016).

In addition to fungicides, there has been considerable interest in the use of soil fumigants for clubroot management. Soil fumigants are active ingredients with relatively high volatility, low water solubility and demonstrable activity against the soil microflora or fauna (White and Buczacki, 1979). Fumigants including chloropicrin (trichloronitromrthane), dazomet, metham sodium, methyl bromide, and methyl isothiocyanate have shown erratic results for clubroot control on canola/oilseed rape (Hwang et al., 2014b), although they appear to be more effective at reducing the disease in vegetable crops such as cabbage, cauliflower, Chinese cabbage, Shanghai pak choi, and swede (White and Buczacki, 1979; Porter et al., 1991; McDonald et al., 2020). In canola, metam sodium and dazomet have proven effective in managing clubroot, especially at high dosages between 0.4-0.8 t a.i $ha^{-1}$ for the former, and 0.4-1.6 mL $l^{-1}$ soil for the latter (Hwang et al., 2017, 2018). Soil fumigants can be very toxic, however, and also expensive to apply, so it is

unlikely they would be adopted in most canola/oilseed rape production systems except for targeted management of isolated infection foci (Al-Mughrabi et al., 2016; Hwang et al., 2017, 2018).

### 2.4.3 Liming

The modification of the soil environment to create conditions less favorable for clubroot development usually involves increasing the soil pH through the application of lime. Agricultural lime can include dolomitic ($CaCO_3$•$MgCO_3$) or calcitic ($CaCO_3$) limestone, calcium oxide (CaO) and hydrated lime ($Ca(OH)_2$) (Havlin et al., 2004). While raising the soil pH may afford some protection against clubroot, it is unlikely to be enough on its own to prevent the disease when inoculum pressure is high and environmental conditions are favourable for the pathogen (Colhoun, 1953; Narisawa et al., 2005; Gossen et al., 2013a). To obtain optimal benefits from liming, soil pH should be maintained above 7.2, and better results are obtained at pH values as high as 8.0 (Myers et al., 1983; Webster and Dixon, 1991b; Donald and Porter, 2004). To attain such alkaline pH, however, large quantities of lime are often necessary, which may be prohibitively expensive and impractical to source, apply and incorporate over multiple fields each year (Hwang et al., 2012a). Additionally, high pH values can be counterproductive due to negative effects on crop nutrition (Wilkinson, 2000). Another limitation of liming as a clubroot control method is the inconsistency in results (Tremblay et al., 2005; Knox et al., 2015; McGrann et al., 2016). The effectiveness of lime application can vary due to multiple factors that affect the capacity of the lime to increase pH uniformly and timely, the most relevant being: i) the extent of mixing of lime with soil, ii) the fineness of the lime, iii) the residual basicity or acidity of nitrogen sources in the rhizosphere, and iv) the timing of application (Dobson, 1983; Donald et al., 2004; Tremblay et al., 2005; McGrann et al., 2016). In general, powder-like limestone has been shown to be more effective, and favours a more uniform pH when mixed well with the soil (Dobson, 1983; Donald et al., 2004).

### 2.4.4 Genetic resistance

As discussed earlier, the host represents one of the three key components of the 'disease triangle', and the deployment of CR cultivars is among the most effective clubroot management practices (Hwang et al., 2014b). In Canada, the first CR canola variety ('45H29') was released in 2009 (Strelkov et al., 2018), and currently there about 28 different CR canola cultivars are available in western Canada (Canola Council of Canada, 2019). While the planting of resistant varieties is the most efficient and convenient method for clubroot management, the extensive cropping of CR canola exerts significant selection pressure on the pathogen (Holtz et al., 2018). This pressure can result in shifts in the virulence of pathogen populations, favoring the emergence of pathotypes that can break or overcome resistance, as has already been observed in Canada and elsewhere (Kuginuki et al., 1999; Diederichsen et al., 2014; Orgeur et al., 2016; Strelkov et al., 2016, 2018).

Neither host resistance nor liming, nor the use of fungicides, nor crop rotation offer a single sustainable solution to managing clubroot, particularly in broad acre crops such as canola/oilseed rape. Therefore, in the face of a fast-evolving population of *P. brassicae* able to breakdown resistance in a time-span of 4 years (Strelkov et al., 2016), integrated crop management strategies should be designed and implemented to minimize crop yield losses in infested fields.

**Table 2-1.** Summary of QTLs identified for clubroot resistance in different populations of *Brassica oleracea.*

| Breeding Population | Plant genotypes | *P. brassicae* isolate | Number of QTLs | Chromosomes where QTLs locate | Reference |
|---|---|---|---|---|---|
| $F_3$ | Breeding line (R) × single plant selection (S) | Pathotype 2 (Williams, 1966) | 2 | C01 and C06 | Landry et al., (1992) |
| $F_2$ | Broccoli (R)× Cauliflower (S) | Pathotype 7 (Williams, 1966) | 7 | C01, C04 and C09 | Figdore et al., (1993) |
| $F_2$ | Kale line (R)× Cauliflower (S) | Pathotype ECD 16/31/31 (Buczacki et al., 1975) | 7 | No specified | Grandclément and Thomas, (1996) |
| DH-lines | DH-line cabbage (R) × DH-line broccoli (S) | Pathotype ECD 16/3/30 (Buczacki et al., 1975) | 2 | C01 and C03 | Voorrips et al., (1997) |
| $F_2$ | Inbreed line cabbage (R) × inbreed line kale (S) | • Field population pathotypes 1 and 3 (Williams, 1966) | 1 | C03 | Moriguchi et al., (1999) |
| $F_3$ | Kale line (R) × DH-line broccoli (S) | • Ms6 and isolate 'eH' (Pathotype P1)<br>• K92 (Pathotype P2)<br>• K92-16 (Pathotype P4)<br>• Pb137-522 (Pathotype P7)<br>(Some et al., 1996) | 5 (Ms6)<br>3 (K92)<br>2 (Pb137-522)<br>3 (K92-15)<br>5 (eH) | C01, C02, C03, C04, C05, C08 and C09 | Rocherieux et al., (2004) |
| DH-lines | Cabbage (R)× Broccoli (S) | Pathotype 4 (Williams, 1966) | 5 | C02, C05, C03 and C07 | Nagaoka et al., (2010) |
| $F_3$ | Inbreed line cabbage (R) × inbreed line cabbage (S) | Korean field isolates YC (Pathotype 2) and GN (Pathotype 9) (Williams, 1966) | 2 (GN)<br>1 (YC) | C02 and C03 | Lee et al., (2016) |
| | 135 *B. oleracea* accessions of broccoli, brussels sprout, cabbage, cauliflower, Chinese kale, kale, kholrabi, and savoy cabbage | • F3-14 (Pathotype 3A)<br>• F-359-13 (Pathotype 5X)<br>(Strelkov et al. 2018) | 3 | C03, C06 and C07 | Farid et al., (2019) |

DH-lines: Population of doubled haploid lines
R: Clubroot resistant
S: Clubroot susceptible

**Table 2-2.** Summary of QTL*s* for clubroot resistance identified in different populations of *Brassica napus*.

| Breeding Population | Plant genotypes | *P. brassicae* isolate | Number of QTL*s* | Chromosomes or linkage group where QTL*s* locate | Reference |
|---|---|---|---|---|---|
| DH-lines | '*Darmor-bzh'* (PR) × *'Yudal'* (S) | Pb137-522 isolate (Pathotype P7) (Some et al., 1996) | 2 | DY2 and DY4 | Manzanares-Dauleux et al., (2000) |
| | | K91-16 (Pathotype P4) (Some et al., 1996) | 2 | DY4 and DY15 | |
| DH-lines | '263/11' (CR)× 'Express' (S) | 01:60 (UP) | 4 | N03, N08 and N13 | Werner et al., (2007) |
| | | 01.07 (UP) | 3 | N03, N08 and N13 | |
| | | e4x04 (UP) | 1 | N19 | |
| | | k (UP) | 3 | N02, N03 and N15 | |
| | | l (UP) | 2 | N03 and N08 | |
| | | a (UP) | 1 | N08 | |
| | | Korporal (UP) | 5 | N09 and N16 | |
| | 472 accessions of *B. napus* | Pathotype 4 (Williams, 1966) | 9 | A04, A10, C03, C06and, C09, | Li et al., (2016) |
| DH-lines | '*Darmor-bzh'* (PR) × *'Yudal'* (S) | 'eH' (Pathotype P1) (Some et al., 1996) | 7 | A05, A07, C02, C03, C09 | Laperche et al., (2017) |
| | | K91-16 (Pathotype P4) (Some et al., 1996) | 5 | A03, A09, C02 and C03 | |
| DH-lines | '*Darmor-bzh'* (PR) × *'Yudal'* (S) | 'eH' (Pathotype P1) (Some et al., 1996) | 4 | A07, C02 and, C09 | Aigu et al., (2018) |
| | 245 accessions of *B. napus* of winter oilseed rape, winter fodder, spring oilseed rape, swede, kale, semi-winter, and not assigned crop type. | Pathotype ECD 17/31/31 (Buczacki et al., 1975) | 9 | A01, A02, A03, A08, C02 and, C07 | Hejna et al., (2019) |
| DH-lines | '*Darmor-bzh'* (PR) × *'Yudal'* (S) | 'eH' (Pathotype P1) (Some et al., 1996) | 8 | A01, A10, C02, C03, C07 and C09 | Wagner et al., (2019) |

DH-lines: Population of doubled haploid lines
CR: Clubroot resistant
PR: Clubroot partially resistant
S: Clubroot susceptible
UP: Unknown pathotype

# Chapter 3 Effect of clubroot (*Plasmodiophora brassicae*) on yield of canola (*Brassica napus*)

## 3.1 Introduction

Canola (*Brassica napus* L.) is one of the most important field crops in Canada. It is grown on more than 8.4 million ha, mainly in the Prairies region (Statistics Canada, 2020), and contributes C$26.5 billion annually to the national economy (LMC international, 2016). One of the major limitations to canola production, however, is clubroot, a soilborne disease of the Brassicaceae family caused by the obligate parasite *Plasmodiophora brassicae* Wor. Infection by *P. brassicae* results in the formation of large galls on the roots of susceptible hosts, which interfere with water and nutrient uptake and can lead to severe yield reductions (Tewari et al., 2005; Pageau et al., 2006). Clubroot first emerged as a disease of the Prairie canola crop in 2003, when it was identified in a dozen fields in central Alberta (Tewari et al., 2005).It has since spread to over 3,300 fields throughout most of the canola-producing regions of the province (Strelkov et al., 2020), and is now also found with increasing prevalence in Saskatchewan and Manitoba (Froese et al., 2018; Ziesman et al., 2018; Strelkov et al., 2020). The costs associated with clubroot and its management have been estimated to be around C$2.1 billion in Alberta alone (Fredua-Agyeman et al., 2018).

The life cycle of *P. brassicae* consists of three main stages: i) survival in the soil, ii) root hair infection, and iii) cortical infection (Kageyama and Asano, 2009). Resting spores are responsible for pathogen survival in the soil; they have a half-life of about 4 years (Wallenhammar, 1996; Hwang et al., 2013), but can persist for up to 17 years (Wallenhammar, 1996) thanks in part to their thick five layered cell walls (Buczacki and Moxham, 1983). Despite this apparent longevity, recent studies suggest that resting spore numbers can decline by up to 90% following a 2-year break from canola, later stabilizing (Peng et al., 2015; Ernst et al., 2019) and resulting in a Type

III survivorship curve (Rauschert, 2010). Root hair infection begins with the germination of the resting spores, which is enhanced in the presence of root exudates, inducing the release of primary zoospores that encyst upon and penetrate the root hairs (Rashid et al., 2013). Following infection, a primary plasmodium is produced and secondary zoospores are released; the secondary zoospores penetrate root cortical tissue and develop into secondary plasmodia, eventually giving rise to a new generation of resting spores that are released back into the soil (Kageyama and Asano, 2009). Visible symptoms of clubroot appear during cortical infection, due to hypertrophy and hyperplasia of the infected tissues, which result in the formation of the root galls (Kageyama and Asano, 2009).

The prolonged survival of *P. brassicae* resting spores in the soil makes clubroot management difficult. As a result, various management strategies have been explored for the disease, including crop rotation, biological control, liming of the soil, and manipulation of the seeding date (Hwang et al., 2014b). The most effective and widely used strategy for clubroot management in Canadian canola, however, is the deployment of clubroot resistant (CR) cultivars (Rahman et al., 2014; Strelkov and Hwang, 2014). Unfortunately, the virulence of *P. brassicae* populations can shift in response to the selection pressure imposed by the planting of CR hosts, and since 2013, novel pathotypes of the clubroot pathogen have been identified that can overcome host resistance (Strelkov et al., 2016; Holtz et al., 2018; Hollman et al., 2021). The loss or erosion of clubroot resistance represents one of the biggest challenges to the management of this disease.

Most canola cultivars available in Canada appear to carry a single resistance gene derived from the European winter oilseed rape 'Mendel' (Strelkov et al., 2018); cultivars carrying this gene possess what is commonly known as '1$^{st}$ generation' resistance (Hollman et al., 2021). Given the emergence of new *P. brassicae* pathotypes that are able to overcome '1$^{st}$ generation' resistance (Strelkov et al., 2014, 2016), new canola hybrids with novel resistance traits have been developed

in recent years. Cultivars with these new CR traits possess so-called '2nd generation' resistance (Hollman et al., 2021), the genetic basis of which is not in the public domain and may differ across hybrids. As of 2019, 28 CR canola cultivars were available in western Canada, of which five were reported to carry '2nd generation' resistance (Canola Council of Canada, 2019).

While clubroot is a cause for concern worldwide, replicated experiments to determine the yield or economic losses associated with infection of canola have been very limited (Rempel et al., 2014). Instead, most yield loss estimates are based on anecdotal reports or approximations of grower-expected yields vs. harvested amounts. In Australia, yield losses in canola were estimated to be around 50% (1.6 t $ha^{-1}$) (Donald and Porter, 2003), while in western Canada, losses have been estimated at 30% in heavily infested soils (Tewari et al., 2005). In at least one canola crop in central Alberta, clubroot was so severe that the crop was not harvested, so the yield loss was effectively 100% (Strelkov and Hwang, 2014). While approximations of yield losses caused by *P. brassicae* are useful to evaluate the general impact of clubroot on canola production, the relationship between disease incidence and severity, pathogen inoculum density, and the genetics of the host and pathogen have not been studied.

It has been observed that as clubroot incidence and severity increase, canola yield diminishes (Wallenhammar et al., 2000; Hwang et al., 2011a; McGrann et al., 2016). In China, the yield of two spring oilseed rape (*B. napus*) crops with a disease incidence of 56.2% or 100% declined by 23.5% and 56.4%, respectively (Ren et al., 2012). These yield losses reflected decreases in the first effective branch number, the effective pod number per plant, the seed number per pod, and 1000-grain weight. In another study where spring oilseed turnip (*Brassica rapa* L.) was tested, yield losses varied between 5% and 8% when the average clubroot incidence was 17%, while at higher levels of disease (> 40% infected plants), yield losses increased (Wallenhammar et al., 2000). In

Quebec, Canada, Pageau et al. (2006) reported yield reductions between 80% and 90% in susceptible canola, and between 69% and 89% in spring oilseed turnip grown in *P. brassicae*-infested fields. Moreover, the 1000-grain weight decreased between 13% and 19%, while oil content was reduced by 2% to 6%. The authors also identified two resistant lines whose yield or yield parameters were not affected when grown in infested soils (Pageau et al., 2006).

In Alberta, Hwang et al. (2011a) evaluated the impact of different *P. brassicae* inoculum densities on clubroot severity and yield loss, and found that disease severity increased while yield decreased at greater inoculum densities. Analysis of the results indicated that inoculum density accounted for 65% of the variance in canola yield. In addition to yield losses, premature ripening was observed in susceptible plants grown in *P. brassicae*-infested soil. While yield decreased as pathogen inoculum increased, no significant differences were observed among inoculated plants when inoculum densities were between $1 \times 10^5$ and $1 \times 10^8$ resting spores $cm^{-3}$ of potting mix (Hwang et al., 2011a). Seed yield was higher in a resistant cultivar, which was not affected by inoculation with *P. brassicae*, while significant declines were observed in a susceptible cultivar (Hwang et al., 2011a).

The effect of clubroot on canola yield needs to be determined more accurately, by conducting experiments of sufficient size under greenhouse and field conditions. Accurate estimates of potential yield loss are necessary to determine action thresholds for clubroot risk assessment, and ultimately to select appropriate management strategies to maintain crop profitability (Shtienberg, 2000). The objective of this research was to evaluate the impact of clubroot on the yield of canola. Specifically, we examined the relationship between *P. brassicae* inoculum density and virulence on clubroot incidence and severity on canola cultivars with different levels of resistance, to assess their effect on yield and yield-related parameters.

## 3.2 Materials and Methods

### 3.2.1 Pathogen and plant material

Experiments were conducted with *P. brassicae* field isolates representing pathotypes 3H (isolate CDCNFG-1035 (field experiments) or 41-14 (greenhouse experiments)) and 5X (isolate LG-2 (field) or a mix of isolates LG-1, LG-2 and LG-3 (greenhouse)), as classified on the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018). Pathotype 3H was the predominant pathotype in Alberta prior to the introduction of CR canola (Strelkov and Hwang, 2014; Strelkov et al., 2018), and is still commonly found in Alberta and Saskatchewan (Hollman et al., 2021). This pathotype is unable to overcome the resistance in any of the CR canola cultivars currently on the market. Pathotype 5X was the first pathotype identified that could overcome the resistance in CR canola, causing clubroot symptoms on host genotypes carrying 1$^{st}$ generation resistance (Strelkov et al., 2016). The pathogen was maintained as frozen root galls on the universally susceptible Chinese cabbage (*B. rapa* subsp. *pekinensis*) cv. Granaat (European Clubroot differential (ECD) 05; Buczacki et al. 1975). Three canola cultivars with different levels of clubroot resistance were included in the experiments: i) the susceptible cultivar '45H31', ii) the 1$^{st}$ generation CR cultivar '45H29', resistant to *P. brassicae* pathotype 3H but susceptible to pathotype 5X, and iii) the 2$^{nd}$ generation CR cultivar 'CS2000', resistant to pathotype 3H and partially resistant to pathotype 5X.

### 3.2.2 Field experiments

#### 3.2.2.1 Experimental design

A two-year field experiment was conducted in 2018 and 2019 at two biosecure disease nurseries at the Crop Diversification Centre North, Alberta Agriculture and Forestry, Edmonton,

AB (53°38'N, 113°21'W), on a Black Chernozemic soil. The experiment was a 2 × 4 × 3 factorial with two *P. brassicae* pathotype treatments (5X and a mixture of pathotypes 3H and 5X), four inoculum densities (0, $5 \times 10^4$, $5 \times 10^6$ and $5 \times 10^8$ resting spores plant$^{-1}$), and three canola cultivars ('45H31', '45H29' and 'CS2000'). One of the nurseries was inoculated with pathotype 5X ("5X nursery"), and the other was inoculated with a mixture of pathotypes 5X and 3H ("mixed nursery"), the experimental design in both nurseries consisted of completely randomized blocks nested within the two years. Each plot consisted of four rows 1 m in length with 0.25 m spacing. Adjacent plots were separated by a 0.5 m buffer zone with 1 m between blocks. The plots were hand-seeded at a rate of 35 seeds per row for a crop density of 120 plants m$^{-2}$. The seeding dates were 13 June 2018, and 3 June 2019.

#### 3.2.2.2 Inoculum preparation and inoculation

Frozen root galls infected with each pathotype were thawed at room temperature and homogenized in distilled water in a home blender at maximum speed for 3 min. The resulting suspension was filtered through eight layers of cheesecloth to remove most of the plant debris, and the resting spore concentration was estimated under a microscope with a haemocytometer (VWR, Mississagua, ON) (Strelkov et al., 2006). The concentration was then adjusted to $1 \times 10^4$, $1 \times 10^6$, and $1 \times 10^8$ resting spores mL$^{-1}$ using distilled water. To prepare inoculum for the mixed nursery, equal volumes of the adjusted suspensions of each pathotype were mixed. Inoculation of both nurseries (5X and mixed) was conducted two weeks after seeding, only in 2018, by applying 5 mL of the resting spore suspension to the base of each seedling; control plots were not inoculated.

Estimates of the pathogen inoculum densities at both nurseries in 2019 were based on the disease severity index (DSI) obtained in 2018 and the resting spore concentration, as determined

by quantitative PCR (qPCR; see below), in soil samples collected from each plot in October of 2018 after harvest. Inoculum densities at the end of the first field season ranged between 0 and 6 × $10^6$ resting spores $g^{-1}$ of soil at the 5X nursery, and from 0 to 3 × $10^6$ resting spores $g^{-1}$ of soil at the mixed nursery. Plots where the DSI was between 0% and 25%, and the pathogen inoculum density was between 0 and 1 × $10^3$ resting spores $g^{-1}$ of soil, were designated as controls (equivalent to 0 resting spores $plant^{-1}$ in 2018); plots where the DSI was between 26% and 50%, and the inoculum densities were between 0 and 1×$10^3$ resting spores $g^{-1}$ of soil, were assigned as the treatment with the lowest expected disease level (equivalent to 5 × $10^4$ resting spores $plant^{-1}$ in 2018); plots where DSI was between 51% and 80%, and the pathogen inoculum density was between 1 × $10^3$ and 1 × $10^4$ resting spores $g^{-1}$ of soil, were assigned as the treatment with intermediate expected disease level (equivalent to 5 × $10^6$ resting spores $plant^{-1}$ in 2018); and plots where DSI was > 80%, and the pathogen inoculum density was > 1 × $10^4$ resting spores $g^{-1}$ of soil, were assigned as the treatment with the highest expected disease level (equivalent to 5×$10^8$ resting spores $plant^{-1}$ in 2018).

#### 3.2.2.3 Soil sampling and quantification of *P. brassicae* inoculum density

One soil sample was collected in each of the plots at both sites (5X nursery and mixed nursery) on 5 October 2018 (fall 2018), 6 June 2019 (spring 2019), and 24 October 2019 (fall 2019). Approximately 500 g of soil was collected from the centre of each plot. Soils were sampled to a depth of 15-cm using a small shovel and placed individually in paper bags and allowed dry at room temperature. The shovel was disinfested between samples by cleaning it with 70% ethanol. To quantify *P. brassicae* inoculum, total genomic DNA was extracted from 0.25 g of each soil sample using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's instructions. The concentration and purity of the DNA were evaluated with a

Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Subsequently, the DNA was diluted 10-fold for quantitative PCR (qPCR) analysis with the primers DR1F and DR1R in a StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA) as per Rennie et al. (2011). Estimation of the number of resting spores per sample was completed by comparison with a standard curve generated with DNA extracted from known quantities of *P. brassicae* resting spores (Rennie et al., 2011). After each qPCR run, a melting point analysis was conducted to identify the amplified product.

#### 3.2.2.4 Accumulated precipitation and air temperature

Information regarding the accumulated precipitation (mm), the monthly average of the highest air temperature (°C), the monthly average of the lowest air temperature (°C), and the monthly average of air temperature (°C) from 13 June 2018 to 25 September 2018, and from 3 June 2019 to 10 October 2019, was obtained from the Alberta Climate Information Service (Alberta Agriculture and Forestry, 2020). The data were collected from the weather station Oliver AGDM (ID 3014921, coordinates: 53°38'36.7" N, 113°21'16.6" W) located within the Crop Diversification Centre North, Edmonton, AB.

#### 3.2.2.5 Disease assessment

In 2018, disease assessment was conducted 8 weeks after inoculation; in 2019, it was conducted 8 weeks after seeding. In both years, all plants from the first row of each plot were pulled, and the roots were washed using tap water. The roots were evaluated for the severity of clubroot symptoms on a 0 to 3 scale, where 0 = no galling, 1 = few small galls (small galls on less than one-third of the roots), 2 = moderate galling (small to medium galls in one third to two-thirds of the roots), and 3 = severe galling (medium to large galls in more than two thirds of the roots).

The severity ratings were used to calculate the disease severity index (DSI) for each plot according to the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006a):

$$DSI = \frac{\sum(n\times0)+(n\times1)+(n\times2)+(n\times3)}{N\times3} \times 100 \quad (1)$$

where $n$ is the number of plants in each severity level; $N$ is the total number of plants evaluated; and 0, 1, 2 and 3 are the symptom severity classes.

