The Impact of Clubroot Resistant Canola Cultivars on *Plasmodiophora brassicae* Resting Spore Concentrations in the Soil

by

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Abstract

The soilborne pathogen *Plasmodiophora brassicae* Woronin, causal agent of clubroot of canola (*Brassica napus* L.), is difficult to manage due to the longevity of its resting spores, its ability to produce large amounts of inoculum, and the prohibitive costs of effective fungicides. The cropping of clubroot resistant (CR) canola cultivars is one of the few effective strategies for clubroot management. This study evaluated the impact of the cultivation of CR canola on *P. brassicae* resting spore concentrations in commercial cropping systems in Alberta, Canada. Soil was sampled pre-seeding and post-harvest at multiple geo-referenced locations within 17 *P. brassicae*-infested fields over periods of up to four years in length. Resting spore concentrations were measured by quantitative PCR analysis, with a subset of samples also evaluated in greenhouse bioassays with a susceptible host. The cultivation of CR canola in soil with quantifiable levels of *P. brassicae* DNA resulted in increased inoculum loads. There was a notable lag in the release of inoculum after harvest, and quantifiable *P. brassicae* inoculum peaked in the spring following years when resistant canola was cultivated. Rotations that included a ≥2-year break from *P. brassicae* hosts resulted in significant declines in soil resting spore concentrations. A strong positive relationship was found between the bioassays and qPCR-based estimates of soil infestation. The results suggest that CR canola should not be used as a tool to reduce soil inoculum loads, and that crop rotations in *P. brassicae* infested fields should include breaks of at least two years away from *B. napus*.

Preface

This thesis is an original work by Thomas William Ernst. No part of this thesis has been published previously other than as preliminary conference abstracts stemming from his presentations as a student. Mr. Ernst conducted all of the experiments described in this document, and wrote the first drafts of all chapters. The chapters were examined by Mr. Ernst's supervisor, Dr. Stephen Strelkov. Dr. Strelkov provided editorial revisions and suggestions on each chapter, which were then incorporated by Mr. Ernst.

Mr. Ernst collected and processed all of the soil samples, with the occasional assistance of summer students or other lab personnel. The 2010 samples were collected by Ms. Christine Pelchat, and the spring 2011 samples were collected by Mr. Ernst (prior to the initiation of his M.Sc. studies), with the assistance of staff at DuPont Pioneer and with the knowledge and consent of cooperating farmers. These samples were placed in storage until Mr. Ernst commenced his M.Sc. program. All other samples were collected over the course of his M.Sc. Mr. Ernst conducted all of the conventional and quantitative PCR analyses, soil bioassays, and data analysis presented in this thesis.

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1. Introduction & Literature Review

1.1. Introduction to *Plasmodiophora brassicae*

1.1.1. Pathogen Life Cycle and Disease Development

Clubroot of crucifers is a soil-borne disease caused by the biotrophic parasite *Plasmodiophora brassicae* (Woronin, 1878). Symptoms of clubroot have been known since the 13th century, long before Woronin identified *P. brassicae* as the causal agent (Karling, 1968, Cook and Schwartz, 1930). Clubroot is characterized by galling or enlargement of infected roots, usually resulting in spindle-shaped root formations or, in more severe infections, larger compound galls (Karling, 1968). A full description of the *P. brassicae* life cycle, adapted from Kageyama and Asano (2009), is provided in Fig. 1-1, and discussed below. Resting spores of the pathogen germinate in the presence of a susceptible host, releasing motile (primary) zoospores. These zoospores encyst and penetrate the root hairs of the host, forming a primary plasmodium which matures into a zoosporangium and then releases secondary zoospores back into the soil matrix. During primary infection (Fig. 1-1. see embedded 3A & 3B), symptoms may not be apparent unless root hairs are viewed under a microscope. The secondary zoospores initiate secondary infection of root cortical tissue. Primary zoospores have also been postulated to contribute to secondary infection (Feng et al., 2013) as have direct movement of primary plasmodia from root hairs into root cortical tissue (Mithen and Magrath, 1992, Graveland et al., 1992). Internally, *P. brassicae* secondary infection of root cortical tissue spreads via an

amoeboid like plasmodium, up and down the cambium, and out into the cortex and xylem (Asano and Kageyama, 2006). In the cortex, infected cells, which should differentiate into functional xylem/medullary rays, instead undergo hyperplasia and hypertrophy resulting in malformed elongated cells that are unable to fulfill their designated purpose (Karling, 1968). Hypocotyl swelling can be observed 15 days after exposure to *P. brassicae* inoculum, while galls or 'root swellings' can be observed after 23 days, and fully developed white galls are observed after 28 days (Agarwal et al., 2009). Once secondary plasmodia fully mature, resting spores form within infected host root cortical cells. When host roots decay, the resting spores are released back into the soil matrix.

Above ground symptoms in a susceptible host include yellowing and wilting of the leaves, pre-mature ripening, and in some cases, atrophy and failure to produce seed. During a three year study looking at the effects of clubroot on various canola (*Brassica napus* L.) cultivars, Pageau et al. (2006) observed yield losses in excess of 80%, as well as reduced straw production, reduced grain oil content, and reduced grain mass. The end result of *P. brassicae* infection is accelerated maturation, stunting, and decreased yield, quality, palatability, and storability of the seed (Dixon, 2009a).

Since the identification of *P. brassicae* as the causal agent of clubroot, various aspects of the life cycle of *P. brassicae* have been elucidated. Host plants of all ages are susceptible, and older plants are almost as susceptible as young ones when growth still occurs (Kunkel, 1918). Kunkel (1918) described plasmodial infection and spread through infected root cortical tissue. Once penetration of cortical tissue occurs, infection spreads throughout the cambium in all directions from the initial point of infection, eventually moving into the cortex/xylem region

and then into the medullary rays. Cook and Schwartz (1930) uncovered the presence of zoospores swarming near the root hairs, and distinguished between primary and secondary zoospores, highlighting the two phases of the *P. brassicae* life cycle: primary infection of the root hairs, with formation of zoosporangia, and secondary infection, consisting of formation of plasmodia in the root cortical tissue, with the eventual production of resting spores. Ledingham (1934) found that *P. brassicae* zoospores have two unequal flagella. These bi-flagellate zoospores typically move no further than 12.7 cm (Chupp, 1917, cited by Karling, 1968). Macfarlane (1970) described *P. brassicae* resting spore germination, including the increased germination success of resting spores from older galls, and increased resting spore germination in the presence of host root exudates. Wallenhammar (1996) studied the longevity of these resting spores, reporting that they have a half-life of 3.6 years in the field, and may persist up to 18 years. Penetration of the host root hairs was described by Aist and Williams (1971), and is achieved mainly via mechanical processes. After the zoospores encyst on the root hairs, a penetrating body known as a Stachel forms within a tubular cavity commonly termed a Rhor. Forced by an expanding cyst vacuole, the Stachel penetrates the host cell wall injecting the parasite within the host.

Study of the progression from primary to secondary infection has been challenging. Difficulty differentiating the origin of *P. brassicae* structures within host cell tissue using existing electron microscopy techniques is a limiting factor in fully understanding the life-cycle (Donald et al., 2008). Observing the transition of *P. brassicae* between phases of its lifecycle, within plant cells in real-time, also remains a challenge. Despite the difficulties, primary plasmodial infection of the root hairs has been observed spreading into root cortical tissue prior to

zoosporangia maturation via a myxamoeboid phase and the migration of small plasmodia (Mithen and Magrath, 1992, Graveland et al., 1992). Mithen and Magrath (1992) described myxamoeba movement, with the aid of pseudopodia-like structures, into the outer cortex of roots via induced breaks in host cell walls. Alternatively, secondary zoospores can infect root cortical tissue directly, after being released into the soil, by movement through infected root hairs, or by a combination of both these methods (Kageyama and Asano, 2009). Asano and Kageyama (2006) also proposed that secondary infection progresses from a myxamoeboid phase in the epidermis, to a secondary plasmodial phase in the internal root vascular tissue, which could spread via cytoplasmic streaming and host-cell division. Furthermore, Feng et al. (2013) suggested that primary zoospores that germinate from resting spores are themselves capable of causing secondary infection directly. It is entirely conceivable that all three methods of pathogen progression occur between zoospores, primary infection, and secondary infection (Fig. 1-1).

P. brassicae influences growth regulating hormonal signaling pathways in the host plant, resulting in a variety of host responses (Ludwig-Müller et al., 2009, Ludwig-Müller, 2014). The pathogen influences cytokinin pathways in the host to produce invertase necessary for pathogen nutrition. Furthermore, the plant hormones cytokinins (CK), indole-3-acetic acid (IAA), and brassinosteroids (BR) are responsible for the hypertrophy and increased cell division associated with *P. brassicae* infection. Hyperplasia and hypertrophy within host roots are responsible for the typical galling symptoms associated with clubroot. Ludwig-Müller (2014) outlines other hormonal pathways influenced by *P. brassicae* infection, and the physiological effects on the host. CK hormone signaling could be responsible for redirection of nutrient

transportation via the phloem to the pathogen. Metabolic changes associated with altered hormone signalling pathways also could be responsible for reduced apical meristem growth (Ludwig-Müller, 2014). Diversion of nutrients to the pathogen, and an inability of the host to properly assimilate water and nutrients due to galling of the roots, results in the typical above-ground symptoms associated with clubroot.

1.1.2. Taxonomic Classification of *Plasmodiophora brassicae*

P. brassicae is a phylogenetically well-supported clade within the plasmodiophorids (Neuhauser et al., 2014). The plasmodial endoparasites of plants form a discrete taxonomic unit known as the Plasmodiophorids, and are considered a monophyletic group (Braselton, 1995). Neuhauser et al. (2010) summarized how protein sequence data from the plasmodiophorids places them in a new hierarchical system outlined by Adl et al. (2005). Based on this new system of classification, the plasmodiophorids are placed within the Phytomyxea, a grouping of protist parasites of plants. Phytomyxea are embedded within the Endomyxea in the Cercozoa, a sister group of the Foraminifera (Keeling, 2001, Archibald and Keeling, 2004), and is composed of all studied filose testate amoeba including the Plasmodiophorida (Cavalier-Smith and Chao, 2003). The Cercozoa and Foraminifera are within the Rhizaria, a monophyletic Eukaryotic group based on Bayesian maximum-likelihood analysis of small-subunit and large-subunit ribosomal DNA markers (Moreira et al., 2007). A full classification of *P. brassicae* lacks complete monophyly but is still useful for the purpose of discussion (Fig. A-1).

1.1.3. Environmental Factors Influencing Clubroot Disease

Many environmental factors influence the development of clubroot disease in susceptible hosts. The effect of soil temperature on the development of clubroot has been discussed widely in the literature. In one of the earliest studies, Monteith (1924) demonstrated that *P. brassicae* infection of cabbage (*Brassica oleracea* L.) could occur within a large temperature range (between 9 ˚C and 30 ˚C). Ideal temperatures for cabbage growth resulted in greater clubroot disease severity and incidence, with the greatest incidence occurring near 20 ˚C and greatest severity occurring near 25 ˚C. In radish (*Raphanus sativus* L.), soil temperatures of approximately 21-22°C resulted in the greatest clubroot severity (Thuma et al., 1983). Temperature also has a specific effect on clubroot infection of canola (*Brassica napus* L.). A review by Gossen et al. (2014) indicates that *P. brassicae* resting spores germinate only at soil temperatures above 14 ˚C, disease development in canola is very slow below 17 ˚C, the fastest disease progression occurs around 23-26 ˚C, and fluctuating temperatures (difference between min/max temperatures of 10 ˚C) have no effect on pathogen success or development. Soil temperature throughout the growth period of a plant appears to be a key predicting variable for clubroot severity, along with rainfall during the seedling stage, and the 'soil moisture' × 'soil temperature' interaction (Thuma et al., 1983).

Soil moisture plays a role in *P. brassicae* development. Zoospore mobility requires moisture between pores within the soil (Cook and Papendic, 1972), and is necessary for the disease to progress from resting spore to primary and secondary infection (Fig. 1-1). High rainfall/soil moisture during the seedling stage is favourable for disease development and increased disease severity (Thuma et al., 1983). The release of zoospores from mature

zoosporangia after primary infection is dependent on the availability of free moisture near the root hairs (Ayers, 1944). The rate of resting spore germination itself is increased under moist conditions (Macfarlane, 1952). Dohms (2013) suggests that soil moisture levels play a greater role in clubroot infection than soil temperature. In one study, infection did not develop when soil water saturation was below 45% of the holding capacity of that soil type (Monteith, 1924). In addition, all of the soil types tested by Monteith (1924) yielded increasing disease incidence and severity when water saturation was greater than 60% of the holding capacity of that soil type. Infection is still possible at low soil moisture levels, and will occur in most soils as long as moisture is adequate. Nonetheless, clubroot severity is likely increased as soil moisture increases, and soils with a greater capacity for moisture retention favor the disease (Gossen et al., 2014). Furthermore, increased rainfall exacerbates clubroot severity in the field in multiple brassica crops, including canola.

Soil pH is another factor influencing *P. brassicae* development. Karling (1968) noted that infection occurs most readily at a pH of 6.0 - 6.5, and that there is a limiting effect on clubroot development when soil pH is 7.2-7.4 with an even greater effect closer to pH 8. However, clubroot infection is still able to occur at a pH above 8.1, and Karling (1968) clearly states that soil pH alone is not a sufficient determining factor of clubroot development. Further research shows that liming of the soil limits *P. brassicae* resting spore germination without effectively decreasing spore longevity (Macfarlane, 1952, Karling, 1968). However, the effectiveness of liming to limit resting spore germination is likely only temporary, and prolonged inoculum availability could eventually result in infection. Increased pH does not kill clubroot resting spores (Karling, 1968), but may only prolong their dormancy until favourable conditions arise

(i.e., pH decreases to favourable levels), potentially increasing the persistence of *P. brassicae* resting spores in the soil (Macfarlane, 1952). The effect of soil pH on clubroot development also has been summarized by Gossen et al. (2014). When soil pH is greater than 7.2, clubroot development may decrease under controlled conditions, but even heavy liming does not prevent disease development in the field when other conditions are favourable (i.e., sufficient spore load, soil moisture, etc.). Alone, increased pH is only weakly correlated with decreased disease severity under field conditions in western Canada (Gossen et al., 2013), however, there is a notable amplifying 'soil pH' X 'soil temperature' interaction, where the greatest levels of infection occur at low pH (~ pH 6) and higher temperatures (~ 25 ˚C).

Another factor affecting *P. brassicae* development is the concentration of nutrients in the soil. The influence of the micronutrients copper, boron, zinc, molybdenum and manganese on clubroot germination and primary infection has been assessed. Copper and boron were found to decrease the levels of root tissue infection (Lewis et al., 2013). Increased rates of boron can inhibit maturation of primary plasmodia into zoosporangia in the root hairs, and maturation of secondary plasmodia into resting spores within root cortical tissue (Gossen et al., 2014). A high rate of boron application (16 - 32 kg ha^{-1}) prior to seeding reduces clubroot severity in muck soil, but is less effective in the mineral soils (where boron leaches out more rapidly) typical of the canola growing region in Canada (Gossen et al., 2014). Calcium salts prolong *P. brassicae* resting spore dormancy (Macfarlane, 1970), and when used to increase soil pH, there is a greater reduction in clubroot symptoms than other soil basification methods alone (Gossen et al., 2014). Nitrogen applied in the form of nitrate along with calcium in the form of calcium cyanamide ($CaCN_2$) or calcium nitrate ($CaNO_3$) inhibits clubroot development

(Gossen et al., 2014). However, the low efficacy of these fertilizer treatments, the high levels of calcium already present in soils across the Canadian prairies, and the costly nature of specialized fertilizer treatments (such as calcium cyanamide) suggest that these strategies will not be useful for clubroot management (Gossen et al., 2014).

1.1.4. *Plasmodiophora brassicae* Host Range

Plasmodiophora brassicae is known to have a very broad host range, including but not limited to all tested cultivated and non-cultivated cruciferous plants (Gibbs, 1932, Karling, 1968). Karling (1968) identified 89 species from 8 crucifer genera that are susceptible to clubroot. Virtually every species from all genera of the Brassicacea family are expected hosts of *P. brassicae (Dixon, 2009a)*. A number of susceptible hosts and non-hosts are outlined in Table 1-1, including brassica crops, cruciferous weeds, and non-host plants previously tested for clubroot symptoms (Gibbs, 1932, Webb, 1949, Macfarlane, 1952, Ludwig-Müller et al., 1999, Feng et al., 2012). Host plants of all ages are susceptible to *P. brassicae* infection, and older plants are almost as susceptible as young ones when growth still occurs (Kunkel, 1918).

Many plasmodiophorids have hosts from two or more plant families, and *P. brassicae* is known to parasitize both monocot and eudicot host species (Neuhauser et al., 2014). Webb (1949) observed the primary infection of non-host grass *Holcus lanatus* root hairs. Ludwig-Müller et al. (1999) highlighted a number of non-brassica hosts with varying degrees of susceptibility, which ranged from only allowing primary *P. brassicae* infection, to being completely susceptible and allowing the pathogen to complete its life cycle (Table 1-1). Primary infection can even occur in non-hosts such as ryegrass (*Lolium perenne* cv. Amazing), and the

secondary zoospores produced from the non-host are capable of infecting susceptible hosts (Feng et al., 2012).

Host plant resistance can limit clubroot infection; however, these plants may still undergo primary infection or even allow development of mature resting spores by the pathogen, effectively propagating inoculum. Primary infection and some secondary stages of infection occur in both susceptible and resistant *Brassica oleracea* hosts. Resistant *B. oleracea* show symptoms of primary and secondary infection that are identical or similar, respectively, to symptoms on susceptible varieties. These include host cell wall breaks, vesicles or inclusion bodies within the host cell walls, cell wall thickening near plasmodesmata and enlarged/disorganized host nuclei (Donald et al., 2008). Resistant genotypes of *B. oleracea* do not, however, experience degradation of xylem cell walls or develop the severe galling observed in susceptible lines. Resistant *B. napus* cultivars produce less galled root mass (Hwang et al., 2012b, Hwang et al., 2015) and produce significantly less inoculum (Hwang et al., 2012b), than susceptible cultivars. In *B. napus*, spheroid galls, once thought to be resistance structures, actually allow very small quantities of *P. brassicae* resting spores to be produced (Rennie et al., 2013). Proliferation of *P. brassicae* within spheroid galls occurs mainly in the extra-stelar space not associated with root vascular tissue, and results in a low incidence of mature resting spore production. This differs from the larger, typical 'spindle galls' observed in highly susceptible cultivars, where *P. brassicae* proliferation occurs within both the stele and extra-stelar region and resting spore maturation was observed in virtually all instances (Rennie et al., 2013). Spheroid galls are regularly observed on resistant and susceptible canola cultivars during field and greenhouse testing (Rennie et al., 2013).

1.2. Impact and Management of *Plasmodiophora brassicae*

1.2.1. Impact

Cultivated brassicas make-up the most important crops economically and nutritionally worldwide after cereals (Dixon, 2014). The agricultural benefits of brassicas are numerous (Dixon, 2007), and their potential importance as model species for the study of molecular biology are still being uncovered (Dixon, 2014). Furthermore, oilseed rape (*Brassica napus*) could become increasingly viable alternatives to fossil fuels considering the positive energy balance for biodiesel production from these crops (Tolmac et al., 2014).

Clubroot is the most important disease of cultivated crucifers worldwide (Karling, 1968, Dixon, 2014). Global crop losses as a result of this pathogen have been estimated at 10 - 15% (Dixon, 2006). In Australia, *P. brassicae* mainly impacts vegetable *Brassica* crops (Donald and Porter, 2014). Clubroot has been reported in Australian canola, but has not yet become widespread in canola-producing fields. Pathotypes virulent on *B. napus* were present in less than 20% of isolated samples collected from clubroot infested areas of Australia (Donald and Porter, 2014). In Germany, clubroot has shifted from affecting primarily *Brassica* vegetables, and is considered now to have a serious impact on oilseed rape or canola (Diederichsen et al., 2014). Clubroot is widespread throughout Sweden, and infection can be found not only in vegetable *Brassica* spp. but also in spring/winter oilseed rapes (Wallenhammar et al., 2014), with up to 78% of surveyed fields testing positive for the presence of clubroot (Wallenhammar, 1996). In China, one third of the total area in which cruciferous crops are grown is affected by clubroot, resulting in average yield losses of 20 - 30% (Chai et al., 2014). Expansion of *Brassica*

vegetable and oilseed rape cultivation in India since the mid-1980s has been accompanied by increased *P. brassicae* incidence and severity, causing estimated crop losses of 32.5% (Bhattacharya et al., 2014).

