The Evaluation of Lime Products as a Clubroot (*Plasmodiophora brassicae*) Management Tool

by

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Abstract

Clubroot, caused by *Plasmodiophora brassicae* Wor., is a soil-borne disease that has become a constraint to canola (*Brassica napus* L.) production in Alberta, Canada. The disease is managed primarily by the planting of clubroot resistant cultivars, but resistance has been overcome in close to 200 fields in the province. Clubroot development is favoured in acidic soils; therefore, increasing soil pH may reduce disease severity in infested soils and serve as another management tool. The efficacy of hydrated lime products in reducing clubroot severity was assessed in replicated field plot experiments in central Alberta in 2017 and 2018. In 2017, the application of moderate to high rates of hydrated lime significantly reduced clubroot severity and increased above-ground biomass in a susceptible canola cultivar 8 weeks after planting. At the highest application rate of 11.4 T ha^{-1} and 12.7 T ha^{-1}, lime treatment reduced the clubroot disease severity index by 35-91%, while increasing above-ground plant biomass by 58-116%. In contrast, no effect of lime treatment was observed in the 2018 field trials, possibly due to a longer interval between lime application and sowing, as well as reduced rainfall received during this time. A greenhouse study also was conducted to compare the efficacy of hydrated lime and limestone in reducing clubroot severity in susceptible and moderately resistant canola cultivars, at different application rates and inoculum concentrations. In the control treatments, indices of disease severity were very high (92-100%) in the susceptible canola and low (9-13%) in the moderately resistant canola. The application of hydrated lime at 4.7, 8.1, 11.4, and 14.8 T ha^{-1} completely eliminated visible clubroot symptoms in both cultivars, whereas limestone decreased disease severity only at the two lowest inoculum concentrations. Other parameters including plant height and root and shoot weight fluctuated closely with the level of disease control. Root tissues from the greenhouse study were analyzed by quantitative-PCR (q-PCR) to measure *P.*

brassicae proliferation *in planta*. Inoculum concentration and the type and rate of lime product applied significantly affected the amount of *P. brassicae* DNA in the root tissue. Pathogen DNA could not be detected in 10-day-old seedlings following the application of hydrated lime, but the rate required to prevent root colonization increased with increasing inoculum concentration. Limestone application also appeared to provide some control, but *P. brassicae* DNA still was detectable in the host roots. Repeated trials with less virulent inoculum revealed similar trends, suggesting that limestone could be applied in soils with lower inoculum concentrations to reduce clubroot severity and root infection. Based on the greenhouse and q-PCR results, hydrated lime appears to be more effective than limestone for clubroot control, but the latter was not evaluated under field conditions. Nonetheless, hydrated lime may represent an effective tool to manage *P. brassicae* in highly infested patches in a field, at field entrances and in acidic soils, by reducing clubroot severity on susceptible and resistant hosts. As such, the application of lime may help to supplement the use of genetic resistance, by reducing disease pressure and the potential for pathotype shifts.

Preface

This thesis is an original work by Nicole Marie Fox. I conducted and analyzed all of the experiments with assistance and training by others. A special thank you goes out to Victor Manolii for helping me with the setup of my greenhouse and field trials, sample collection and disease ratings, to Tiesen Cao and Ileana Strelkov for teaching me the molecular protocols in the lab, and to the summer students and staff at the University of Alberta and Crop Diversification Centre North (Alberta Agriculture and Forestry) in sample collection, washing roots and harvesting plants. While some abstracts related to this work have been presented at various scientific and industry meetings, none of the chapters are yet published. The first draft to all chapters within this thesis were written by myself, and then reviewed by Drs. Stephen Strelkov and Sheau-Fang Hwang who suggested revisions.

Dedication

I would like to dedicate my thesis and determination in academia to my parents. Mom and Dad, you have been an amazing support system and my biggest fans amongst my minor and major achievements in life, for that I am forever grateful. A special thank you goes to the rest of my family and friends for your support, interest and always cheering me on.

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Chapter 1: Introduction and Literature Review

1.1 General Introduction

1.1.1 Clubroot background

Clubroot is a soil-borne disease of cruciferous plants worldwide. The clubroot pathogen (*Plasmodiophora brassicae*, Woronin) causes hyperplasia and hypertrophy of the host root tissue, resulting in club-shaped galls of various sizes on infected tap and lateral roots. These malformations interfere with the ability of plants to absorb nutrients and water from the soil, leading to above-ground symptoms such as wilting, stunting, and premature ripening. In canola (*Brassica napus* L.), significant reductions in seed yield and oil content may occur when symptoms are severe (Pageau *et al*., 2006; Cao *et al*., 2009). Worldwide, crop losses from clubroot are reported to be at least 10–15% (Dixon, 2006). The theory of a Mediterranean centre of diversity for *P. brassicae* has been difficult to validate (Dixon, 2009a). The first historical reports of the pathogen date back to the 13th century in Europe, and its migration always seems to be associated with agriculture (Dixon, 2009a). This suggests that clubroot is a 'disease of cultivation' and becomes established where its hosts are mass produced (Feng *et al*., 2014).