#### 3.2.2.6 Assessment of yield and yield parameters

One week before harvest, all pods from five plants randomly selected from each plot were counted. Plots in both nurseries were harvested at maturity, 105 days after seeding in 2018, and 127 days after seeding in 2019. The aboveground parts of all plants from the remaining three rows of each plot were cut, placed in fabric bags, and allowed to dry at room temperature. Once dry, the pods from each plot were threshed manually, and the seeds cleaned and weighed. After recording the yield per plot, the 1000-grain weight also was measured.

### 3.2.3 Greenhouse experiment

#### 3.2.3.1 Experimental conditions and measured variables

A greenhouse experiment was conducted in winter 2020-2021 at the University of Alberta, Edmonton, AB. Plants were grown in 32 cell trays (cell dimensions: 6 cm upper diameter and 7.5 cm height) filled with Sunshine LA4 potting mixture (Sunshine Growers, Vancouver, BC) consisting of 60.75% Canadian sphagnum peat moss, horticultural grade perlite and dolomitic limestone. Two seeds were sown per cell and thinned to one plant per cell after germination. The plants were maintained at 20°C ± 2°C with a 16 h photoperiod under natural light supplemented by artificial lighting. Inoculum of *P. brassicae* was prepared as described above for the field

experiments, with the experiment arranged as a $3 \times 4 \times 3$ factorial with three *P. brassicae* pathotype treatments (5X, 3H and an equal mixture of both pathotypes), four inoculum densities (0, $1 \times 10^3$, $1 \times 10^4$ and $1 \times 10^6$ resting spores plant$^{-1}$), and three canola cultivars ('45H31', '45H29', and 'CS2000'). The experimental design consisted of completely randomized blocks with a nested structure. The large plots comprised a combination of the pathotype and inoculum densities, where three subsamples (trays) of the canola cultivars were nested. Clubroot severity was assessed on eight plants per tray 8 weeks after inoculation and a DSI was calculated as described for the field experiments. The soil was kept saturated for the first week after inoculation, and then watered and fertilized as needed with 20 N: 20P: 20 K. One week before harvest, all pods of eight randomly selected plants of each tray were counted. The plants were harvested at maturity, 95 days after seeding. The aboveground parts of the remaining plants of each tray were cut, placed in paper bags, and allowed to dry for one week. The pods from each tray were threshed manually, seeds were cleaned and weighed. After recording the yield per tray, the 1000-grain weight was determined.

### 3.2.4 Statistical analysis

Statistical analyses were performed with R (R Core Team, 2019). Mixed linear models and simple linear models were estimated to analyse the effect of *P. brassicae* inoculum density and pathotype on the DSI of the three canola cultivars; and the effect of the canola cultivar, pathotype, and DSI on yield, number of pods per plant (PPP), and 1000-grain weight. The *nlme* (Pinheiro et al., 2019) and *lsmeans* (Lenth, 2016) packages were used to test both random and fixed effects. To compare the models that assessed the effect of *P. brassicae* pathotype, DSI and canola cultivar on yield and yield components, the response variables were standardized with a min-max normalization using the package *scales* (Wickham and Seidel, 2020).

In the field experiments, inoculum density and canola cultivars were considered as fixed effects; pathotype was considered both as a random and fixed effect since pathotype 5X and the mixture of the pathotypes 5X and 3H were inoculated at different sites; year and block were considered as random effects. In the greenhouse experiment, pathotype, inoculum density and canola cultivar were considered as fixed effects; pathotype, block and subsamples were considered as random effects. The most parsimonious models were selected, and to assess the models' fit, the marginal and conditional $R^2$ were estimated for the mixed linear models using the *MuMIn* package (Barton, 2020) and $R^2$ was estimated for the simple linear models. The resulting models were used to obtain the regression equations to estimate the losses caused by clubroot in yield, PPP and 1000-grain weight.

### 3.3 Results

#### 3.3.1 Disease

Disease levels increased along with inoculum density in both the field and greenhouse experiments. In the field experiment, only the canola cultivar ($p = 0.0004$) and the inoculum density ($p < 0.0001$) had a significant effect on the DSI. The highest disease levels were observed in the susceptible '45H31', while DSI was statistically equal in both CR cultivars (Figure 3-1). Lower levels of clubroot were observed in the first year of the experiment. In 2018 at the 5X nursery, the lowest DSI when the pathogen was not inoculated was 23% in the susceptible cultivar '45H31', 5% in the 1$^{st}$ generation CR '45H29', and 11.3% in the 2$^{nd}$ generation CR 'CS2000'. In 2019, the lowest DSI increased to 28.8%, 19.4% and 22.3% in '45H31', '45H29' and 'CS2000', respectively. At the mixed nursery, the lowest DSIs in 2018 were 5.9% in the susceptible cultivar and 0% in both CR cultivars, while in 2019, DSIs increased to 35.3% in the susceptible cultivar,

and to 25.9% and 35.3% in the CR cultivars ‘45H29’ and ‘CS2000’. In 2018, the highest DSI in the susceptible ‘45H31’ was 100% at the 5X nursery, and 89% at the mixed nursery. In the CR cultivars ‘CS2000’ and ‘45H29’, the highest DSIs were 93% and 62.2%, respectively, at the 5X nursery, and 75.5% and 44.4% at the mixed nurseries. In 2019, DSI reached a maximum of 100% in all cultivars at both nurseries.

In the greenhouse experiments, there was a significant effect of *P. brassicae* pathotype ($p = 0.0127$), inoculum density ($p < 0.0001$), canola cultivar ($p < 0.0001$) and their interaction ($p < 0.0001$) on DSI. The lowest DSIs were observed when only pathotype 5X was inoculated. When pathotypes 3H and 5X were inoculated together, DSIs were equal to those observed with pathotype 3H alone. Canola cultivars also showed differences, with the highest DSIs developing on the susceptible ‘45H31’, followed by the second-generation CR cultivar ‘CS2000’. The lowest DSIs were obtained in the 1st generation CR ‘45H29’ (Figure 3-2). When inoculated at the highest inoculum density with pathotype 3H, DSI in the susceptible ‘45H31’ was 87.5%, vs. 13.8% with pathotype 5X and 82% with the mixture; in the CR cultivars ‘45H29’ and ‘CS2000’, the DSIs at the highest inoculum density were 14.4% and 19.6% with pathotype 3H, 7% and 4.8% with pathotype 5X, and 10.5% and 27% with the pathotype mix, respectively.

### 3.3.2 Yield and yield components

In both the field and greenhouse experiments, the yield, PPP and 1000-grain weight decreased as the DSI increased. Yield was affected by the DSI ($p < 0.0001$) and canola cultivar ($p < 0.0001$ in the greenhouse and $p = 0.0163$ in field) but not by pathotype ($p = 0.6944$ in the greenhouse and $p = 0.6093$ in the field). In both the field and greenhouse, the highest yield was observed in the CR cultivar ‘45H29’, while the lowest was found in the susceptible cultivar ‘45H31’. Under

greenhouse conditions, the susceptible '45H31' and the 2nd generation CR cultivar 'CS2000' had similar yields, while in the field experiments, the yield of 'CS2000' yield was not significantly different from '45H31' or '45H29'.

When the treatments were not inoculated, the average yield of the 1st generation CR cultivar '45H29' was 129.15 g $m^{-2}$ in the field and 12.42 g $tray^{-1}$ in the greenhouse. In the 2nd generation CR cultivar 'CS2000', these values were 113.66 g $m^{-2}$ and 10.21 g $tray^{-1}$. In the susceptible cultivar '45H31', the average yield in the absence of added inoculum was 110.68 g $m^{-2}$ in the field and 10.58 g $tray^{-1}$ in the greenhouse. In the field experiments, where the highest DSI observed was 100% for all three canola genotypes, the lowest adjusted mean yield across all inoculation levels in both years was 39.68 g $m^{-2}$ for '45H31', 58.15 g $m^{-2}$ for '45H29' and 42.66 g $m^{-2}$ for 'CS2000'. In the greenhouse experiment, where the highest DSI observed for '45H29' was 25%, the lowest average yield was 9.67 g $tray^{-1}$, while for 'CS2000', where the maximum DSI was 45.8%, the lowest average yield was 5.17 g $tray^{-1}$. In the susceptible cultivar '45H31', the yield was 0 g $tray^{-1}$ when the DSI was 100%.

Regression analysis indicated that under greenhouse conditions, an increment of 1% in the DSI resulted in a decrease of 0.49% in yield; under field conditions, this percentage was reduced to 0.26% (Figure 3-3). The regression model for the greenhouse experiment had a marginal $R^2$ ($mR^2$) and a conditional $R^2$ ($cR^2$) of 0.23 and 0.39 (Figure 3-4A), whereas the regression model for the field experiment had an $mR^2$ = 0.09 and a $cR^2$ =0 .82 (Figure 3-4B). Although the yield reduction rate was equal among cultivars in both experiments, yield losses in the susceptible cultivar '45H31' were greater compared with the CR canola genotypes, since they developed less severe clubroot. Under greenhouse conditions, in which neither of the CR cultivars had a DSI > 45.8%, maximum yield losses were 22% for '45H29' and 41.8% for 'CS2000' (Figure 3-5).

PPP and 1000-grain were higher in the field experiments. In the field experiments when plots were not inoculated, the adjusted mean of PPP across all cultivars was 45 and 1000-grain weight was 3.15 g, 2.95 g and 3.06 g, respectively, in '45H31', '45H29' and 'CS2000'. Under greenhouse conditions, PPP and 1000-grain weight in the control treatments across all cultivars were 18 and 2.21 g, respectively (Figure 3-6). In the field experiments, PPP was affected only by the DSI ($p < 0.0001$), while 1000-grain weight was affected by DSI ($p < 0.0001$) and cultivar ($p = 0.0029$). The highest 1000-grain weight was observed in '45H31', while this value was intermediate in 'CS2000' and lowest in '45H29' (Figure 3-6). Under greenhouse conditions, PPP was affected by DSI ($p < 0.0001$) and cultivar ($p < 0.0001$), while 1000-grain weight was affected only by DSI ($p < 0.0001$). The lowest number of pods per plant was observed in 'CS2000', while PPP was similar in '45H31' and '45H29'.

The mixed linear models that estimated the effect of DSI and canola cultivar on PPP in the field experiments had an $mR^2 = 0.12$ and a $cR^2 = 0.57$; similarly, the $mR^2$ for the models evaluating 1000-grain weight also was 0.12, while the $cR^2 = 0.68$ (Figure 3-6). Simple linear models were selected as the most parsimonious to assess the effect of DSI and canola cultivar on PPP and 1000-seed weight for the greenhouse experiment. The regression model for PPP had a $R^2 = 0.4$, while the model for 1000-grain weight had a $R^2 = 0.21$. Regression analysis of the standardized values of the PPP indicated that under greenhouse conditions, an increment of 1% in the DSI resulted in a decrease of 0.48% in the PPP and of 0.34% in 1000-grain weight; under field conditions, these percentages were reduced to 0.27% and 0.23%, respectively (Figure 3-3).

### 3.3.3 Inoculum density of *P. brassicae* in field experiment

Inoculum density increased in both nurseries consistently from fall 2018 to fall 2019. At the 5X nursery in fall 2018, inoculum density ranged from 0 to $6 \times 10^6$ resting spores g of soil$^{-1}$. In spring 2019, it was between 0 and $5 \times 10^7$ resting spores g of soil$^{-1}$, and increased to densities between $8 \times 10^3$ and $1 \times 10^8$ resting spores g of soil$^{-1}$ at the end of the second growing season in the fall of 2019. In the mixed nursery, inoculum density ranged from 0 to $3 \times 10^6$ resting spores g of soil$^{-1}$ in the samples collected in the fall of 2018. In the spring of 2019, densities were between 0 and $3 \times 10^7$ resting spores g of soil$^{-1}$; by fall 2019, at the end of the second growing season, inoculum densities were between 0 and $1 \times 10^8$ resting spores g of soil$^{-1}$.

Statistical analysis did not show an effect of the number of resting spores of *P. brassicae* inoculated in the first growing season or the canola cultivar on the inoculum density. Nonetheless, overall differences were observed between both nurseries, with the mixed nursery having a lower concentration of resting spores than the 5X nursery ($p = 0.003$). Furthermore, at the 5X nursery, each season was different from the previous one, while at the mixed nursery, inoculum densities in the fall of 2018 and spring of 2019 were statistically equal (Figure 3-7). In both nurseries, the highest inoculum densities were observed in fall 2019, at the end of both growing seasons.

### 3.3.4 Weather conditions

Overall, 2019 was colder relative to 2018 from June to August, but warmer in September. The largest difference in monthly average air temperature was observed in June, when the temperature was 3.5°C higher in 2018 vs. 2019. During the first growing season (2018), average air temperatures were between 6.16°C and 17.2°C, while during the second growing season (2019), they ranged between 10.7°C and 15.4°C. The average air temperatures between June and August,

when the disease was assessed, oscillated between 15.4°C and 17.2°C in 2018, and between 13.7°C and 15.9°C in 2019 (Figure 3-8). Accumulated precipitation over the 2019 growing season was 308 mm, which was 108.7 mm greater than the 199.3 mm of precipitation received in 2018 (Figure 3-9). Most of the precipitation in 2019 (77.2% or 241.5 mm) fell in June and July, while in 2018, only 52.3% (105.5) mm of the precipitation fell during this period (Alberta Agriculture and Forestry, 2020).

### 3.4 Discussion

The aim of this study was to evaluate the impact of clubroot disease on the yield of canola. Clubroot was found to be significantly affected by *P. brassicae* inoculum density and the genetics of the canola cultivar grown. The DSI in all cultivars increased with increasing inoculum density, with the susceptible genotype '45H31' developing the highest disease levels. Indeed, under greenhouse conditions, '45H31' was the only cultivar for which a DSI > 50% was observed.

In the field experiment, clubroot severity was lower in 2018 than in 2019. This can be attributed to changes in the inoculum density from one year to the next, as well as weather conditions more favourable for disease development in 2019. It is likely that resting spore density in the soil increased after the first year of the trial, reaching levels higher than what was initially inoculated in 2018 across all of the plots. While such an increase was not detected in the inoculum density quantification conducted immediately after harvest in October 2018, the spore density did increase from October 2018 to June 2019. This increase in spore levels in the spring following cultivation of a host is consistent with a recent analysis of *P. brassicae* resting spore dynamics in commercial canola crops, and may reflect the decomposition of infected gall tissue over the fall and spring (Ernst et al., 2019). Further increases in inoculum density were detected in October 2019, after the

second growing season when clubroot symptoms were more severe. In addition to an increase in pathogen inoculum densities, precipitation in 2019 was higher than in 2018; more precipitation would have favored clubroot development, since the incidence and severity of the disease increase together with increasing moisture levels (Samuel and Garrett, 1945; Hamilton and Crête, 1978; Dobson et al., 1982). In addition, in 2019 most (77.2%) of the precipitation that fell over the growing season was in June and July, while in 2018, only 52.3% of the precipitation fell during this period (Alberta Agriculture and Forestry, 2020). The timing of rainfall is particularly important for clubroot development, with early rains favoring infection and the onset of symptoms (Dobson et al., 1982; Dixon, 2009a). Average air temperatures between June and August, when the disease was assessed, were slightly below the optimum range (20°C to 25°C) for clubroot development (Ayers, 1944; Thuma et al., 1983; Sharma et al., 2011b, 2011a; Gossen et al., 2012, 2013a; Luo et al., 2014) in both 2018 and 2019. This does not imply adverse conditions for disease development, since clubroot symptoms can occur at temperatures > 10°C (Sharma et al., 2011b, 2011a; Gossen et al., 2012; Luo et al., 2014). Moreover, it is important to note that mean soil temperatures may be 1-11°C higher than air temperatures (Zhang et al., 2005; Barman et al., 2017).

In the field experiments, mild symptoms of clubroot (DSI < 30%) were observed in non-inoculated plots in both years at both nurseries. This suggests the presence of pre-existing *P. brassicae* inoculum at both sites, despite the fact that symptoms had not been reported there previously. The pathotype(s) present in the nurseries prior to the inoculations conducted in this study is not known. However, the presence of a pre-existing pathotype, which could have obscured the differences between pathotype 5X and the mix of pathotypes 3H and 5X, may help to explain why pathotype did not show an effect on DSI in the field experiments. It is also possible that differences in the environmental conditions at the 5X and mixed nurseries affected crop and

disease development. In contrast, pathotype affected DSI under greenhouse conditions. This effect, however, was not necessarily consistent with the results expected based on the virulence/resistance profiles of the pathotypes and cultivars used. A higher DSI was expected on all cultivars whenever pathotype 5X was inoculated, since it has been reported to be highly virulent and capable of overcoming clubroot resistance (Strelkov et al., 2016). Nonetheless, the lowest disease levels were observed in all canola cultivars when challenged with 5X. Furthermore, co-inoculation of the pathotypes 5X and 3H resulted in disease levels equal to inoculation with pathotype 3H alone. One of the possible causes for lower disease levels following inoculation with pathotype 5X could be related to reduced inoculum viability, but staining with the vital stain Evan's blue (Harding et al., 2019) ruled this possibility out (results not shown). Another, perhaps more likely explanation, is that there was a shift in the virulence or aggressiveness of the pathotype 5X isolate following repeated multiplication on the universally susceptible Chinese cabbage host 'Granaat'. While it has been standard practice to maintain *P. brassicae* stocks on a universally susceptible host (Buczacki et al., 1975) to avoid virulence shifts associated with hosts carrying a resistance trait, this could cause issues in cases where increased virulence carries a cost for the pathogen. Indeed, shifts in pathotype structure have been reported recently in *P. brassicae* cycling experiments with pathotype 5X (Cao et al., 2020) and remain the focus of further study.

Regardless of the virulence of specific isolates, a wide range of disease levels were obtained, which could then be linked back to yield and yield-related parameters to model the impact of clubroot on yield. Clubroot was found to have an inverse relationship with yield, PPP, and 1000-grain weight in all three canola cultivars. While the yield varied in each cultivar, the rate at which yield was reduced (0.49% under field conditions and 0.26% under greenhouse conditions) for each 1% increase in DSI was consistent. While yield losses may appear to be minimal for CR

hosts, this does not mean that the occurrence of disease will not affect yield. In a study conducted with the winter oilseed rape cultivars 'Visby' (susceptible) and 'Mendelson' (resistant), yield losses in the resistant genotype were not significant, but the maximum DSI observed was only 24% (Strehlow et al., 2014). The present results indicate, however, that once clubroot symptoms begin to develop on a CR genotype, the relative declines in yield per unit increase in disease will be equivalent to those of a susceptible host.

Previous studies have examined the impact of clubroot on the yield of canola and oilseed rape (Wallenhammar et al., 2000; Hwang et al., 2011a, 2011b; Ren et al., 2012; McGrann et al., 2016), but few have modelled yield and yield parameters against DSI. In the UK, a linear model to assess the impact of clubroot on yield of susceptible and resistant winter oilseed rape cultivars was estimated after a series of field experiments (McGrann et al., 2016). Similar to the results of this study, a common yield loss rate, estimated at 0.03 t $ha^{-1}$ per 1% increase in DSI, was calculated for all of the cultivars (two resistant and one susceptible). Separate yield loss models that resulted in different estimated yield loss rate for each cultivar were also developed, but the authors did not mention if these models were statistically different or if only one model was sufficient to estimate yield losses caused by clubroot (McGrann et al., 2016). Additionally, quadratic and negative exponential models have been fitted to yield losses caused by clubroot in susceptible and resistant cultivars of canola in Canada (Hwang et al., 2011a, 2011b). In one study, a susceptible cultivar was evaluated, with a minimum DSI of 42.9% obtained (Hwang et al., 2011a), while in another study, the maximum DSI in the resistant cultivar was just 5.6% (Hwang et al., 2011b); those maximum and minimum DSI represent a narrow range of the independent variable in the models, imposing a limitation in their estimation.

In contrast to DSI, previous analyses of the effect of clubroot on PPP and 1000-grain weight are scarcer to find and the results more erratic. One study with winter oilseed rape conducted under semi-controlled conditions did not find an effect of *P. brassicae* inoculation on PPP or 1000-grain, independent of the resistance level of the host (Strehlow et al., 2014). Another study conducted under field conditions with canola found that as clubroot incidence increased both, PPP and 1000-grain weight were negatively affected (Ren et al., 2012). That study, however, did not include disease severity in the analysis, and since the experiments were carried out in the field and inoculum levels were not controlled, their reproducibility was affected (Ren et al., 2012).

This research indicated that clubroot levels are highly dependent on *P. brassicae* inoculum density, host genetics, and pathogen virulence. Yield losses were negatively affected by DSI, and independently of the host genetics, they can be between 0.26% and 0.49% for each 1% increment in DSI. These yield reductions reflect a decrease in the number of pods per plant and 1000-grain weight. Moreover, in susceptible canola, lower pathogen inoculum densities can cause a higher DSI compared with resistant cultivars, and thus yield losses in the former are usually higher.

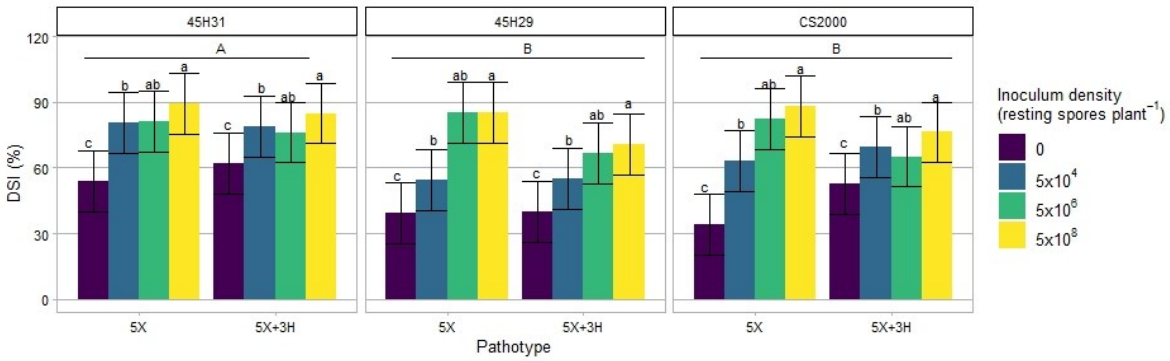


**Figure 3-1**. Adjusted mean clubroot disease severity index (DSI) obtained at two sites in a two-year field experiment in Edmonton, Alberta. Three canola cultivars, '45H31', '45H29' and 'CS2000', were inoculated with *Plasmodiophora brassicae* pathotype 5X or a mix of pathotypes 5X and 3H (5X+3H) at inoculum densities of $5 \times 10^4$, $5 \times 10^6$, and $5 \times 10^8$ resting spores plant$^{-1}$. Different lower-case letters indicate significant differences in DSI according to Tukey's test at $p > 0.05$. Average DSI across canola cultivars with different upper-case letters are different according to Tukey's test at $p > 0.05$. Error bars represent the standard error of the mean.

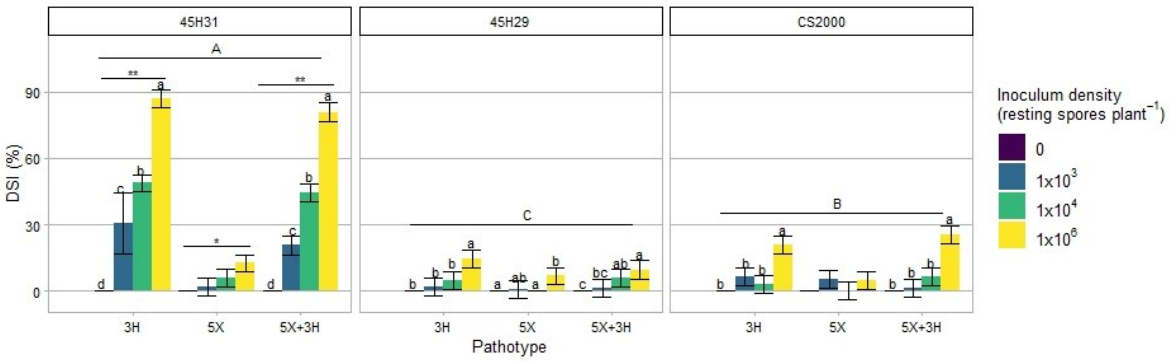


**Figure 3-2.** Adjusted mean clubroot disease severity index (DSI) obtained in a greenhouse experiment. Three canola cultivars '45H31', '45H29, and 'CS2000' were inoculated with *Plasmodiophora brassicae* pathotype 5X, 3H or a mix of pathotypes 5X and 3H (5X+3H) at inoculum densities of $1 \times 10^3$, $1 \times 10^4$, and $1 \times 10^6$ resting spores plant$^{-1}$. Different lower-case letters indicate significant differences in DSI according to Tukey's test at $p > 0.05$. Average DSI across canola cultivars with different upper-case letters are different according to Tukey's test at $p > 0.05$. Average DSI across pathotypes within cultivars with different number of asterisks are different according to Tukey's test at $p > 0.05$. Error bars represent the standard error of the mean.