The importance of canola (*Brassica napus*) in Canada cannot be underestimated. Developed in Canada via conventional breeding techniques, canola products are widely used and consumed (Rempel et al., 2014). Canola acreage continues to increase since 2000 (8.6 million hectares harvested in 2012), with Canada-wide yield averages of 1980 kg ha^{-1} (2009), 1920kg ha^{-1} (2011), and 1550kg ha^{-1} (2012), compared with only 3.6 million hectares harvested in 2003 (Rempel et al., 2014). In 2015, 8.08 million hectares of canola were harvested (Canola Council of Canada, 2016a), with yields of 2100 kg ha^{-1} (Canola Council of Canada, 2016b). In Canada, clubroot was restricted to vegetable *Brassicas* until 1997, when it was identified on canola (*Brassica napus* L.) in Quebec, which was followed by the identification of clubroot of canola near Edmonton, Alberta, in 2003 (Tewari et al., 2005, Strelkov and Hwang, 2014). At the time of identification, all cultivated canola cultivars tested were 'highly susceptible' to the pathotypes present in Alberta, which included European Clubroot Differential (ECD) 16/15/12 or pathotype 3, and ECD 16/15/0 or pathotype 5, as defined on the hosts of the ECD differential set (Buczacki et al., 1975) and the Williams differential set (Williams, 1966), respectively (Strelkov et al., 2006b, Strelkov et al., 2007). Pathotyping of single-spore isolates of *P. brassicae* collected from Alberta indicated there are likely four pathotypes present in the province (Xue et al., 2008). In canola fields in Alberta, disease incidences as high as 94% have been reported, resulting in yield losses of approximately 30% (Tewari et al., 2005). In some severely infected canola fields, yield loss may reach up to 100% if the crop is not suitable for harvest. However,

severe cases such as this make up less than 10%-15% of surveyed fields in the province (Strelkov and Hwang, 2014). The spread of *P. brassicae* throughout Alberta has been tracked through annual clubroot surveys which have uncovered new cases every year, resulting in an almost 90-fold increase in confirmed field infestations from 2003 to 2013 (Strelkov and Hwang, 2014). These cases include a significant number of infested fields previously surveyed and found to be free of clubroot, indicating that the observed spread of clubroot in the province is not simply a result of increased/focused survey efforts. Currently, the region with the greatest incidence of clubroot remains central Alberta, where the disease was first observed on Canadian canola cultivars. Nonetheless, a small yet increasing number of cases have been reported in southeastern Alberta, Saskatchewan, Manitoba, and North Dakota (Gossen et al., 2015).

1.2.2. Introduction to the Management of *Plasmodiophora brassicae*

In general, the management of *P. brassicae* is extremely difficult despite over 100 years of research directed towards all forms of control (Howard et al., 2010). The difficulty lies within the nature of the pathogen itself. The longevity of the resting spores (Wallenhammar, 1996), its below-ground nature, intracellular lifestyle, and late above-ground display of symptoms, make successful management of *P. brassicae* very challenging.

In Alberta, the identification of clubroot on canola is relatively recent, observed for the first time in 2003 (Tewari et al., 2005), and as such, the pathogen has not yet completely proliferated within its potential range throughout the Canadian Prairies and Northern Great Plains of the United States. As such, the principal method of clubroot management in canola

still may revolve around preventing further spread. Resting spores of *P. brassicae*, the primary source of inoculum, spread via infested soil and plant material; even a single spore is capable of causing infection (Buczacki, 1977). The spread of clubroot occurs mainly by the movement of infested soil attached to field equipment (Cao et al., 2009), but other factors appear to play a role as well. Fairly high numbers of *P. brassicae* resting spores have been detected in wind-borne dust from infested fields, likely contributing to the spread of clubroot (Rennie et al., 2015). Indeed, Gossen et al. (2014) suggested, based on the amount of infested soil potentially moved in a single wind-storm, that some dissemination of clubroot throughout the Canadian prairies may be the result of the spread by wind. Water erosion of infested soil could also spread *P. brassicae* inoculum (Strelkov et al., 2011). Irrigation water and the sediments in irrigation ponds can contain *P. brassicae* resting spores (Dixon, 2015). Disturbing sediment when abstracting water from reservoirs near infested fields could re-suspend the resting spores, enabling pathogen spread into irrigation systems and throughout irrigated fields. Finally, to a lesser extent, the surface infestation of seeds and tubers (Rennie et al., 2011) could potentially result in *P. brassicae* inoculum dispersal. Methods of dissemination, the potential relative risk of each method, and suggested mitigation strategies were summarized by Strelkov and Hwang (2014a) (Fig. 1-2) and will be discussed further along with additional methods of control. There is ongoing research focused on minimizing the spread and economic impact of *P. brassicae* throughout Canada (Howard et al., 2010).

An integrated pest management (IPM) strategy should focus on the long-term management of *P. brassicae* infestations to maximize economic benefit using any/all available methods, while minimizing risks to human health, beneficial and non-target organisms, and the

environment. The methods available for the management of clubroot infestation are discussed below.

1.2.2.1. Sanitation

Currently, the best clubroot management approach in any area is the practice of good sanitation. Clubroot spreads mainly by the movement of infested soil attached to field equipment (Cao et al., 2009), therefore, good sanitation practices involve cleaning soil and crop debris from all equipment before leaving a clubroot infested field or entering an un-infested field (Howard et al., 2010). Specifically, Howard et al. (2010) suggest: removing bulk soil and crop debris, blowing/scrubbing/pressure washing remaining particles, and applying a disinfectant to the cleaned area, allowing a contact time of at least 20 minutes. Sanitation practices help prevent the transfer of inoculum and slow the spread of *P. brassicae*.

Any method that reduces the movement of infested soil between fields can be useful (Strelkov et al., 2011). A general reduction in traffic on an infested field can be beneficial, and where infestation is more severe near an approach or field entrance, the creation or use of an alternative field entrance may be justified (Howard et al., 2010). Another useful strategy to prevent inoculum spread could be managing the sequence in which field work is performed. Conducting farming operations on infested land after un-infested land has been worked could help prevent the transfer of infested soil between fields. Considering that upwards of 200 kg of soil can attach to tractor-cultivator units under moist field conditions, infested fields are best left unworked when field conditions are conducive to the attachment of soil to tools and equipment (Canola Council of Canada, 2012).

Inoculum also can be transferred on the surface of seeds and tubers (Rennie et al., 2011); therefore, it is important to use clean certified seed and avoid using common untreated seed of any kind. Seed crops should not be cultivated in *P. brassicae*-infested fields. Harvested seed could become surface-contaminated with soil and dust containing *P. brassicae* resting spores, which could potentially help to spread the pathogen (Howard et al., 2010).

1.2.2.2. Genetic Resistance

Genetic resistance to *Plasmodiophora brassicae* infection has been found in many *Brassica* genotypes (Diederichsen et al., 2009). Walker (1939) identified lines of turnip and rutabaga that were resistant to clubroot. Early screening efforts in Canada identified *Brassica* lines resistant to *P. brassicae* infection for use as cabbage breeding stock (Chiang and Crete, 1972). Some *B. rapa* and *B. juncea* genotypes from China have been identified as having resistance to clubroot (Zhang et al., 2015). Generally, clubroot resistance in canola is conferred by a single gene and is mainly race- or pathotype-specific (Diederichsen et al., 2009). Current resistance in *B. napus* is likely based on a gene-for-gene model (Feng et al., 2014). Primary gene pool genetic resistance can be bred into open pollinated or hybrid cultivars of spring canola from source winter canola cultivars with known clubroot resistance, such as *B. napus* cv. Mendel (Rahman et al., 2011) or cv. Tosca. Many other sources of genetic resistance have also been identified and characterized against *P. brassicae* pathotypes prevalent in Canada (Hasan et al., 2012, Rahman et al., 2014). For example, primary gene pool germplasm sources like the rutabaga (*B. napus* subsp. *napobrassica*) cultivars Wilhelmsburger, Brookfield 9005, Polycross 9006, and York could serve as effective sources of resistance (Hasan et al., 2012, Rahman et al.,

2014). Secondary gene pool sources, including multiple turnip (*B. rapa* var. *rapifera*) genotypes, could also contribute to clubroot resistance in open pollinated or hybrid cultivars of canola (Hasan et al., 2012, Rahman et al., 2014).

Clubroot resistance genes, present in a variety of cultivars such as those listed above, have been identified, mapped, and molecular markers have been developed. The clubroot resistance gene *CRb* derived from *B. rapa* has been mapped (chromosome R3) (Piao et al., 2004, Zhang et al., 2014) and molecular markers have been identified (Kato et al., 2012, Kato et al., 2013). Many more clubroot resistance genes exist, with at least three additional loci present in *B. rapa* including *Crr1*, *Crr2*, and *Crr3* (Hirai et al., 2004) on chromosomes R8, R6, and R3, respectively (Hirai, 2006). The resistance genes *CRa* (chromosome R3) (Matsumoto et al., 1998, Ueno et al., 2012), *CRk* (chromosome R3), and *CRc* (chromosome R2)(Sakamoto et al., 2008) have also been identified and mapped in the *B. rapa* genome. Marker assisted selection (MAS) could be an invaluable tool for integrating or pyramiding qualitative resistance into desired canola lines. Using MAS, Matsumoto et al. (2012) produced lines that were homozygous for three different clubroot resistance genes, effectively pyramiding or stacking multiple major-genes for clubroot resistance into *B. rapa*. Due to the small number of major resistance genes, the prospect of pyramiding resistance is practical, achievable, and desirable (Hwang et al., 2014, Peng et al., 2014, Rahman et al., 2014). Recently, one Canadian crop production company registered 'PV 580 GC', the first canola cultivar in western Canada with multiple clubroot resistance genes (H.R. Rahman, personal communication, 2016).

The link between specific CR genes, physiological processes, and their contribution to resistance is still unresolved. During *P. brassicae* infection, flavonoid levels may affect auxin

transport, leading to a plant stress response (Paesold et al., 2010), or arginase activity could limit hormonal-triggered hyperplasia of host root cells (Gravot et al., 2012). Also, up-regulation of salicylic acid biosynthesis likely plays a role in the defense response of resistant *Brassica* spp. (Agarwal et al., 2011, Lovelock et al., 2013). More defense responses may exist, and the specific role CR genes (e.g., *Crr1*, *Crr2*, *Crr3*, *CRa*, *CRb*, *CRk*, *CRc*) play in host defense remains unknown. The incomplete understanding of resistance mechanisms and their role, as well as a lack of knowledge linking clubroot resistance genes to function, limit breeders' ability to select for mechanisms of resistance. However, progress is being made, and the genome of *P. brassicae* has been recently sequenced by two independent groups (Schwelm et al., 2015, Rolfe et al., 2016). Analysis of the genome not only helps to clarify the taxonomic relationship between the Rhizaria and other Eukaryotes, but could also elucidate other important characteristics of *P. brassicae*. The complete genome may clarify/illuminate aspects of the (often difficult to study) obligate parasite's metabolism, as well as determine which proteins, CAZymes, and phytohoromones *P. brassicae* is capable of producing.

The potential for quantitative resistance to clubroot also is being explored. A combination of qualitative resistance within a background of quantitative resistance may prove to be more durable to a diverse *P. brassicae* population (Rahman et al., 2014). The *B. napus* winter rapeseed line Darmor-*bzh* has demonstrated partial quantitative resistance to a single-spore isolate (K92–16) of pathotype 4, as defined on the differentials of Williams (1966), and the resistance response has been linked to at least two quantitative trait loci (Manzanares-Dauleux et al., 2000). Ideally, if a set of clubroot resistance QTLs are identified, breeding efforts

would benefit by including a background of quantitative clubroot resistance with additional stacked qualitative resistance.

All Canadian canola cultivars were susceptible to *P. brassicae* infection (Strelkov et al., 2006b, Strelkov and Hwang, 2014) until 2009-10, when a number of private companies released canola cultivars with clubroot resistance suitable for the Canadian prairies, including: '45H29' from Pioneer Hi-Bred, '9558C'from Viterra, 'D3152' from DuPont, '73-67 RR' and '73-77 RR' from Monsanto, '1960' from Canterra, and 'L135C' from Bayer Crop Science. Use of these clubroot resistant (CR) cultivars is widespread throughout Alberta. The cultivars are grown to produce healthy crops in fields known to be clubroot-infested. They are also used in fields that are apparently free of the clubroot pathogen, but which are located in areas of high clubroot incidence, in order to decrease the likelihood of initial disease establishment. These cultivars are not completely immune, and volunteers and genetic off-types in seed lots may result in the growth of some susceptible host plants. Nevertheless, genetically resistant cultivars remain one of the most effective tools available for the management of clubroot.

Studies from France have shown that *P. brassicae* exhibits extensive genetic diversity in the field, and it is suggested that breeding strategies focus on developing cultivars with durable clubroot resistance to multiple pathotypes (Manzanares-Dauleux et al., 2001). There is a diverse population of *P. brassicae* in Alberta as well, at least with respect to virulent phenotypes (Strelkov et al., 2006b, Xue et al., 2008), and currently, clubroot resistance is likely introduced from a limited number of sources available to breeders (Rahman et al., 2011, Rahman et al., 2014). Therefore, resistance will need to be managed carefully because there is a fairly high potential for resistance erosion and breakdown (LeBoldus et al., 2012, Hwang et al.,

2014). Recently, reports have identified six fields where CR cultivars were grown, yet higher than expected clubroot incidence and severity were observed (Strelkov et al., 2014b, Strelkov et al., 2015). In greenhouse testing, four *P. brassicae* populations collected from two of these six fields were highly virulent on a set of CR canola cultivars available in Canada (Strelkov et al., 2016). The identification of new virulence phenotypes over the last couple of years serves to highlight the continued risk posed by this pathogen.

1.2.2.3. Bait Crops and Weed Control

Feng et al. (2014) summarize the current understanding of *P. brassicae* host recognition. Environmental factors likely contribute to *P. brassicae* germination, but host exudates can also trigger resting spore germination (Macfarlane, 1970) and allow the pathogen to recognize potential hosts (Macfarlane, 1970, Feng et al., 2014). Non-host and host exudates or 'germination stimulating factors' (GSFs) (Feng et al., 2014) are the basis of so-called 'bait crops', which functionally promote *P. brassicae* resting spore germination while avoiding any consequential infection that may increase existing inoculum levels, or which are destroyed before significant infection occurs. The goal of planting bait crops is to promote the maximum amount of *P. brassicae* resting spore germination while avoiding any further production of inoculum.

Greenhouse studies, identified ryegrass (*Lolium perenne*) as a potential non-host bait crop capable of causing increased germination of *P. brassicae* resting spores (indeed, induced germination rates were comparable to or even superior to those induced by the host Chinese cabbage (*B. rapa* var. *pekinensis*)) (Friberg et al., 2005). However, under field conditions, it is

not likely that ryegrass would provide any significant short term decrease of *P. brassicae* inoculum load in infested soils (Friberg et al., 2006). Various other promising bait crops, including lettuce (*Lactuca sativa*), result in decreased levels of inoculum when grown on heavily infested fields. These decreases, however, were not significantly different than those associated with fallow treatments without bait plants (Ikegami, 1985). The CR host Japanese radish (*Raphanus sativus* var. *long-ipinnotu*) can significantly decreased inoculum levels in heavily infested fields (Ikegami, 1985). Resistant hosts, including radish, toria, and sarson, are the most effective bait crops in India when grown for 30-days prior to cultivation of main crops, such as cabbage, cauliflower, and rayosak (Bhattacharya and Dixon, 2010).

Bait crops in canola cropping systems were assessed by Ahmed et al. (2011) and appear to be of limited use for managing clubroot in canola on a field scale. Under heavily infested field conditions (>1.0 x 10^6 spores g^{-1} soil), inoculum levels were only slightly reduced by bait crops (canola, Chinese cabbage, bentgrass, orchardgrass, perennial ryegrass, red clover, barley, and wheat) and no difference in subsequent disease severity was observed. The benefits of bait crops may not be sufficient to warrant their use in heavily infested fields. Nevertheless, the possibility exists that at low to medium inoculum levels, bait crops may have some benefit as part of an IPM strategy (Ahmed et al., 2011). Due to the large scale of canola field production in the Canadian prairies, however, the cost of seeding and managing a bait crop might negate any economic benefit (i.e., a small yield increase or inoculum decrease). Also, effective bait cropping practices may not be possible given the shorter growing season in much of the region cultivated to canola in Canada. Early seeding is generally practiced to maximize yield, and a pre-seeding of any bait crop may not be feasible. Nonetheless, cruciferous bait crops proved slightly

more effective than non-cruciferous bait crops or cereals for decreasing clubroot incidence (10% lower) and disease index (8% lower) in heavily infested fields in Alberta (Ahmed et al., 2011). If these benefits could be applied to fields with low to medium levels of infestation, in conjunction with other cultural practices (i.e. crop rotation), bait crops could be considered as part of an effective IPM strategy.

Since *P. brassicae* can infect virtually all members of the Brassicaceae family (Dixon, 2009b), proper weed management helps to prevent the build-up of pathogen inoculum during years of a rotation in which canola is not grown. Thus, the strict control of cruciferous weeds and volunteer *B. napus* plants should be practiced, in order to prevent weedy cruciferous host species from propagating the resting spores of *P. brassicae* and reducing the effectiveness of crop rotations.

1.2.2.4. Cultural Control

The ability of *P. brassicae* resting spores to persist in the soil for long periods of time (Wallenhammar, 1996) suggests that there are limitations to 'crop rotation' as a clubroot management tool. The pathogen resting spores have been estimated to have a half-life in soil of 3.6 years (Wallenhammar, 1996). Nevertheless, various aspects of practicing a good crop rotation are very helpful in mitigating the effects of clubroot in an infected area. Both the Canola Council of Canada and the 'Alberta Clubroot Management Plan' (Alberta Agriculture and Rural Development and Alberta Clubroot Management Committee, 2007), recommend a crop rotation where canola is grown no more than once every four years. There are a number of benefits associated with a long/varied crop-rotation, including improved soil fertility and

improved disease management. Peng et al. (2015) demonstrated that on heavily infested fields, a break of 2- to 4-years without a susceptible host can result in yield increases of 32% - 76% when a resistant cultivar is grown. Resting spore loads declined fairly quickly with a 2-year break from a susceptible host (Peng et al., 2015). Lower inoculum levels, where host crops are grown once in four years, result in decreased disease pressure. There will also be lower selection pressure for inoculum capable of overcoming host plant resistance. Documented shifts in the virulence of *P. brassicae* populations and an erosion or loss of host resistance have been observed around the world, including CR white cabbage in Poland (Wesolowska, 2014), CR Chinese cabbage and CR broccoli in Japan (Tanaka et al., 1991, Tanaka et al., 1997), and in the CR oilseed rape 'Mendel' in Germany (Diederichsen et al., 2014) and the UK (Oxley, 2007). Shifts in the virulence of *P. brassicae* have been observed already in canola fields in Alberta (Strelkov et al., 2014b, Strelkov et al., 2015, Strelkov et al., 2016), weakening the effectiveness of genetic resistance as a clubroot management tool in the Canadian prairies. The best method to prevent virulence shifts is the use of a proper rotation.

Many other clubroot management strategies may be considered when planting canola and other potential host crops. Karling (1968) and Thuma et al. (1983) noted that high soil moisture during the seedling stage facilitates zoospore infection of the root; therefore, selecting fields with well-drained soil and no previous clubroot infestation will help to prevent disease (Howard et al., 2010). Zero-till, reduced-till, or any soil conservation practice that reduces soil erosion will help to minimize the movement of inoculum within a field or to neighbouring fields (Howard et al., 2010). Resting spores also are found in straw, hay/greenfeed, silage, and manure collected from infested fields. Avoiding use of these

materials when they come from a clubroot infested field will help to prevent the spread of inoculum to non-infested fields (Howard et al., 2010). Clubroot resting spores germinate at temperatures above 12-14˚C (Gossen et al., 2014), therefore, early seeding into cooler soil may help prevent infection of host plants when they are most susceptible to disease. This practice is also consistent with efforts to maximize the length of the growing season.

1.2.2.5. Soil Amendments, Biological Controls and Chemical Controls

The management of *P. brassicae* is inherently difficult as a consequence of the robustness and below ground nature of its resting spores. Indeed, inoculum can be detected at depths down to 30-40 cm (Naiki et al., 1985). Even if the bulk of viable inoculum can be found closer to the soil surface, it is difficult to permeate treatments evenly throughout soil at any depth, and the amount of inputs required for large field scale applications could be quite prohibitive both economically and environmentally. Nonetheless, in terms of clubroot management, some treatments do show potential.

As previously discussed, soil pH has an influence on *P. brassicae* development. Karling (1968) reported that infection occurs most readily at a pH of 6.0 - 6.5, and that there is a limiting effect on clubroot development when soil pH is 7.2-7.4, with an even greater effect closer to pH 8.0. *P. brassicae* infection may still occur at pH > 8.1, and increased pH does not eliminate resting spores, but instead slows the rate of spore germination (Macfarlane, 1952, Karling, 1968). Given this effect, the treatment of the soil with wood ash or calcium cyanamide, which would increase pH, has been assessed (Hwang et al., 2011c) as a clubroot management strategy for the canola cropping systems in Alberta. It was determined that despite yield

increases, even in heavily infested fields, the application costs would outweigh the yield gain benefit (Hwang et al., 2011c, Strelkov et al., 2011). To further undermine the use of these soil treatments as a management practice, warm soil temperatures (Gossen et al., 2013) or sufficient soil moisture (Colhoun, 1953, Gossen et al., 2013, Gossen et al., 2014) could negate the beneficial effects of increasing the soil pH.

The application of boron can reduce root hair infection by *P. brassicae* and clubroot severity in canola (Deora et al., 2011). If applied at low doses, the phytotoxic effects of boron on canola could be avoided, and it could be used as part of a clubroot management plan (Deora et al., 2011, Gossen et al., 2014). However, boron is less effective in mineral soils, which represent the majority of canola hectares in Canada, because leaching from the soil occurs more rapidly (Gossen et al., 2014).

There are currently no soil fumigants registered for use in *P. brassicae* infested canola fields. However, preliminary work with Vapam (dithiocarbamate; sodium N-methyldithiocarbamate) has shown that it has potential as a treatment to reduce resting spore levels and disease severity in infested soils (Hwang et al., 2014). Vapam treatment improved plant growth and decreased disease severity in infested soil, especially under moist conditions (Hwang et al., 2014). The treatment cost is still prohibitive when large scale application is required, but Vapam could be a tool used to limit or eradicate localized infestations. If small patches of highly infested soil are identified in a field, the application of Vapam over these limited areas may be justified (Zuzak, 2016, Unpublished data).

Peng et al. (2014) suggested that some rhizobacteria and endophytic fungi are able to reduce clubroot severity by >75% when applied as a soil drench under controlled conditions.

Commercially available biocontrol agents registered for other soil-borne diseases have been tested for their efficacy in controlling *P. brassicae* (Peng et al., 2011). Biocontrol agents tested included: *Bacillus subtilis*, *Gliocladium catenulatum*, and *Streptomyces griseoviridis* among others. At low to moderate inoculum pressure, biocontrol agents applied as a soil drench reduced clubroot severity and were more effective than biocontrol seed treatments. Despite weaker clubroot control, seed treatments would represent a more practical method to apply biocontrol agents, given the greater ease and lower cost associated with the application of treatments directly to the seed. The biocontrol agents evaluated by Peng et al. (2011) were not optimized for seed treatment, yet still showed some activity, indicating potential for future improvements to this method of biocontrol application. Nevertheless, neither soil drenches nor seed treatments were effective at high inoculum levels, indicating that biocontrols may be useful only as part of an IPM strategy. As the formulations of both biofungicidal seed treatments and soil drenches improve, they may become increasingly useful management tools for the control of clubroot in varying soil types, while demonstrating reliable viability, efficacy, and ease of application (Peng et al., 2014).

Potential synthetic *P. brassicae* pesticides such as the non-ionic surfactants: Agral (90% nonylphenoxy-polyethoxy ethanol), Citowett Plus (50% octylphenoxypolyethoxy ethanol), AquaGro 2000-L (80% ethoxylated alkyl phenols, 5% fatty acid esters), and the granular formulation AquaGro 2000-G (24% ethoxylated alkyl phenols, 1.5% fatty acid esters, 70% vermiculite carrier), have been shown to increase Chinese cabbage yield in field plots heavily infested with *P. brassicae*. However, these chemicals can be phytotoxic to host plants (Hildebrand and McRae, 1998). No synthetic fungicides are currently registered in Canada for

use on *P. brassicae* in canola (Howard et al., 2010), although some preliminary testing of Terraclor 75% WP (PCNB, pentachloronitrobenzene) and Ranman (cyazofamid) has shown some reduction in root symptom severity. In Canada, fluazinam (Allegro, Omega) is registered for clubroot control in vegetables, and cyazofamid (Ranman), which is registered for other diseases of vegetables, can substantially decrease clubroot disease severity in highly infested soil (Peng et al., 2014). The greatest limiting factor to the use of any synthetic fungicide is the large amount of water necessary for proper application over an entire field, the prohibitive cost of large quantities of these chemicals, and the general ineffectiveness of the fungicides at higher levels of infestation in field settings (Peng et al., 2014).

Seedlings are less susceptible to clubroot infestation after 4 weeks of growth, with infection by *P. brassicae* resulting in lower disease severity, taller plants, and greater yields when plants are infected after this time (Hwang et al., 2011a). If a fungicide or biocontrol agent is developed that can protect canola seedlings for at least 4 weeks of development, it may be a useful management tool in an IPM strategy for clubroot.

1.2.2.6. Scouting, Detection, & Quantification: Tools Used for Risk Assessment

Because avoidance is such an important component of *P. brassicae* management strategies, proper scouting for the pathogen and the disease is an invaluable tool. Scouting is important for the identification of new cases of clubroot, and to monitor the spread of disease within or between infested fields (Howard et al., 2010). Farmers with fields that are known to be infested with *P. brassicae* may implement alternative crop-rotations, avoiding the cultivation of susceptible hosts, and also may work those fields last in order to reduce spread of the

pathogen. Most often, scouting for clubroot involves the identification of foliar and root symptoms (Section 1.1.1), but testing of the soil for the presence of *P. brassicae* resting spores also is feasible.

It is possible to detect *P. brassicae* in plant tissue or soil at extracted DNA concentrations as low as 100 fg, or the equivalent 1 x 10^3 resting spores g^{-1} soil, with high specificity, using a simple polymerase chain reaction (PCR) (Cao et al., 2007). Indeed, PCR-based tests for the detection of *P. brassicae* are now commercially available (Faggian and Strelkov, 2009). The economic and environmental costs of *P. brassicae* infection and control could be mitigated by a more focused risk assessment. Areas at greater risk for infestation by the pathogen, for example soil patches near field entrances (Cao et al., 2009), could be tested and if found to be positive for *P. brassicae* could be avoided or undergo targeted treatment, potentially saving costs and preventing extensive negative environmental consequences to the rest of the field.

Quantitative polymerase chain reaction (qPCR)-based tests for *P. brassicae* also exist. DNA extracted form soil or root material can be assessed to determine the quantity of *P. brassicae* DNA (Wallenhammar et al., 2012) or resting spores (Rennie et al., 2011) present in a given sample. These methods are sensitive down to approximately 1.0 x 10^3 resting spores g^{-1} soil and could help to indicate the level of infestation, helping to make field management decisions that are more evidence-based. Wallenhammar et al. (2012) suggested the following guidelines based on quantified levels of inoculum using qPCR. If *P. brassicae* DNA cannot be detected, it is safe to plant *Brassica* crops. When less than 3000 spores g^{-1} soil is detected yield loss in susceptible cultivars may occur, but it is safe to plant resistant *Brassica* cultivars. If

between 3000 and 130 000 spores g^{-1} soil is detected yield loss in a susceptible cultivar is likely, while it is still safe to plant resistant *Brassica* cultivars. If more than 130 000 spores g^{-1} soil is detected, planting of any *Brassica* crop is not advised because the potential for disease propagation is too great. Also, at these levels of soil infestation, selection pressure for *P. brassicae* strains capable of overcoming host plant resistance is significant.

When costs are warranted, a number of clubroot risk assessment tools could be used to mitigate the impact of the disease. These tools include soil testing for the presence of *P. brassicae* inoculum prior to seeding, which would help to guide crop selection (e.g., canola, CR canola, non-host crop, etc.) decisions. However, in high risk areas, CR canola and non-host crops should be used exclusively. Similarly, the scouting of fields throughout the growing season for root symptoms, as well as inspection of patches in a field where wilting and/or premature ripening of the crop canopy is observed, will help to identify new cases of clubroot and shifts in virulence patterns of the pathogen. Eventually, qPCR-based quantification of *P. brassicae* spores in the soil may help growers determine when it is safe to plant a resistant host crop.

1.3. Conclusions, Statement of Hypothesis, and Research Objectives

1.3.1. Purpose of Research

Many methods to manage *P. brassicae* have been evaluated. The use of a crop rotation, with no hosts planted for at least 2-years, and the sowing of *P. brassicae* resistant canola cultivars, are currently the most effective strategies for the management of clubroot in fields

that have already been infested. Planting non-host crops or canola cultivars with genetic resistance can help decrease the likelihood of disease establishment in a commercial field, as well as lower disease severity in previously infested fields. However, in a field setting, these CR cultivars are not 100% effective, and some infection occurs even in pure seed-lots. For example, two CR cultivars developed a disease index of 1-5 % when grown in fields heavily infested with *P. brassicae* (1.0×10^8 spores g^{-1} soil) (Hwang et al., 2015). Cruciferous weeds, canola volunteers and genetic off-types in seed lots also can result in the growth of some susceptible host plants within a resistant crop. The presence of these susceptible genetic off-types and volunteers will increase the inoculum levels compared with the growth only of resistant canola (Hwang et al., 2012a). The cultivation of resistant and susceptible canola genotypes in heavily infested fields has been shown to increase inoculum levels, but resistant cultivars contributed significantly less inoculum compared with susceptible cultivars (Hwang et al., 2011b). Moreover, preliminary evidence from a greenhouse study indicates that when these same cultivars are grown at low to medium inoculum densities, the subsequent inoculum concentration is far less for soil seeded with resistant versus susceptible genotypes. Subsequent soil inoculum concentrations (as assessed by qPCR analysis) following the planting of a resistant canola cultivar did not differ significantly from a fallow control treatment (Hwang et al., 2011b). These findings suggest that CR cultivars could potentially prevent increases in soil inoculum levels, when cultivated in soil with an initial *P. brassicae* inoculum load below some threshold value. This suggestion requires further investigation, particularly under field conditions, and is the focus of the current M.Sc. project.

General Hypothesis

The general hypothesis underlying this project is that the cultivation of a clubroot resistant canola genotype in a clubroot infested field will not result in increased *P. brassicae* inoculum loads, and may contribute to an inoculum decrease if the initial concentration of resting spores falls below some threshold level.

Clubroot resistant canola cultivars generate less *P. brassicae* inoculum due to their low susceptibility to infection, which results in very limited root galling and limited proliferation of the pathogen within host tissues. If the presence of a resistant host can induce *P. brassicae* resting spore germination in the soil, but then support only very limited generation of new resting spores within infected roots, then potentially the cultivation of this resistant host could help deplete field inoculum levels. I would, however, predict that resistant canola cultivars, under naturally infested field conditions in Alberta, will further increase inoculum levels if the initial *P. brassicae* spore loads are high, based on greenhouse results (Hwang et al., 2011b). Nonetheless, there could be a 'threshold clubroot resting spore concentration' at which resistant canola cultivars will not add additional inoculum to the soil. Below this spore concentration, it is possible that a resistant cultivar could stimulate *P. brassicae* resting spore germination while avoiding substantial infection, thereby resulting in a net decline in the spore concentration in the soil.

1.3.2. Thesis Objectives

The specific research objectives of this thesis are:

- To determine the effect of resistant cultivars on *P. brassicae* soil inoculum loads at various initial levels of infestation
- To determine the effect of resistant cultivars on *P. brassicae* soil inoculum loads under various field conditions and crop rotation regimes throughout Alberta
- To determine the level of clubroot incidence and severity in resistant cultivars planted in soils with varying levels of naturally occurring *P. brassicae* inoculum in Alberta
- To determine if there is a threshold level of *P. brassicae* inoculum at which it is reasonable to plant resistant cultivars and expect no subsequent increase in spore load

1.3.3. Null Hypotheses

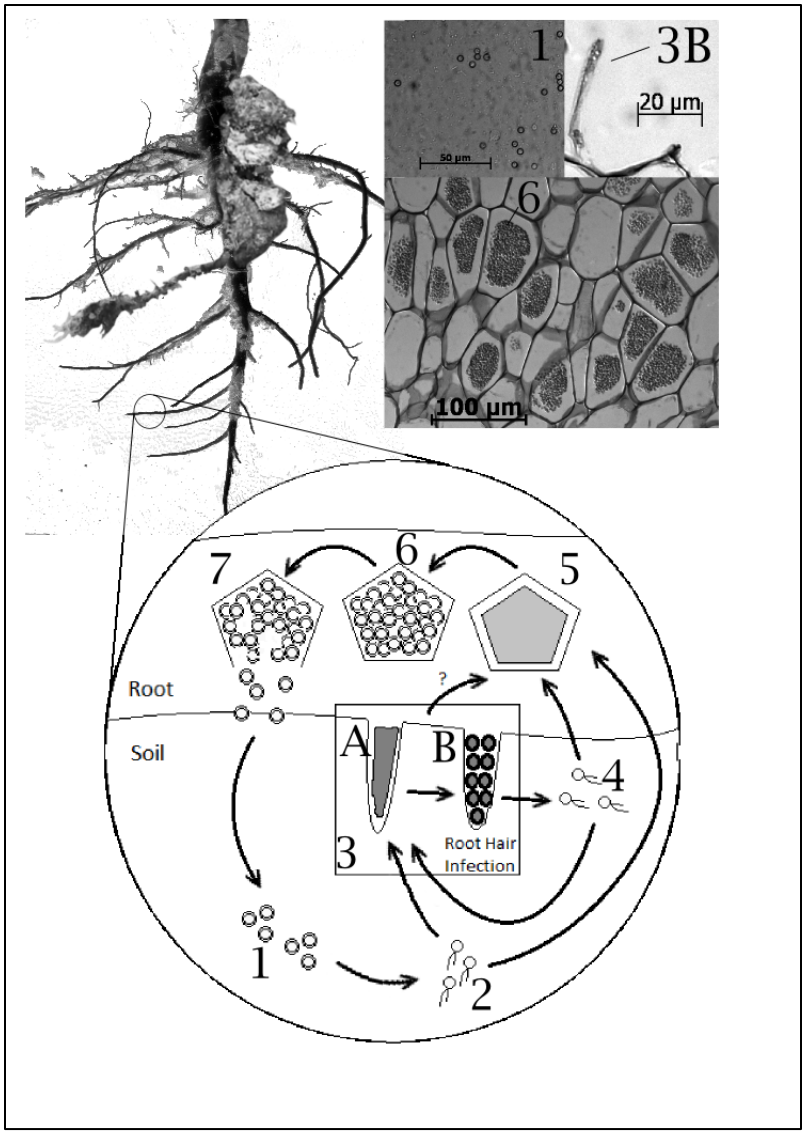
The null hypotheses of this thesis are:

- Growing resistant cultivars has no effect on the inoculum load in *P. brassicae* infested soil regardless of the initial level of infestation
- Growing resistant cultivars has no effect on the inoculum load in *P. brassicae* infested soil regardless of field conditions and crop rotation
- Clubroot incidence and severity in resistant canola cultivars is not affected by various naturally occurring inoculum loads typical of what is found throughout Alberta
- Resting spore load following cultivation of resistant cultivars is unaffected by the initial level of *P. brassicae* inoculum

Table 1-1. Compilation of hosts/non-hosts of *Plasmodiophora brassicae*. Some of the species undergo *P. brassicae* infection without secondary infection (NON-HOST), while others can exhibit up to complete susceptibility including secondary infection and the development of pathogen resting spores (HOST).

Species	Cv.	Common Name	Author	Host Status
Agrostis alba stolonifera Roth		Black Bent, Redtop	Macfarlane (1952)	NON-HOST
Allium porrum L.		Garden Leek	Dixon (2009b)	NON-HOST
Armoracia rusticana Lam.		Horseradish	Chai et al. (2014)	HOST
Beta vulgaris L.	Kawemono	Beet	Ludwig-Müller et al. (1999)	HOST
Brassica carinata A. Braun		Ethiopian mustard	Howard et al. (2010)	HOST
Brassica juncea L.		Indian Mustard	Bhattacharya et al. (2014)	HOST
Brassica juncea L. var. *megarrhiza*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica juncea L. var. *crassicaulis*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica juncea L. var. *tumida*		Big Stem Mustard	Chai et al. (2014)	HOST
Brassica juncea L. var. *gemmifera*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica juncea L. var. *multisecta*		Thousand-Head Mustard	Chai et al. (2014)	HOST
Brassica juncea L. var. *leucanthus*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica juncea L. var. *longepetiolata*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica juncea L. var. *linearifolia*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica juncea L. var. *strumata*		Large-Petiole Mustard	Chai et al. (2014)	HOST
Brassica juncea L. var. *latipa*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica juncea L. var. *involuta*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica juncea L. var. *multiceps*		Multi-Shoot Mustard	Chai et al. (2014)	HOST
Brassica juncea L. var. *rugosa*		Head Mustard	Chai et al. (2014)	HOST
Brassica juncea L. var. *foliosa*		Leaf Mustard	Chai et al. (2014)	HOST
Brassica juncea L. var. *utilis*		Indian Mustard var.	Chai et al. (2014)	HOST
Brassica napus L.		Oil seed rape, canola	Gibbs (1932)	HOST
Brassica napus L. var. *napobrassica*		Rutabaga, swede turnip	Gibbs (1932)	HOST
Brassica nigra L.		Black Mustard	Howard et al. (2010)	HOST
Brassica oleracea L.		Wild Cabbage	Gibbs (1932)	HOST
Brassica oleracea L. var. *acephala*	Thousand-Headed, Buda	Kale/Collard Greens	Gibbs (1932)	HOST
Brassica oleracea L. var. *botrytis*		Cauliflower	Gibbs (1932), Bhattacharya et al. (2014)	HOST
Brassica oleracea L. var. *capitata*		Cabbage	Gibbs (1932)	HOST
Brassica oleracea L. var. *gemmifera*		Brussels sprouts	Gibbs (1932)	HOST
Brassica oleracea L. var. *ramosa*		Perennial Kale	Gibbs (1932)	HOST
Brassica rapa L. ssp. *chinensis* var. *utilis*		Chinese cabbage var., Bok Choy var.	Chai et al. (2014)	HOST
Brassica rapa L. ssp. *chinensis* var. *communis*		Chinese cabbage, Bok Choy	Chai et al. (2014)	HOST
Brassica rapa L. ssp. *chinensis* var. *rosularis*		Chinese cabbage, Bok Choy	Chai et al. (2014)	HOST
Brassica rapa L. ssp. *chinensis* var. *purpurea*		Chinese cabbage, Bok Choy	Chai et al. (2014)	HOST
Brassica rapa L. ssp. *chinensis* var. *taitsai*		Chinese cabbage, Bok Choy	Chai et al. (2014)	HOST
Brassica rapa L. ssp. *pekinensis*	Granaat	Chinese cabbage, Napa cabbage	Ludwig-Müller et al. (1999),	HOST
Brassica rapa L. ssp. *pekinensis* var. *dissoluta*		Chinese cabbage, Napa cabbage	Chai et al. (2014)	HOST
Brassica rapa L. ssp. *pekinensis* var. *infarcta*		Chinese cabbage, Napa cabbage	Chai et al. (2014)	HOST
Brassica rapa L. ssp. *pekinensis* var. *laxa*		Chinese cabbage, Napa cabbage	Chai et al. (2014)	HOST
Brassica rapa L. ssp. *pekinensis* var. *cephalata*		Chinese cabbage, Napa cabbage	Chai et al. (2014)	HOST
Brassica rapa L. var. *rapa*		Turnip	Gibbs (1932)	HOST

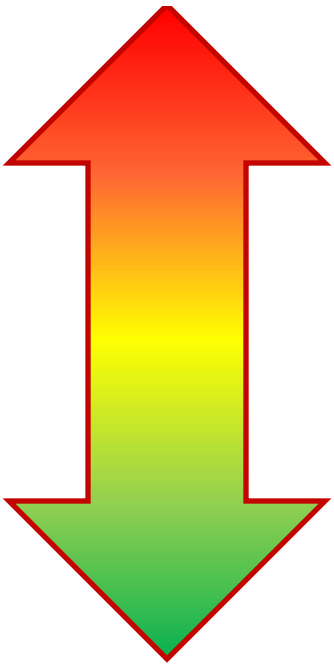
Brassica rapa L. ssp. *sylvestris*		Wild Turnip	Gibbs (1932)	HOST
Brassica rapa L. syn. campestris		Annual Turnip Rape	Chai et al. (2014)	HOST
Brassica rapa L. var. *trilocularis*		Yellow Sarson	Bhattacharya et al. (2014)	HOST
Brassica rapa L. ssp. *toria*		Tori Sarson	Bhattacharya et al. (2014)	HOST
Camelina sativa (L.) Crantz		False Flax	Seguin-Swartz et al. (2009)	HOST
Capsella bursa-pastoris L.	Medik	Shepherd's Purse	Gibbs (1932)	HOST
Carica papaya L.		Papaya	Ludwig-Müller et al. (1999)	NON-HOST
Dactylis glomerata L.		Orchard Grass	Macfarlane (1952)	NON-HOST
Diplotaxis muralis (L.) DC.		Annual Wall-Rocket	Gibbs (1932)	HOST
Eutrema wasabi (Siebold) Maxim.		Wasabi	Chai et al. (2014)	HOST
Erysimum cheiranthoides L.		Treacle-mustard	Karling (1968)	HOST
Holcus lanatus L.		Velvet Grass	Webb (1949)	NON-HOST
Lepidium africanum (burm. f.) DC.		African Pepperwort, Peppercress	Gibbs (1932)	HOST
Lepidium campestre (L.) W.T. Aiton		Field Pepperwort	Gibbs (1932)	HOST
Lepidium sativum L.		Cress, Garden Cress	Karling (1968)	HOST
Lolium perenne L.		Perennial Rye-Grass	Macfarlane (1952), Dixon (2009b)	NON-HOST
Lolium perenne L.	Amazing	Ryegrass	Feng et al. (2012)	NON-HOST
Matthiola longipetala ssp. *bicornis* (Vent.) DC.		Night-Scented Stock	Macfarlane (1952)	NON-HOST
Matthiola incana (L.) W.T. Aiton		Hoary Stock	Macfarlane (1952)	NON-HOST
Nasturtium officinale W.T. Aiton		Watercress	Chai et al. (2014)	HOST
Papaver rhoeas L.		Common Poppy	Macfarlane (1952)	NON-HOST
Raphanus sativus L. var. *longipinnatus*		Daikon	Chai et al. (2014)	HOST
Raphanus sativus L. var. *radiculus*		Raddish	Chai et al. (2014)	HOST
Reseda alba L.		White Mignonette	Ludwig-Müller et al. (1999)	NON-HOST
Reseda odorata L.		Garden Mignonette	Macfarlane (1952)	NON-HOST
Secale cereale L.		Rye, Winter Rye	Dixon (2009b)	NON-HOST
Sinapis alba L.		White Mustard	Gibbs (1932)	HOST
Sinapis arvensis L.		Wild Mustard	Karling (1968)	HOST
Sisymbrium altissimum L.		Tall-mustard, Tumble-mustard	Karling (1968)	HOST
Sisymbrium officinale (L.) Scop.		Hedge Mustard	Gibbs (1932)	HOST
Thlaspi arvense L.		Stinkweed	Howard et al. (2010)	HOST
Tropaeolum majus L.	Nanum	Nose-Twister	Ludwig-Müller et al. (1999)	HOST



Illustrated portions of Fig. 1-1 are not to scale.