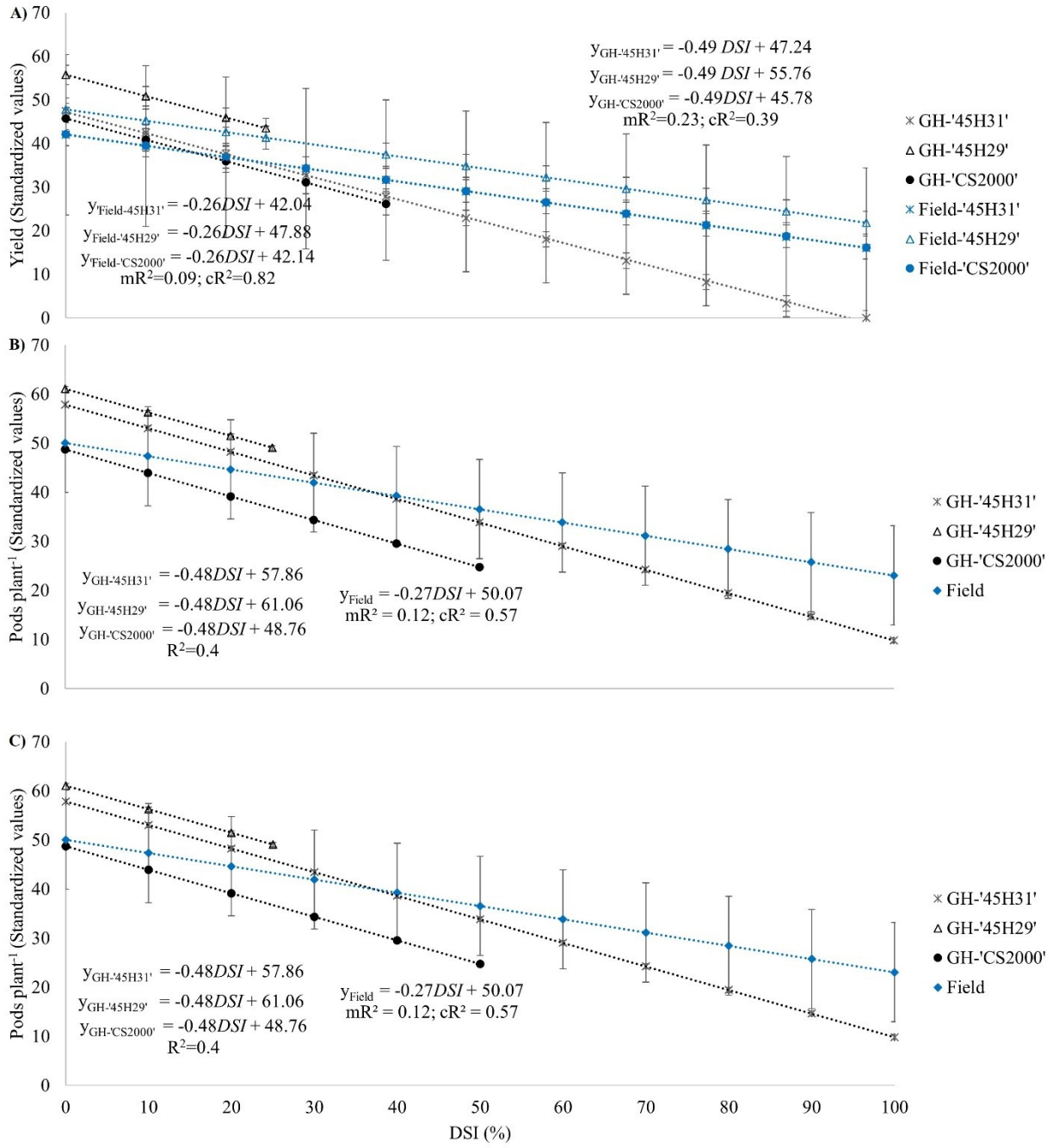


**Figure 3-3.** Relationship between clubroot disease severity index (DSI) and yield (A), number of pods per plant (B), and 1000-grain weight (C), standardized with a min-max normalization, in three canola cultivars '45H31', '45H29, and 'CS2000' under field (blue lines) and greenhouse (GH) conditions (black lines). The lines represent the regression of the most parsimonious models that estimate the effect of the DSI and the canola cultivar on the min-max standardized values of yield, pods per plant, and 1000-grain weight. $cR^2$ is the conditional $R^2$, and $mR^2$ is the marginal $R^2$ estimated from the linear mixed effect models. Error bars represent the standard error of the mean.

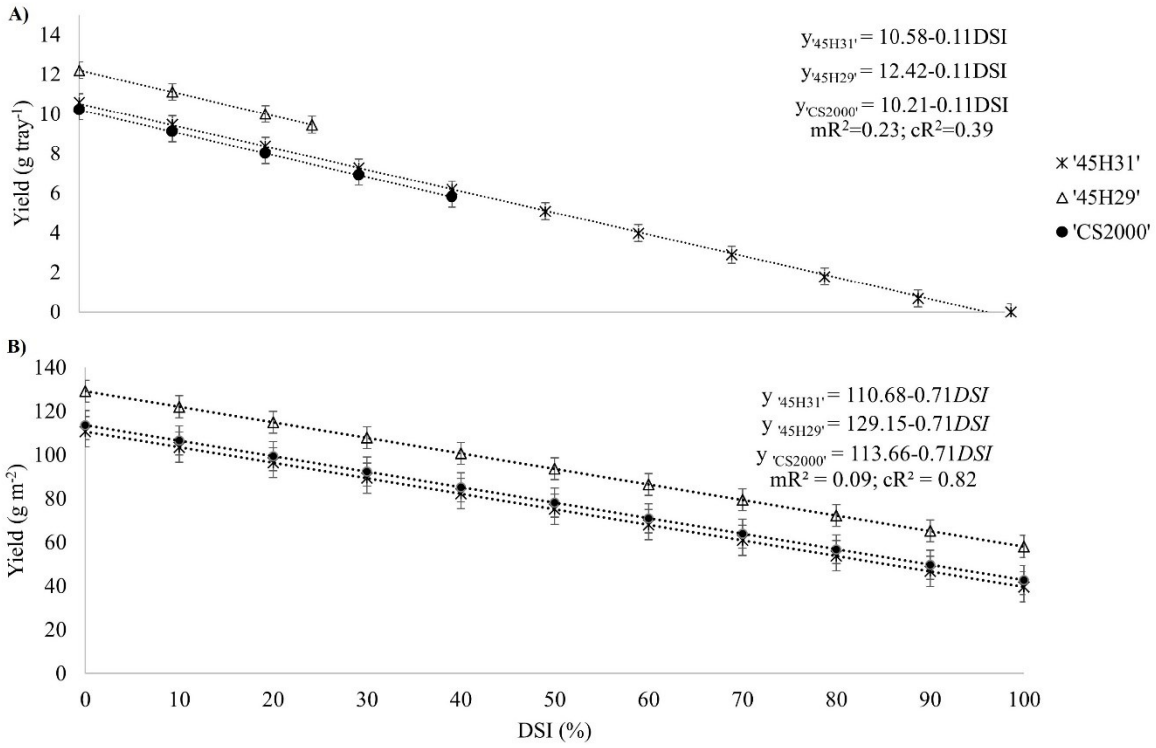


**Figure 3-4.** Relationship between clubroot disease severity index (DSI) and yield in three canola cultivars '45H31', '45H29, and 'CS2000' under greenhouse (A) and field (B) conditions. The lines represent the regression of the most parsimonious models that estimate the effect of the DSI and canola cultivar on yield. $cR^2$ is the conditional $R^2$, and $mR^2$ is the marginal $R^2$ estimated from the linear mixed effect models. Error bars represent the standard error of the mean.

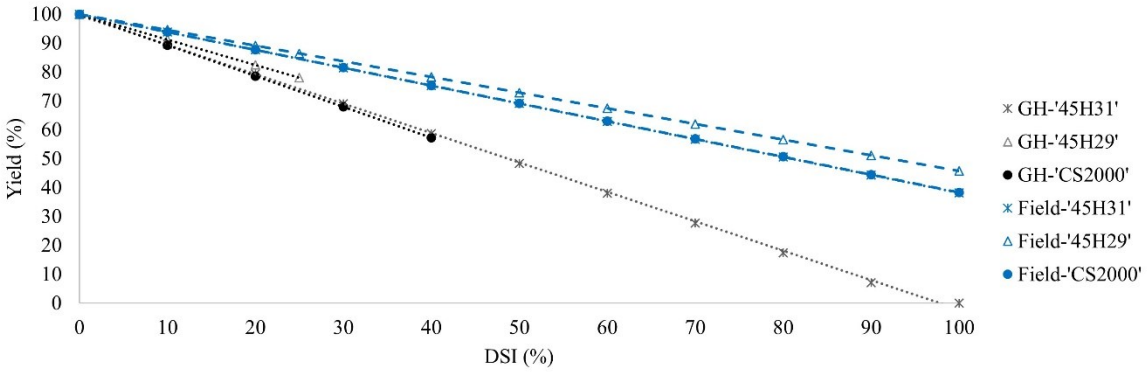


**Figure 3-5.** Average yield losses caused by clubroot in the canola cultivars '45H31', '45H29, and 'CS2000' under field (blue lines) and greenhouse (GH) conditions (black lines) with increasing disease severity index (DSI). Yield losses were calculated using the parsimonious linear mixed models for the greenhouse and field experiments that evaluated the effect of DSI on the yield of the cultivars. Both models were estimated using min-max standardized yield.

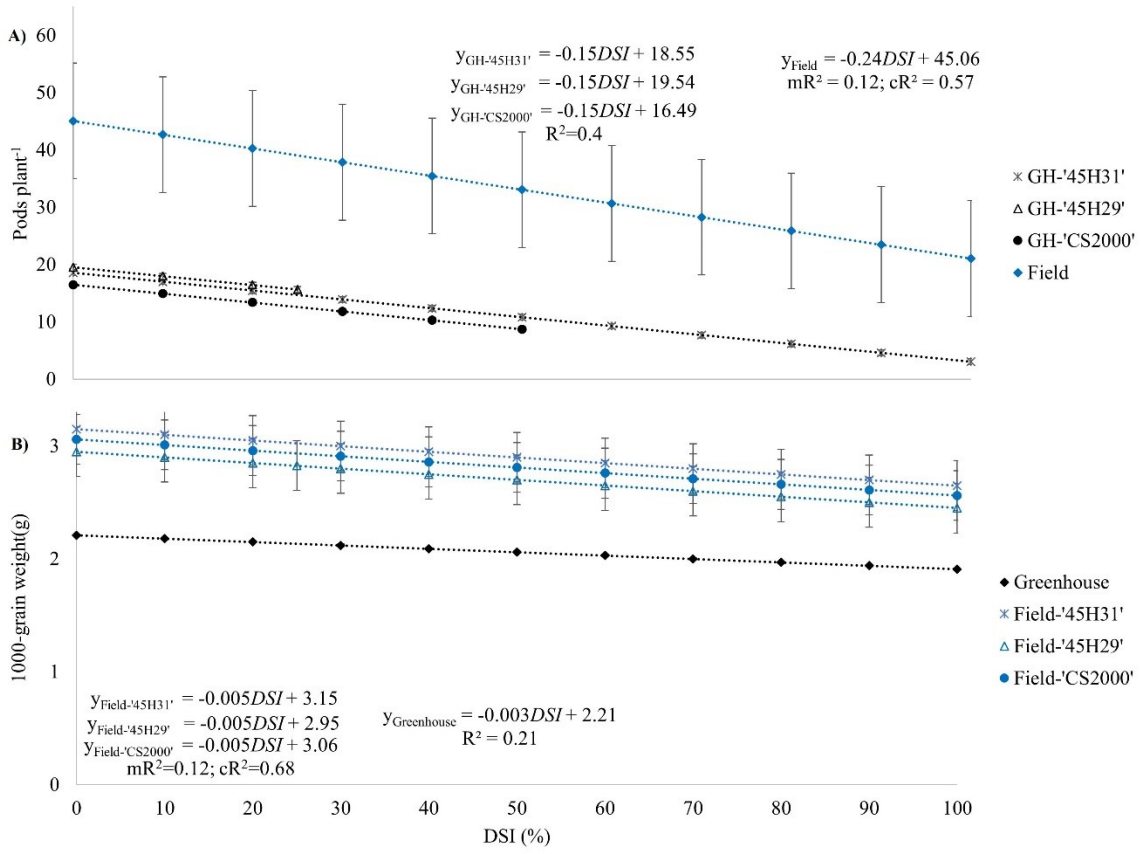


**Figure 3-6.** Relationship between clubroot disease severity index (DSI) and number of pods per plant (A), and 1000-grain weight (B) in three canola cultivars '45H31', '45H29, and 'CS2000' under field (blue lines) and greenhouse (GH) conditions (black lines). The lines represent the regression of the most parsimonious models that estimate the effect of clubroot DSI and canola cultivar on the number of pods per plant and 1000-grain weight. $cR^2$ is the conditional $R^2$, and $mR^2$ is the marginal $R^2$ estimated from the linear mixed effect models. Error bars represent the standard error of the mean.

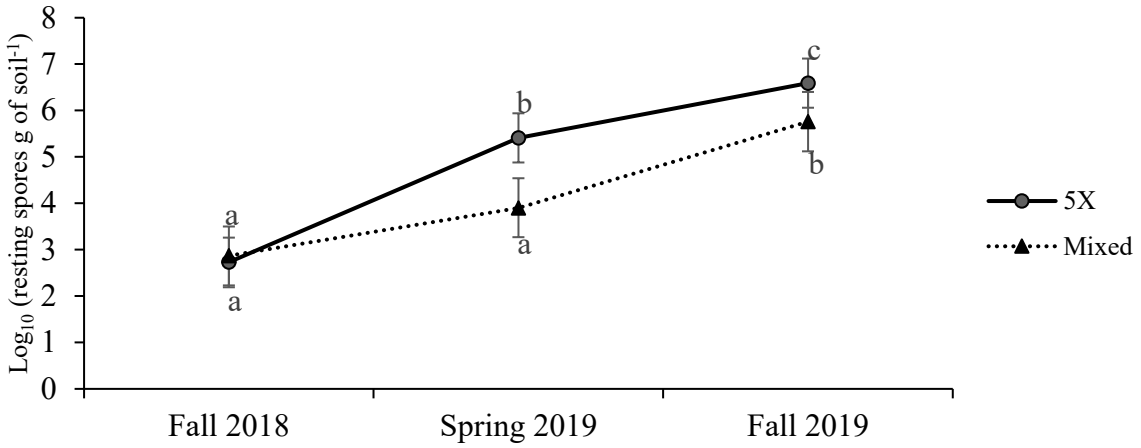


**Figure 3-7.** Concentration of *Plasmodiophora brassicae* resting spores in soil from nurseries at the Crop Diversification Centre North, Edmonton, Alberta, inoculated with pathotype 5X (solid line) or a mixture of pathotypes 5X and 3H (dotted line). Each point represents the adjusted mean of the inoculum density across 48 plots in October 2018 (Fall 2018), June 2019 (Spring 2019), and October 2019 (Fall 2019). Points with different lower-case letters show differences among inoculum densities quantified in different seasons according to Tukey's test at $p > 0.05$. Error bars represent the standard error of the mean.

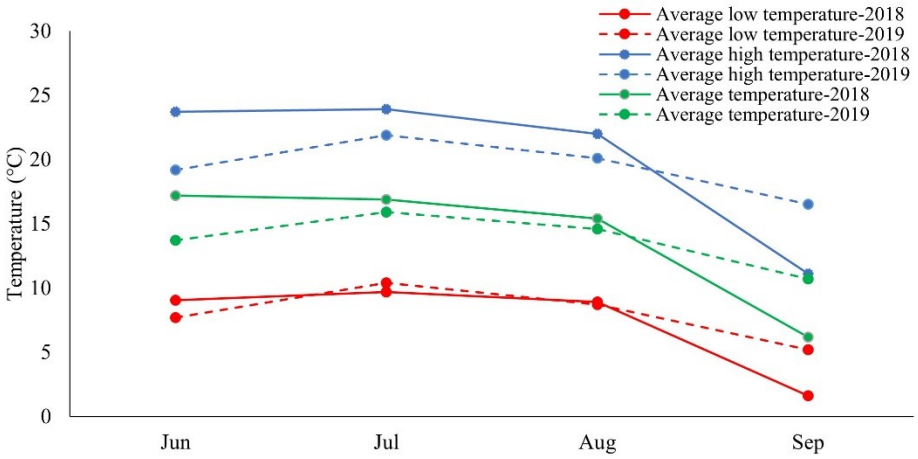


**Figure 3-8.** Average monthly air temperature from 13 June 2018 to 31 September 2018, and from 3 June 2019 to 31 September 2019, according to data collected from the weather station Oliver AGDM (ID 3014921, coordinates: 53°38'36.7" N, 113°21'16.6" W), in Edmonton, Alberta. The monthly average air temperature is shown in green, the monthly average highest air temperature is shown in blue, and the monthly average lowest air temperature is shown in red. Solid lines represent the data recorded in 2018, while dotted lines represent the data recorded in 2019.

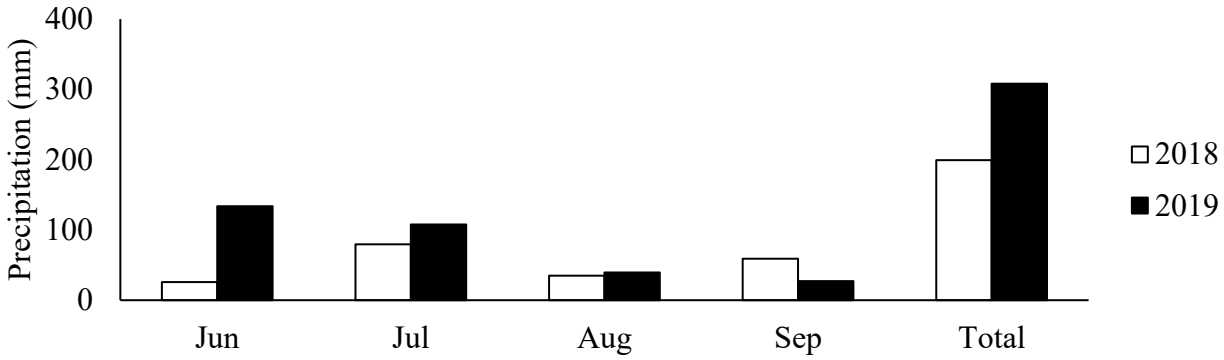


**Figure 3-9.** Accumulated monthly precipitation from 13 June 2018 to 31 September 2018, and from 3 June 2019 to 31 September 2019, according to data collected from the weather station Oliver AGDM (ID 3014921, coordinates: 53°38'36.7" N, 113°21'16.6" W), in Edmonton, Alberta. White bars represent the monthly accumulated precipitation for 2018, black bars represent the monthly accumulated precipitation for 2019.

# Chapter 4 *Plasmodiophora brassicae* inoculum density and spatial patterns at the field level and relationship to soil-related characteristics

## 4.1 Introduction

Canola (*Brassica napus* L.) is one of the most important field crops in Canada, contributing C$26.5 billion annually to the national economy (LMC international, 2016). In 2019, the crop was grown on approximately 8.6 million hectares, mostly in the western provinces of Alberta, Saskatchewan and Manitoba (Statistics Canada, 2019). The sustainable production of canola is threatened, however, by the increasing prevalence of *Plasmodiophora brassicae* Wor., a soilborne parasite that causes clubroot disease of crucifers. Clubroot development is associated with a deformation of the host root system, resulting in major yield and quality losses as water and nutrient uptake from the soil is reduced (Tewari et al., 2005; Pageau et al., 2006). In western Canada, yield losses as high as 30-100% have been reported in severely infected canola crops (Tewari et al., 2005).

The pathogen survives in the soil as long-lived resting spores, which under moist conditions and temperatures of 15-30°C germinate to release primary zoospores (Hamilton and Crête, 1978; Takahashi, 1994; Gossen et al., 2012). Germination of the resting spores is enhanced by the presence of host root exudates (Friberg et al., 2005; Rashid et al., 2013). The primary zoospores infect host root hairs, forming primary plasmodia from which secondary zoospores are released back into the soil. The secondary zoospores penetrate cortical root tissue and develop into intracellular secondary plasmodia, which eventually cleave to produce a new generation of resting spores (Kageyama and Asano, 2009). It has been calculated that between $1 \times 10^7$ and $1 \times 10^{10}$ resting spores per plant can be produced over a single infection cycle (Murakami et al., 2004; Hwang et al., 2013; Aigu et al., 2018). Visible symptoms of clubroot appear during cortical tissue

infection, when hyperplasia and hypertrophy result in formation of the root galls (Kageyama and Asano, 2009). One of the major challenges associated with clubroot management is the persistence of *P. brassicae* resting spores in the soil. The half-life of resting spores is around 4 years (Wallenhammar, 1996; Hwang et al., 2013), although some may survive for up to 17 years (Wallenhammar, 1996). Recent studies from Canada have found that resting spore levels may decline by up to 90% after 2 years in the absence of a host crop, and that only a subset of the spores persist for much longer periods (Peng et al., 2015; Ernst et al., 2019), with the pattern resembling a Type III survivorship curve (Rauschert, 2010).

As a soilborne pathogen, the physical and chemical conditions of the soil affect *P. brassicae* survival and infectivity, and therefore, clubroot development (Dixon, 2009a). Alkaline soils tend to be less favorable for clubroot and are associated with milder levels of the disease (Donald et al., 2004; Tremblay et al., 2005; Niwa et al., 2007). This effect appears to result from reduced germination of the *P. brassicae* resting spores, declines in root hair infection, and inhibition of maturation of plasmodia, sporangia, and zoosporangia (Macfarlane, 1958; Webster and Dixon, 1991b; Shinoda et al., 2006; Niwa et al., 2008; Rashid et al., 2013). As such, liming of the soil to increase alkalinity has often been suggested as a clubroot management strategy, particularly for vegetable Brassicas grown over smaller areas (Tremblay et al., 2005; Gossen et al., 2013b). Nonetheless, it has also been reported that acidic soils can negatively affect *P. brassicae* resting spore survival, likely due to stimulation of resting spore germination in absence of a host (Macfarlane, 1952; Friberg et al., 2005), possibly resulting in a more rapid decline in inoculum levels.

High concentrations of nutrients such as boron, calcium, and magnesium also have been reported to reduce clubroot, but the effective quantities of those cations appear to be inversely

related to soil pH (Fletcher et al., 1982; Myers and Campbell, 1985; Webster and Dixon, 1991a; Donald and Porter, 2004; Niwa et al., 2007; Ruaro et al., 2010). Reductions in clubroot under high boron, calcium or magnesium concentrations have been attributed mainly to a decrease in the maturation of the primary plasmodia, which prevents the release of secondary zoospores (Myers and Campbell, 1985; Webster and Dixon, 1991b, 1991a). Furthermore, the effect of calcium on clubroot has been ascribed to reduced resting spore germination and the inhibition of sporangial development and dehiscence (Webster and Dixon, 1991b; Niwa et al., 2008), as well as to its involvement in the induction of defense-related compounds and *P. brassicae*-induced cell death in the host (Takahashi et al., 2002, 2006). Boron diminishes clubroot by suppressing or delaying primary infection and cortical colonization (Webster and Dixon, 1991a; Deora et al., 2011), but its effects have been reported to be erratic and highly dependent on the soil type and the dosage, with phytotoxicity a major risk (Deora et al., 2011, 2014).

The impact of soil properties on *P. brassica*e and clubroot development are dependent on soil inoculum density, and consequently under high inoculum concentrations, their effect is hidden (Webster and Dixon, 1991b, 1991a). As a result, it has been proposed that the longevity and viability of *P. brassicae* inoculum is determined by soil type, pH, ion concentration and host susceptibility, which ultimately influence pathogen inoculum density at a particular site (Dixon, 2009a). Studies directly addressing the relationship between chemical soil properties and inoculum density of *P. brassicae* are, however, scarce. The development and implementation of improved practices for clubroot management require a better understanding of the epidemiology of this disease (Savary and Cooke, 2006; Kranz, 2012). From a practical perspective, spatial epidemiological studies can help to enhance knowledge of pathogen biology and ecology, which

is essential for refined and more effective sampling methods and disease management programs (Nicot et al., 1984; Campbell and Noe, 1985; Chellemi, 1988; Rekah et al., 1999; Kranz, 2012).