Figure 1-1. Life cycle of *Plasmodiophora brassicae* adapted from Kageyama and Asano (2009). Resting spores germinate (**1**), releasing bi-flagellate motile primary zoospores (**2**) which move through water films in the soil towards host roots. Primary infection occurs in the host root hairs. After the zoospores encyst and penetrate the host root hairs, a primary plasmodium (**3A**) matures into a zoosporangium (**3B**) within the root hair, which releases secondary zoospores (**4**). Secondary infection occurs via primary/secondary zoospore penetration of root cortical tissue, where secondary plasmodia (**5**) form within root cortical cells. Primary plasmodia also may migrate into root cortical tissue via myxamoeboid movement through induced breaks in host cell walls, resulting in secondary infection. As secondary plasmodia mature, resting spores (**6**) form within host root cortical cells. When host roots decay, resting spores (**7**) are released back into the soil completing the life cycle.

High Risk



Infested soil on field equipment
Risk: Large amounts of soil can quickly establish new infestations
Mitigation: Equipment cleaning and sanitation

Erosion of infested soil
Risk: Function of the amount of soil eroded and distance travelled
Mitigation: Minimize erosion processes

Irrigation and disturbed irrigation pond sediments
Risk: Irrigation ponds near clubroot infested land may act as a new source of inoculum, or spread the disease more completely in infested fields
Mitigation: Avoid disturbing sediment when drawing water from irrigation ponds
*not an issue throughout most of the canola growing regions of Alberta, which are under dryland agriculture

Soil tag on seeds and tubers
Risk: Potential for long distance dispersal
Mitigation: Seed cleaning and seed treatments

Low Risk

Figure 1-2. Possible *Plasmodiophora brassicae* dissemination mechanisms in western Canada, adapted from Strelkov and Hwang (2014a). The relative risk and possible mitigation strategies associated with each mechanism are indicated.

Chapter 2

2. *Plasmodiophora brassicae* Resting Spore Dynamics in Clubroot Resistant Canola (*Brassica napus*) Cropping Systems

2.1. Introduction

Clubroot, caused by the soilborne, obligate parasite *Plasmodiophora brassicae* Woronin (Woronin, 1878), is one of the most important diseases of cultivated crucifers worldwide (Karling, 1968, Dixon, 2014). Global crop losses as a result of this pathogen have been estimated at 10 - 15% (Dixon, 2006). Clubroot has been reported from the majority of regions cultivated to cruciferous crops, including oilseed rape or canola (*Brassica napus* L.) (Donald and Porter, 2014, Diederichsen et al., 2014, Wallenhammar et al., 2014, Hwang et al., 2014). Clubroot of canola was first identified in the Canadian prairies near Edmonton, Alberta, in 2003, and has since spread widely throughout central Alberta, resulting in a nearly 90-fold increase in confirmed field infestations (Tewari et al., 2005, Strelkov and Hwang, 2014). The level of *P. brassicae* infestation in most fields is low to moderate, but losses ranging from 30-100% have been recorded in some canola crops nearly every year (Tewari et al., 2005, Strelkov and Hwang, 2014). The area seeded to canola across the prairies continues to expand, and yield losses resulting from *P. brassicae* infection could have a serious impact on the Canadian economy (Rempel et al., 2014).

Various methods of clubroot management have been evaluated, which have varying degrees of efficacy and economic practicality. The planting of clubroot resistant (CR) canola cultivars is the management approach most commonly adopted by producers (Diederichsen et

al., 2009, Rahman et al., 2011, Rahman et al., 2014). Preventing the movement of *P. brassicae*-infested soil through the proper sanitization of field equipment (Howard et al., 2010) is an additional strategy occasionally implemented by producers, but is often viewed as being too labor intensive. The low efficacy and/or prohibitive cost of soil amendments, biological controls and chemical treatments reduces their feasibility as clubroot management tools in canola cropping systems (Peng et al., 2014, Gossen et al., 2014). Longer rotations away from susceptible cruciferous hosts are often suggested as a clubroot management strategy (Alberta Agriculture and Rural Development and Alberta Clubroot Management Committee, 2007), but these are not always implemented. The high economic value of canola (Rempel et al., 2014) and limited cropping options in many regions deter most producers from adopting longer rotations.

The management of clubroot is further complicated by the longevity of *P. brassicae* resting spores (Wallenhammar, 1996). In many cases, farmers find it difficult to abstain from planting *Brassica* crops long enough to significantly reduce soil inoculum levels, particularly in heavily infested fields (Wallenhammar et al., 2012). When soil infestation levels exceed ~1.0 x 10^5 *P. brassicae* resting spores g^{-1} soil under field conditions, even the planting of resistant *Brassica* cultivars is not recommended (Wallenhammar et al., 2012). Nonetheless, recent work by Peng et al. (2015) demonstrates the potential effectiveness of an extended rotation away from canola in heavily infested fields. After a ≥2-year break from canola, resting spore loads dropped by up to 90% from initial levels, resulting in yield gains of 32-76% (Peng et al., 2015).

The clubroot disease cycle (Kageyama and Asano, 2009) begins with the germination of *P. brassicae* resting spores in the presence of a susceptible host, releasing motile, bi-flagellate, primary zoospores into the soil matrix. Primary infection occurs in the root hairs of a

susceptible host, where zoospores encyst and penetrate the root hairs, form primary plasmodia which mature into zoosporangia, and release secondary zoospores back into the soil matrix. Secondary zoospores initiate secondary infection of the root cortical tissue (Kageyama and Asano, 2009), although primary zoospores and other mechanisms also have been suggested to contribute to this process (Mithen and Magrath, 1992, Feng et al., 2013). Within root cortical tissue, amoeboid-like plasmodia form and spread up and down the cambium, and out into the cortex and xylem. In the cortex, infected cells which should differentiate into functional xylem/medullary rays instead undergo hyperplasia and hypertrophy resulting in malformed elongated cells that are unable to fulfill their designated purpose (Karling, 1968). As secondary plasmodia mature, resting spores form within the host root cortical cells. When host roots decay, resting spores are released back into the soil matrix (Fig. 1-1).

Even at very high concentrations of *P. brassicae* resting spores (>1.0 x 10^8 spores g^{-1} soil), resistant host cultivars contribute significantly less inoculum to the soil matrix in subsequent years than do susceptible cultivars (Hwang et al., 2011b). Resistant *Brassica* spp. may still undergo primary infection (Donald et al., 2008, Hwang et al., 2011b). However, secondary infection and the production of mature resting spores may be reduced significantly, if not completely prevented (Hwang et al., 2011b, Rennie et al., 2013). It is reasonable to hypothesize that, if initial spore loads are low enough, resistant cultivars may induce resting spore germination while supporting limited production of new inoculum, thereby serving to deplete spore loads in the soil. The objective of this study was to evaluate the impact of growing CR canola cultivars on *P. brassicae* resting spore dynamics under field conditions in commercial cropping systems.

2.2. Materials and Methods

2.2.1. Soil Sampling & Processing

2.2.1.1. Soil Sampling Regime

Monitoring of *P. brassicae* resting spore concentration in the soil was carried out in 17 fields across Alberta (Fig. 2-1). Fields were selected from regions where the incidence of clubroot had been detected prior to the 2010 growing season (Strelkov et al., 2009). Cooperating canola producers from these regions identified fields, and areas within each field, that were infested with *P. brassicae* to varying degrees. Soil sampling was conducted in the spring close to seeding and in the fall after harvest at no less than 5 fixed points within each field regardless of cultivated crop. Fixed points within each field were located in areas identified by the cooperating producer as having greater clubroot incidence in the past. Where this information was not available, the fixed points were randomly selected throughout the field in a "W" pattern. This sampling pattern is commonly used during clubroot scouting and disease surveys (Strelkov et al., 2009) (Fig. 2-1). A sample position near any entrance to the field was often included since the incidence of clubroot is generally greatest in field approaches (Cao et al., 2009). The sampled fields consisted of Black Solodized Solonetz (central Alberta) and Orthic Brown Chernozem (southern Alberta) soils (Agriculture and Agri-Food Canada, 1998).

Each fixed position in a field was georeferenced using a global positioning system (GPS) receiver (+/- 1 m accuracy) and consisted of a 3 m x 3 m square from which 10 individual ~500 g soil samples were collected into paper bags using a small shovel. Sampling depth was up to 15 cm deep, and included all surface material present. Soil sampling at each fixed position was

performed twice annually from 2010-2013 regardless of cultivated crop. The initial 'spring' sampling occurred as early as May 22 or as late as June 28, but was generally performed less than 2-weeks after seeding depending on field conditions (sampling was not conducted while seeding was underway, or when a field was excessively wet). The second 'fall' sampling occurred as early as Oct. 4 or as late as Oct. 25, always after swathing and harvest were complete.

A minimum of two 3 m x 3 m control blocks, completely free of susceptible hosts, were set up in each field, either adjacent to the test plots or at the field edges (when adjacent plots were not permitted) (Fig. 2-1). These control blocks were georeferenced, and soil samples were collected pre-seeding and post-harvest in accordance with the previously mentioned sampling regime. The control blocks were denuded of all plants with periodic spray treatments of Liberty (glufosinate ammonium) (1.2L): RoundUp (glyphosate) (0.3L): water (18L) using a backpack sprayer. Where it was not possible to denude the blocks of plants, soil was collected pre-seeding from the georeferenced control blocks and part of the sample was weathered by exposure to a full growing-season of natural environmental conditions, free of any plants, prior to processing.

All tools used for collection of samples were sanitized between sampling points by washing with bleach or KleenGrow disinfectant (Pace 49 Inc., 2012 Pace Chemicals Inc., Delta, BC, Canada). All soil samples were kept in paper bags and stored at -20 ˚C within 24 h of collection for future analysis.

The number of fields sampled differed by year, as field ownership occasionally changed. In 2010, 11 fields were sampled. In 2011, one of these fields was not sampled because of a change

in ownership, but six new fields were added totalling 16 fields sampled. In 2012 and 2013, the same 14 fields were sampled. A total of nine fields were sampled continuously over the entire 4-year duration of the study, and included fields in which CR canola was grown 1-in-4, 2-in-4, and 3-in-4 year rotations.

2.2.1.2. Soil Processing

The distribution of *P. brassicae* is often very heterogeneous within infested soils. In order to ensure accurate representation of resting spore load within a sample, it was necessary to homogenize each soil sample prior to DNA extraction. The processing and homogenization of soil samples was performed as follows. Samples were defrosted at room temperature and soil was allowed to dry for one week. Sub-samples (60 g) were taken from each of the 10 (~500 g) soil samples associated with a specific field location/sampling point and time (e.g., spring 2010), and pooled to form a composite sample, making processing/analysis more manageable and efficient. Composite samples were homogenized using a barrel sieve (1.5 mm-diam. pores) and rolling bars which were cleaned with air and brushes, and washed with ethanol between samples, or ground in a mortar with a pestle which were cleaned with ethanol between every sample.

2.2.2. Field Clubroot Disease Rating

During years when a CR canola cultivar was grown in a field, post-harvest soil sampling was accompanied by incidence and severity ratings of 50 plants within a 1 m^2 area at each fixed position. The roots were gently dug out from the ground, and the soil was carefully removed to

inspect for symptoms of galling. The sampled roots were rated using the scale of Kuginuki et al. (1999), where: 0 = no galls, 1 = small/minor galling, 2 = moderate galling, and 3 = severe galling. Disease incidence and severity ratings were then used to calculate an index of disease (ID) using the formula of Strelkov et al. (2006b):

$$ID\ (\%) = \frac{\sum(n\ \times 0 + n\ \times 1 + n\ \times 2 + n\ \times 3)}{N\ \times 3} \times 100$$

Where: n is the number of plants of a class, N is the total number of plants, and 0, 1, 2, and 3 are the symptom severity classes.

2.2.3. DNA Extraction

Dried and homogenized composite soil samples were processed individually. Total genomic DNA was extracted from 0.25 g of soil per sample with a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) as per the manufacturer's instructions. Briefly, homogenization and lysis of cells within soil samples was achieved with a vortex adapter and specialized PowerBead tubes (MO BIO Laboratories) with a cell lysing anionic detergent (MO BIO Laboratories). Non-DNA organic and inorganic matter, including humic acid, cell debris, and proteins were then precipitated by the addition of proprietary 'inhibitor removal reagents' (MO BIO Laboratories) followed by centrifugation and collection of the supernatant containing DNA in 2.0 mL collection tubes. The salt concentration of the resulting solution was increased so that DNA would bind tightly to a silica membrane, and the solution was added to a spin filter column within a 2.0 mL collection tube. The DNA bound to the silica membrane of the spin filter columns was then washed with 100% ethanol and an additional proprietary reagent (MO BIO

Laboratories) using a PowerVac Manifold (MO BIO Laboratories). Sterile, DNA-free PCR-grade water was used to elute the DNA from the filter column membranes. The quality of the purified DNA was verified on a NanoDrop spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA, USA) prior to conducting PCR assays.

2.2.4. Conventional & Quantitative PCR Analysis

2.2.4.1. Verification of DNA Extraction Success

The success of total DNA extraction from the soil samples was evaluated by PCR amplification with the primers ITS1 (5´ - TCC GTA GGT GAA CCT TGC GG - 3´) and ITS4 (5´ - TCC TCC GCT TAT TGA TAT GC - 3´) of Korabecna et al. (2007), which flank the repeating segment of DNA that codes for the ribosomal RNA (rRNA) 5S region in fungi. These primers are non-specific and well conserved in the majority of fungi, plants, and some other Eukaryotes. Reaction conditions were as described by Korabecna et al. (2007) with some minor modifications. Briefly, a 50 µL reaction volume contained: 1X PCR buffer (Invitrogen), 10µM dNTP, 1.5 mM of $MgCl_2$ (Invitrogen), 0.5µM of ITS1 and 0.5µM ITS4 primers, 1U Platinum *Taq* DNA polymerase (Invitrogen), and 10 ng of template DNA, diluted in sterile distilled (sd) water. In the positive controls, 10 ng of *P. brassicae* DNA replaced the template DNA to ensure conditions were conducive to DNA amplification. In the negative controls, the template DNA was replaced with an equivalent volume of sd water to ensure an absence of contamination in the reaction mix. PCR amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The amplification cycle consisted of an initial denaturation at 95˚C for 15 min, followed by 35 cycles of denaturation at 94˚C for 1 min, annealing at 55.5˚C for 2 min,

extension at 72˚C for 2 min, and a final extension at 72˚C for 10 min. DNA loading dye (Thermo Scientific) was added to the mixture following PCR, and amplification products were resolved in 1% agarose gels stained with 1X SYBR safe DNA gel stain (Invitrogen). Binary categorical data, where DNA in the extract is either 'present or absent', was used to determine DNA extraction success. The expected proportion of successful extractions from the samples was 100% while the expected proportion for the negative control (sd water) was 0%. From a total of more than 900 samples, a randomly selected subsample of 241 DNA extracts more than ensured a power of >80% at a 5% significance level (Campbell et al., 1995).

2.2.4.2. *P. brassicae*-specific Conventional PCR Analysis

The method of Cao et al. (2007) was used to specifically amplify *P. brassicae* DNA in a conventional PCR assay. A 25 µl reaction volume, including the primers TC1F (5' - GTG GTC GAA CTT CAT TAA ATT TGG GCT - 3') and TC1R (5' - TTC ACC TAC GGA ACG TAT ATG TGC ATG TG - 3'), contained: 1X PCR buffer (minus Mg)(Invitrogen), 0.2 mM dNTP, 2.0 mM of Magnesium Chloride ($MgCl_2$) (Invitrogen), 0.4 µM of TC1F and 0.4 µM of TC1R primers, 1 U Platinum Taq DNA polymerase (Invitrogen), and 10 ng of template DNA. In the positive controls, 10 ng of *P. brassicae* DNA was used as a template to ensure conditions were conducive to DNA amplification. In the negative controls, the template DNA was replaced with an equivalent volume of sd water. The PCR amplification cycle consisted of an initial denaturation at 94˚C for 2 min, followed by 45 cycles of denaturation at 94˚C for 30 sec, annealing at 65˚C for 1 min, extension at 72˚C for 1 min, and then a final extension at 72˚C for 10 min. DNA loading dye (Thermo Scientific) was added to each PCR product, with amplicons resolved on 1% agarose

gels stained with 1X SYBR safe (Invitrogen) and run at 4-5V/cm for 30 min. All conventional PCR amplifications were carried out with a GeneAmp PCR System 9700 (Applied Biosystems).

2.2.4.3. *P. brassicae*-specific Quantitative PCR Analysis

If any samples tested positive by conventional PCR analysis, the quantity of *P. brassicae* resting spores was determined by quantitative PCR (qPCR) analysis. When a sample tested positive for the presence of *P. brassicae*, all other samples, from all seasons and years, collected from the same location were assessed as well.

The qPCR method developed by Rennie et al. (2011) was used to measure the concentration of resting spores (spores g^{-1} soil) in a sample. Amplification was conducted using the *P. brassicae*-specific primers DC1F (5' - CCT AGC GCT GCA TCC CAT AT - 3') and DC1R (5' - CGG CTA GGA TGG TTC GAA AA - 3') in a StepOnePlus Real Time PCR System (Applied Biosystems). The 10 µl reaction volume contained: 0.8 µM DC1F/DC1R primer mix (0.4 µM of each DC1F/DC1R primer, 2.5 µl of stock 3.2µM DC1F/DC1R added per 10 µl reaction volume), 5µl of qPCR Mastermix (Molecular Biology Service Unit, University of Alberta, Edmonton, AB, Canada) containing SYBR Green (detection dye) and Thermo-Start DNA Polymerase in a proprietary reaction buffer (ThermoFisher Scientific) (36 µl of ROX (reporter dye) was added per 1 mL of master mix), and 2.5 µl of template DNA (diluted 10-fold prior to qPCR to help manage PCR inhibitors). In the negative controls, 2.5 µl of sd water was added instead of the template DNA solution.

Standard curves for the quantification of *P. brassicae* resting spores were generated with serial dilutions of DNA extracted from spore suspensions isolated from galled root material

using the methods of Cao et al. (2007) and Rennie et al. (2011). Briefly, suspensions of 1.0 x 10^8 resting spores mL^{-1} (determined by counting in a haemocytometer) were centrifuged and DNA was extracted from the pellet as described above, with the spore pellet replacing the soil sample. The DNA extracts were serially diluted at 10-fold intervals with sd water. Five standards, the DNA equivalent of 1.0 x 10^6 - 1.0 x 10^2 spores mL^{-1}, were then treated as templates in the qPCR assay. The negative control, standards, and templates were run in triplicate under the following reaction conditions: initial heat denaturation at 95˚C for 2 min, then 35 cycles of 95˚C for 15 s and 60˚C for 60 s. Each cycle included a melting point analysis and identification of any amplified product. A standard curve was generated based on the cycle threshold values (Ct) of the standards, and used to calculate resting spore concentrations (spores g^{-1} soil) in the samples as per the method of Rennie et al. (2011). Predictions outside of the standard curve were not incorporated into the analysis, and therefore the detectable range of *P. brassicae* resting spore concentrations was 4.0 x 10^6 - 4.0 x 10^3 spores g^{-1} soil.

2.2.5. Greenhouse Bioassays

In addition to assessment by qPCR analysis, the inoculum potential of selected soil samples also was evaluated in greenhouse bioassays with the universally susceptible Chinese cabbage (*Brassica rapa* ssp. *pekinensis* L.) cv. Granaat. A subset of all of the soil samples analyzed, consisting of at least 25 composite soil samples from each of the following categories (over 150 soil samples in total), was included in the bioassays: (1) no *P. brassicae* DNA detected by conventional or quantitative PCR analysis, (2) *P. brassicae* DNA detected by conventional PCR analysis, but not quantifiable by qPCR (< 4.0 x 10^3 spores g^{-1} soil), (3) spore concentration

between 4.0 x 10^3 - 3.2 x 10^4 spores g^{-1} soil as determined by qPCR analysis, (4) spore concentration between 3.2 x 10^4 - 1.6 x 10^5 spores g^{-1} soil, (5) spore concentration between 1.6 x 10^5 - 8.0 x 10^5 spores g^{-1} soil, and (6) spore concentration between 8.0 x 10^5 - 4.0 x 10^6 spores g^{-1} soil. Samples from each category were evenly selected from across all sampling seasons and years. A minimum of 25 soil samples from each of the six categories were analyzed to ensure 80% power at a significance level of 0.05.

Bioassays were conducted using a method modified from Strelkov et al. (2006b). Briefly, Chinese cabbage seeds were placed on moistened filter paper in Petri dishes for 1 week to allow germination. The seedlings were then transplanted into standard 12-cell plastic inserts (5.79 cm depth) (Grower Supply - FarmTek, Dyersville, Iowa, USA) and filled with a mixture (1:1 v:v) of soilless potting mix (Sunshine Mix #4, SunGro Horticulture, Seba Beach, AB, Canada) and homogenized *P. brassicae*-infested field soil. Each composite sample of field soil included in the bioassay was tested with one repetition of 12 seedlings in a single 12-cell insert. Each 12-cell insert was kept in its own tray of standing water (pH adjusted to 6.0 with 10% HCl) for the first week after seedlings were transplanted, after which the water in each tray was discarded and the plants were watered and fertilized as required. Sets of plants corresponding to different soil samples remained separated during watering to avoid cross-contamination. The plants were grown for 6 weeks in a greenhouse maintained at 19 ˚C to 24 ˚C with a 16 h photoperiod, and were then rated for clubroot symptom development as described above (Kuginuki et al., 1999, Strelkov et al., 2006b).