Soilborne diseases are characterized by aggregated spatial patterns, with dynamic patches that vary in size over time, and which may reflect local soil conditions that favour the disease or inhibit crop growth (Campbell and Noe, 1985; Gilligan, 1995; Madden et al., 2007). Observations of in-field clubroot spatial patterns have indicated that disease incidence is higher at the field entrances (Cao et al., 2009; Řičařová et al., 2017) or field margins (Wallenhammar et al., 2012; Vojtěch et al., 2017), suggesting that disease spread occurs mainly through the movement of infested soil on farm machinery (Cao et al., 2009). However, while it is widely known that clubroot tends to have a patchy spatial pattern (Cao et al., 2009; Wallenhammar et al., 2012; Řičařová et al., 2017), detailed evaluations of within field spread over time or the effect of soil properties on inoculum density have not been conducted.

While maps can provide intuitive and rapid summaries of complex spatial patterns, statistical analyses are important in relating these patterns to disease dynamics (Campbell and Noe, 1985; Real and McElhany, 1996; Elliott and Wartenberg, 2004). Spatial autocorrelation coefficients such as Moran's *I* are useful to measure the magnitude, intensity, and extent of spatial patterns (El-Shaarawi and Piegorsch, 2002; Madden et al., 2007). Moreover, geostatistics (by the estimation of sample and fitted semivariograms) have proven helpful in quantifying the direction, degree, and range of the spatial dependency of variables. In plant pathology, semivariograms have been used to quantitatively characterize the spatio-temporal dynamics of plant diseases (Rekah et al., 1999; Musoli et al., 2008). On the other hand, an understanding of the ecological processes and interactions that generate spatial patterns involves the estimation of statistical models able to account for spatio-temporal variation and correlation, enabling reliable inferences regarding the

process under study (Arab, 2015; Blangiardo and Cameletti, 2015; Souris, 2019b). Bayesian hierarchical modeling represents a good approach to account for variability in data sampling, spatio-temporal autocorrelation and parameter uncertainty, by partitioning complex problems into data model(s), process model(s) and parameter models (Arab, 2015; Zuur et al., 2017).

The current study had two main objectives: i) to identify and describe the spatial patterns of *P. brassicae* inoculum density and their temporal variation in four clubroot-infested fields in Alberta, Canada, where canola is regularly grown; and ii) to evaluate the effect of soil pH, boron, calcium, and magnesium concentration on the pathogen inoculum density. Knowledge of the spatial dynamics of *P. brassicae* inoculum and the influence of soil properties on these dynamics can be useful to improve understanding of clubroot epidemiology and management.

## 4.2 Materials and Methods

### 4.2.1 Soil sampling

Four clubroot-infested fields located in Sturgeon and Westlock counties in central Alberta (Figure 4-1), Canada, near the center of the clubroot outbreak (Strelkov and Hwang, 2014), were selected for this study. Fields 1 and 2, in Sturgeon County, had an area of 37.6 ha and 37.2 ha, respectively; Fields 3 and 4, in Westlock County, had an area of 34.6 ha and 39.2 ha, respectively. The soil in Fields 1, 2 and 4 was an Eluviated Black Chernozem, while Field 3 included three different soil types, approximately 58.7% of the field was a Gray Solodized Solonetz, 31.9% was an Eluviated Black Chernozem and the remaining 9.4% was an Orthic Humic Gleysol (Alberta Agriculture and Rural Development, 2015). The crop rotation from 2017 to 2019 included canola-wheat-peas (Field 1), wheat-canola-wheat (Field 2), canola-barley-canola (Field 3) and barley-canola-oats (Field 4).

Soil sampling in each field was conducted in Oct. 2017 and Oct. 2019 (Table 4-1). In 2017, each field was sampled extensively in a regular grid pattern (80 m × 80 m) (Figure 4-2), with approximately 500 g of soil collected at each node of the grids. Soils were sampled to a depth of 15-cm using a small shovel and placed individually in paper bags. All sampling locations were georeferenced with a smartphone and the geocoordinates were recorded using the mobile application MapIt Spatial (Mapit GIS Ltd, 2017). Ninety-nine samples were collected from Field 1, 97 from Field 2 and 100 from each of Fields 3 and 4 (Table 4-1). In 2019, sampling in Fields 1, 2 and 3 was intensified around the field entrances and points that had tested positive for *P. brassicae* in 2017 (Figure 4-3). However, since the pathogen inoculum was more widespread in Field 4, the sampling strategy for this field was kept the same as in 2017. Eighty-six samples were collected in Field 1, 81 in Field 2, 76 in Field 3, and 100 in Field 4 in 2019 (Table 4-1).

After collection, the soil samples were air-dried and stored at 4°C until processing. All soil samples were ground and homogenized in a mortar with a pestle or in a commercial spice grinder WSG 60 (Waring commercial, Stamford, CT), which were washed with ethanol between samples. Three subsamples were taken from each homogenized soil sample, including 0.25 g for DNA extraction, 10 g for pH measurement and 200 g for nutrient quantification, as described below.

### 4.2.2 Soil chemical properties

Soil pH was measured in all samples collected in 2017 and 2019 using a commercial pH meter Orion STAR A111 (ThermoScientific, Walthman, MA) with an Orion 8172BNWP Ross Sure-Flow pH electrode (ThermoScientific). Soil samples were homogenized with distilled water in solution at a 1:1 (w/w) ratio, agitated for 30 min in an oscillating table and left to settle for 30 min. Prior to taking measurements, the pH meter was calibrated with pH 4 and 7 buffer solutions.

Available soil boron, calcium, and magnesium were measured in half of the samples collected in 2017, selected from each field to maintain a regular 160 m × 160 m grid. Quantification of soil nutrients was conducted by Exova Canada Inc., Edmonton, Alberta. Calcium and magnesium were extracted by the ammonium acetate method, while boron was extracted via the hot water method (Pansu and Gautheyrou, 2006). The nutrients were quantified by inductively coupled plasma optical emission spectrometry (OCP-OES).

### 4.2.3 Presence and quantity of *P. brassicae* in the soil samples

Genomic DNA was extracted from 0.25 g of each soil sample using a DNeasy PowerSoil Kit (Qiagen, Germantown, MD) following the manufacturer's instructions. The concentration and purity of the DNA were evaluated with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Subsequently, the DNA was diluted using nuclease-free water to a concentration of 2 ng $\mu L^{-1}$ for conventional PCR or diluted 10-fold for quantitative PCR (qPCR) analysis.

Conventional PCR was conducted following Cao et al. (2007) with the primers TC1F and TC1R. All amplifications were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster city, CA). Positive controls included 10 ng of *P. brassicae* DNA as a template, while 5 µL of nuclease-free water was substituted in place of the template in the negative controls. Amplicons were resolved on 2% agarose gels stained with 1X SYBR Safe (Invitrogen, Carlsbad, CA). All samples that tested positive for the presence of *P. brassicae* DNA, along with all adjacent samples from the field (regardless of conventional PCR result), were evaluated further by qPCR analysis (Table 4-2). Quantification of the *P. brassicae* inoculum level in the soil samples was conducted by qPCR with the primers DR1F and DR1R as per Rennie et al. (2011) in a StepOnePlus Real

Time PCR System (Applied Biosystems, Foster City, CA). Estimation of the number of resting spores per sample was completed by comparison with a standard curve generated with DNA extracted from known quantities of resting spores (Rennie et al., 2011). After each qPCR run, a melting point analysis to identify the amplified product was conducted.

### 4.2.4 Prevailing wind direction

Information regarding the prevailing wind direction from Apr. 2017 to Oct. 2019 was obtained from the Alberta Climate Information Service (Alberta Agriculture and Forestry, 2020). The data were collected from weather stations surrounding the sampled fields: Busby AGCM station (ID 3010979, coordinates: 53.9309, -113.9216), St. Albert Research (ID 3025750, coordinates: 53.6920, -113.6196), Legal AGCM (ID 3013790, coordinates: 54.0030, -113.4744), Dapp AGDM (ID 3061975, coordinates: 54.3290, -113.9345), and Barrhead CS (ID 3060535, coordinates: 54.1000, -114.4500).

### 4.2.5 Spatial analysis

#### 4.2.5.1 Spatial autocorrelation

Statistical analyses were performed with *R* (R Core Team, 2019). Inoculum density was log transformed prior to analysis since the distribution of this variable was highly skewed to the left (Blangiardo and Cameletti, 2015). Spatial autocorrelation in the *P. brassicae* inoculum density was evaluated by Moran's *I*, estimated using the package *spdep* (Bivand and Wong, 2018). Moran's *I* is a classic correlation index that ranges from -1 to 1, the absolute value of which increases as the autocorrelation does; values near 0 indicate an absence of spatial autocorrelation, positive values indicate positive autocorrelation, and negative values indicate negative autocorrelation (Souris, 2019a).

Experimental semivariograms for each field in both sampling years were generated by plotting semivariance versus lag distance (distance between pairs at which the semivariogram is calculated) with the package *gstat* (Pebesma, 2004). The presence or absence of anisotropic patterns was determined by examination of the semivariograms at 0°, 22.5°, 45°, 67.5°, 90°, 112.5°, 135° and 157.5°, where 0° represents the N direction. Semivariograms represent the average squared differences in values between pairs of samples, and include statistical information on the observed differences between values, depending on the distance between individuals (Souris, 2019a). Semivariance values that increase with the lag distance indicate spatially dependent samples (Larkin et al., 1995). Afterwards, a spherical model was fitted to each sample semivariogram using the least square approach of Cressie (1993). Fitted semivariograms allow for the description of spatial patterns through the estimation of the autocorrelation parameters: (i) the spatial range, which indicates the maximum distance at which spatial autocorrelation is present and can be regarded as an index of the average patch diameter (Perry et al., 2002); (ii) the nugget ($C_0$), which is the estimate of the error in the measurements and environmental variability; and (iii) the sill ($C_0+C$), which quantifies the spatial pattern intensity (El-Shaarawi and Piegorsch, 2002).

#### 4.2.5.2 Models

A Bayesian hierarchical spatial approach was used to model the relationship between *P. brassicae* inoculum density and soil pH, and concentration of boron, calcium, and magnesium in the soil. In this approach, a stochastic spatial effect was added to a generalized linear model. Hurdle models (also known as Zero-Altered Poisson models) were fitted since the data presented a problem of zero-inflation, and we aimed to identify the possible effect of each covariate on the presence/absence of the pathogen and, when it was present, on the number of *P. brassicae* resting spores (Zuur and Ieeno, 2018). Misalignment of soil nutrient concentrations and resting spore

numbers occurred, since boron, calcium, and magnesium were quantified in half of the points where *P. brassicae* inoculum was quantified; therefore, spatial variation in the nutrient concentration and the number of resting spores were modelled jointly (Krainski et al., 2018). Posterior distributions for model parameters were approximated with the Integrated Nested Laplace Approximation using the package *INLA* (Rue et al., 2009). The fitted model can be written as:

$$RS_i \sim ZAP(\mu_i, \pi_i) \quad (1)$$
$$E(RS_i) = \pi(\frac{\mu_i}{1-e^{-\mu^i}}) \quad (2)$$
$$var(RS_i) = \frac{\pi_i}{1-e^{-\mu^i}}(\mu_i + \mu_i^2) - (\frac{\mu_i \pi_i}{1-e^{-\mu^i}})^2 \quad (3)$$
$$log(\mu_i) = \beta_1 + \beta_2 pH_i + \beta_3 Ca_i + \beta_4 B_i + \beta_5 Mg_i + u_i \quad (4)$$
$$logit(\pi_i) = \gamma_1 + \gamma_2 pH_i + \gamma_3 Ca_i + \gamma_4 B_i + \gamma_5 Mg_i + v_i \quad (5)$$

Where, $RS_i$ is the logarithm of the number of *P. brassicae* resting spores per g of soil; $ZAP(\mu_i, \pi_i)$ corresponds to a Zero-Altered Poisson distribution with parameters $\mu_i$ and $\pi_i$; $\mu_i$ is the population mean and $\pi_i$ is the probability of the presence of *P. brassicae*; $\beta_1$ is the intercept for the count (Poisson) component of the model; $\gamma_1$ is the intercept for the binary component of the model (presence/absence of *P. brassicae*); $\beta_{2,3,4,5}$ are the estimators for each covariate in the count part of the model; $\gamma_{2,3,4,5}$ are the estimators for each covariate in the binary part of the model; $pH$ is the soil pH; $B, Ca$ and $Mg$ are the concentrations (mg $kg^{-1}$) of boron, calcium and magnesium in the soil; and $u_i$ and $v_i$ are spatially correlated random effects in the count and binary parts of the model, respectively. The spatial terms $u_i$ and $v_i$ were assumed to have a multivariate Gaussian distribution whose covariance matrix depended on the distance between locations. The spatial fields were resolved with the explicit link between Gaussian Markov random fields and continuous Gaussian fields with a Matérn covariance structure via a weak solution to a stochastic partial differential equation (SPDE) (Lindgren et al., 2011).

Models including all possible combinations among covariates were compared using the Watanable Akaike Information Criterion (WAIC) and the Deviance Information Criterion (DIC) (Zuur and Ieeno, 2018). Hypothesis testing was conducted by checking whether zero fell within the 95% Bayesian credibility interval (CI) of the parameter estimators (Wang et al., 2018). If zero fell in the CI, the null hypothesis was accepted, and therefore, the covariate was not assumed to have an important effect on *P. brassicae* inoculum density. Prediction surfaces of the mean of the posterior distribution of *P. brassicae* inoculum density were mapped using the packages *ggplot2* (Wickham, 2016) and *ggmap* (Kahle and Wickham, 2013). Additional modeling was conducted using the *nlme* package (Pinheiro et al., 2019) to test, by a frequentist approach, the effect of maximum inoculum density and rotation scheme on clubroot patch diameter. Results from both the frequentist and Bayesian approach were compared.

## 4.3 Results

### 4.3.1 Inoculum density

Between 53% and 99% of the soil samples tested negative for the presence of *P. brassicae* (Table 4-3). The maximum inoculum density observed differed among fields, ranging from $1.7 \times 10^3$ resting spores/g soil in Field 3 (2017) to $3.2 \times 10^7$ resting spores/g soil in Field 4 (2019) (Table 4-4). The percentage of positive samples as well as the maximum inoculum density increased from 2017 to 2019, which at first glance suggested an expansion of the infested area in all fields from one year to the other. Maximum inoculum density variation was within the same order of magnitude in Fields 1 and 2, but increased by two orders of magnitude in Fields 3 and 4 between 2017 and 2019 (Table 4-4).

### 4.3.2 Soil chemical properties

All fields had acidic soil, with mean pH values between 5.03 and 6.23. Mean pH in Fields 1, 2, 3, and 4 was 5.34, 5.03, 5.49, and 5.83 in 2017, respectively. In 2019, these increased to 5.96, 5.46, 5.83 and 6.23 (Table 4-5). Within field variation was observed in all fields. The difference between the maximum and minimum pH in Fields 1 and 4 was 2.0 in 2017 and 2.2 in 2019. In 2017, Field 3 had the same variation range as Fields 1 and 4, but in 2019, a larger difference was observed (2.9). The lowest variation was found in Field 2, where the differences were 1.5 and 1.2 in 2017 and 2019, respectively (Table 4-5).

Calcium, boron, and magnesium varied among fields. Mean calcium concentration in Fields 1, 2, 3, and 4 was 4648, 4129, 3853, and 4247 mg $kg^{-1}$, respectively. Mean boron concentration was 1.97 (Field 1), 2.22 (Field 2), 1.52 (Field 3), and 2.34 mg $kg^{-1}$ (Field 4), respectively, while mean magnesium concentration was 756.8 (Field 1), 477.6 (Field 2), 374.1 (Field 3), and 319.7 mg $kg^{-1}$ (Field 4), respectively. Within field variation was also observed (Table 4-6).

### 4.3.3 Spatial patterns

Soil samples that tested positive for the presence of *P. brassicae* were located mostly at the field edges (the field edge was considered to be 10 m from the most external edge of cultivated soil) and/or adjacent to the entrance (Figure 4-4). Nonetheless, there was some variation in each field. For example, in Field 1 in 2017, *P. brassicae* was detected only in one sample on the southern border of the field (Figure 4-4A), while in 2019, most of the positive samples were on the western edge closer to the entrance (Figure 4-4B). In Field 2, most of the positive soil samples were identified along the northern edge, with a few positives also found along the eastern side of the field, in both 2017 and 2019 (Figures 4-4C and 4-4D). In Field 3, only one *P. brassicae*-infested

soil sample was identified on the western edge of the field near the entrance; this patch grew in size between 2017 and 2019 (Figures 4-4E and 4-4F). In Field 4 in 2017, most positive samples were towards the southern edge of the field (Figure 4-4G), while in 2019 more positive samples were detected at the center of the field (Figure 4-4H).

As only one positive sample was detected in Fields 1 and 3 in 2017, it was not possible to detect spatial autocorrelation with Moran's *I* or with the semivariograms. The low number of positive samples also impeded the adjustment of any model to evaluate the relationship between the density of *P. brassicae* inoculum and soil properties. Therefore, for the purposes of analysis, to draw conclusions about patch growth in Fields 1 and 3, half of the distance between sampling points was regarded as the range size (40 m) for 2017. This was taken as the range size since only one sample tested positive for the presence *P. brassicae*, and it is likely that if samples would have been taken less than 80 m apart, the pathogen could have been detected, allowing to measure more accurately the patch size in those fields. In Field 1, Moran's *I* was not significant in 2019; it also was not significant in Field 4 in 2017. A small positive spatial autocorrelation was detected in Field 2 in both years and in Fields 3 and 4 in 2019. Moran's *I* for Field 2 was 0.05 in 2017 and 0.18 in 2019, while for Fields 3 and 4 it was 0.05 and 0.06, respectively, in 2019. Positive values for this index suggest aggregation of the inoculum density to some extent, but further confirmation was required since values were close to zero.

Fitted semivariograms indicated spatial autocorrelation in all fields with a small nugget effect (error). Structural variance $(C/(C_0+C))$ ranged from 77% to 100%, suggesting high spatial dependency of the inoculum density in all of the fields. In Fields 2 and 4, where semivariograms for both years were fitted, the slope of the semivariogram curve in the spatial range was higher in 2019 than in 2017, indicating an increase in the spatial autocorrelation. The patch size, measured

by the spatial range, changed between years in all of the fields, with average patch growth of 221.3 m (Table 4-7). Patch growth in Field 3 (37.7 m) was lower than in Fields 1, 2 and 4. In Fields 1 and 2, patch growth was 249.3 m and 288.8 m, respectively. The greatest increase in patch size was observed in Field 4, with a patch growth of 309.3 m between 2017 and 2019.

Statistical analysis indicated a positive effect of the maximum inoculum density observed on the patch size ($p = 0.015$); in contrast, patch size was not significantly affected by the number of years when canola was grown in 2017, 2018 and 2019 ($p = 0.308$). Isotropic spread of the pathogen was observed only in Field 3. Anisotropy in Field 1 was observed at 45° (W direction). In Fields 2 and 4, it was predominant at 157.5° (NW direction), although some anisotropy was observed in Field 2 in 2019 at 90° (S direction) and in Field 4 in 2017 at 22.5° (NE direction).

Models to evaluate the effect of soil properties on inoculum density did not show an important effect of pH, boron, calcium, or magnesium on *P. brassicae* inoculum density. Means of the posterior distribution of pH, boron and calcium were erratic in all fields in both years, while for magnesium it was negative in all fields for both years, suggesting that although this nutrient is not a critical factor defining *P. brassicae* inoculum density, it may influence it to some extent.

**4.4 Discussion**

Maximum *P. brassicae* inoculum densities varied from $1.7 \times 10^3$ to $3.2 \times 10^7$ resting spores/g soil in the fields evaluated. These values are similar to inoculum densities previously observed in commercial fields in Alberta and Europe where canola or rapeseed, respectively, was grown (Wallenhammar et al., 2012; Řičařová et al., 2017; Vojtěch et al., 2017; Ernst et al., 2019). It has been reported that inoculum densities between $1 \times 10^3$ and $1 \times 10^5$ resting spores/g soil are sufficient to cause clubroot symptoms under field conditions (Faggian and Strelkov, 2009), and

that concentrations between $3 \times 10^3$ and $1.3 \times 10^5$ resting spores/g soil caused yield losses in susceptible hosts (Wallenhammar et al., 2012). In the current study, *P. brassicae* resting spore numbers, as opposed to clubroot severity, were used to assess inoculum density and spatial patterns, since spore numbers are not influenced by the resistance or growing conditions of the particular crop. Nonetheless, the infestation levels observed in some of the fields suggested that significant levels of clubroot would have developed on a susceptible canola crop.

A few studies have investigated the spatial patterns of *P. brassicae* at a field level, but (to our knowledge) none has evaluated changes in these patterns over time, nor their relationship with soil chemical properties. A patchy spatial pattern has been described for the pathogen in fields in Canada, Sweden and the Czech Republic, based on anecdotal observations, descriptive statistics (Cao et al., 2009; Wallenhammar et al., 2012) and Spatial Analysis by Distance Indices (SADIE) (Řičařová et al., 2017; Vojtěch et al., 2017). Clubroot also occurs more frequently at field entrances (Cao et al., 2009; Řičařová et al., 2017) and margins (Vojtěch et al., 2017). Similarly, in the current analysis, a patchy pattern also was found with respect to *P. brassicae* inoculum density in the evaluated fields, with most patches located at the field edges adjacent to the entrance. While this pattern was readily observable on the maps, based on the statistical analyses the spatial aggregation of *P. brassicae* inoculum was not as strong as expected.

The patchiness of the *P. brassicae* inoculum was confirmed with the semivariograms. In fields where the semivariograms were fitted in both years, a higher spatial autocorrelation was observed in 2019 compared with 2017. Greater spatial autocorrelation was caused mainly by an increase in the number of positive samples and larger patches, which produced an increase in the extent of aggregation between samples. This phenomenon has been described in tomato crown and root rot (*Fusarium oxysporum* f. sp. *radicis-lycopersici*) (Rekah et al., 1999), bell pepper crown and root

rot (*Phytophthora capsici*) (Chellemi, 1988), microsclerotia of *Macrophomina phaseolina* causing root and stalk rot (Campbell and Van der Gaag, 1993), chestnut ink disease (*Phytophthora cinnamomi* and *Phytophthora cambivora*) (Martins et al., 2007), and coffee wilt (*Fusarium xylarioides*) (Musoli et al., 2008). The limited number of soil samples that tested positive for the presence of *P. brassicae* in Fields 1 and 3 in 2017 impeded the identification of patchiness by Moran's *I*, since autocorrelation can be detected only on a larger scale than the sampling quadrat (Nicot et al., 1984; Campbell and Van der Gaag, 1993; Gottwald et al., 1998). This suggests that a lower lag distance should have been used to sample those fields during the first year to accurately detect aggregation.

In fields where Moran's *I* could be estimated, indices were slightly greater than zero, indicating a lack of aggregation of the inoculum density, as previously reported by Řičařová et al. (2017) when estimating spatial autocorrelation using SADIE indices. This does not mean a lack of spatial autocorrelation, but rather it may indicate the need for additional analysis using tools such as geostatistics, since different spatial processes can create bias in the estimation of spatial autocorrelation indices (Chellemi, 1988). Firstly, spatial aggregation indices are sensitive to extreme observations, especially over large lag distances (Campbell and Van der Gaag, 1993) and secondly, those indices assume equal spatial autocorrelation in all directions (isotropy). When spatial correlation shows anisotropy, the detection of patchiness via Moran's *I* may not be possible. Inconsistencies between Moran's *I* and the true spatial pattern were reported by Chellemi et al. (1988) in spatial analyses of *Phytophthora nicotianae* var. *parasitica* in pineapple, who suggested that the fact that inoculum was not aggregated equally in all directions could explain the discrepancies between the semivariograms and Moran's *I* results.

Patch diameters ranged between 40 m and 346.1 m in 2017, consistent with a previous report by Cao et al. (2009) who observed an average patch size of approximately 300 m. By 2019, the patch diameters had grown an average of 221.3 m, with diameters ranging from 77.7 m to 634.9 m. A positive relationship was identified between the maximum inoculum density and patch diameter. Collectively, the results are consistent with what was found for *M. phaseolina*, where patch growth occurred through a combination of the spread of pathogen propagules from existing infection foci and local differences in the inoculum density (Campbell and Van der Gaag, 1993).