2.2.6. Statistical Analysis

The results of the qPCR assays were assessed using a mixed model repeated measurements analysis (RMA) (proc mixed, SAS/STAT 14.1, SAS institute Inc., Cary, North Carolina, USA) in order to evaluate the effect of CR canola cultivation on soil *P. brassicae* resting spore concentrations over time. Mixed model RMA with an appropriate covariate structure (in this case consistently type = UN or unstructured) properly accounted for unequal variance resulting from temporally correlated observations (i.e., repeated soil sampling/resting spore quantification over time) (Wang and Goonewardene, 2004). Non-linear regression (proc NLIN, SAS/STAT 14.1) best modeled the relationship between initial *P. brassicae* resting spore concentration and the resulting concentration after CR canola was cultivated. The effect of CR canola cultivation on *P. brassicae* resting spores was compared to fallow, or cultivation of non-hosts, in adjacent control plots. Finally, the non-normal distribution of ID in the field, as well as in greenhouse bioassays when placed into categories based on initial *P. brassicae* resting spore concentrations, favoured the use of Kruskal-Wallis analyses. All plots with quantifiable levels of *P. brassicae* resting spores, from all years (2010-2013), as well as the corresponding control plots were included in these assessments.

2.3. Results

2.3.1. Sample collection

Soil samples were collected twice annually between spring 2010 - fall 2013 from a total of 182 GPS marked locations (i.e., the experimental units) within 17 different fields. Over 8500

soil samples were collected, and formed a total of 895 composite soil samples. The response variable was the presence and quantity of *P. brassicae* DNA found in each of the 895 composite samples. These 895 composite samples represented all repeated visits to each experimental unit (i.e., 182 GPS marked locations) included in the study. The fixed treatment effects were crop rotation (Table 2-1) and time (spring/fall from 2010-2013). In 2010, 111 GPS marked locations sampled in the spring and fall totaled 222 composite samples. In 2011, 129 GPS marked locations sampled in the spring and fall totaled 253 composite samples. In 2012, 110 GPS marked locations sampled in the spring and fall totaled 200 composite samples. In 2013, 110 GPS marked locations sampled in the spring and fall totaled 220 composite samples. The crop rotations observed on all 17 fields (including fields observed only for some of the study years) are summarized in Table 2-1.

2.3.2. DNA Extraction from Soil Samples

Total genomic DNA was extracted from 895 composite soil samples. DNA was successfully isolated from all soil samples regardless of soil type. The quantity and quality of the extracted DNA was evaluated by spectrophotometric analysis, and confirmed by PCR amplification of the ITS region with the universal primers ITS1 and ITS4 (Korabecna, 2007) in a subset of 241 samples. DNA was amplified successfully from all of the samples, confirming the successful extraction of DNA from the soil matrix. The amplicons varied in size from approximately 400 bp to 800 bp, reflecting the non-specific nature of the ITS primers used, and numerous discrete bands were observed in many of the samples. Amplicons also were

obtained from each of the positive controls, while no amplification of product was detected in the negative controls (Fig. A-2).

2.3.3. Detection of *P. brassicae* DNA by Conventional PCR Analysis

The DNA extracts of 895 composite soil samples, collected from 17 different fields across Alberta between May 2010 - October 2013, were tested by conventional PCR analysis for the presence of *P. brassicae* DNA (Cao et al., 2007). A single amplicon of ~500-600 bp (consistent with the expected size of 548bp) was obtained from 140 of the samples analyzed with the *P. brassicae*-specific primers TC1F and TC1R (Fig. 2-2) (Cao et al. 2007). These 140 positive observations were from 75 discrete experimental units (i.e., georeferenced sampling locations) from 16 different fields, indicating that in 75 of the 182 experimental units, at least one observation over time (2010-2013) was positive for *P. brassicae* DNA. Only one field sampled had 0% detected *P. brassicae* DNA in all years at all georeferenced points, while the others ranged from 3.51% to 49.1%. There were cases within the 895 composite samples where eight consecutive composite samples from the same experimental unit or georeferenced location (collected from 2010-2013) tested uniformly positive or negative. Out of the 182 experimental units sampled, 107 (from 16 different fields) had no detectable levels of *P. brassicae* DNA in any year sampled. In some instances, at a single experimental unit (GPS marked sample location), positive *P. brassicae* DNA was not detected in the complete set of soil samples collected. Of all 182 experimental units sampled, 60 tested positive for *P. brassicae* DNA in ≤ 50% of the periods when soil was sampled (i.e., 2010-2013, spring and fall), 5 experimental units were positive in > 50% to < 100% of samples collected, and 10 cases

resulted in 100% of samples testing positive. In fields where sampling locations were selected by producers (9 fields) based on the identification of clubroot symptoms prior to 2010 (or whichever year the field was first included in our study), a greater percentage of samples (spring/fall, 2010-2013) from experimental units tested positive for *P. brassicae* DNA (19.89%, $t_{(156)}$=1.655, p<0.0004) than when sampling locations were selected randomly within infested fields (8 fields, 6.32%).

2.3.4. qPCR Assessment of Soil Samples

General qPCR results

As previously noted, conventional PCR analysis was used to confirm the presence of *P. brassicae* DNA in 16 fields and at 75 georeferenced points within those fields. Once the presence of pathogen DNA was verified, the DNA extracted from all composite soil samples (all seasons, years, and crops) collected at these 75 georeferenced points was assessed individually for the concentration of *P. brassicae* resting spores in the soil. A total of 343 composite samples had a quantifiable amount of resting spores, while an additional 29 samples had detectable but not quantifiable levels of resting spores. When all DNA extractions with quantifiable levels of *P. brassicae* resting spores were considered, the average measurable concentration was 1.30×10^5 resting spores g^{-1} soil.

Sampling locations (i.e., experimental units) where soil samples consistently tested positive for *P. brassicae* DNA tended to have higher average resting spore concentrations over the duration of sampling (2010-2013, spring and fall). Sampling locations where > 0% - ≤ 25% of the soil samples collected from 2010-2013 were positive for *P. brassicae* DNA averaged 1.94 x

10^4 spores g^{-1} soil among positive soil samples. Sampling locations where > 25% - ≤ 50% of samples tested positive had an average resting spore concentration of 9.84 x 10^4 spores g^{-1} soil among positive soil samples, while sampling locations where > 50% - ≤ 75% of samples tested positive had an average resting spore concentration of 1.11 x 10^5 spores g^{-1} soil among positive soil samples. Finally, where > 75% of samples tested positive, the average resting spore concentration was 4.52 x 10^5 spores g^{-1} soil among positive soil samples. Despite the upwards trend, the average resting spore concentrations were not quite significantly different from one another ($F_{(1,123)}$=3.797, p=0.0536).

Quantifiable P. brassicae *resting spore concentration & cultivation of CR canola*

The effect of CR canola cultivation on *P. brassicae* resting spore concentration in the soil within a single growing season was assessed. The results of the qPCR assays were analyzed and the effect CR canola cultivation on resting spore concentration was compared with the effect of fallow periods, or the cultivation of non-hosts, in adjacent plots. The analysis included experimental units (i.e., GPS marked locations) where *P. brassicae* resting spore concentration was quantified in the spring and fall and CR canola was cultivated. The number of fields where CR canola was cultivated varied by year. The experimental units included in the analysis (i.e., sample locations where CR canola cultivation occurred) consisted of: 33 experimental units from 8 fields in 2010, 9 experimental units from 2 fields in 2011, 13 experimental units from 6 fields in 2012, and 10 experimental units from 3 fields in 2013. In all years assessed individually (i.e., 2010, 2011, 2012, 2013),mixed model RMAs of variance failed to demonstrate significant treatment effects (2010: $F_{(1,31)}$=0.03, p>0.05, 2011: $F_{(1,7)}$=0.07, p>0.05, 2012: $F_{(1,11)}$=0.34, p>0.05, 2013: $F_{(1,8)}$=1.7, p>0.05) or treatment by time interaction (2010: $F_{(1,31)}$=0.8, p>0.05, 2011:

$F_{(1,7)}$=0.24, p>0.05, 2012: $F_{(1,11)}$=3.29, p=0.0971, 2013: $F_{(1,8)}$=0, p>>0.05) (Fig. 2-3). Within a single growing season, resting spore concentration did not significantly differ between control plots (where no susceptible host was cultivated) and sample locations (where CR canola was cultivated). However 'season' or 'time' was significant in 2010 ($F_{(1,31)}$=6.04, p= 0.0198), where the average quantifiable resting spore concentration decreased in both sample locations (from 5.30 x 10^4 spores g^{-1} soil to 1.07 x 10^4 spores g^{-1} soil, a 79.81% decrease) and control plots (from 3.84 x 10^4 spores g^{-1} soil to 1.87 x 10^4 spores g^{-1} soil, a 51.3% decrease)(Fig. 2-3).

When the cumulative resting spore concentration results from 2010-2013 were evaluated together, again, no significant treatment effect ($F_{(1,63)}$=0.55, p>0.05) or treatment by time interactions ($F_{(1,63)}$=0.12, p>0.05) were observed. However, there was a nearly significant effect of time ($F_{(1,63)}$=3.76, p=0.057), where the average quantifiable resting spore concentration decreased in both sample locations (from 3.58 x 10^4 spores g^{-1} soil to 1.89 x 10^4 spores g^{-1} soil, a 47.2% decrease) and control plots (from 2.46 x 10^4 spores g^{-1} soil to 1.28 x 10^4 spores g^{-1} soil, a 48.0% decrease)(Fig. 2-3).

The concentration of *P. brassicae* resting spores in the soil also was monitored over the course of two growing seasons, in which CR canola was cultivated only in the first year. All test plots seeded to CR canola in year-1, followed by the cultivation of a non-host (wheat, barley, or flax) in year-2, along with the corresponding control plots which remained host-free for both years, were assessed. This included 50 georeferenced sampling points from 13 field locations across Alberta and their corresponding control plots. The plots assessed included CR canola cultivation from 2010-2012 as year-1 samples, along with corresponding non-host cultivation from 2011-2013, and their respective control plots from all years. Mixed model RMA resulted in

nearly significant treatment effect at $F_{(1,48.4)}$=3.03, p=0.0882, and treatment by time period interaction at $F_{(3,45.1)}$=2.74, p=0.0543. The *P. brassicae* resting spore concentration was affected by a 'treatment' (Cultivation of CR canola/Control) x 'time' interaction. Spore concentration appeared to increase by sampling period with the cultivation of CR canola. In contrast, in the controls, where no susceptible host was cultivated over the entire duration of the study, the spore concentration remained constant or decreased slightly. The test plots, in which CR canola was cultivated, experienced a 2.4-fold increase in average *P. brassicae* resting spore concentration, from 6.5 x 10^4 spores g^{-1} soil in the spring of year-1 to 1.57 x 10^5 spores g^{-1} soil in the spring of year-2 (Fig. 2-4). Control plots, in which no susceptible host was cultivated, experienced a 2.6-fold decrease in average *P. brassicae* resting spore concentration, from 5.6 x 10^4 spores g^{-1} soil in the spring of year-1 to 2.19 x 10^4 spores g^{-1} soil in the fall of year-2 (Fig. 2-4).

Modelling P. brassicae *inoculum response to initial resting spore concentration*

The increase in *P. brassicae* resting spore concentration was not apparent until the year following cultivation of CR canola. *P. brassicae* resting spore concentration in year-2, resulting from an initial quantifiable resting spore concentration in the spring of the previous year (e.g., spring year-1), was analyzed. Quantifiable resting spore concentration in year-2, following the cultivation of CR canola, peaked at different times depending on location. In most cases, the spore concentration was greatest in the spring of year-2 following a year when CR canola was cultivated, although occasionally the greatest concentration was observed in the fall of year-2. Based on a multiple comparisons analysis, treatment plots (where CR canola was cultivated in year-1) did not show any significant difference in quantifiable *P. brassicae* resting spore

concentration between spring year-2 and fall year-2 samples (p>0.05) (Fig. 2-4). In order to assess any increase in *P. brassicae* resting spore concentration, the peak inoculum level had to be included in the analysis and, therefore, an average resting spore concentration in year-2 was used as the dependent variable. Linear regression did not sufficiently explain the relationship between year-1 and year-2 spore loads ($F_{(1,13)}$=5.65, p=0.0335, R^2=0.2492)(Fig. 2-5, Fig. A-3). Instead, a non-linear model was better able to explain the variation in average resting spore concentration in year-2 based on the initial resting spore concentration prior to cultivation of CR canola in year-1 ($F_{(2,14)}$=9.91, p=0.0021, R^2=0.50381)(Fig. 2-5). A model was developed where:

$$\text{AVERAGE\_SPORE}_{year2} = \frac{((5.37007 * 10^5) * \text{SPORE}_{year1})}{((1.0375 * 10^5) + \text{SPORE}_{year1})}$$

and in which the response variable '$\text{AVERAGE\_SPORE}_{year2}$' is the expected average quantifiable *P. brassicae* resting spore concentration in a plot following cultivation of CR canola (expected average spore concentration was modelled based on actual spore concentration of soil samples collected in spring and fall of year-2), and 'SPORE_{year1}' is the quantifiable resting spore concentration in a plot prior to cultivation of CR canola in year-1.

Clubroot incidence and severity in fields of CR canola

Clubroot disease levels in the CR canola, under field conditions, were generally very low. The variable 'initial *P. brassicae* resting spore concentration' did not sufficiently explain variation in observed ID in sampled CR canola roots (see section 2.2.2, Strelkov et al., 2006) despite a minor increase in ID as initial *P. brassicae* resting spore concentration increased ($F_{(1,68)}$=6.04, p=0.0165, r^2=0.08). The greatest observed ID was 13.33% and corresponded with

an initial *P. brassicae* resting spore concentration of 2.88 x 10^4 spores g^{-1} soil. Occasionally, no clubroot symptoms were observed on CR canola roots (7 cases) from sampling locations with quantifiable levels of *P. brassicae* resting spores (7.22 x 10^3 spores g^{-1} soil up to 2.49 x 10^5 spores g^{-1} soil; mean=1.52 x 10^4 spores g^{-1} soil, median= 2.27 x 10^3 spores g^{-1} soil), resulting in IDs of 0%. Also, plants growing in some locations (13 cases) that had non-detectable or unquantifiable levels of *P. brassicae* resting spores still had very low levels of disease in the field (IDs from 0.667% to 2.66%, mean=1.38%, median=1.33). When we consider only cases where there was a quantifiable concentration of *P. brassicae* resting spores and clubroot symptoms were observed (28 cases), the initial spore concentration still failed to have a significant impact on observed infection of CR canola in our sample plots ($p>0.05$). In many sample locations *P. brassicae* could not be detected and the ID was 0%, but only negative sample locations from within fields in which at least one location tested positive were included in the analysis (29 cases).

The variable 'initial *P. brassicae* resting spore concentration' was assessed categorically to include unquantifiable yet detectable *P. brassicae* resting spore samples in the analysis. Categories mirrored the positive controls used in the creation of a standard curve during qPCR analysis. The categories of 'initial *P. brassicae* resting spore concentration' used included: 1) undetected, 2) detected but unquantifiable (< 4.0 x 10^3 spores g^{-1} soil), 3) ≥ 4.0 x 10^3 spores g^{-1} soil - < 4.0 x 10^4 spores g^{-1} soil, and 4) ≥ 4.0 x 10^4 spores g^{-1} soil. Non-normal distribution of the data favoured the use of a Kruskal-Wallis analysis. At least one category of 'initial *P. brassicae* resting spore concentration' resulted in different ID ($F_{(3, 66)}$=8.81, $p<0.0001$). Based on multiple comparisons analysis, the two highest categories of 'initial *P. brassicae* resting spore

concentration' (corresponding to ≥ 4.0 x 10^3 spores g^{-1} soil, and ≥ 4.0 x 10^4 spores g^{-1} soil) resulted in greater ID in CR canola compared with sample plots with undetectable levels of *P. brassicae* resting spores (p=0.0122, p=0.0013, respectively)(Fig. 2-6).

Crop rotation and P. brassicae resting spore concentration

The concentration of *P. brassicae* resting spores in the soil was assessed under various crop rotation regimes. A rotation where CR canola was planted once in 2-years was assessed with a mixed model - RMA. There was no significant effect of any kind over the entire sampling window (spring year-1 to spring year-3, Fig. 2-7) (P>0.05), although the control plots differed from sample plots in the periods spring year-2 ($t_{(45.1)}$=-1.94, p=0.0585) and fall year-2 ($t_{(25.7)}$=-2.05, p=0.0503) in terms of *P. brassicae* resting spore concentration (Fig. 2-7).

A mixed model - RMA also was used to assess a rotation where CR canola was planted once in a 4-year period. There was no significant treatment effect over the entire sampling window (spring 2010- fall 2013, Fig. 2-8) (P>0.05), and due to high variation within the control plots during year-2 (spring 2011 and fall 2011), no significant difference in *P. brassicae* resting spore concentration was observed between sample and control treatments in 2011. There was, however, a significant effect of time period on *P. brassicae* resting spore concentration $F_{(7,5)}$=4.98, p=0.0498 over the entire 4-year sampling window. Given two years without the cultivation of a susceptible host (2011, 2012) following the cultivation of CR canola (in 2010), *P. brassicae* resting spore concentrations decreased more than 8-fold, from an average of 1.17 x 10^5 spores g^{-1} soil (spring 2010) to 1.45 x 10^4 spores g^{-1} soil (fall 2013) even in highly infested fields (initial resting spore concentration > 1.0 x 10^5 spores g^{-1} soil). From the maximum average *P. brassicae* resting spore concentration (2.97 x 10^5 spores g^{-1} soil) observed in spring-2011, a

≥2-year break without *Brassica* cultivation resulted in a 20.5-fold decrease in resting spore concentration to 1.45 x 10^4 spores g^{-1} soil in fall-2013.

2.3.5. Greenhouse Bioassay

Of 164 soil samples evaluated in the greenhouse bioassays, 141 samples had detectable levels of *P. brassicae* DNA based on PCR analysis (including 111 with quantifiable levels), while 23 samples had no detectable *P. brassicae* DNA. Of the 141 PCR positive samples tested, 101 (71.63%) caused visible symptoms of clubroot (ID > 0%) on at least some of the bait plants (Chinese cabbage cv. Granaat). Of the 111 samples tested that had quantifiable levels of *P. brassicae* DNA, 95 (85.59%) caused visible symptoms (ID > 0%) on at least some of the bait plants. The IDs ranged from 1.01% - 100% when all samples were considered. Soil samples with undetectable levels of *P. brassicae* DNA rarely produced symptoms of clubroot in susceptible bait plants. Of the 23 PCR negative samples tested, only 2 produced symptoms on bait plants, resulting in IDs of 7.78% and 25.93%.

The lowest concentration of resting spores that could be measured reliably by qPCR analysis was 4.0 x 10^3 spores g^{-1} soil, which was slightly greater than the limit of detection by conventional PCR (Cao et al. 2007). Therefore, samples testing positive for *P. brassicae* DNA based on conventional PCR analysis, but at non-quantifiable levels using the qPCR method, favoured a categorical assessment of the data. Data was grouped into five categories based on initial *P. brassicae* resting spore concentration, as described in the materials and methods and Fig. 2-9. These categories were: 1) no detected *P. brassicae* DNA, 2) detected but not quantifiable (< 4.0 x 10^3 spores g^{-1} soil), 3) 4.0 x 10^3 spores g^{-1} soil - < 4.0 x 10^4 spores g^{-1} soil, 4) ≥

4.0 x 10^4 spores g^{-1} soil - < 4.0 x 10^5 spores g^{-1} soil, and 5) ≥ 4.0 x 10^5 spores g^{-1} soil. The non-normal distribution of the data further favoured the use of a Kruskal-Wallis analysis. At least one category of '*P. brassicae* resting spore concentration' resulted in a different ID in the Chinese cabbage cv. Granaat ($F_{(4,159)}$=36.98, p<0.0001). Based on multiple comparisons analysis, all resting spore categories differed from each other in terms of the ID induced in the bait plants (P<0.05), except the two lowest (Fig. 2-9); the IDs observed in the Chinese cabbage plants grown in soil samples with no detectable levels of *P. brassicae* spores, and samples with detected but unquantifiable levels (< 4.0 x 10^3 spores g^{-1} soil), did not differ significantly (P>0.05).

2.4. Discussion

The main purpose of this study was to evaluate the effect that cultivation of CR canola has on *P. brassicae* resting spore concentrations in naturally infested fields. This included an examination of the effect of CR canola on *P. brassicae* inoculum within a single season, as well its effect over various cropping sequences and multiple years. A second goal of the study was to better understand how initial *P. brassicae* resting spore concentrations in the soil can influence clubroot development in CR canola.

An accurate assessment of the presence or concentration of *P. brassicae* resting spores in soil samples is critical for this type of study. The extraction of DNA from soil samples needed to be highly effective to avoid false negatives due to poor extraction. Confirmation of DNA extraction success via PCR amplification was attempted initially with random or actin primers, which yielded poor or inconsistent results, respectively (data not shown). In contrast, the

universal primers ITS1 and ITS4 (Korabecna, 2007) proved very useful for verifying the success of DNA extraction from composite soil samples. Since amplification products could be obtained from all samples in the subset tested, with no visible product in the negative controls, it appears that the extraction methods used were effective for the recovery of DNA from soil. This extra step of verification helped to mitigate the potential risk of making a type-2 error, where a sample would be identified as negative for *P. brassicae* DNA when it was in fact positive but the DNA extraction failed.

The strong positive relationship between the bioassay results and the qPCR-based estimates of soil infestation reflects the accuracy achieved when quantifying soil resting spore concentrations by qPCR analsyis. Clubroot severity on susceptible host plants increased with increasing *P. brassicae* spore concentration. Nevertheless, in spite of the general success of resting spore quantification, there were two cases in which plants grown in a soil sample that had tested negative by PCR analysis still developed symptoms of clubroot. The distribution of *P. brassicae* inoculum in the soil is known to be very heterogeneous (Strelkov et al., 2006a, Cao et al., 2007, Wallenhammar et al., 2012), and despite strategies such as the collection of multiple soil samples from each sampling location, it is likely that these two false negatives reflected this heterogenity. It is also possible that the presence of PCR inhibitors or other artefacts were responsible for the false negatives. DNA extracted from soil can be contaminated with humic acid, an organic polymer found in humus that can act as a PCR inhibitor even at very low concentrations (e.g., 0.1 ng of humic acid per qPCR reaction volume) (Green and Field, 2012). Multiple purification steps and other precautions were taken during the DNA extraction process in order to minimize the impact of humic acid and other PCR

inhibitors. Therefore, notwithstanding the rare occurrence of false negatives, DNA extraction and *P. brassicae* quantification were successful in the vast majority of the samples, as corroborated by the bioassay results.

Consistent detection of *P. brassicae* DNA at a single sampling location tended to increase when the average resting spore concentration was high throughout the sampling period from 2010-2013. Cooperating producers identified areas within their fields where a high incidence of clubroot had been observed in previous canola crops. These areas tended to yield a higher percentage of samples testing positive for the presence of *P. brassicae*. In contrast, when experimental units were selected at random throughout a field, *P. brassicae* infestation was detected in a lower percentage of the samples. In some cases, *P. brassicae* DNA could not be detected at a location that had previously yielded positive results. This may have reflected decreases in resting spore concentrations over time at those locations, at least to levels that were not detectable. Given the extensive sampling that was conducted within and across fields and years, a wide range of initial *P. brassicae* resting spore concentrations were identified, from which subsequent analyses were possible.

The cumulative evaluation of resting spore concentration within a single season revealed that spore concentrations decreased from spring to fall in fields where CR canola was grown, and in control plots where no crop was grown. While there was a noticeable decrease in the number of quantifiable resting spores per gram of soil, no significant treatment effect was observed on spore concentration within a single growing season (any and all year(s) from 2010-2013). When evaluated year-by-year, the average *P. brassicae* resting spore concentration in CR canola fields either decreased slightly (in 2010) or was not significantly

different in spring and fall samples (in 2011-2013). It is possible that the impact of CR cultivars on *P. brassicae* resting spore germination rates is not significantly different from germination rates under fallow conditions, or from germination rates in the presence of the non-host crops observed in this study (i.e., barley, wheat, flax). As such, CR canola does not appear to function as a useful bait crop for the reduction of *P. brassicae* infestations, at least under field conditions in Alberta. This finding is consistent with a report by Ahmed et al. (2011), who found that the planting of bait crops (including canola) did not reduce clubroot severity in subsequent crops under field conditions, and only had a minimal effect on *P. brassicae* resting spore concentrations.

When levels of *P. brassicae* inoculum in the soil were evaluated in the year following a crop of CR canola, significant increases in resting spore concentrations were detected, only in those fields that had been planted to CR canola. Indeed, the resting spore concentrations peaked in the year following cultivation of CR canola. It can be hypothesized that there is a lag in the release of new mature *P. brassicae* inoculum (resting spores) into the soil after the cultivation of CR canola. The initial decline in resting spore concentration within a single growing season (between spring year-1 and fall year-1) may reflect widespread germination of pre-existing spore inoculum in the presence of a host such as CR canola, while new mature resting spores remain in the galled root material until it becomes sufficiently decomposed or incorporated into the soil matrix the following year.

It has been suggested that the *P. brassicae* resting spore concentration at which planting a CR *Brassica* crop does not result in excessive inoculum production is about 1.3×10^5 spores g^{-1} soil (Wallenhammar et al., 2012). Based on the model developed in this study, an

initial spore load of 1.0 x 10^5 spores g^{-1} soil can result in a concentration of approximately 2.6 x 10^5 spores g^{-1} soil in the year following the planting of CR canola. Despite the propagation of inoculum by CR canola when below the recommended threshold of 1.3 x 10^5 spores g^{-1} soil (Wallenhammar et al., 2012), these increases in inoculum could be considered minor compared with the potential amplification of inoculum by a susceptible host (Hwang et al., 2012b) to high levels capable of more substantial infection (> 1.0 x 10^8 spores g^{-1} soil) (Hwang et al., 2011b). A 2.6-fold increase in resting spore concentration from 1.0 x 10^5 spores g^{-1} soil to 2.6 x 10^5 spores g^{-1} soil may be acceptable to many producers growing CR canola cultivars, since it is near the range of resting spore concentrations (5.7 x 10^4 - 2.1 x 10^5 spores g^{-1} soil) found to have a smaller impact on yield following a ≥2-year break from *P. brassicae* hosts (Peng et al., 2015).

The data in the current study were obtained from natural *P. brassicae* infestations under field conditions, and a variety of factors may influence the resting spore concentration in subsequent years. The presence of susceptible weeds, such as shepherd's purse (*Capsella bursa-pastoris* (L.) Medik.) and stinkweed (*Thlaspi arvense* L.), may play a role in propagating *P. brassicae* inoculum (Howard et al., 2010). Also, although there is usually much more than the minimum 80% hybridity required (Canadian Seed Growers' Association, 2005) in commercial seed lots, off-types or the presence of volunteers may result in some susceptible plants within a CR canola crop. The predictive capability of the model developed in this study decreases near the highest *P. brassicae* resting spore concentrations observed (e.g., 3.4 x 10^5 spores g^{-1} soil). A data set including soil samples with resting spore concentrations exceeding 1.0 x 10^6 to 1.0 x 10^8 spores g^{-1} soil could help elucidate the resting spore response (average year-2) after CR canola is cultivated in fields with higher initial resting spore concentrations. Nevertheless,

based on the current model, an initial spore concentration of 1.0 x 10^5 spores g^{-1} soil would maintain the inoculum load below 2.6 x 10^5 spores g^{-1} soil, and may represent an inoculum level at which the cultivation of CR canola is acceptable, especially where a proper rotation is implemented. This model was evaluated only within the limits of the current data set, and model predictions when considering an initial spore concentration >3.50 x 10^5 spores g^{-1} soil (the maximum observed initial resting spore concentration) may not be biologically significant. At higher initial resting spore concentrations than those in this study, a new non-linear regression model should be calculated, which may be able to better explain the relationship between year-1 and year-2 spore loads.

In fields with *P. brassicae* infestations, the clubroot severity observed in plant stands seeded with CR canola was very low. The ID was rarely greater than 10%, and was generally lower than 4.15% even when inoculum levels were as high as 4.0 x 10^5 spores g^{-1} soil. The mild symptoms of clubroot that developed even in the presence of relatively high levels of *P. brassicae* inoculum likely reflected the fact that this study was focused on CR canola, which would not be expected to develop much disease (Hwang et al., 2012b). Nevertheless, greater initial concentrations of inoculum did result in slightly higher levels of disease in CR canola crops. It is possible that at high initial levels of inoculum, further increases in spore concentration in the soil occur from a combination of infected weedy hosts (Howard et al., 2010), genetic off-types in seed-lots, volunteers, and also, occasionally, resistant genotypes in which heavy inoculum pressure is capable of producing symptoms. The risk of selecting for pathotypes or stains of *P. brassicae* capable of overcoming the genetic resistance in CR canola is of particular concern, especially in fields with heavy infestations. Indeed, the virulence of

clubroot pathogen populations appears to shift fairly rapidly in response to the selection pressure associated with the planting of resistant host genotypes (Tanaka et al., 1991, Tanaka et al., 1997, Oxley, 2007, Diederichsen et al., 2014, Strelkov et al., 2014b, Wesolowska, 2014, Strelkov et al., 2016). In Alberta, strains of *P. brassicae* exhibiting greatly increased virulence on CR canola have been recently identified, after only a few years of exposure to the host genotypes, and represent a threat to the sustainable management of clubroot (Strelkov et al. 2016).

The propagative effect of the cultivation of CR canola when initial *P. brassicae* resting spore concentrations are high, as well as the risk of resistance erosion, highlights the need to implement longer rotations out of canola, in order to allow time for the depletion of pathogen resting spores in the soil (Peng et al., 2015). Results presented by Peng et al. (2015) suggest that a rotation away from a *Brassica* host as short as 2 years is associated with declines in soil *P. brassicae* resting spore concentrations as high as 90% relative to a single year break from canola or continual canola cropping. In the current study, a single year break from CR canola cultivation resulted in resting spore concentrations that were similar to the concentrations detected prior to the initial planting of CR canola. Given additional time, resting spore concentrations may decrease even further. Consistent with the findings of Peng et al. (2015), the current study demonstrated large declines in resting spore concentrations given a ≥2-year break from *Brassica* cultivation (specifically CR canola). Although many producers are hesitant to incorporate longer rotations away from canola into their farming practices (Smith et al., 2013, Rempel et al., 2014, Strelkov and Hwang, 2014), they may find that the increased yields associated with longer rotations (and reduced *P. brassicae* inoculum loads) may offset any

perceived loss of canola-generated revenue, particularly if these benefits can be achieved in as little as 2-years without a host crop.

Despite an initial increase in *P. brassicae* resting spore concentration the year following cultivation of CR canola, 3-years after the harvest of said crop the resting spore concentrations were similar to those of host-free control plots. Initial increases in pathogen inoculum may include many resting spores that are immature and disappear quickly from the soil. Nonetheless, a subset of *P. brassicae* resting spores must either reach full maturity, or exhibit some higher level of resilience, making it possible for them to survive much longer periods. It is apparent that some inoculum persists for many years at levels sufficient to initiate clubroot development in a susceptible host (Wallenhammar, 1996).

Combining cultivar resistance with a proper crop rotation is part of an effective clubroot management strategy (Hwang et al., 2014, Peng et al., 2015). The results of this study suggest that CR canola can result in increases in *P. brassicae* resting spore concentrations in commercial fields, although these increases may be acceptable if the initial spore concentration is below 1.0 x 10^5 spores g^{-1} soil. It is important, however, to plant CR canola in rotations longer than 1-in-2 years; even a 2 year rotation away from a susceptible host may be sufficient to prevent excessive build-up of soil inoculum. By implementing longer rotations and having an awareness of clubroot severity or inoculum levels in their fields, producers may be able to prolong the effectiveness of CR cultivars and thereby contribute to the more sustainable management of clubroot of canola.

Table 2-1. Crop rotations in 17 fields in Alberta, Canada, which were evaluated for *Plasmodiophora brassicae* resting spore concentrations in the soil from 2010-2013.

Years observed[a]	CR canola frequency[b]	Rotation[c]	Frequency[d]
4	3 - in - 4	CR canola - Wheat - CR canola - CR canola	1
4	2 - in - 4	CR canola - Wheat - CR canola - Wheat	2
4	2 - in - 4	CR canola - Wheat - CR canola - Barley	3
4	2 - in - 4	Wheat - CR canola - CR canola - Wheat	1
4	1 - in - 4	CR canola - Wheat - Barley - Flax	2
3	1 - in - 3	Peas - Barley - CR canola	1
3	1 - in - 3	Wheat - CR canola - Barley	2
3	1 - in - 3	Peas - CR canola - Wheat	1
3	2 - in - 3	CR Canola - Wheat - CR Canola	1
2	1 - in - 2	CR canola - Wheat	3

[a]*Years observed* is the number of years a field was part of the trial

[b]*CR canola frequency* is the number of times CR canola was grown - in - the number of years the field was observed

[c]*Rotation* is the exact rotation of crops observed

[d]*Frequency* is the number times (i.e., the number of fields) that were observed using each rotation

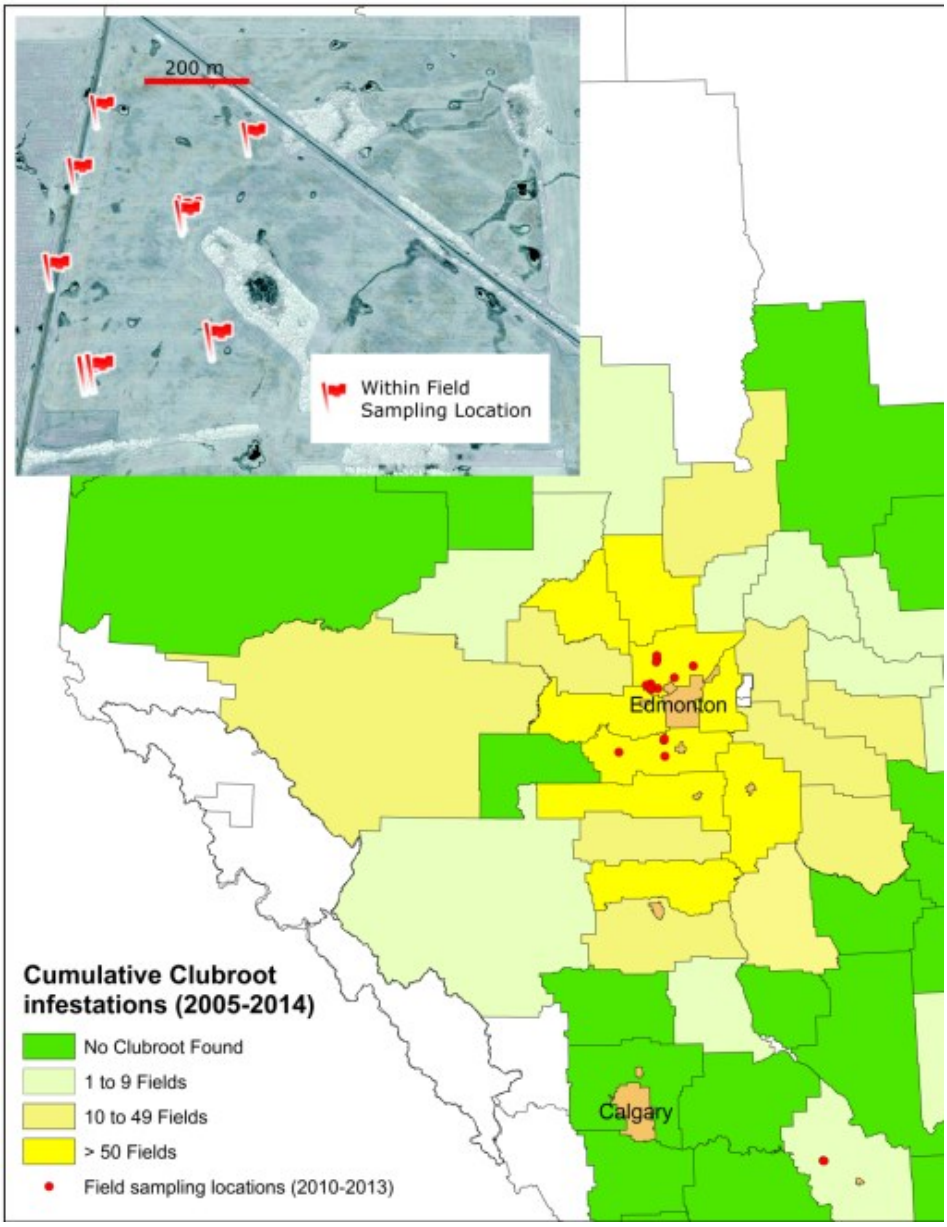


Figure 2-1. Distribution of fields monitored for *Plasmodiophora brassicae* resting spore concentration from 2010-2013 in Alberta, Canada. Embedded image (top left) illustrates the within-field distribution of sampling points for one sample field. In the example provided, sampling points followed a 'W' sampling pattern, and two control sampling points were included along the western field edge. In some fields, sampling points also were selected based on the reported occurrence of clubroot, when this information was available. Cumulative infestations (main panel) reflect total number of confirmed clubroot infestations in specific counties or municipalities, as assessed in yearly surveys (adapted from Strelkov and Hwang, 2014a).

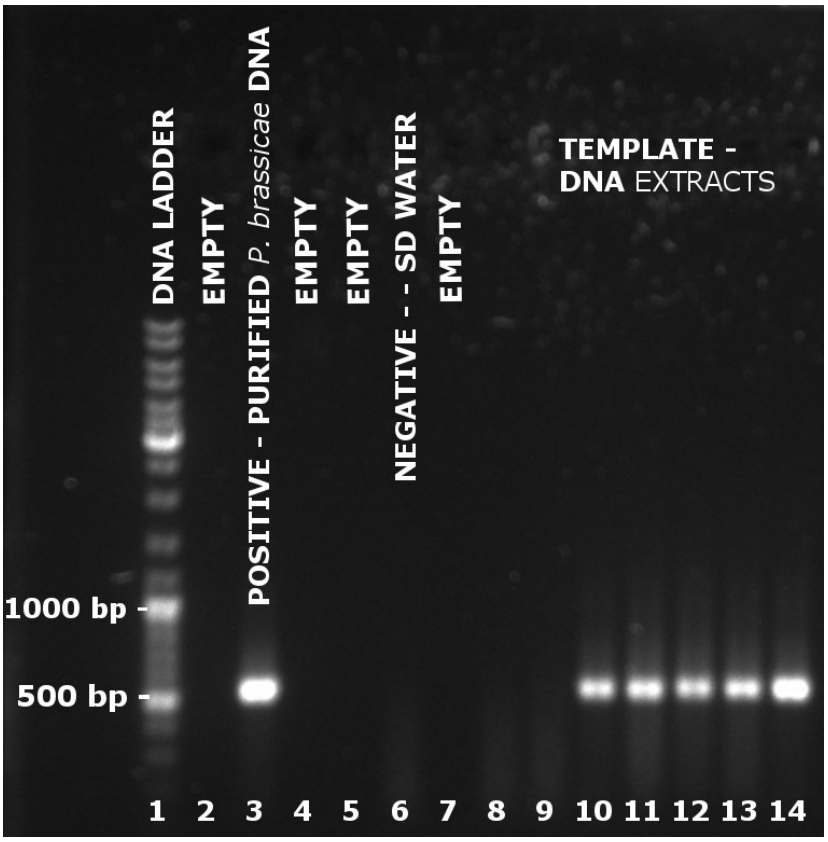


Figure 2-2. Polymerase chain reaction (PCR) products amplified using the *Plasmodiophora brassicae*-specific primers TC1F and TC1R (Cao et al., 2007) from total genomic DNA extracted from soil samples from Alberta, Canada. The amplicons were resolved in 1% agarose gels stained with 1X 'SYBR safe DNA gel stain (Invitrogen). All DNA extracted from soil samples collected from 2010-2013 across Alberta was subjected to PCR analysis with the TC1F and TC1R primers, but only a sample image is shown. Lane 1: DNA ladder (GeneRuler 1 kb Plus DNA Ladder, Fermentas Life Sciences), Lane 3: Positive control (purified *P. brassicae* SACAN-SS2, extracted DNA), Lane 6: Negative control (sterile distilled H_2O), Lane 8 - 14: template DNA (Lane 8 – 9 = negative for *P. brassicae* DNA, Lane 10 - 14 = positive for *P. brassicae* DNA), Lanes 2, 4, 5 and 7: empty wells.