Patch growth indicates within-field pathogen dispersal. While the main mechanism for *P. brassicae* spread between fields is the movement of infested soil on farming equipment (Cao et al., 2009), within-field dispersal mechanisms are less well understood. The active dispersal of soil microorganisms through the soil matrix is generally very limited (depending on the taxon, in the order of a few millimeters to centimeters per day), although passive dispersal over larger distances is possible via accidental or specialized animal vectors, water and wind (Ettema and Wardle, 2002). In the case of *P. brassicae*, active spread is restricted because zoospore motility is limited (Hwang et al., 2012b), while passive dispersal methods include movement in dust (Rennie et al., 2015), via water or water-mediated soil erosion (Datnoff et al., 1984), and as an external contaminant of seeds and tubers (Rennie et al., 2011). The current study indicated anisotropic movement of *P. brassicae* inoculum in all fields with the exception of Field 3, where the spatial patterns were isotropic. Changes in the direction of spread can help to identify the most important pathogen dispersal methods within a field (Larkin et al., 1995; Gottwald et al., 1998). For example, an analysis of the direction of peak winds from Apr. 2017 to Oct. 2019, as recorded in weather stations surrounding the sampled fields, indicated that wind speeds > 35 km/h were mostly oriented in the NW direction, corresponding to the anisotropic movement of the pathogen at 157.5°

NW in Fields 2 and 4. Those results support the suggestion of Rennie et al. (2015) that wind dispersal over short distances could expand resting *P. brassicae* infestations within an infested field or between immediately adjacent fields. Farming operations conducted with large equipment such as tractors and seeders also could contribute to within-field spread. The anisotropy detected at 45° in Field 1 could have reflected such operations, as was found for *Verticillium dahliae* (Xiao et al., 1997) and *M. phaseolina* (Olanya, 1988) in earlier reports.

One of the main difficulties when sampling soil pathogens is defining an appropriate sampling strategy able to capture variability between individual soil cores (Ophel-Keller et al., 2008). When defining a sampling strategy, different issues arise, among them the area to be sampled, the definition of the sampling unit, the timing of the sampling effort, and the size of sample required to answer the questions posed in relation to the pathogen populations (Hughes and Madden, 2002). In our research, different locations of *P. brassicae* patches were observed Field 1 in 2017 vs. 2019. Those results may reflect large variations in and a skewed distribution of the pathogen DNA between individual soil cores (Ophel-Keller et al., 2008). Therefore, although our sampling strategy was aimed at capturing most of the within-field variation in pathogen inoculum, the sampling intensity was not enough, especially in 2017. Previous results suggest that the choice of sampling strategy is one of the main challenges associated with accurately testing a field for the presence of this pathogen (Cao et al., 2007; Wallenhammar et al., 2012). Better and more rational sampling schemes require information on spatial patterns to evaluate both the pathogen and the disease (Xiao et al., 1997; Faggian and Strelkov, 2009). Previously proposed soil sampling strategies for the detection *P. brassicae* suggest the gathering of soil samples along a diagonal (Wallenhammar, 1996), or the collection of 40 subsamples in a ‘W’ transect that should be pooled in a composite sample and complemented with samples from high moisture areas, headlands and

the field entrance (Wallenhammar et al., 2012). Clubroot symptom-based sampling strategies propose evaluation for the presence of galled roots in at least 50 plants collected from a 20-30$m^2$ area near the field entrance; if symptoms are observed, further sampling is conducted along the field following a 'W' transect, whilst if no symptomatic plants are found, no further sampling is conducted (Strelkov et al., 2019). Although previous sampling strategies may be appropriate in many cases, based on the spatial patterns observed in this study, sampling should be more intense at the field edges, converging at the field entrance.

The mean calcium, boron and magnesium levels in the sampled fields were generally consistent with what has been reported as sufficient to support adequate crop development (Westerman, 1990; McKenzie, 1992; Canola Council of Canada, 2021). Calcium and magnesium levels were similar to the average concentrations in soils from the Canadian prairies (Canola Council of Canada, 2021), while boron concentrations were slightly higher than average (Karamanos et al., 2003). Regardless, soil pH, boron, calcium, and magnesium concentrations were not found to have an important effect on the inoculum density of *P. brassicae*. These results do not necessarily indicate that none of the soil chemical properties affect pathogen inoculum density, but rather suggest that other underlying spatial processes have a greater influence on spatial patterns. More specifically, the lack of an effect of pH on the pathogen inoculum density may reflect inconsistencies that have been reported in the relationship between pH and clubroot, since severe disease symptoms can still occur in alkaline soils under high spore loads and favorable moisture and temperature conditions (Colhoun, 1953; Fletcher et al., 1982; Myers and Campbell, 1985; Gossen et al., 2013b). Only weak negative correlations were found between soil pH and clubroot severity on canola in surveys of *P. brassicae*-infested fields in Alberta (Strelkov et al., 2007; Gossen et al., 2013b).

Deviations from uniform inoculum spatial patterns and disease levels may occur in homogeneous fields as a result of the temporal and spatial dynamics of the pathogen population (Gilligan, 1995). Distinction of the spatial aggregates as a result of the population interaction versus environmental heterogeneity have been recognized in plant pathology, and those two forms of infectious disease processes have been designated as 'truly contagious' or 'apparently contagious' processes (Campbell and Madden, 1990). In truly contagious processes, epidemics begin from a focal point by infection of a few, often randomly spaced, individuals, and the pathogen spread results mainly from the decomposition of infected host tissue that is randomly dispersed (Nicot et al., 1984). As a result, aggregation develops around the initial infection point due to the limited dispersal of the pathogen, which generates regions of high disease prevalence around the focal point (Zadoks and van den Bosch, 1994). On the other hand, in apparent contagion processes, the pathogen is uniformly dispersed and randomly connected to individuals across the network, and therefore, aggregation results from heterogeneity in the environment (Real and Biek, 2007). Since no effect of soil properties on the inoculum density of *P. brassicae* was observed in this study, a truly contagion process may explain the patchiness of the pathogen inoculum.

This study indicated that *P. brassicae* inoculum occurred mostly at the field margins, converging at the field entrance. Infested patches grew each year, with this growth determined by the maximum inoculum density and active dispersal methods. In the fields evaluated, wind and possible mechanical operations contributed to in-field dispersal of the pathogen. These results suggest that adoption of field sampling strategies based on the likely spatial patterns of the pathogen, with more intense sampling towards the field edges and scattered sampling at the center of the field, may be warranted. Nonetheless, further research is required to improve understanding

of the underlying processes determining *P. brassicae* spatial patterns, limit further spread of the pathogen, and optimize in-field management practices.

**Table 4-1.** Sampling dates, locations and number of samples collected in each of four fields sampled in central Alberta, Canada, in 2017 and 2019.

| Field | County | Date of sampling | Number of collected samples |
|---|---|---|---|
| Field 1 | Sturgeon County | October 12, 2017 | 99 |
| | | October 17, 2019 | 86 |
| Field 2 | Sturgeon County | October 12, 2017 | 97 |
| | | October 17, 2019 | 81 |
| Field 3 | Westlock County | October 13, 2017 | 100 |
| | | October 18, 2019 | 76 |
| Field 4 | Westlock County | October 13, 2017 | 100 |
| | | October 18, 2019 | 100 |

**Table 4-2**. Number of samples that tested positive for the presence of *Plasmodiophora brassicae* DNA by conventional PCR and the number of samples where the pathogen inoculum density was quantified by qPCR.

| Field | Year | Number of positive samples | Number of samples quantified for inoculum density |
|---|---|---|---|
| Field 1 | 2017 | 1 | 6 |
| | 2019 | 13 | 40 |
| Field 2 | 2017 | 23 | 45 |
| | 2019 | 38 | 54 |
| Field 3 | 2017 | 1 | 5 |
| | 2019 | 8 | 33 |
| Field 4 | 2017 | 28 | 53 |
| | 2019 | 47 | 65 |

**Table 4-3.** Number and percentage of soil samples that tested negative for the presence of *Plasmodiophora brassicae* DNA by conventional PCR. Soil samples were collected from four clubroot-infested fields located in Sturgeon and Westlock counties in central Alberta, Canada.

| Field/Year | 2017 | | | 2019 | | |
|---|---|---|---|---|---|---|
| | Number of samples collected | Number of negative samples | Percentage of negative samples | Number of samples collected | Number of negative samples | Percentage of negative samples |
| Field 1 | 99 | 98 | 99% | 86 | 73 | 85% |
| Field 2 | 97 | 74 | 76% | 81 | 43 | 53% |
| Field 3 | 100 | 99 | 99% | 76 | 68 | 89% |
| Field 4 | 100 | 72 | 72% | 100 | 53 | 53% |

**Table 4-4**. Minimum and maximum *Plasmodiophora brassicae* inoculum density quantified by qPCR in soil samples collected from four clubroot-infested fields located in Sturgeon and Westlock counties in central Alberta, Canada.

| Field/Year | 2017 | | 2019 | |
|---|---|---|---|---|
| | Minimum (resting spores/g soil) | Maximum (resting spores/g soil) | Minimum (resting spores/g soil) | Maximum (resting spores/g soil) |
| Field 1 | na* | $1.4 \times 10^5$ | $4.3 \times 10^3$ | $2.7 \times 10^5$ |
| Field 2 | $1.1 \times 10^4$ | $1.7 \times 10^7$ | $6.7 \times 10^3$ | $9.9 \times 10^6$ |
| Field 3 | na | $1.7 \times 10^3$ | $4.3 \times 10^3$ | $1.7 \times 10^5$ |
| Field 4 | $5.4 \times 10^3$ | $1 \times 10^5$ | $5 \times 10^3$ | $3.2 \times 10^7$ |

*na, not applicable.

**Table 4-5.** Minimum, maximum, and mean pH of soil samples collected from four clubroot-infested fields located in Sturgeon and Westlock counties in central Alberta, Canada. Soil pH was measured in a soil water: solution ratio 1:1 (w/w).

| pH | Year | Min | Max | Mean |
|---|---|---|---|---|
| Field 1 | 2017 | 4.7 | 6.72 | 5.34 |
| | 2019 | 4.94 | 7.14 | 5.96 |
| Field 2 | 2017 | 4.49 | 5.97 | 5.03 |
| | 2019 | 4.83 | 6.05 | 5.46 |
| Field 3 | 2017 | 4.64 | 6.67 | 5.49 |
| | 2019 | 4.45 | 7.32 | 5.83 |
| Field 4 | 2017 | 4.93 | 6.95 | 5.83 |
| | 2019 | 5.24 | 7.42 | 6.23 |

**Table 4-6.** Minimum, maximum, and mean concentration of calcium, boron, and magnesium in soil samples collected in 2017 from four clubroot-infested fields located in Sturgeon and Westlock counties in central Alberta, Canada.

| Field | Element | Min (mg $kg^{-1}$) | Max (mg $kg^{-1}$) | Mean (mg $kg^{-1}$) |
|---|---|---|---|---|
| Field 1 | Ca | 2990 | 6470 | 4648 |
| Field 2 | | 3440 | 5880 | 4129 |
| Field 3 | | 2090 | 5140 | 3853 |
| Field 4 | | 3780 | 6190 | 4247 |
| Field 1 | B | 0.97 | 3 | 1.969 |
| Field 2 | | 1.6 | 3 | 2.222 |
| Field 3 | | 0.79 | 2.7 | 1.515 |
| Field 4 | | 1.2 | 4.2 | 2.336 |
| Field 1 | Mg | 432 | 1200 | 756.8 |
| Field 2 | | 291 | 681 | 477.6 |
| Field 3 | | 214 | 513 | 374.1 |
| Field 4 | | 159 | 614 | 319.7 |

**Table 4-7.** Semivariogram parameters describing the spread of the log-transformed *Plasmodiophora brassicae* inoculum density in four clubroot-infested fields located in Sturgeon and Westlock counties in central Alberta, Canada.

| Field | Year | Nugget ($C_0$) | Sill ($C+C_0$) | $C/(C+C_0)$ | Range (m) |
|---|---|---|---|---|---|
| Field 1 | 2017* | NA | NA | NA | 40.0† |
| | 2019 | 0.01 | 0.20 | 0.96 | 289.2 |
| Field 2 | 2017 | 0.12 | 0.72 | 0.83 | 346.1 |
| | 2019 | 0.00 | 4.00 | 1.00 | 634.9 |
| Field 3 | 2017* | NA | NA | NA | 40.0† |
| | 2019 | 0.00 | 0.10 | 1.00 | 77.7 |
| Field 4 | 2017 | 0.00 | 0.912 | 1.00 | 113.6 |
| | 2019 | 0.58 | 2.49 | 0.77 | 422.9 |

*Identification of only one positive sample did not allow fitting of a semivariogram, and hence parameters are not presented.
†When only one positive sample was observed, half of the distance between sampling points was assumed as the range (40 m).

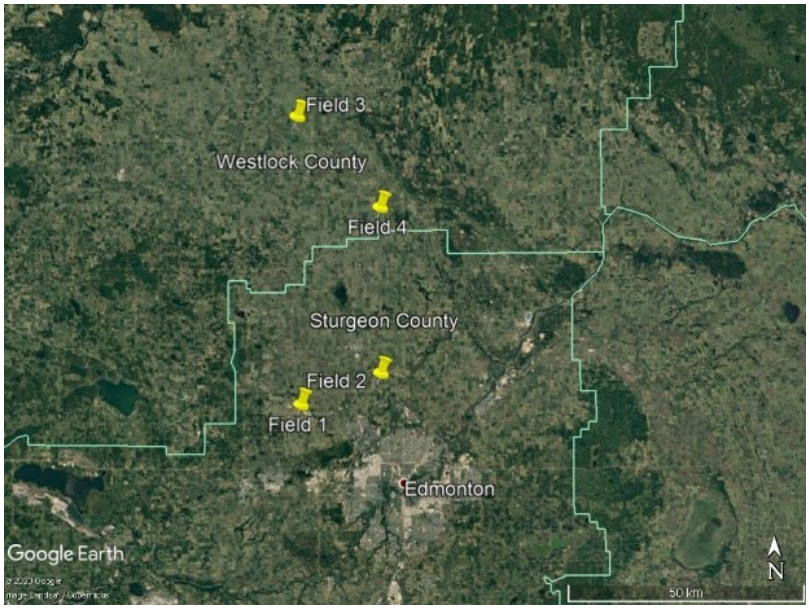


**Figure 4-1.** Location of fields tested for the presence of *Plasmodiophora brassicae* in 2017 and 2019 in Sturgeon and Westlock counties in central Alberta, Canada**.**

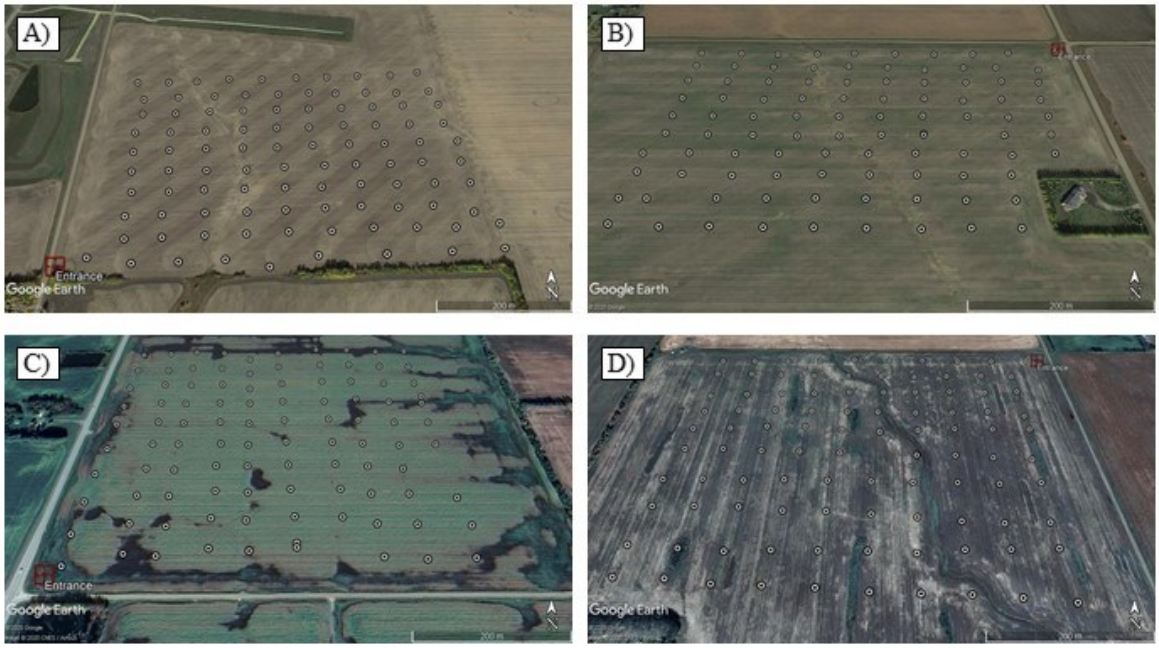


**Figure 4-2.** Sampling strategy in fields tested for *Plasmodiophora brassicae* inoculum in central Alberta, Canada, in 2017. The red square represents the field entrance, and each of the white points represents the location where a soil sample was collected for pathogen detection by conventional PCR and quantification by qPCR analysis in A) Field 1; B) Field 2; C) Field 3; D) Field 4.

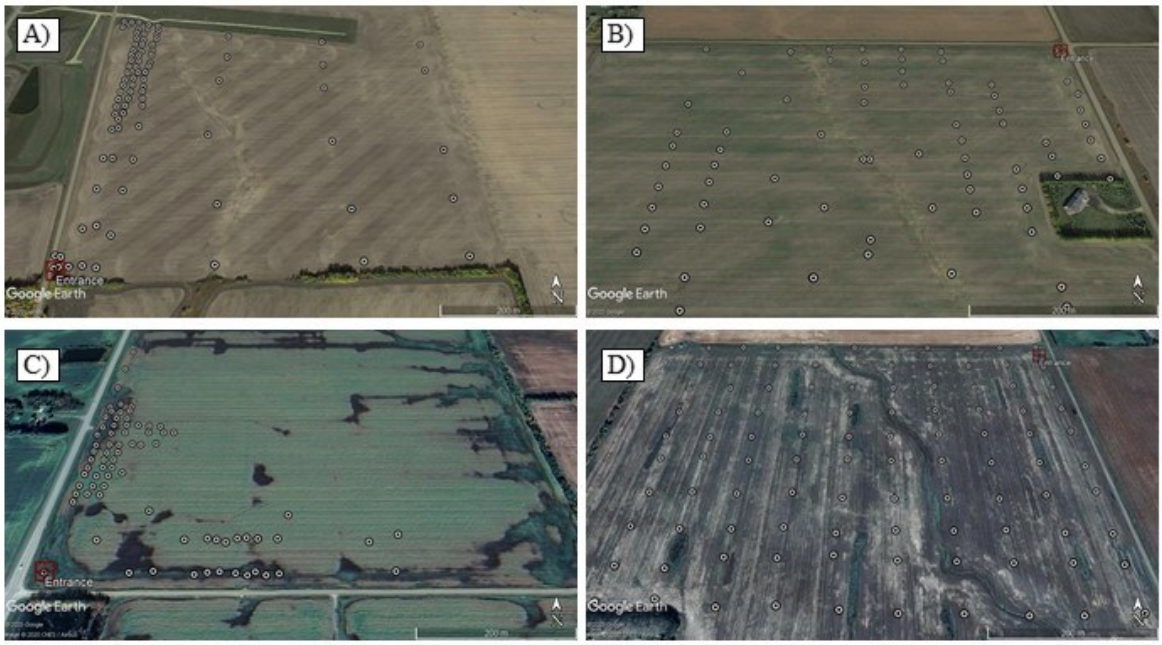


**Figure 4-3.** Sampling strategy in fields tested for *Plasmodiophora brassicae* inoculum in central Alberta, Canada, in 2019. The red square represents the field entrance, and each of the white points represents the location where a soil sample was collected for pathogen detection by conventional PCR and quantification by qPCR analysis in A) Field 1; B) Field 2; C) Field 3; D) Field 4.

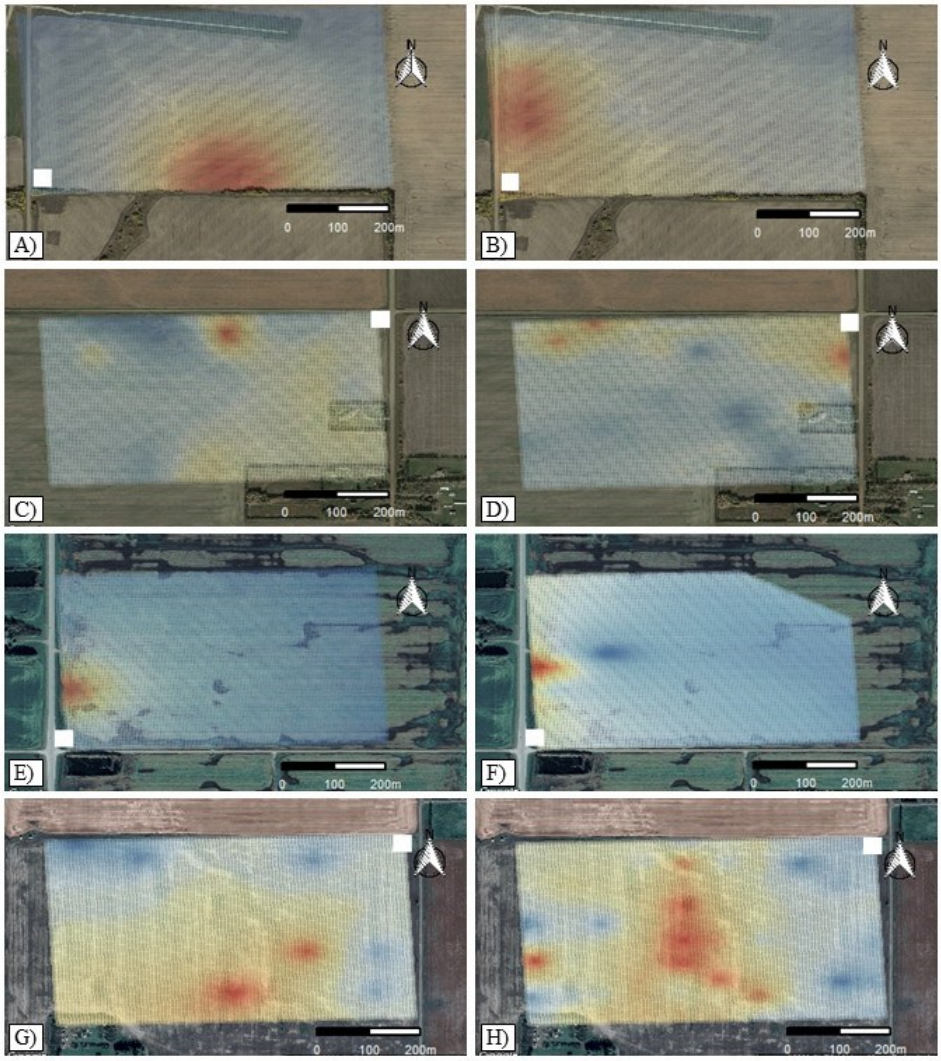


**Figure 4-4.** Prediction surface of the posterior mean of the log-transformed *Plasmodiophora brassicae* inoculum density for: A) Field 1 in 2017; B) Field 1 in 2019; C) Field 2 in 2017; D) Field 2 in 2017; E) Field 3 in 2017; F) Field 3 in 2019; G) Field 4 in 2017, and H) Field 4 in 2019. The white squares represent each field entrance. Color scale is relative within each field and is a representation of the *P. brassicae* inoculum density. Blue shading represents areas where *P. brassicae* was not detected, yellow shading indicates areas with intermediate inoculum densities, and red shading indicates areas with the highest inoculum densities.

# Chapter 5 Clubroot symptoms and resting spore production in a doubled haploid population of oilseed rape (*Brassica napus*) are controlled by four main QTLs

## 5.1 Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is one of the most important diseases of cruciferous crops worldwide, causing significant yield and quality losses in oilseed rape (canola; *Brassica napus* L.) and other Brassicas (Dixon, 2009b). A soilborne disease, clubroot is associated with the formation of large galls on the roots of susceptible hosts, which impede water and nutrient uptake. The life cycle of *P. brassicae* consists of three main stages: (i) survival in the soil, (ii) root hair infection, and (iii) cortical infection. The pathogen survives in the soil as long-lived resting spores (Kageyama and Asano, 2009). Under favorable conditions, the resting spores germinate to produce primary zoospores, which infect the host root hairs. The presence of plant root exudates can enhance resting spore germination (Macfarlane, 1970; Friberg et al., 2005; Rashid et al., 2013). Primary plasmodia develop within the infected root hairs, eventually giving rise to secondary zoospores. The secondary zoospores penetrate the cortical tissue and develop into intracellular secondary plasmodia, which cleave to produce a new generation of resting spores (Kageyama and Asano, 2009). As the galls decompose, these resting spores are released back into the soil, where they serve as inoculum for future infections. As many as $1\times10^{10}$ resting spores per g of gall tissue may be produced on a susceptible *B. napus* host (Hwang et al., 2013).