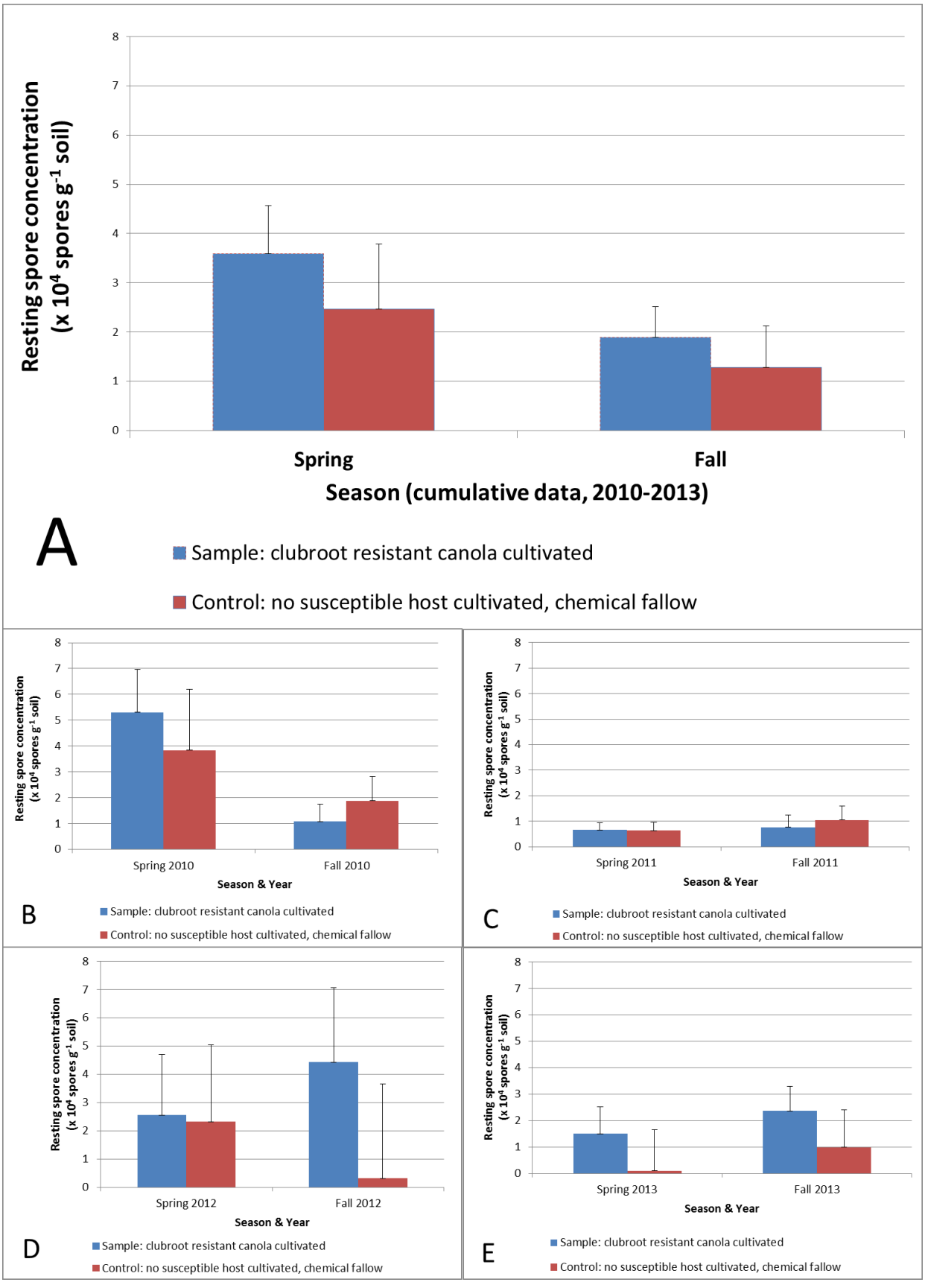

Resting spore concentration (x 10⁴ spores g⁻¹ soil)
0
1
2
3
4
5
6
7
8
Spring
Fall
Season (cumulative data, 2010-2013)
A
Sample: clubroot resistant canola cultivated
Control: no susceptible host cultivated, chemical fallow
Spring 2010
Fall 2010
Season & Year
B
Spring 2011
Fall 2011
C
Spring 2012
Fall 2012
D
Spring 2013
Fall 2013
E

Figure 2-3. Average concentration of *Plasmodiophora brassicae* resting spores in the soil within each individual year of the study. Graphs illustrate average resting spore concentrations in the spring and fall at sample locations where CR canola ("Sample") was cultivated and at corresponding plots that were kept fallow ("Controls") . Bars represent the standard error. Panel 'A' illustrates the average resting spore concentrations in spring and fall over all of the years of the study (i.e., cumulative average resting spore concentrations from 2010-2013). Panel 'B' illustrates the average resting spore concentration of 33 sample locations from 8 fields in 2010. Panel 'C' illustrates the average resting spore concentration of 9 sample locations from 2 fields in 2011. Panel 'D' illustrates the average resting spore concentration of 13 sample locations from 6 fields in 2012. Panel 'E' illustrates the average resting spore concentration of 10 sample locations from 3 fields in 2013. The only significant variable was time period (i.e., season) in panels 'A' ($F_{(1,63)}$=3.76, p=0.057) and 'B' ($F_{(1,31)}$=6.04, p= 0.0198).

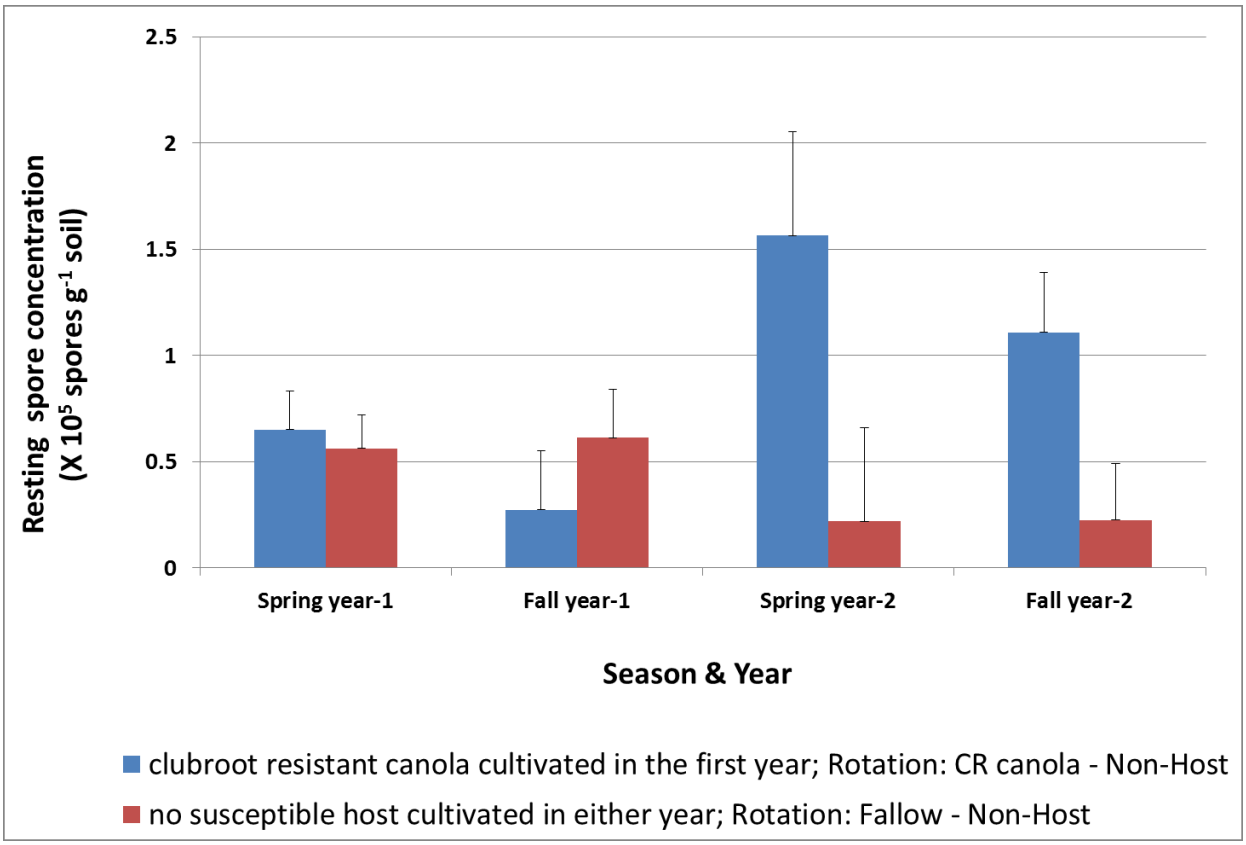


Figure 2-4. Mean concentration of *Plasmodiophora brassicae* resting spores in the soil over any two year period within 2010-2013. All instances in which 'Clubroot resistant' (CR) canola is cultivated in the first year followed by a non-host in the second year (as well as controls, in which no susceptible host was cultivated) were analyzed. This included 25 experimental units or sampling points (with corresponding control plots) from 6 different fields, where spores were reliably quantified over all 4 sampling periods. Resting spore concentration is increase by sampling period after the cultivation of CR canola. In the control, where no susceptible host was cultivated, spore concentration remains constant or decreases slightly by sampling period. This represents an interaction effect of 'treatment' (CR canola cultivation/Control) x 'time' (Seasone/Year) $F_{(3,45.1)}$=2.74, p=0.0543. Bars represent the positive standard error value.

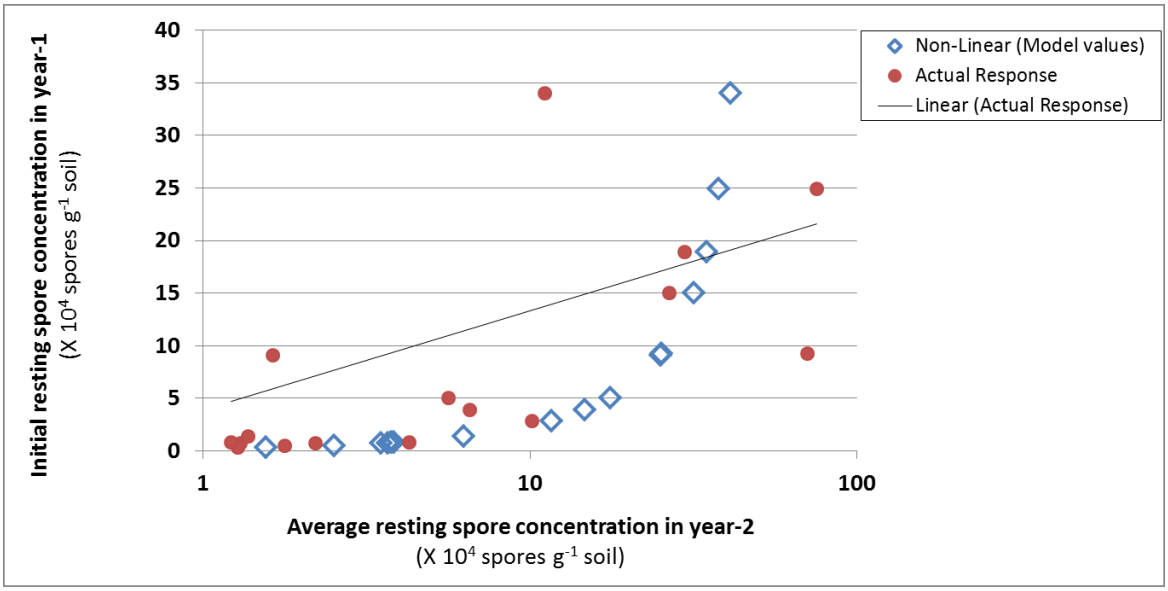


Figure 2-5. Graphical representation of the linear and non-linear relationship between initial *Plasmodiophora brassicae* resting spore concentration in the soil, and the average resting spore concentration observed in the following year. Average resting spore concentration observed in year-2, followed the cultivation of clubroot resistant canola in year-1. Data collected over four years (2010-2013) were used to generate these models. Non-Linear (◇, Model values): represent values generated using a best fit non-linear model ($F_{(2,14)}$=9.91, p=0.0021, R^2=0.50381) and initial *P. brassicae* resting spore concentrations observed in the spring of year-1. Actual Response (●): represents a scatter plot of the average *P. brassicae* resting spore concentrations observed in year-2, in relation to resting spore concentrations in the spring of year-1 when CR canola was cultivated. —— Linear (Actual Response): is the linear regression explaining the relationship between year-1 and average year-2 spore loads ($F_{(1,13)}$=5.65, p=0.0335, R^2=0.2492).

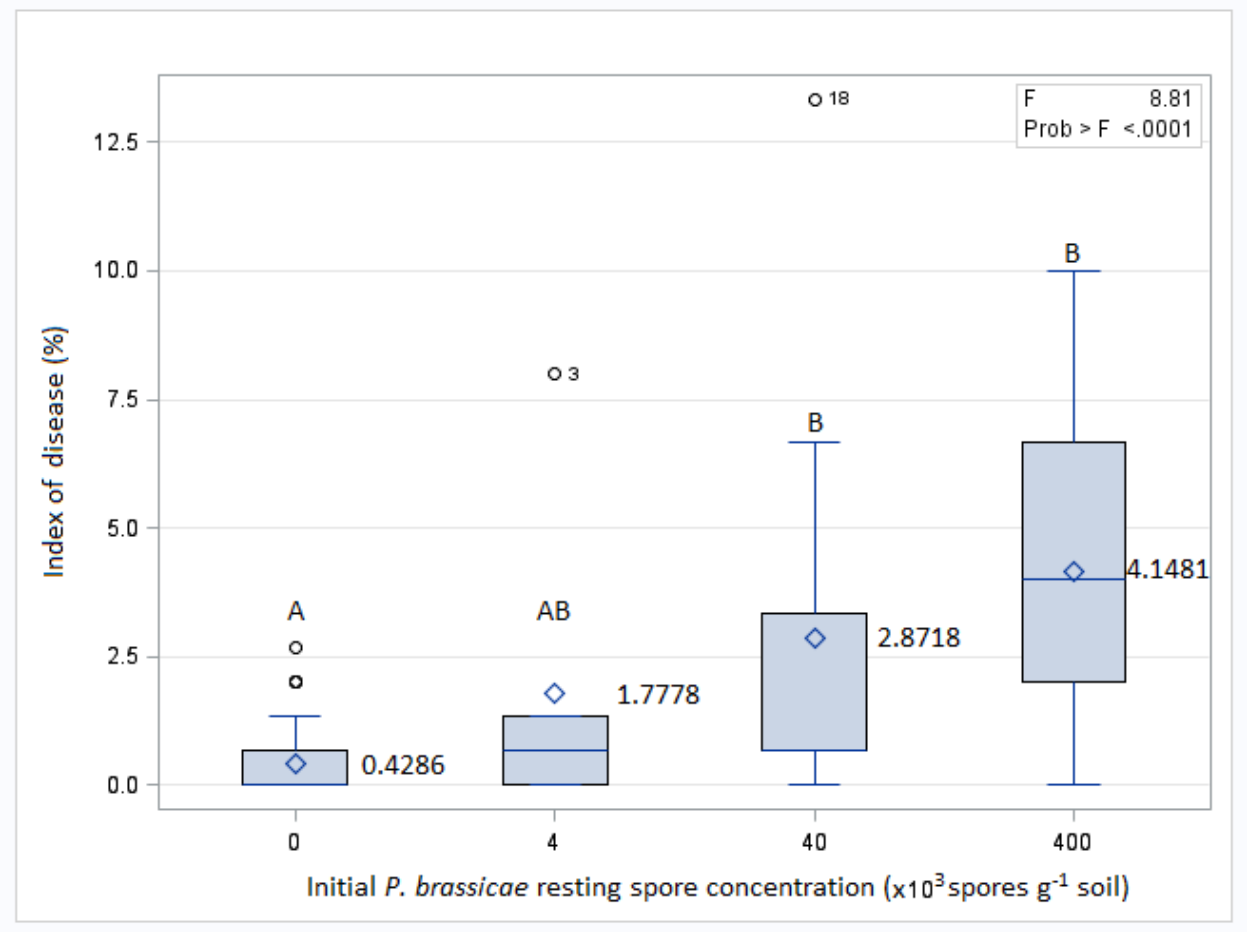


Figure 2-6. Index of disease (ID, %) in clubroot resistant canola plants sampled from soil in which the 'initial *P. brassicae* resting spore concentration' was classified into one of four categories, where: 0 = undetected, 4 x 10^3 = detected but unquantifiable (< 4.0 x 10^3 spores g^{-1} soil), 40 x 10^3 = ≥ 4.0 x 10^3 spores g^{-1} soil - < 4.0 x 10^4 spores g^{-1} soil, and 400 x 10^3 = ≥ 4.0 x 10^4 spores g^{-1} soil. The blue box represents the interquartile range (25th and 75th percentile), ◇ represents the group mean, the horizontal line in the box represents the group median, and bars represent the group minimum and maximum values. The symbols ○, ○^{3}, and ○^{18}, represent extreme values. Letters represent significantly different ID between groups ($p < 0.05$, adjusted for multiple comparisons). Resting spore concentrations and corresponding IDs were based on 70 samples collected throughout 2010-2013 (only when CR canola was cultivated) from 35 GPS marked locations in 9 different fields.

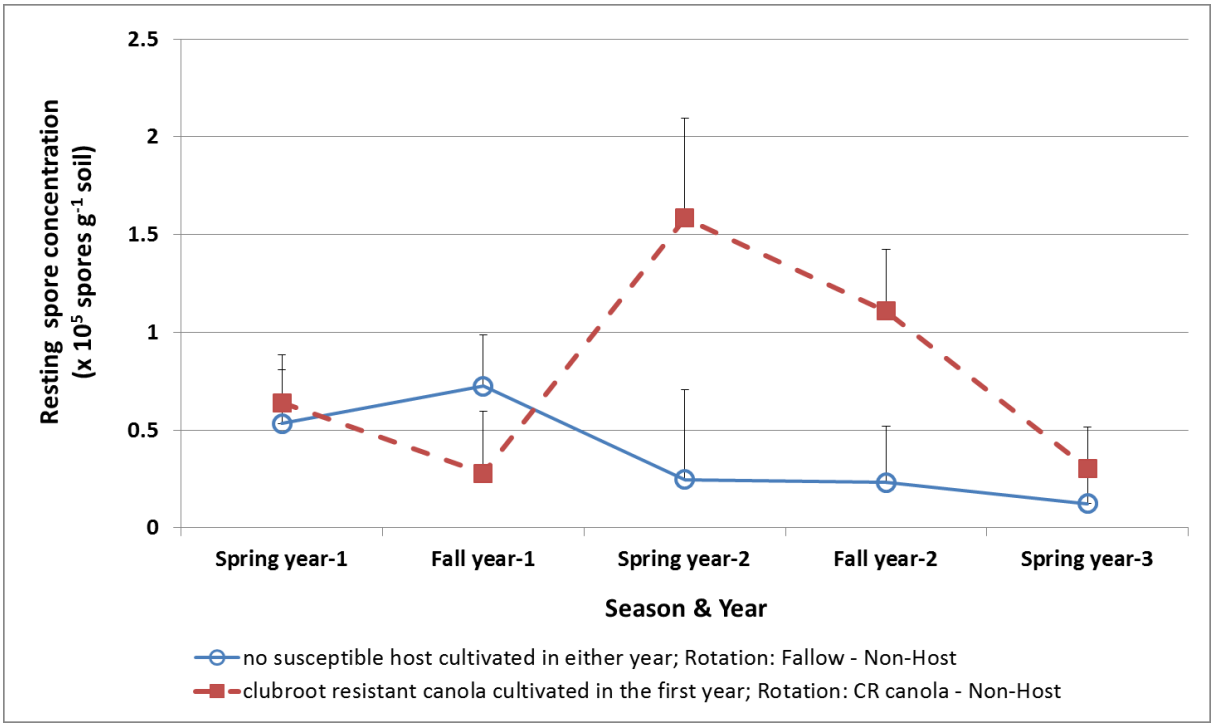


Figure 2-7. Concentration of *Plasmodiophora brassicae* resting spores in the soil of fields with a rotation that includes clubroot resistant (CR) canola every second year (1-in-2 year rotation). Over a two year period, CR canola was cultivated only once in the first year of the two year rotation. In the control plots, no susceptible host was grown over the duration of the trial. Soil sampling was conducted over 5 periods: Spring of year-1, Fall of year-1, Spring of year-2, Fall of year-2, Spring of year-3. Mean resting spore concentrations are presented, and bars indicate standard error. Samples were collected from each of the 5 consecutive time periods at 22 different GPS marked locations (experimental units) from 8 different fields. The actual year represented by 'year-1' varied by field, depending on when CR canola was cultivated between the years 2010-2013.

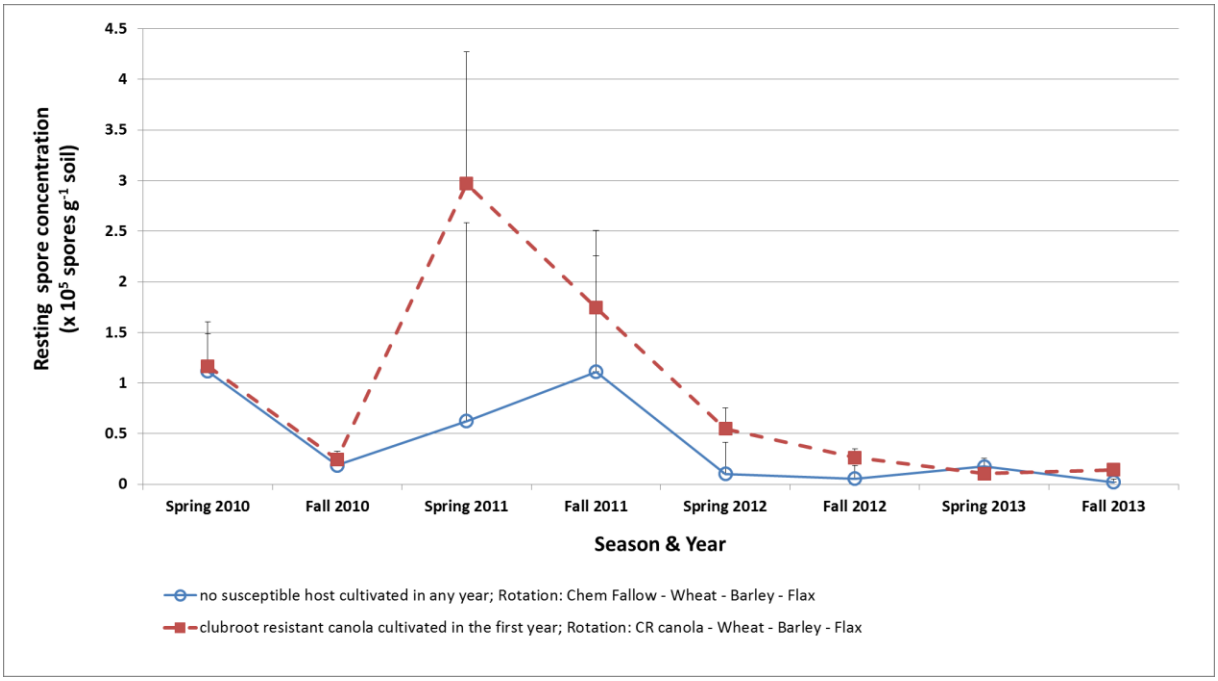


Figure 2-8. Concentration of *Plasmodiophora brassicae* resting spores in the soil in a 1-in-4 year clubroot resistant (CR) canola rotation. Over a four year period, CR canola was cultivated only once in the first year. In the control plots, no susceptible host was planted at any point over the duration of the study. Soil sampling was conducted in the spring and fall of each year from 2010-2013. Mean resting spore concentrations are presented, and bars indicate standard error. Samples were collected at all 8 time periods (spring and fall 2010-2013) from 9 different GPS marked locations (experimental units) within 2 fields.