The persistence of *P. brassicae* resting spores in the soil (Wallenhammar, 1996) makes clubroot a particularly difficult disease to manage. Various strategies have been proposed, including crop rotation, biological control, liming of the soil and manipulation of the seeding date

(Hwang et al., 2014b). The most effective strategy for clubroot management, however, is the deployment of clubroot-resistant (CR) host cultivars (Rahman et al., 2014). The resistance in most CR oilseed rape cultivars is monogenic and derived from the European winter oilseed rape 'Mendel' (Diederichsen et al., 2014; Fredua-Agyeman et al., 2018). 'Mendel' was one of the first CR winter oilseed rape cultivars with acceptable agronomic performance released in Europe (Diederichsen et al., 2003, 2009). The clubroot resistance in 'Mendel' is based on one dominant and race- or pathotype-specific gene (Diederichsen et al., 2006). While genetic resistance is the most effective and convenient method to manage clubroot, the selection pressure it imposes on *P. brassicae* populations can cause rapid shifts in virulence. These shifts may result in a loss of resistance, as has already been documented in oilseed rape/canola in Europe (Diederichsen et al., 2014; Orgeur et al., 2016) and Canada (Strelkov et al., 2016, 2018).

The diversification and introduction of novel resistance sources can reduce the risk of resistance loss, as can the development of cultivars with polygenic resistance and the implementation of strategies such as gene pyramiding and the planting of multilines or cultivar mixtures (Parlevliet and Zadoks, 1977; Pink and Puddephat, 1999). For example, a combination of major resistance genes and quantitative trait loci (QTLs) could result in CR cultivars with resistance that is more durable. The development of cultivars with more diverse resistance, however, requires a deeper understanding of the genetic control of clubroot resistance (Manzanares-Dauleux et al., 2000).

Most genetic studies of clubroot resistance have focused on understanding the resistance harboured in *Brassica oleracea* and *Brassica rapa,* the ancestral parents of *B. napus.* In *B. rapa*, multiple major genes controlling clubroot resistance have been identified, including *Crr1a, Crr1b (Crr1a* and *Crr1b* were initially identified as a single locus, *Crr1*), *Crr2, Crr3, Crr5A, CRa, CRb,*

*CRd, CRs, Rcr1, Rcr2, Rcr3, Rcr5,* and *Rcr9$^{WA}$* (Suwabe et al., 2003; Hirai et al., 2004; Saito et al., 2006; Sakamoto et al., 2008; Hatakeyama et al., 2013, 2017; Mingguang Chu et al., 2014; Huang et al., 2017; Yu et al., 2017; Laila et al., 2019). In addition, two QTLs were reported to control resistance to a Korean isolate of *P. brassicae* classified as per Williams (1966) pathotype 2 (Choi et al., 2020), and two QTLs were found to control resistance to a Chinese isolate of pathotype 7 (Zhu et al., 2019). Another QTL (*Rcr4*) was found to control resistance to Canadian isolates of the pathogen representing pathotypes 2, 3, 5, 6, and 8, classified as per Williams (1966), and two QTLs (*Rcr8* and *Rcr9*) controlled resistance to an isolate of pathotype 5X, as defined on the Canadian Clubroot Differential set (Yu et al., 2017; Strelkov et al., 2018). Three loci (*Crr4, CRc,* and *CRk*) controlled resistance to non-pathotyped isolates of *P. brassicae* (Suwabe et al., 2006; Sakamoto et al., 2008). In *B. oleracea*, clubroot resistance has been found to be quantitative and is controlled mainly by QTLs with both major and minor effects (Rocherieux et al., 2004; Neik et al., 2017).

Genetic analyses of the control of clubroot resistance in *B. napus* have identified the presence of a major resistance gene (*Cra*) (Zhang et al., 2016) and nearly 30 different QTLs in various plant populations harbouring resistance to multiple *P. brassicae* strains (Manzanares-Dauleux et al., 2000; Werner et al., 2007; Li et al., 2016; Laperche et al., 2017; Aigu et al., 2018; Hejna et al., 2019; Wagner et al., 2019). A series of studies by our group using a doubled haploid (DH) population derived from the varieties 'Darmor-*bzh'* and 'Yudal' allowed the identification of a major QTL controlling resistance to *P. brassicae* isolate Pb137-522, while two QTLs were found to govern resistance to isolate K92-16 (Manzanares-Dauleux et al., 2000). In addition, when this DH population was tested against the pathogen isolate 'eH', two major and one minor QTLs were found to control resting spore production (Aigu et al., 2018), one moderate and three minor QTLs controlled the pathogen-plant genomic DNA ratio (Wagner et al., 2019), and nine QTLs regulated

clubroot severity, measured as a disease index (DI) (Laperche et al., 2017; Aigu et al., 2018; Wagner et al., 2019). Moreover, other groups have identified different QTLs controlling clubroot resistance in one DH population challenged with seven different isolates of *P. brassicae* (Werner et al., 2007), in an associative transcriptome analysis of 245 accessions inoculated with the European Clubroot Differential (ECD) pathotype 17/31/31 (Hejna et al., 2019), and in a genome wide association study of 472 host accessions inoculated with Williams' pathotype 4 (Li et al., 2016).

Previous studies have included very diverse genetic materials (spring, old winter lines), whose use in breeding programs may be difficult given potential issues such as linkage drag (Zamir, 2001; Yousef and Juvik, 2002; Lecomte et al., 2004). The challenges associated with the introduction of polygenic partial resistance from non-elite materials into elite oilseed rape genetic backgrounds may be one reason for the limited introduction of QTLs into new CR cultivars. Therefore, the identification of loci conferring partial clubroot resistance in recent cultivars with good agronomic performance may be of interest, as breeders could use these more readily.

Sustainable clubroot management requires both short and long-term approaches. In the short term, the focus should be on minimizing the impact of the pathogen on the host and therefore on crop production; in the longer term, the aim should be on reducing inoculum levels. Resistant cultivars are a convenient and highly effective disease management tool in the short-term, but may not prove durable over the long-term. The loss of effective pathogen control may reflect the strong selection pressure imposed on the pathogen, particularly since plants with low disease severity do not necessarily produce low amounts of resting spores (Siemens et al., 2002). A recent evaluation of *P. brassicae* resting spore dynamics in response to the cropping of CR *B. napus* cultivars indicated increases in soil inoculum loads (Ernst et al., 2019).

Plant disease epidemics are highly influenced by pathogen virulence (measured as disease severity) and fitness (measured as the reproductive rate of the pathogen), both of which are generally assumed to be positively correlated (Sacristán and García-Arenal, 2008); clubroot is no exception. However, studies with respect to the relationship between pathogen virulence and reproductive rate have produced contradictory results in some pathosystems (Fox and Williams, 1984; Kover and Schaal, 2002; Montarry et al., 2006). Indeed, there are reports where pathogen inoculum production and virulence are not correlated, or even negatively correlated (Robert et al., 2002; Sacristán et al., 2005; Montarry et al., 2010; Aigu et al., 2018).

Given the assumption of a high correlation between disease severity and pathogen fitness, most genetic studies of clubroot resistance have focused only on disease severity (Manzanares-Dauleux et al., 2000; Werner et al., 2007; Laperche et al., 2017). However, while there is a relationship between *P. brassicae* resting spore production and root gall size (disease severity), these variables are not necessarily correlated, and resting spore production can be affected by host resistance and environmental factors (Murakami et al., 2004; Gravot et al., 2016; Aigu et al., 2018). Therefore, both traits should be considered for genetic analyses and in breeding programs. The new CR ideotypes should combine low resting spore production and low DI. Selection for both traits will facilitate improved disease management over the short and long-term. In the short-term, such an approach will minimize the direct impact of the pathogen on disease severity and hence on crop production, while in the long-term, it will reduce the number of resting spores released into the soil, thereby limiting inoculum build-up and the potential for future epidemics.

This research had two objectives. First, it aimed to identify the QTLs involved in the control of resting spore production per plant (RSP) and clubroot symptoms (DI) in a segregating doubled haploid (DH) population from the cross of ‘Aviso’× ‘Montego’, two modern winter oilseed rape

cultivars partially resistant to clubroot with good agronomic characteristics. Second, it aimed to identify some recombinant lines of potential interest for breeding efforts, i.e., carrying favourable alleles at multiple QTLs to decrease DI and limit resting spore production.

## 5.2 Materials and Methods

### 5.2.1 Pathogen material and inoculum preparation

The *P. brassicae* selection isolate 'eH' (Fähling et al., 2003), classified as pathotype $P_1$ on the system of Somé et al. (1996), was used for all the experiments. To prepare inoculum, resting spores were extracted from frozen root galls of the universally susceptible Chinese cabbage (*B. rapa* subs. *pekinensis*) cv. Granaat (European Clubroot differential ECD 05; Buczacki et al., 1975) inoculated with the isolate. Briefly, the galls were thawed at room temperature and then homogenized at maximum speed in a home blender. The resulting homogenate was filtered sequentially, first through cheesecloth and then through 56 and 100-µm diameter pore stainless steel sieves (Retsch, Haan, Germany). The resting spore concentration of the filtered suspension was estimated by counting in a Malassez cell and adjusted to a final concentration of $1\times10^7$ resting spores $mL^{-1}$ with sterile distilled water.

### 5.2.2 Greenhouse experiments and disease assessment

A two-year experiment was conducted under greenhouse conditions in 2015 and 2016. The experimental design consisted of completely randomized blocks nested within the two years; in total, 114 genotypes (treatments) with four replicates (blocks) were established, with the experimental unit comprising six plants per genotype. Seeds of each genotype were sown in in 4-cm-diameter pots (1 seed per pot) filled with "Falienor 922016F3" potting mix (Falienor, Vivy, France), which consists of 65% Irish peat, 20% black peat, 15% perlite and 2% clay (pH = 6.2).

The greenhouse was maintained at temperatures between 19 and 22°C under a 16/8 day/night cycle. Plants were fertilized with "Liquoplant FD 134 hiver" nutrient solution (Plantin,Courthéson, France) once or twice a week by sub-irrigation. Inoculations were conducted 7 days after sowing, by applying 1 mL of the *P. brassicae* resting spore suspension to the base of each seedling.

Disease assessment was conducted 54 days after inoculation (dai) on a 0 to 3 scale following Manzanares-Dauleux et al. (2000), where 0 = no visible galling, 1 = very light galling usually confined to lateral roots, 2 = moderate galling on lateral roots and the taproot, 2+ = severe galling on all roots but some roots remain healthy, and 3 = one large gall with no remaining healthy roots. The individual severity ratings were then used to calculate a disease index (DI) using Equation 1:

$$DI = \frac{[(0\times n_0)+(25\times n_1)+(50\times n_2)+(75\times n_{2+})+(100\times n_3)]\times 100}{N} \qquad (1)$$

Where, $n_0$, $n_1$, $n_2$, $n_{2+}$ and $n_3$ represent the number of plants in each severity class and $N$ is the total number of plants evaluated. To confirm the pathotype designation of the isolate 'eH' as $P_1$, the isolate was inoculated on the differential hosts of Somé et al. (1996) [*B. napus* cv. 'Nevin' (ECD 06), *B. napus* cv. 'Wilhelmsburger' (ECD 10), and *B. napus* cv. 'Brutor']. The Chinese cabbage ECD 05 was also included as a susceptible check in all of the experiments.

After disease assessment, all roots in the experimental unit were pooled and stored at -20°C until processing. Resting spores in the pooled root samples were quantified by flow cytometry following (Aigu et al., 2018). Briefly, the roots were thawed at room temperature and weighted. Each sample was homogenized in 100 mL of distilled water in a home blender, and the resulting suspension was filtered as described for the inoculum preparation. The resting spore suspensions were diluted in a 1:20 ratio with distilled water. Resting spores were quantified in a CyFlow flow

cytometer (Sysmex partec, Görlitz, Germany) equipped with a 20-mW blue laser (488 nm) and a Forward-Scatter (FCS) detector to determine particle size (Aigu et al., 2018).

A standard curve was generated from a suspension of resting spores, produced as described above for inoculum preparation, with the spore concentration estimated by counting in a Malassez cell. This solution was then diluted as needed to generate nine spore suspensions with concentrations ranging from $1 \times 10^4$ to $6 \times 10^5$ resting spores·mL$^{-1}$. Particle counting was completed for each of the dilution points with the flow cytometer and an injection speed of 20. The measured resting spore concentrations were adjusted with a regression Equation (2) obtained from the standard curve. The obtained regression curve had an $R^2$ of 95%. Spore levels were expressed as resting spores per plant (RSP) by dividing the total number of resting spores in the suspension by the number of plants in the pooled sample.

$$RS = 2 \times 10^{-6}x^2 + 2.2283x + 41716 \quad (2)$$

Where, $RS$ is the adjusted resting spore count in the suspension and $x$ is the particle count obtained with the flow cytometer.

### 5.2.3 Plant material and genetic map

A population of 114 doubled haploid (DH) lines obtained from the cross of the winter oilseed rape cultivars ‘Aviso’ x ‘Montego’ was chosen for linkage analysis because previous experiments indicated that both parents are partially resistant to clubroot, and therefore the presence of new QTLs controlling the disease was suspected. The parental lines were used as controls in all experiments. The genetic map used for the analysis was described by Delourme et al. (2013). Additional genotyping was conducted with the 60K infinitum array (Clarke et al., 2016), thus leading to an updated map. Given the population size, recombination was not always possible,

resulting in a high number of markers at the same genetic position. Since this is not desirable for QTL mapping, we only kept a single marker or unique loci to represent each cluster. The genetic map covers 1947 cM (892 cM for the A genome and 1055 cM for the C genome) at a density of 1.18 markers each cM; it comprises 2301 SNPs representing 831 unique loci. The linkage groups (LGs) with the highest proportion of loci distortion were A02, A03, A09, C01 and C09.

### 5.2.4 Statistical analyses

Statistical analyses were performed with R (R Core Team, 2019). Spearman's rank correlation coefficient was estimated to evaluate the correlation between RSP and DI. The mixed linear model presented in Eqn 3 was estimated to analyse RSP and DI. The *nlme* (Pinheiro et al., 2019) and *lsmeans* (Lenth, 2016) packages were used to test both random and fixed effects:

$$Y_{ijk} = \mu + G_i + Y_j + B_{k(j)} + GY_{ij} + \varepsilon_{ijk} \qquad (3)$$

Where, $Y_{ijk}$ is either RSP or DI in each genotype ($i^{th}$) observed in the $j^{th}$ year and in the $k^{th}$block; $\mu$ is the population mean; $G_i$ is the effect of the $i^{th}$ genotype; $Y_j$ is the effect of the $j^{th}$ year; $B_{k(j)}$ is the effect of the $k^{th}$ block nested with the $j^{th}$ year; $GY_{ik}$ is the effect of the interaction between the $i^{th}$ genotype and the $j^{th}$ year; and $\varepsilon_{ijk}$ are the residuals. $B_{k(j)}$ was considered as random.

Broad sense heritability as defined by Holland et al. (2003) (Eqn 4) was estimated using the model in Eqn 3.

$$H^2 = \frac{\sigma^2 G}{\sigma^2 G + \frac{\sigma^2 Gy}{y} + \frac{\sigma^2 e}{y*b}} \qquad (4)$$

Where, $H^2$ is the broad sense heritability, $\sigma^2 G$ is the variance of the genotype effect, $\sigma^2 Gy$ is the variance of the genotype × year effect, $y$ is the number of years, $b$ is the number of blocks, and $\sigma^2 e$ is the residual variance.

Adjusted means were calculated using the *lsmeans* package (Lenth, 2016) for each genotype across years and replications and used as phenotypic values for QTL analyses.

The reactions of the progeny were grouped based on genotypes which possessed the identified QTLs for DI and RSP, and the phenotypic responses were compared using Duncan's new multiple range test at $p \leq 0{\cdot}05$.

### 5.2.5 Linkage analysis

Linkage analysis was conducted with the R/qtl package (Broman et al., 2003). At first, a simple interval mapping (SIM) was performed to get a rough estimate of the QTLs controlling each trait (RSP and DI). The LOD threshold for these analyses was 3.17, estimated by a 1000 permutation test (α=0.05). Afterwards, multiple QTL mapping was conducted. Manual selection of the QTL model was completed with the functions *addqtl, addintqtl* and *fitqtl*, allowing for QTL-pairwise interaction using the multiple imputation regression method. The QTLs were added one by one and at each step, and two models were compared: one with the already validated QTLs, and the same model including the tested QTL and the corresponding QTL by QTL interactions. Only QTLs whose effect was significant (α=0.05) according to the ANOVA table were retained in the model. LOD values and $R^2$ values for each QTL were also obtained by the *fitqtl* function. The confidence intervals of the QTLs were estimated with a LOD drop of one unit.

For each QTL, the genes present in the confidence interval were gathered using the reference genome v4 of 'Darmor-*bzh'* (Chalhoub et al., 2014) by using the physical anchorage of the confidence interval flanking SNP markers.

## 5.3 Results

### 5.3.1 Characterization of the phenotypic response

The frequency distribution for DI (Figure 5-1) and RSP (Figure 5-2) indicated a continuous distribution, suggesting polygenic control of both variables. In both cases, the parents 'Aviso' and 'Montego' showed intermediate clubroot responses. Mean DI was 36.8 for 'Aviso' and 46.5 for 'Montego'; the RSP was $7.2 \times 10^7$ resting spores plant$^{-1}$ for 'Aviso' and $7.6 \times 10^7$ resting spores plant$^{-1}$ for 'Montego'. Resting spores per plant was significantly correlated with DI ($p$ value <0.001) (Figure 5-3), resulting in a Spearman's coefficient of 0.65. The heritability for both variables was high (85.2% for DI and 84.4% for RSP).

Among the 114 recombinant progeny lines tested, 18% had a DI < 20; 53% had intermediate levels of disease (DI between 20 and 50), while the remaining 29% had a DI between 50.7 and 98. Resting spores per plant ranged from $5.6 \times 10^7$ to $1.4 \times 10^8$, with about 54.2% of the lines exhibiting a higher RSP than both parents. Only 30% of the lines produced fewer resting spores than the parental lines (Figure 5-2).

Lines 88 and 189 developed mild symptoms of clubroot (DI of 7.3 and 10.5, respectively) and had significantly low resting spore production (RSP of $5.6\times10^7$ and $5.9\times10^7$ resting spores plant$^{-1}$, respectively) (Figure 5-3). Similarly, although lines 27, 64, 52 and 127 had a DI < 20, the RSP was fairly high, ranging from $8 \times 10^7$ in lines 52 and 127 to $9 \times 10^7$ resting spores plant$^{-1}$ in lines 27 and 64. Some lines with intermediate DI also showed high resting spore production, including

line 83 (DI 30.5 and $1.2\times10^9$ resting spores plant$^{-1}$), line 148 (DI 44.8 and $1.2\times10^9$ resting spores plant$^{-1}$), line 120 (DI 36.3 and $9.2\times10^8$ resting spores plant$^{-1}$), line 74 (DI 37.3 and $9.7\times10^8$ resting spores plant$^{-1}$), line 168 (DI 44.8 and $9.3\times10^8$ resting spores plant$^{-1}$), line 48 (DI 46.8 and $9.7\times10^8$ resting spores plant$^{-1}$), and lines 109 and 39 (DI 48.9 and $9.2\times10^8$ resting spores plant$^{-1}$). In contrast, some lines with an intermediate DI had a lower RSP, including lines 76 (DI 20.8 and $5.8\times10^7$ resting spores plant$^{-1}$) and 164 (DI 32.8 and $5.7\times10^7$ resting spores plant$^{-1}$) (Figure 5-3).

### 5.3.2 QTLs controlling DI and number of resting spores per plant

QTLs were assigned names consisting of three parts separated by underscores. The first part of each name includes the initials Pb and Bn to indicate *P. brassicae* and *Brassica napus*, respectively. The second part indicates the trait controlled by the QTL, DI or RSP, in lowercase letters. The third part indicates the chromosome on which the QTL is located. Simple interval mapping (SIM) indicated the presence of two main QTLs controlling DI on chromosomes A04 and C03 (Figure 5-4), PbBn_di_A04 and PbBn_di_C03. Further analysis allowed the identification of an additional QTL on chromosome A02, PbBn_di_A02, and the final fitted model accounted for 78.4% of the total variation. Most of the variance in DI was controlled by PbBn_di_C03 (51.0%) followed by PbBn_di_A04 (18.1%). A minor effect was detected for PbBn_di_A02, which explained 4.5% of the total variation. Since the population under investigation was a DH, only homozygous lines were tested, and thus only additive effects were detectable, leading to a strong relationship between the percentage of the variance accounted for by the QTLs and their additive effect. It was observed that the QTL with the strongest effect (PbBn_di_C03) also had the highest additive effect (Table 5-1). No epistasis was found among the QTLs.

Only one QTL controlling RSP, PbBn_rsp_C03 on the chromosome C03, was detected either by SIM or by fitting a multiple QTL model (Figure 5-5). The fitted model accounted for 18.3% of the total variation for that variable. PbBn_di_C03 and PbBn_rsp_C03 overlapped. On PbBn_di_C03 and PbBn_rsp_C03, the 'Aviso' allele contributed to phenotypes with lower DI and RSP, respectively, while on PbBn_di_A04 and PbBn_di_A02, the 'Montego' alleles were more favourable for reducing DI (Table 5-1). The lines that did not have any favourable allele for disease reduction presented the highest DI; an intermediate DI was observed only when the favourable allele of PbBn_di_C03 was present, and the highest reductions in DI occurred whenever the PbBn_di_C03, PbBn_di_A04 and PbBn_di_A02 favourable alleles were present together (Figure 5-6).

It was confirmed that RSP was not controlled by the QTLs PbBn_di_A02 or PbBn_di_A04, since the presence of their favourable alleles did not cause a difference in this value (Figure 5-7).

### 5.3.3 Physical anchorage of the confidence intervals on the *B. napus* genome

The markers names, positions, start and end-points for all QTLs are indicated in Table 5-1.

Only three of the four identified QTLs could be mapped on the B. napus genome: PbBn_di_A02, PbBn_di_C03 and PbBn_rsp_C03. The QTL PbBn_di_A04 could not be mapped because the region where it was positioned corresponded to a chimeric region on the reference genome (v4). PbBn_di_A02 covered 661.48 kb in a region encoding 101 genes, while PbBn_di_C03 covered 788.420 kb in total, encoding 147 genes. Four genes related to plant resistance and defence were found in QTL PbBn_di_A02. Two were annotated as hypersensitive-induced response protein (HIR), two as a cyclic nucleotide gated channel, and the fourth as a Mlo-like protein 6. In PbBn_di_C03, six genes related to disease resistance or defence were identified,

including five TIR-NBS-LRR class disease resistance proteins and one LRR protein kinase-like protein.

The only QTL related to RSP, PbBn_rsp_C03, covered 1614.43 kb. In this QTL, 346 genes were encountered, of which 10 were related to disease resistance or defence, including five TIR-NBS-LRR class disease resistance proteins, three leucine-rich repeat (LRR) protein kinase-like proteins, one defensin-like protein 203 and one WRKY transcription factor 18. PbBn_rsp_C03 QTL overlapped with PbBn_di_C03 in the region from 28.10 to 29.01 cM (corresponding to a 0.39 Mb physical region). In the common region between PbBn_di_C03 and PbBn_rsp_C03 (4.09-4.50 Mb), three TIR-NBS-LRR class disease resistance proteins were found (Table 5-2).

**5.4 Discussion**

Research on clubroot resistance has focused mainly on disease severity expressed as a disease index (DI). Resting spore production in host genotypes has not been examined to the same extent, although this is an important measurement of pathogen fitness, affecting inoculum build up in infested fields. In the 'Aviso' × 'Montego' population, the highest observed RSP ($1.4 \times 10^8$ resting spores plant$^{-1}$) was 2.5 times greater than the lowest ($5.6 \times 10^7$ resting spores plant$^{-1}$). Such variation may be important from an epidemiological perspective, since doubling the number of resting spores released into the soil could result in faster and more significant inoculum increases for future crops.