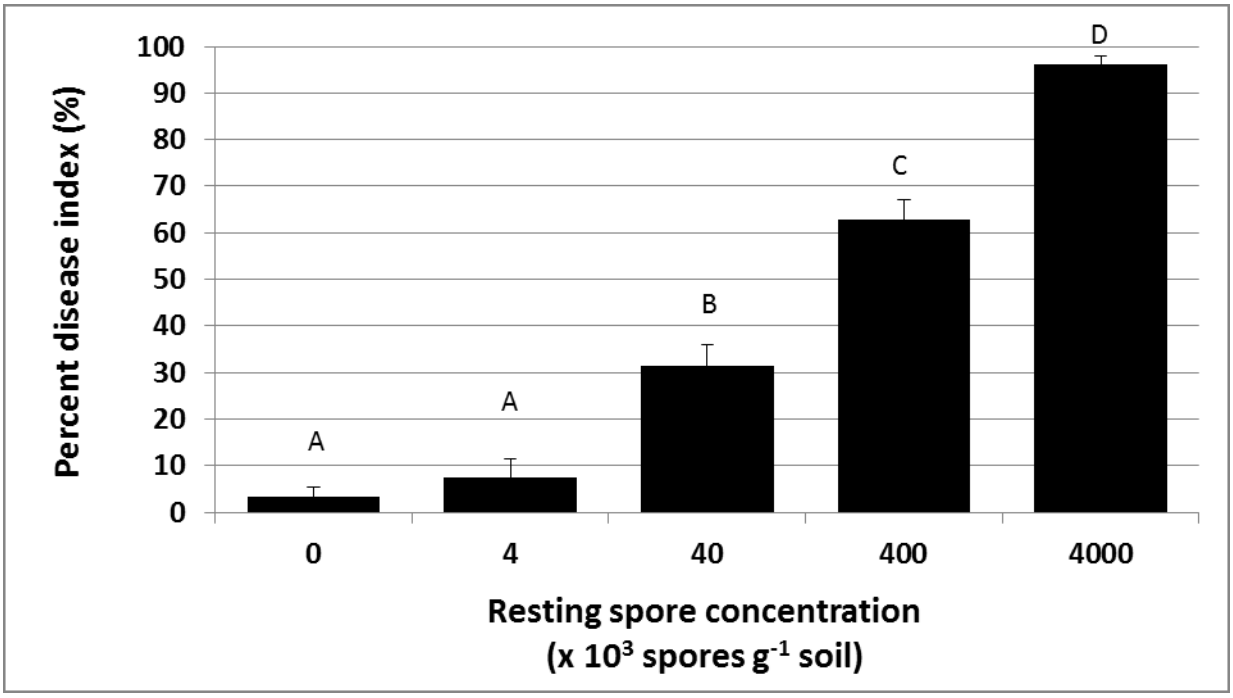


Figure 2-9. Relationship between *Plasmodiophora brassicae* resting spore concentration in infested soil as determined by quantitative PCR (qPCR) and index of disease (ID) severity on the susceptible *Brassica napus* cv. Granaat in greenhouse bioassays ($F_{(4,159)}$=36.98, p<0.0001). Soil samples were grouped into five categories based on initial *P. brassicae* resting spore concentration, where: 0 = pathogen not detected, 4 x 10^3 = detected but not quantifiable (< 4.0 x 10^3 spores g^{-1} soil), 40 x 10^3 = ≥ 4.0 x 10^3 spores g^{-1} soil - < 4.0 x 10^4 spores g^{-1} soil, 400 x 10^3 = ≥ 4.0 x 10^4 spores g^{-1} soil - < 4.0 x 10^5 spores g^{-1} soil, and 4000 x 10^3 = ≥ 4.0 x 10^5 spores g^{-1} soil. Letters represent significantly different ID between groups (p < 0.05, adjusted for multiple comparisons). Bars represent standard error. A total of 141 homogenized soil samples with detectable levels of *P. brassicae* DNA (111 of which had quantifiable levels) and an additional 23 homogenized soil samples with no detectable *P. brassicae* were included in the analysis. Samples were randomly selected for each category from all fields (where at least one sample location tested positive for *P. brassicae* DNA) and from all seasons and years (spring/fall, 2010-2013).

Chapter 3

3. General Conclusions and Recommendations

3.1. The Selection of Appropriate Management Strategies

An integrated pest management (IPM) strategy should focus on the long-term management of *Plasmodiophora brassicae* infestations to maximize economic benefit using any/all available methods, while at the same time minimizing risks to human health, beneficial and non-target organisms, and the environment. Selecting the most appropriate choice of management strategies requires an initial assessment of risks. In the case of clubroot in canola, some of the major risks include losses in crop yield and quality, potential spread/propagation of *P. brassicae* inoculum, and even the breakdown of genetic resistance in host cultivars. The costs associated with various management tools must also be taken into account. In general, the planting of certified hybrid canola seed is warranted, since the latest canola traits can add value for producers (Malla and Brewin, 2015). Disease resistance traits, such as clubroot resistance (CR), are also economically justified in *P. brassicae* infested fields. The planting of canola cultivars with CR results in lower infection, greater yields, and reduced propagation of pathogen inoculum (Hwang et al., 2011b, Hwang et al., 2014, Peng et al., 2014, Rahman et al., 2014). The use of certified clean seed also mitigates the possibility of spread of *P. brassicae* as a seedborne contaminant (Rennie et al., 2011), and reduces the likelihood of susceptible volunteers or genetic off-types being planted in *P. brassicae* infested fields (Canadian Seed Growers' Association, 2005).

Numerous management strategies have been evaluated for the control of clubroot in canola, including the application of chemical fungicides and soil amendments. However, the costs associated with chemical management of *P. brassicae* may be too great for the widespread use of this strategy. The cost to purchase and apply pesticides over the large acreages associated with canola cropping systems would be prohibitively expensive and require large amounts of water (Peng et al., 2014). Also, the potential risk of harmful effects towards beneficial soil microflora, humans, and the crop itself would restrict their use (Hildebrand and McRae, 1998, Hwang et al., 2014, Peng et al., 2014). Nonetheless, the application of selected pesticides may be warranted for isolated spot treatments (e.g., spot treatment of *P. brassicae* infestations with Vapam (dithiocarbamate; sodium N-methyldithiocarbamate) under plastic covers to prevent evaporative air pollution) (Zuzak, 2016, Unpublished data).

Cruciferous bait crops including canola and Chinese cabbage, which can induce *P. brassicae* resting spore germination and thereby deplete soil inoculum levels, have shown promise as clubroot management tools in greenhouse bioassays (Ahmed et al., 2011). Similarly, other CR cruciferous crops (e.g., Japanese radish (Ikegami, 1985), toria sarson (Bhattacharya and Dixon, 2010)) have been effective at reducing the concentration of *P. brassicae* resting spores in infested fields. However, under field conditions in Alberta, the planting of bait crops (including canola and Chinese cabbage) resulted in only a slight decrease in *P. brassicae* resting spore concentration, and no change in the disease severity of canola plants grown in the subsequent year (Ahmed et al., 2011). The current study corroborates the results of Ahmed et al. (2011). In commercial fields, CR canola failed to reduce *P. brassicae* resting spore

concentration in the soil any more than control plots denuded of plants (Chapter 2). As such, CR canola does not appear to function as a bait crop in Alberta.

Another strategy evaluated for the management of clubroot has been crop rotation. Crop rotation is regularly used to manage other agricultural plant pathogens (e.g., *Leptosphaeria maculans* (Guo et al., 2005), *Sclerotinia sclerotiorum* (Koch et al., 2007)). However, the longevity of *P. brassicae* resting spores in the soil (Wallenhammar, 1996) makes crop rotation less effective as a management tool for this pathogen. A crop rotation of CR canola grown 1-in-4 years has been recommended for infested fields, but is rarely implemented due to the high value of canola compared with other crops (Rempel et al., 2014, Strelkov and Hwang, 2014). It is currently more profitable for producers to maintain a short-duration canola rotation with disease resistant cultivars, given the high price of canola (Smith et al., 2013). Peng et al. (2015) recently demonstrated the benefits of an extended rotation away from CR canola in infested fields. A ≥2-year break from CR canola cultivation was found to increase subsequent CR canola yields 32-76% and also significantly reduced soil inoculum loads (i.e., from ~2.7 - 2.9 x 10^6 spores g^{-1} soil to ~ 5.7 x 10^4 - 2.1 x 10^5 spores g^{-1} soil) (Peng et al., 2015). The current study corroborates these results, at least with respect to *P. brassicae* resting spore dynamics, and supports a ≥2-year break from CR canola in *P. brassicae* infested fields. Indeed, high inoculum loads were depleted relatively quickly when non-host cultivars were grown for at least 2-years (i.e., from 1.17 - 2.97 x 10^5 spores g^{-1} soil to 1.45 x 10^4 spores g^{-1} soil) (Chapter 2). In both studies (this thesis and Peng et al. 2015), there appeared to be an initial steep decline in resting spore concentration associated with the cultivation of non-hosts. It is possible that a large proportion of immature resting spores (or resting spores that are otherwise less fit) decay

or germinate within 2-years, leaving only the more durable resting spores, those capable of inducing infection at least 17.3-years as suggested by Wallenhammar (1996). Longer rotations have the additional advantage of mitigating the risk of shifts in *P. brassicae* populations towards more virulent pathotypes (LeBoldus et al., 2012, Hwang et al., 2014, Strelkov and Hwang, 2014, Strelkov et al., 2016).

3.2. Threshold *P. brassicae* Resting Spore Concentration and Risk Assessment

In many cases, CR canola is the only tool used to manage *P. brassicae* infestations. In heavily infested fields (> 1.0 x 10^6 spores g^{-1} soil), CR canola enables the production of this crop with very low infection and yield loss while minimizing further pathogen propagation (Hwang et al., 2012b). As such, genetic resistance is widely implemented as a management tool for clubroot, although there may be risks associated with its use in these cases. Peng et al. (2015) reported that under a short canola rotation (0 - 1 year without a susceptible host), yield was lower (1740 - 2080 kg ha^{-1}) and *P. brassicae* resting spore concentration was higher (2.7 - 2.9 x 10^6 spores g^{-1} soil) than under a longer rotation (≥2-years without a susceptible host) (yield = 2290 - 2390 kg ha^{-1}, resting spore concentration = 5.7 x 10^4 - 2.1 x 10^5 spores g^{-1} soil). Wallenhammar et al. (2012) recommended refraining from planting even CR *Brassica* crops at resting spore concentrations > 1.3 x 10^5 spores g^{-1} soil, as the risk of pathogen propagation becomes too great at this point. Based on the model developed in the current study (Chapter 2), an initial resting spore concentration of 1.0 x 10^5 spores g^{-1} soil can result in a concentration of approximately 2.6 x 10^5 spores g^{-1} soil after the cultivation of CR canola. This is still near the range of resting spore concentrations (5.7 x 10^4 - 2.1 x 10^5 spores g^{-1} soil) resulting in greater

yields (2290 - 2390 kg ha^{-1}) following a ≥2-year break from *P. brassicae* hosts (Peng et al., 2015). This value is also within the range recommended by Wallenhammar et al. (2012) at which it is safe to grow CR *Brassica* crops (i.e., 3.0 x 10^3 spores g^{-1} soil to 1.3 x 10^5 spores g^{-1} soil). Thus, it may be reasonable to recommend that producers refrain from cultivating any *Brassica* host (including CR canola) on infested soils when resting spore concentrations are > 1.0 x 10^5 spores g^{-1} soil. Even in heavily infested fields (> 1.0 x 10^6 spores g^{-1} soil), avoiding *P. brassicae* hosts for 2-years can reduce resting spore concentrations to these levels (i.e., 5.7 x 10^4 - 2.1 x 10^5 spores g^{-1} soil) (Peng et al., 2015), suggesting that such a recommendation would not be so difficult for farmers to accept.

Risk assessment with respect to *P. brassicae* infestations requires an accurate identification of the pathogen and a reliable measure of its concentration. PCR-based methods (Cao et al., 2007, Rennie et al., 2011) are effective tools for these purposes, but the timing of their use also can have an impact on the results obtained. In the current study, *P. brassicae* resting spore concentration increased following the cultivation of CR canola, but peak levels were not observed until the following year (Chapter 2). There is a notable lag in the release of resting spores, likely because infected root material needs time to decompose and for the spores to be freed from the host tissues. Any future soil-based risk assessments developed to aid in making decisions regarding the cultivation of CR canola, or to determine if spot treatment of *P. brassicae* infestations with fungicides is warranted, would have to account for such a lag.

3.3. Concluding Remarks

CR canola represents an important clubroot management tool, but one which must be used responsibly. Resistant cultivars of this crop do not appear to function as effective bait crops, given that resting spore concentrations in fields sown to CR canola did not differ significantly from fallow control treatments. Moreover, spore concentration appeared to increase by sampling period with the cultivation of CR canola, while the spore concentration remained constant or decreased slightly in the controls. These results suggest that the cultivation of CR canola should be managed carefully, in order to avoid increases in pathogen inoculum levels and selection pressure for more virulent pathotypes. Based on the current study, it may be advisable for producers to refrain from planting CR canola (or any CR *Brassica*) until the *P. brassicae* inoculum concentration in a field declines below 1.0×10^5 spores g^{-1} soil. A rotation out of a *Brassica* host for 2-years could be sufficient to reduce spore loads below this threshold. Thus, producers and crop advisors should consider carefully whether or not the planting of a CR canola cultivar is warranted, or if it could do more harm than good to long-term clubroot management efforts.

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Appendix

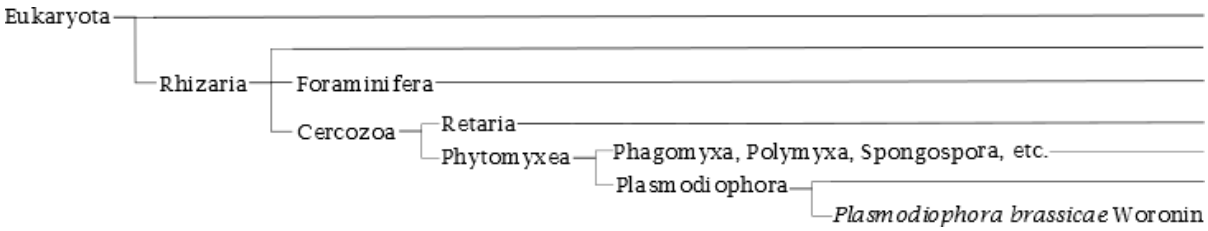

Figure A-1. Taxonomic classification of *Plasmodiophora brassicae*. *P. brassicae* is a Eukaryote within the Rhizaria, based on ribosomal DNA marker analysis, and is placed with other filose testate amoeba within the Cercozoa. The pathogen belongs to a group of Protist Cercozoan parasites of plants known as the Phytomyxea. *P. brassicae* is within the monophyletic clade known as Plasmodiophora comprised of plasmodial endoparasites of plants.

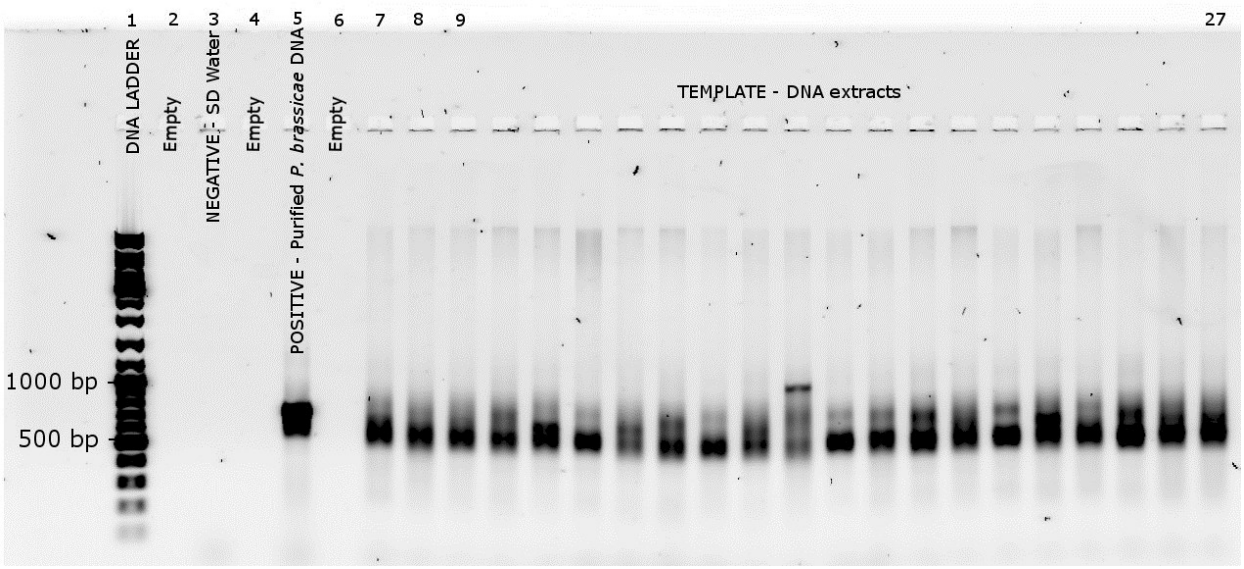

Figure A-2. Example of a 1% agarose gel stained with 1X 'SYBR safe DNA gel stain' (Invitrogen™) used to visualize polymerase chain reaction (PCR) products amplified using the primers ITS1 and ITS4 (Korabecna, 2007). A subsample of all DNA extracted from soil samples collected between 2010-2013 across Alberta were amplified with ITS1 and ITS4 primers based on the methods outlined in section 2.2.4. Lane 1: DNA ladder (GeneRuler™ 1 kb Plus DNA Ladder, Fermentas Life Sciences), Lane 3: Negative control (sterile distilled H_2O), Lane 5: Positive control (purified *P. brassicae* SACAN-SS2, extracted DNA), Lanes 7 - 27: template DNA, Lanes 2, 4 and 6: Empty.

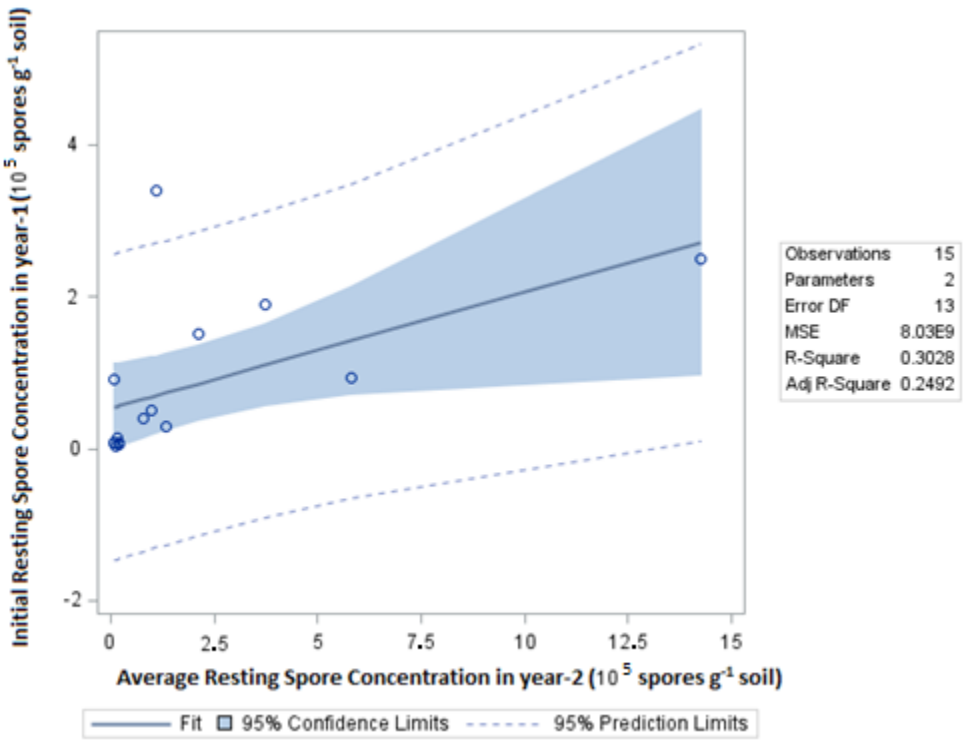

Figure A-3. Graphical representation of the linear relationship between initial *Plasmodiophora brassicae* resting spore concentration in the soil, and the average resting spore concentration observed in the following year. Average resting spore concentration observed in year-2, followed the cultivation of clubroot resistant canola in year-1. Data collected over four years (2010-2013) were used to generate these models. Fit (——): represents the best fit linear regression ($F_{(1,13)}$=5.65, p=0.0335, adj-R^2=0.2492).