Our results indicate that RSP is not as strongly correlated with DI (Spearman's coefficient of 0.65) as might be expected based on the trade-off hypothesis, where within-host multiplication, within-host transmission and virulence of pathogens are positively correlated traits (Frank, 1996); and thus, higher disease levels would result in higher within host reproduction rates (Sacristán and

García-Arenal, 2008). Nonetheless, these results are consistent with previous reports on the clubroot pathosystem. In a study with *Arabidopsis thaliana*, Siemens et al. (2002) observed that correlation between DI and spore number per root weight was between 0.7 and 0.9, and that one of the main factors determining those correlation values was host resistance. Similarly, Murakami et al. (2004) concluded that resting spore production is host-specific and, therefore, clubroot severity cannot account for resting spore production on its own. This was especially true for intermediate disease severities; for example, Chinese cabbage and broccoli plants with intermediate levels of clubroot produced approximately $1\times10^9$ resting spores plant$^{-1}$, while cabbage plants with the same amount of disease produced only $1\times10^8$ resting spores plant$^{-1}$ (Murakami et al., 2004). More recently, Aigu et al. (2018) reported that *P. brassicae* was able to produce high numbers of resting spores in some *B. napus* genotypes with mild symptoms of clubroot, and highlighted the partially resistant genotype 'Darmor-*bzh*' ($2.7\times10^8$ resting spores plant$^{-1}$ and DI = 30).

Linkage analysis showed that the genetic control of RSP and DI are related, since the QTLs PbBn_di_C03 and PbBn_rsp_C03 co-localized, suggesting that the C03 locus controls both traits. The importance of this genomic region is highlighted by the fact that PbBn_di_C03 explains the highest proportion of the variation for DI. The other loci, PbBn_di_A04 and PbBn_di_A02, only control DI and not RSP. We have reported this type of genetic architecture previously, where all QTLs controlling RSP also control DI, but not all DI QTLs control RSP (Laperche et al., 2017; Aigu et al., 2018). Co-localization of two QTLs with an intermediate effect on DI (QTL controlling <20% of the variation) and a high effect on RSP (QTL controlling >50% of the variation) also has been reported (Laperche et al., 2017; Aigu et al., 2018). Collectively, these studies suggest that co-

localization of DI and RSP QTLs does not depend on the QTL effect (percentage of the variance of the trait that the QTL explains).

In a recent study of the genomic regions controlling DI and RSP in response to *P. brassicae* isolate 'eH' in another *B. napus* DH population ('Darmor-*bzh*' × 'Yudal'), five QTLs controlling DI were located on chromosomes A05, A07, C02, C03 and C09 (Laperche et al., 2017). Two of these QTLs, on chromosomes C02 and C09, were also found to control RSP (Aigu et al., 2018). In the current study, we also identified QTLs on chromosome C03, but ours were in the regions 4.09-4.88 Mb (PbBn_di_C03) and 2.89-4.5 Mb (PbBn_rsp_C03), while the one found by Laperche et al. (2017) occurred in the region 4.6-5.0 Mb. This suggests that the same genomic region may be controlling clubroot resistance in both populations; however, further analysis is required for confirmation. The percentage of the variation explained by PbBn_di_C03 in our study and the QTL on chromosome C03 reported by Laperche et al. (2017) in the 'Darmor-*bzh*' × 'Yudal' population was very different. The QTLs we detected on C03 controlled 51.4% of DI and 18.3% of the variation in RSP, while Laperche et al. (2017) found that the QTL on chromosome C03 controlled 7.75% of DI. No QTL controlling RSP was found on chromosome C03 in the 'Darmor-bzh' × 'Yudal' population (Aigu et al., 2018). Similarly, Werner et al., (2007) found QTLs in the linkage groups N13 and N02 (chromosomes C03, and A02 respectively) in a DH population of *B. napus* obtained from a cross of the DH line '263/11' and the oilseed rape cultivar 'Express' challenged with the *P. brassicae* isolates '*01:60', '01:07'* and '*k'*. QTLs on chromosome C03 were identified when plants were inoculated with isolates '*01:60'* and *'01:07'* and explained 28.6% and 11.7% of the variance in DI, respectively. A QTL on chromosome A02 was also found after inoculation with the isolate '*k'*, explaining 17.6% of the variance in DI. In addition, QTLs at syntenic positions have been identified on *B. oleracea* on chromosome C03 (Nagaoka et al., 2010;

Lee et al., 2016; Li et al., 2016) and in *B. rapa* on chromosome A02 (Yu et al., 2017), suggesting that the C03 genomic region merits further investigation. Indeed, the QTLs on the chromosome C03 seem to be involved in the genetic control of response to different isolates, as has been observed in multiple genetic backgrounds harbouring different effects (minor to major) depending on both the plant genotype and the *P. brassicae* isolate.

Most genetic analyses of the resistance harboured by oilseed rape against clubroot disease have been performed with very diverse genetic material (spring, old winter lines), and few resistance sources appear to be lines with good agronomic value. In the current study, the parents of the DH population were the cultivars 'Aviso' and 'Montego', with the former released by Danisco seeds (Holeby, Denmark) in 2000 and the latter released by Limagrain (Saint-Beauzire, France) in 2002. 'Aviso' is a variety with good agronomic performance that shows resistance to other diseases such as blackleg (*Leptosphaeria maculans*) (Stonard et al., 2007; Jestin et al., 2015). These cultivars may represent suitable donors of clubroot resistance in oilseed rape breeding programs, considering that with their good agronomic value, less intensive backcrossing with elite lines would be required, facilitating the transfer of polygenic traits into new cultivars.

The potential utility of the 'Aviso' × 'Montego' population in breeding programs is underscored by the fact that the resting spore production was lower relative to values reported in previous studies. The 'Aviso'× 'Montego' DH population produced between $5.6 \times 10^7$ and $1.45 \times 10^8$ resting spores plant$^{-1}$. In contrast, in the 'Darmor-*bzh*' × 'Yudal' DH population, the RSP values ranged from $1 \times 10^8$ and $5 \times 10^9$ (Aigu et al., 2018). Hence, the 'Aviso'× 'Montego' DH population used in this research appears promising for the selection of parental lines with low DI and RSP (lines 189 and 88) for the transfer of the identified QTLs into new cultivars. While it has been reported that the simultaneous transfer of even four unlinked QTLs is possible (Hospital

and Charcosset, 1997; Lecomte et al., 2004; Steele et al., 2006), the use of at least three markers for each QTL is recommended to make them useful in marker-assisted selection (Hospital and Charcosset, 1997). The transfer of the four QTLs identified in the current study is therefore possible, although additional refinement and validation of the markers is required for marker-assisted selection.

The search we conducted of the genes underlying the QTLs in the 'Darmor-*bzh*' reference genome allowed the identification of a set of genes, which might be involved in the response of the 'Aviso' × 'Montego' population to *P. brassicae*, on chromosomes A02 and C03. Several of the putative gene products belonged to protein families involved in plant defence and resistance, including hypersensitive-induced response proteins (HIR), cyclic nucleotide gated channel, Mlo-like protein 6, TIR-NBS-LRR (TNL) class disease resistance proteins, Leucine-rich repeat (LRR) protein kinase-like, defensin-like protein 203, and WRKY transcription factor 18 (Rushton et al., 1995; Song et al., 1995; Buchanan and Gay, 1996; García-Olmedo et al., 1998; Clough et al., 2000; Eulgem et al., 2000; Dangl and Jones, 2001; Kanzaki et al., 2002; Choi et al., 2010; Zhou et al., 2010; Duan et al., 2013). The presence of a cluster of seven TNL-encoding genes is of special interest. The two clubroot-resistance loci (*CRa* and *Crr1*) that have been molecularly resolved so far (Ueno et al., 2012; Hatakeyama et al., 2013) both encode for TNLs. In addition, the fine mapping of the *Rcr1* locus (Yu et al., 2016) and GWAS have highlighted the recurrent presence of NLR-gene clusters in clubroot-resistance loci, supporting the importance of this protein family in driving clubroot resistance (Stotz et al., 2018).

The 'Aviso'× 'Montego' population characterized in this study holds promise for the development of new CR oilseed rape cultivars, since the parental lines were recent varieties with good agronomic traits, and some of the tested lines presented very low DI accompanied by low

RSP. While much of the emphasis in clubroot resistance breeding traditionally has been on selection based on low disease severity, resting spore production in plant material should also be considered. While breeding based on DI is critical for producing cultivars that perform well in *P. brassicae*-infested fields, the incorporation of lower RSP as a selection criterion will enable more sustainable clubroot management, by selecting plant genotypes where pathogen multiplication (fitness) is reduced.

**Table 5-1.** QTLs controlling clubroot disease index (DI) or number of *Plasmodiophora brassicae* resting spores per plant (RSP), and their position in the physical map, identified by multiple QTL mapping in a doubled haploid population obtained from a cross of the oilseed rape cultivars 'Aviso' × 'Montego'.

| QTL name | Trait | Chromosome | Position (cM) | CI (cM) | Markers CI | LOD | $R^2$ (%) | Favourable allele source | Additive effect | Position in physical map (Mb) | Number of genes | Unannotated or proteins with unknown function | Genes related with plant resistance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PbBn_di_A02 | DI | A02 | 28.9 | 28.0-34.0 | BS008863-BS009106 | 4.7 | 4.5 | Montego | -6.93 | 4.84-5.50 | 101 | 23 | 5 |
| PbBn_di_A04 | | A04 | 57.6 | 57.6-59.4 | BS006202-BS006447 | 15.2 | 18.1 | Montego | -7.737 | 1.34- | . | . | . |
| PbBn_di_C03 | | C03 | 30.8 | 28.1-32.6 | BS007532 – Bn-C3-p5080881 | 30.3 | 51 | Aviso | 13.193 | 4.09-4.88 | 147 | 29 | 6 |
| PbBn_rsp_C03 | RSP | C03 | 24.5 | 19.1-29.0 | BS012716-Bn-C3-p4469843 | 5.1 | 18.3 | Aviso | $8.5 \times 10^5$ | 2.89-4.50 | 346 | 72 | 10 |

CI = Confidence interval

**Table 5-2.** Annotation of the genes related to resistance and defence for the QTLs controlling clubroot disease index (DI) or number of *Plasmodiophora brassicae* resting spores per plant (RSP), in a doubled haploid population obtained from a cross of the oilseed rape cultivars 'Aviso' × 'Montego'.

| QTL name | Trait | Chromosome | Position in physical map (Mb) | Gene name | Position (bp) | Gene annotation | Homoeologous gene name | *Arabidopsis thaliana* orthologs | *Brassica rapa* orthologs | *Brassica oleracea* orthologs |
|---|---|---|---|---|---|---|---|---|---|---|
| PbBn_di_A02 | DI | A02 | 4.84-5.50 | BnaA02g09860D | 4945996 - 4948245 | Hypersensitive-induced response protein (HIR) 2 | BnaC02g13810D | AT5G54100 | Bra022696 | Bo2g041840 |
| | | | | BnaA02g09870D | 4950703 - 4954201 | Hypersensitive-induced response protein (HIR) 2 | BnaC02g13820D | AT5G54095 | Bra022695 | Bo2g042150 |
| | | | | BnaA02g09790D | 4902667 - 4907022 | Cyclic nucleotide gated channel | BnaC02g44680D | AT5G54250 | Bra022702 | Bo2g042020 |
| | | | | BnaA02g10440D | 5360323 - 5362868 | Cyclic nucleotide gated channel | BnaC02g14560D | AT5G53130 | Bra022632 | . |
| | | | | BnaA02g10080D | 5102140 - 5105226 | Mlo-like protein 6 | BnaC02g14090D | AT5G53760 | Bra022673 | Bo2g044430 |
| PbBn_di_C03 | | C03 | 4,50-4.88 | BnaC03g09410D | 4510238 - 4510891 | TIR-NBS-LRR class disease resistance proteins | . | . | Bra006487 | Bo3g013450 |
| | | | | BnaC03g09420D | 4511004 - 4512486 | TIR-NBS-LRR class disease resistance proteins | . | . | . | . |
| | | | | BnaC03g10100D | 4867978 - 4869203 | Leucine-rich repeat protein kinase-like protein | . | AT5G20480 | Bra006560 | Bo3g015190 |
| PbBn_rsp_C03 | RSP | C03 | 2.89-4.09 | BnaC03g08650D | 4082610 - 4087660 | TIR-NBS-LRR class disease resistance proteins | . | AT5G17880 | . | Bo3g012820 |
| | | | | BnaC03g08660D | 4089063 - 4094327 | TIR-NBS-LRR class disease resistance proteins | . | AT5G17890 | . | Bo3g012830 |
| | | | | BnaC03g06310D | 3057621 - 3060722 | Leucine-rich repeat protein kinase-like proteins | BnaA03g04770D | AT5G14210 | Bra006243 | Bo3g009670 |
| | | | | BnaC03g07510D | 3539026 - 3542690 | Leucine-rich repeat protein kinase-like proteins | BnaA03g05790D | AT5G16000 | Bra006335 | Bo3g010820 |
| | | | | BnaC03g07920D | 3723953 - 3729620 | Leucine-rich repeat protein kinase-like proteins | . | . | Bra006366 | Bo3g012140 |
| | | | | BnaC03g08510D | 4041438 - 4041833 | Defensin-like protein 203 | . | . | . | Bo3g012680 |
| | | | | BnaC03g06770D | 3236365 - 3238839 | WRKY transcription factor 18 | BnaA03g05230D | AT5G15130 | Bra006283 | Bo3g010100 |
| Common region between PbBn_di_C03 and PbBn_rsp_C03 | | C03 | 4.09-4.50 | BnaC03g08900D | 4222525 - 4224460 | TIR-NBS-LRR class disease resistance proteins | . | . | . | . |
| | | | | BnaC03g08920D | 4227171 - 4232582 | TIR-NBS-LRR class disease resistance proteins | BnaAnng27760D | AT5G18370 | . | . |
| | | | | BnaC03g09010D | 4264274 - 4265687 | TIR-NBS-LRR class disease resistance proteins | BnaA03g07110D | . | Bra006458 | Bo3g013130 |

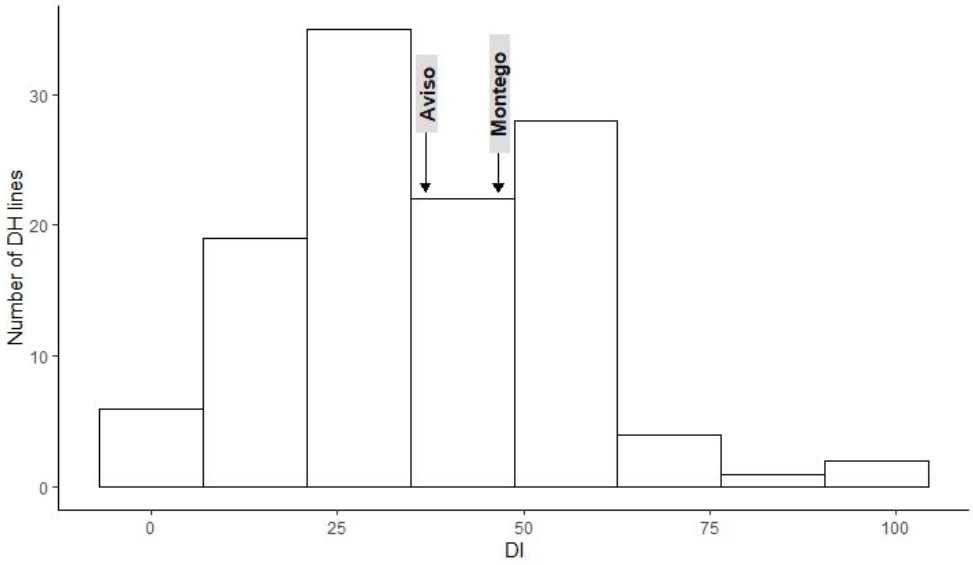


**Figure 5-1.** Distribution of the clubroot disease index (DI) for the doubled haploid progeny from a cross of the oilseed rape cultivars 'Aviso' × 'Montego' following inoculation with *Plasmodiophora brassicae* isolate ‘eH’. The parents 'Aviso' and 'Montego' are highlighted.

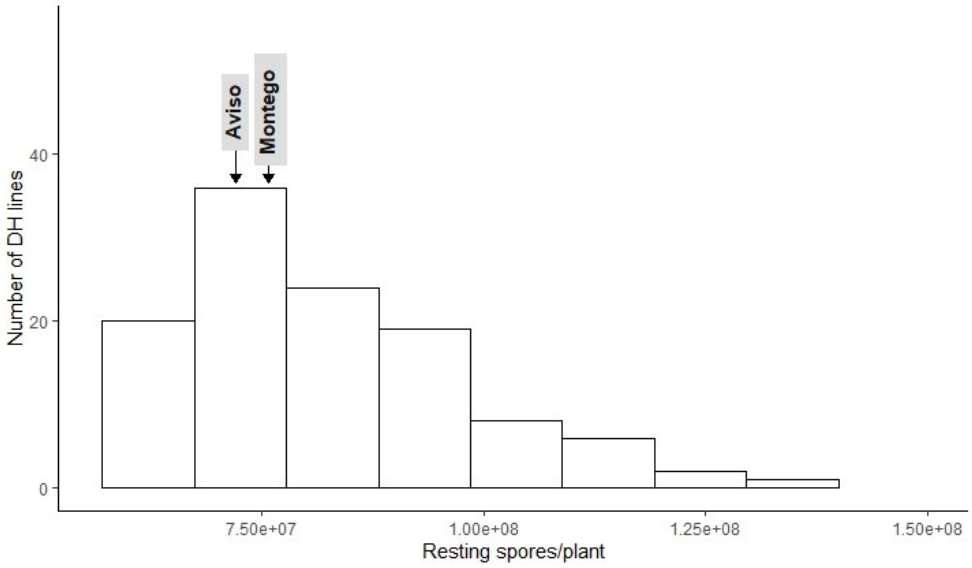


**Figure 5-2.** Distribution of number of *Plasmodiophora brassicae* resting spores produced per plant for the doubled haploid progeny from a cross of the oilseed rape cultivars 'Aviso' × 'Montego' following inoculation with the pathogen isolate ‘eH’. The parents 'Aviso' and 'Montego' are highlighted.

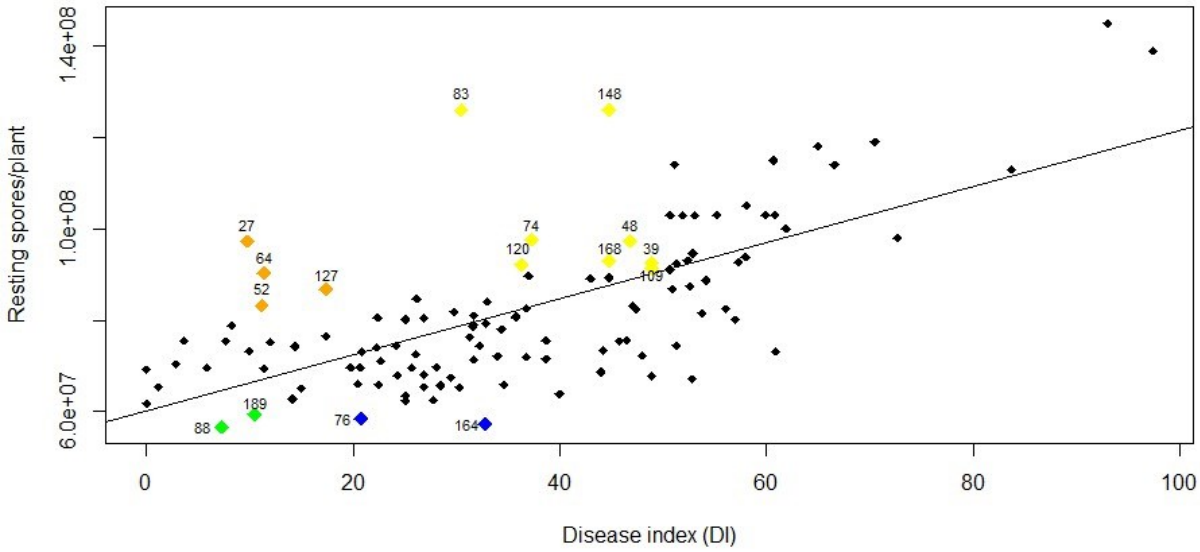


**Figure 5-3.** Relationship between disease index (DI) and number of resting spores per plant (RSP) for the doubled-haploid progeny from a cross of the oilseed rape cultivars 'Aviso' × 'Montego'. The Spearman's rank correlation coefficient was 0.65 (*p* value <0.001). Numbers over the highlighted points indicate the code of each line with atypical behaviour. Green points represent lines with a DI < 20 and an RSP < $5.9 \times 10^7$ resting spores plant$^{-1}$; blue points represent lines with a DI between 20 and 40 and an RSP < $5.9 \times 10^7$ resting spores plant$^{-1}$; orange points represent lines with DI < 20 and an RSP > $8 \times 10^7$ resting spores plant$^{-1}$; yellow points represent lines with DI between 30 and 50 and an RSP > $9 \times 10^7$ resting spores plant$^{-1}$.

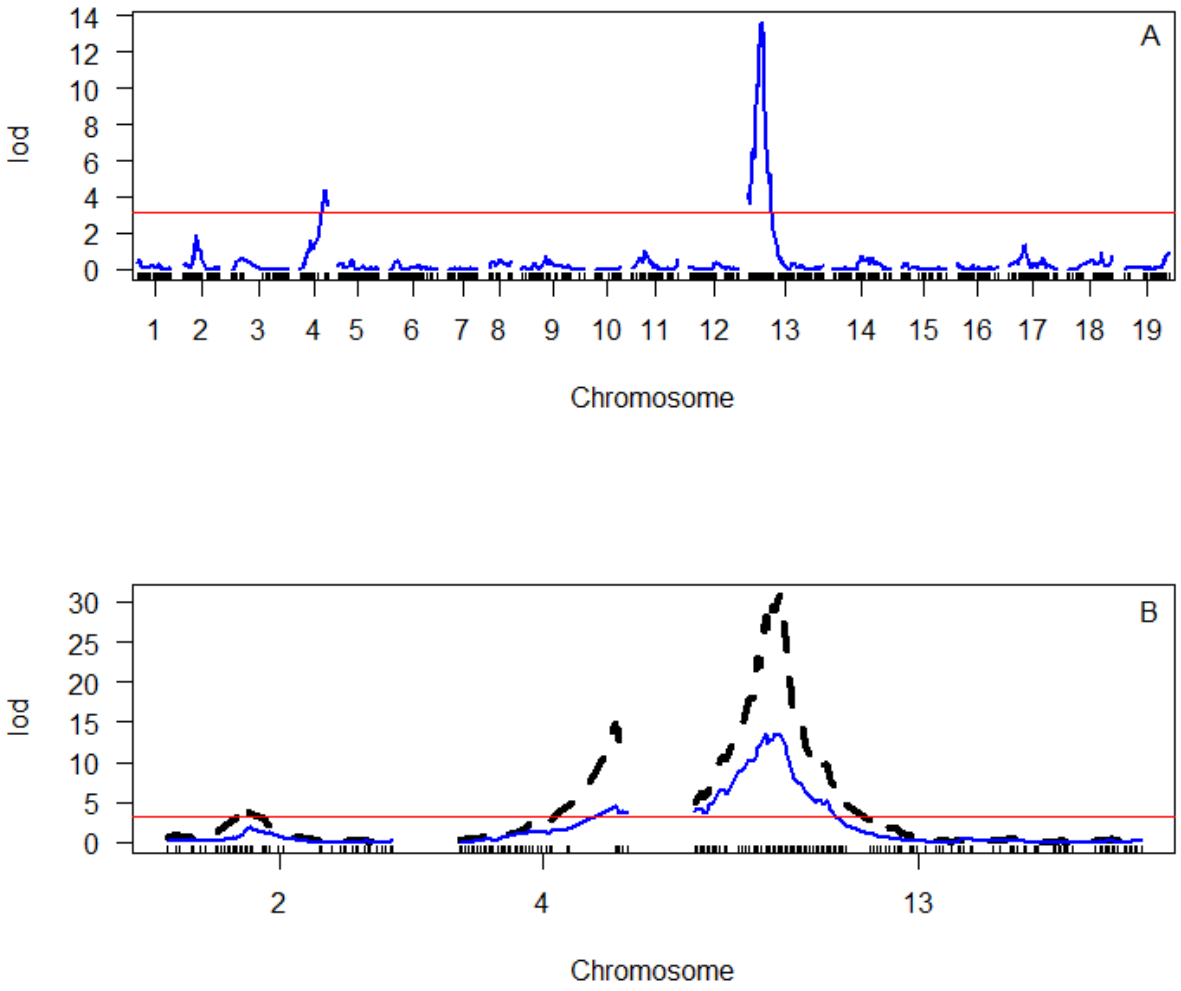


**Figure 5-4.** Genome scan of LOD scores for disease index (DI). A shows the LOD scores estimated by simple interval mapping (SIM). B shows the LOD scores for the chromosomes where QTLs were identified (A02 = chromosome 2, A04 = chromosome 4, C03 = chromosome 13); the LOD scores estimated by multiple QTL mapping are indicated in black, while the LOD scores estimated by SIM are indicated in blue. The red line in both graphs represents the LOD threshold determined by 1000 permutations.

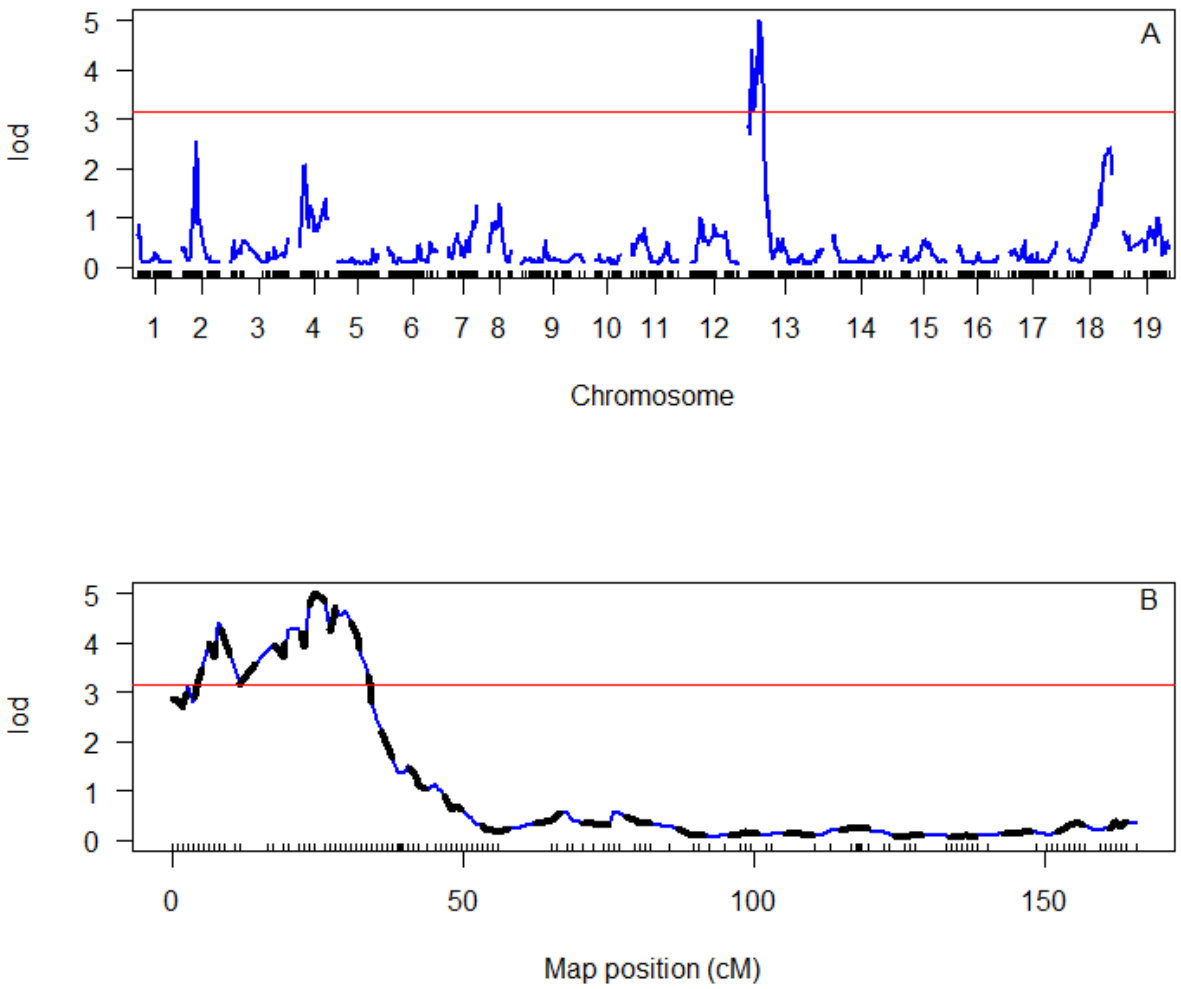


**Figure 5-5.** Genome scan of LOD scores for the number of resting spores per plant. A shows the LOD scores estimated by simple interval mapping (SIM). B shows the LOD scores for the chromosome C03 where one QTL was identified (C03 = chromosome 13); the LOD scores estimated by multiple QTL mapping are indicated in black, while the LOD scores estimated by SIM are indicated in blue. The red line represents the LOD threshold determined by 1000 permutations.

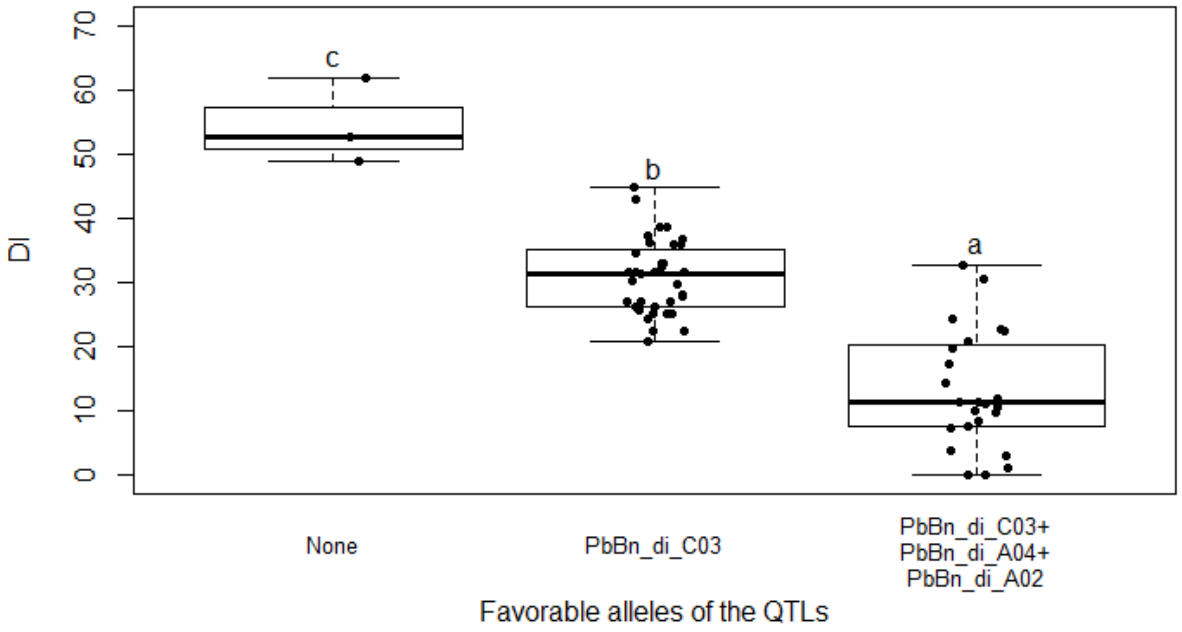


**Figure 5-6.** Boxplot showing clubroot disease index (DI) in the doubled-haploid progeny from a cross of the oilseed rape cultivars 'Aviso' × 'Montego'. The reactions of the progeny are grouped based on genotypes which possess none of the favourable QTL alleles (‘None’); the favourable allele of the QTL PbBn_di_C03 (‘PbBn_di_C03’); and the favourable alleles of the QTLs PbBn_di_C03, PbBn_di_A04, and PbBn_di_A02 (‘PbBn_di_C03 + PbBn_di_A04+ PbBn_di_A02’). Boxes with the same letter within the graph do not differ according to Duncan's new multiple range test at $p > 0{\cdot}05$.

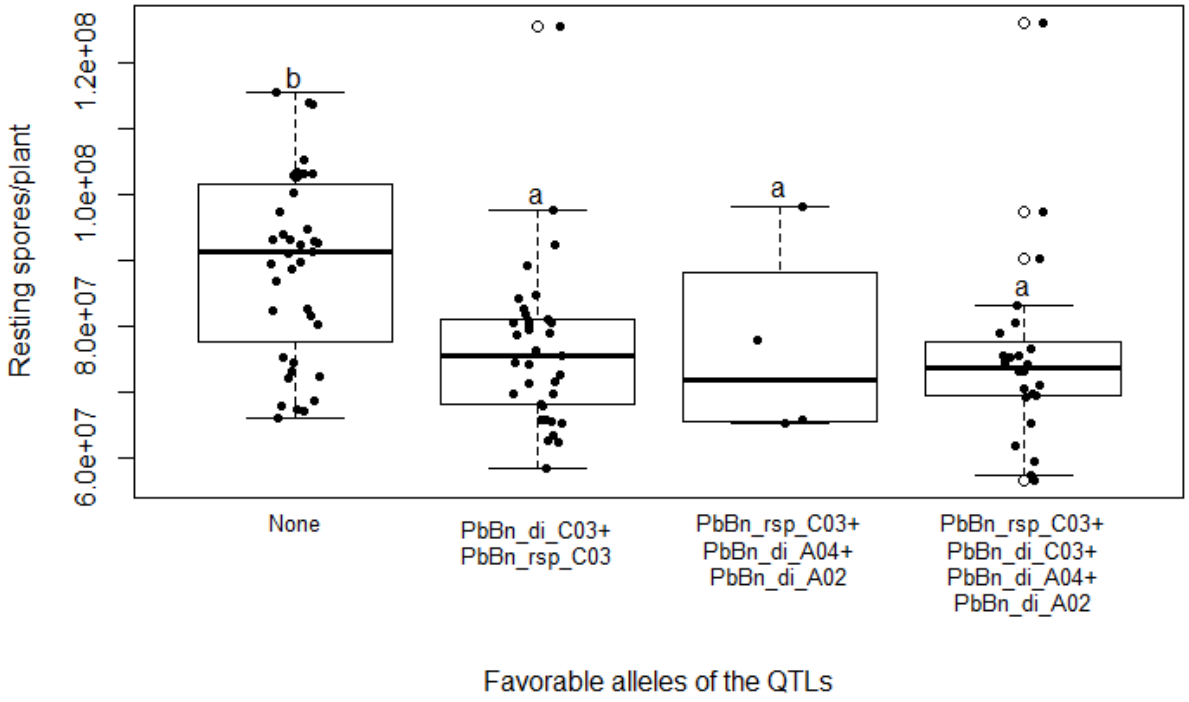


**Figure 5-7.** Boxplot showing *Plasmodiophora brassicae* resting spores per plant in the doubled-haploid progeny from a cross of the oilseed rape cultivars 'Aviso' × 'Montego'. The progeny are grouped based on genotypes which possess the favourable alleles of the QTLs PbBn_rsp_C03, PbBn_di_A04, and PbBn_di_A02 ('PbBn_rsp_C03 + PbBn_di_A04 + PbBn_di_A02'); the favourable alleles of the QTLs PbBn_rsp_C03 and PbBn_di_C03 ('PbBn_rsp_C03 + PbBn_di_C03'); the favourable alleles of the QTLs PbBn_rsp_C03 + PbBn_di_C03 + PbBn_di_A04+ PbBn_di_A02 ('PbBn_rsp_C03 + PbBn_di_C03 + PbBn_di_A04 + PbBn_di_A02'); or none of the favourable alleles ('None'). Boxes with the same letter within the graph do not differ according to Duncan's new multiple range test at $p > 0{\cdot}05$.

# Conclusion

Clubroot is one of the most important threats associated with canola/oilseed rape (*Brassica napus* L.) production. While multiple strategies have been proposed for the management of this disease, the most effective tactic to date has been the deployment of cultivars with genetic resistance (Diederichsen et al., 2006, 2009). Unfortunately, the planting of clubroot resistant (CR) cultivars imposes a strong selection pressure on *Plasmodiophora brassicae* Wor., causing rapid shifts in the population structure of the pathogen (Holtz et al., 2018). These shifts favor an increase in new virulence phenotypes, resulting in a loss of effective resistance, as has already occurred in Canada and Europe (Kuginuki et al., 1999; Diederichsen et al., 2014; Orgeur et al., 2016; Strelkov et al., 2016; Hollman et al., 2021).

This dissertation presented three studies to improve understanding of *P. brassicae* epidemiology and the genetic control of the resistance harbored by specific host genotypes. In the first study, a model was developed, by comparing two *P. brassicae* pathotypes and canola cultivars with different levels of resistance, to describe the relationship between clubroot severity and yield loss. In the second study, in-field inoculum dynamics and spatial patterns were examined with respect to soil characteristics and wind direction. Finally, in the third study, an analysis of the genomic regions involved in the resistance harbored by a doubled haploid population of winter oilseed rape was conducted, to identify quantitative trait loci (QTLs) involved in the control of clubroot severity and resting spore production. In the following sections, the conclusions and recommendations stemming from these studies will be addressed.

## 6.1 Effect of clubroot on canola yield

Maintaining crop profitability is the main objective behind any integrated plant disease management program. To achieve this goal, it is important to estimate the impact of disease accurately, thereby enabling the establishment of economic thresholds for risk assessment and the selection of appropriate management strategies (Shtienberg, 2000). While clubroot is one of the most damaging diseases of canola, replicated experiments to determine the yield or economic losses associated with *P. brassicae* infection have been very limited (Rempel et al., 2014).

In Chapter 3 of this thesis, the relationships among clubroot incidence and severity, inoculum density, and host resistance were evaluated in connection with canola yield and yield-related parameters. The results indicated that clubroot levels varied depending on inoculum density, the environment (rainfall), host resistance, and pathogen virulence. Nonetheless, once clubroot developed on a host, the yield losses were similar for each unit increase in disease severity index (DSI). Independently of the host cultivar, yield was estimated to decline by 0.26% in the field and 0.49% in the greenhouse with each 1% increment in DSI. These yield reductions reflected a decrease in the number of pods per plant and 1000-grain weight.

Regardless of the virulence of specific isolates, a wide range of clubroot levels were obtained, which could be linked back to yield and yield-related parameters to model the impact of clubroot on yield. Such a wide range of disease allowed us to fit linear and linear mixed models capable of explaining reductions in seed weight, pods per plant (PPP) and 1000-grain weight based on the DSI. It is worth noting that these models may be applicable to most canola cultivars, since the slope (yield loss rate for each 1% increase in DSI) was equal in all of the hosts evaluated in this study. Indeed, once clubroot symptoms begin to develop on a CR genotype, the relative declines

in yield per unit increase in disease will be equivalent to those of a susceptible host. The only reason that they would be expected to suffer less yield loss is that they develop less disease.

### 6.2 Inoculum density and spatial patterns and relationship to soil characteristics

Epidemiological studies on pathogen spatial patterns and spread can help to improve understanding of *P. brassicae* biology and ecology. Such knowledge is essential for the development of improved and more effective sampling methods and disease management programs (Nicot et al., 1984; Campbell and Noe, 1985; Chellemi, 1988; Rekah et al., 1999; Kranz, 2012). Currently, however, only a few studies have conducted any spatial analysis of clubroot disease at the field level (Cao et al., 2009; Wallenhammar et al., 2012; Řičařová et al., 2017).

In Chapter 4, *P. brassicae* inoculum dynamics and spatial patterns, and their relationship to soil pH, boron, calcium and magnesium concentration, as well as prevalent wind direction, were examined over time in four fields in central Alberta. The results indicated that inoculum of the pathogen occurred mostly at the field margins, converging at the field entrance. The diameter of infested patches grew an average of 221.3 m from 2017 to 2019, with this growth determined by the maximum inoculum density and active dispersal methods. In the evaluated fields, wind and possibly mechanical operations contributed to in-field dispersal of *P. brassicae*. In contrast, soil pH, boron, calcium, and magnesium concentrations were not found to have an important effect on the inoculum density. These results do not necessarily indicate that these soil chemical properties do not affect pathogen inoculum density, but rather suggest that other underlying spatial processes have a greater influence on spatial patterns, at least in the fields evaluated.

Furthermore, the results in Chapter 4 underscore the importance of conducting multiple statistical analyses when assessing spatial aggregation of soilborne diseases. While patchiness in

the *P. brassicae* inoculum distribution was evident in maps, Moran's *I* indices were slightly greater than zero, indicating a lack of aggregation. However, this does not mean a lack of spatial autocorrelation, but rather may indicate the need for additional analysis using tools such as geostatistics, since different spatial processes can create bias in the estimation of spatial autocorrelation indices (Chellemi, 1988). In the present case, further analyses through semivariograms confirmed the spatial aggregation observed in the maps.

One of the main difficulties when sampling soil pathogens is defining an appropriate sampling strategy to capture variability between individual soil cores (Ophel-Keller et al., 2008). When defining a sampling strategy, different challenges may arise, among them identification of the area to be sampled, the definition of the sampling unit, the timing of the sampling effort, and the size of sample required (Hughes and Madden, 2002). In Chapter 4, different locations were observed for *P. brassicae*-infested patches in Field 1 in 2017 vs. 2019. These results may reflect a large variation in and a skewed distribution of the pathogen DNA between individual soil cores (Ophel-Keller et al., 2008). Therefore, while the sampling strategy in Chapter 4 was aimed at capturing most of the within-field variation in *P. brassicae* inoculum, the sampling intensity was not sufficient, especially in 2017. Previous results suggest that the choice of sampling strategy is one of the main issues associated with accurately evaluating a field for the presence of this pathogen (Cao et al., 2007; Wallenhammar et al., 2012).

Better and more rational sampling schemes require information on spatial patterns to evaluate both the pathogen and the disease (Xiao et al., 1997; Faggian and Strelkov, 2009). Previously proposed soil sampling strategies for the detection *P. brassicae* suggest sampling along a diagonal (Wallenhammar, 1996), or the collection of 40 subsamples in a 'W' transect that should be pooled in a composite sample and complemented with samples from high moisture areas, headlands and

the field entrance (Wallenhammar et al., 2012). Clubroot symptom-based sampling strategies propose evaluation for the presence of galled roots in at least 50 plants collected from a 20-30$m^2$ area near the field entrance; if symptoms are observed, further sampling is conducted along the field following a 'W' transect, whilst if no symptomatic plants are found, no further sampling is conducted (Strelkov et al., 2019). This strategy is justified based on the higher prevalence of *P. brassicae* infestation reported at field entrances (Cao et al., 2009).

While previous sampling strategies may be appropriate in many cases, based on the spatial patterns observed in this study, sampling should be more intense at the field edges, converging at the field entrance. Therefore, a sampling strategy is proposed wherein the field is divided in three quadrats that should be sampled independently, with two smaller quadrats that correspond to the field edges and which converge at the field entrance, and one larger quadrat that covers the other margins and the centre of the field (Figure 6-1). In the two smaller quadrats, sampling should be conducted in a regular grid of 40 m × 80 m, while in the larger quadrat, nine samples should be collected following a 'W' transect. For the purposes of PCR testing for *P. brassicae* DNA, all samples from each quadrat could be pooled together, to generate three composite samples for each field, particularly if there are cost or personnel limitations. It is worth noting, however, that the ability to detect the clubroot pathogen in pooled samples can diminish due to inoculum dilution effects, for example, if uninfested subsamples are pooled with mildly infested ones (Cao et al., 2007). For mapping purposes, the detection and quantification of inoculum should be kept separate.

## 6.3 Control of clubroot symptom development and resting spore production in a doubled haploid population of oilseed rape

Given the efficacy and ease of use of CR varieties, genetic resistance should remain as an option for clubroot management. Ideally, however, the durability of the resistance could be enhanced via strategies such as the deployment of polygenic resistance, gene pyramiding, and the planting of multilines and cultivar mixtures (Parlevliet and Zadoks, 1977; Pink and Puddephat, 1999). These approaches require a deeper understanding of the genomic regions involved in the resistance harbored by specific host genotypes. As such, in Chapter 5, linkage analysis was carried out to identify the QTLs involved in the control of DSI and resting spore production per plant (RSP) in a segregating doubled haploid (DH) population from the cross of 'Aviso'× 'Montego', two modern winter oilseed rape cultivars partially resistant to clubroot with good agronomic characteristics.

Analysis of the DSI and RSP in the 'Aviso' × 'Montego' population indicated that RSP is not correlated as strongly with DSI as might be expected. As a result, some lines with mild clubroot symptoms had a high resting spore production, and some lines with intermediate symptoms had a low resting spore production. Furthermore, the highest observed RSP was 2.5× greater than the lowest. These results suggest the need for the inclusion of RSP as a trait to evaluate in breeding programs for CR cultivars, since such variation may be important from an epidemiological perspective. For instance, a doubling of the number of resting spores produced and released into the soil could result in faster and more significant inoculum increases for future crops.

Linkage analysis showed that the genetic control of DSI in the evaluated population relies on two minor QTLs on the chromosomes A02 and A04 (PbBn_di_A02 and PbBn_di_A04 and), and a major QTL on chromosome C03 (PbBn_di_C03). Only one QTL was found to control the

production of resting spores per plant (PbBn_rsp_C03). It was inferred that genetic control of RSP and DSI are related, since the QTLs PbBn_di_C03 and PbBn_rsp_C03 co-localized, suggesting that the C03 locus controls both traits. The importance of this genomic region is highlighted by the fact that PbBn_di_C03 explains the highest proportion of the variation for DSI. In addition, QTLs at syntenic positions have been identified in *Brassica oleracea* L. on chromosome C03 (Nagaoka et al., 2010; Lee et al., 2016; Li et al., 2016), suggesting that the C03 genomic region merits further investigation.

The parents of the DH population, 'Aviso' and 'Montego', were released by Danisco Seeds (Holeby, Denmark) in 2000 and by Limagrain (Saint-Beauzire, France) in 2002, respectively. 'Aviso' is a variety with good agronomic performance that shows resistance to other diseases such as blackleg (*Leptosphaeria maculans*) (Stonard et al., 2007; Jestin et al., 2015). These cultivars may represent suitable donors of clubroot resistance in oilseed rape breeding programs, considering that with their good agronomic qualities, less intensive backcrossing with elite lines would be required, facilitating the transfer of polygenic traits into new cultivars. The potential utility of the 'Aviso' × 'Montego' population in breeding programs is enhanced by the fact that resting spore production was lower relative to values reported in previous studies.

### 6.4 Final conclusion and impact

The results presented in this thesis contribute knowledge that may be useful for mitigating the impact of clubroot on canola and oilseed rape. It is clear that once *P. brassicae* is introduced into a field, the spread of infection foci can be quick. Therefore, strategies such as patch management can be helpful in slowing their growth over time, and ultimately in reducing the losses caused by clubroot at the field scale. While various soil parameters have often been implicated in affecting

in-field spread of the disease, wind, mechanical operations, and inoculum density may play particularly significant roles. Longer rotations out of susceptible crops will help to reduce inoculum density, and hence should be practiced whenever possible. Once the disease is established, the yield losses associated with clubroot development on canola can be very significant. For example, even a mild level of disease, such as a DSI = 10% (found in many fields in western Canada), would result in a yield reduction of about 2.5% based on the yield loss model presented. This is not necessarily an inconsequential figure, particularly when canola prices are high. Multiplied over hundreds or thousands of fields across Alberta and western Canada, one can appreciate the potential impact of clubroot on overall productivity. Finally, while most of the emphasis in clubroot resistance screening has focused on DSI, the evaluation of the 'Aviso' × 'Montego' population suggests that control of RSP is also important, and should not be overlooked.

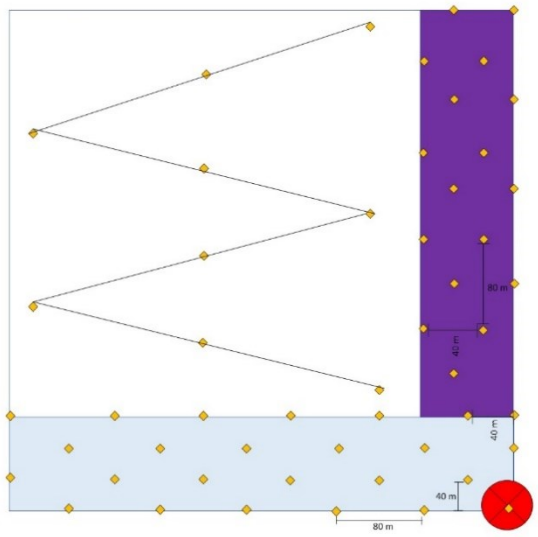


**Figure 6-1.** Proposed sampling strategy for the detection of inoculum of *Plasmodiophora brassicae* in soil samples. Red circle represents the field entrance, blue rectangle is the first sampling quadrat, purple rectangle is the second sampling quadrat, and white rectangle is the third sampling quadrat; yellow diamonds represent the points where samples should be collected.

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