1.1.2 Clubroot in Alberta, Canada

In Canada, clubroot was historically found on cruciferous vegetables from Ontario to the Maritimes, as well as in British Columbia (Howard *et al*., 2010). The disease was not identified on canola in the Prairie Provinces until 2003, when 12 infested fields were found near Edmonton, Alberta (Tewari *et al*., 2005). The number of confirmed field infestations has increased quite rapidly since then. By 2009-2010, when clubroot resistant (CR) canola cultivars first became available to producers, the number of known infested fields was 566 (Strelkov *et al*., 2011). As of 2018, 3,044 fields had been identified as clubroot-infested in Alberta (Strelkov *et*

al., 2019). Clubroot management has been made more complicated by the emergence, beginning in 2013, of new strains of *P. brassicae* that can overcome the resistance in CR canola (Strelkov *et al*., 2016; Strelkov *et al*., 2018). At present, there are about 170 fields in Alberta where the effectiveness of resistance has been reduced or completely overcome (S. E. Strelkov, *personal communication*). As the struggle to control this pathogen continues, extensive research has been conducted to identify additional management methods.

1.2 Biology of *Plasmodiophora brassicae*

1.2.1 Life cycle

Plasmodiophora brassicae is a protist belonging to the Class Phytomyxea (Hirani and Li, 2015). This parasite is placed in the Order Plasmodiophorales, which contains only obligate parasites (Ingram and Tommerup, 1972; Braselton, 1995). The Genus *Plasmodiophora* exhibits unique features such a cruciform nuclear division, parasitism and biflagellate zoospores with uneven anterior flagella (Braselton, 1995). Along with these key features, *P. brassicae* produces long-lived resting spores that possess a high degree of resistance to degradation by the environment (Braselton, 1995).

The life cycle of *P. brassicae* is monocyclic and can be summarized in three key stages: resting spores, primary (root hair) infection and secondary (cortical) infection (Ayers, 1944; Ingram and Tommerup 1972; Naiki *et al*., 1987). The resting spores of *P. brassicae* are highly resistant due to a complex layer of five spore walls composed of chitin and carbohydrates (Moxham and Buczaki, 1983). The durability of these spores allows them to remain dormant for at least 20 years in the absence of a host, with an estimated half-life of 3.6 to 4.4 years (Wallenhammer, 1996; Hwang *et al*., 2013). The optimal conditions for resting spore germination include soil temperatures between 20-25°C with a high degree of free moisture

(Ikegami *et al*., 1981). Resting spore germination is affected by spore maturity and environmental factors including pH, humidity, temperature, inorganic ions and biological factors (Takahashi, 1994; Friberg *et al*., 2005). The germination of resting spores is reportedly triggered by the release of calcium ions, and by host and nonhost root exudates (Yano *et al*., 1991; reviewed in Kageyama and Asano, 2009). Upon germination, the resting spores release a delicate, single-walled zoospore (primary zoospore) equipped with two flagella of unequal lengths, which swim through the free water in the soil towards susceptible roots (Dixon, 2009b). These primary zoospores can measure from 2.8 to 5.9 µm in diameter (Ayers, 1994). Unlike the resting spores, the zoospores are not resilient and will die in the absence of a host.

Once the motile primary zoospores find a host plant, they infect the root hairs and epidermal root cells (Ayers, 1944). Upon successful entry, each primary zoospore develops into a plasmodium and then into a zoosporangial cluster (Ayers, 1944). The primary infection stage does not cause any macroscopic symptoms or yield loss (Howard *et al*., 2010). The contents of the zoosporangium divide and 4 to 16 secondary zoospores are discharged into the rhizosphere (Tommerup and Ingram, 1971). The secondary zoospores re-infect the host roots, entering the cortical infection stage of the *P. brassicae* life cycle. Invasion by the secondary zoospores is followed by the formation of secondary plasmodia; it is at this time that the typical macroscopic, clubbed root symptoms begin to develop (Kageyama and Asano, 2009). Eventually, the secondary plasmodia cleave to produce new resting spores, which are released back into the soil as the clubs decompose (Kageyama and Asano, 2009). These resting spores serve as the inoculum for subsequent infections in the following years.

1.2.2 Host range

Host crops

P. brassicae is believed to infect all members of the Family Brassicaceae, which contains 330 genera and 3,700 species of potential hosts (Dixon, 2009a). As a 'disease of cultivation', cultivated crops appear to be notably susceptible, including *B. napus*, *Brassica oleracea* L. and *Brassica rapa* L. (Dixon, 2009a). Cruciferous weeds including, stinkweed (*Thlaspi arvense* L.), and shepherd's purse (*Capsella bursa-pastoris* (L.) Medik) also are susceptible to the disease (Buczacki and Ockendon, 1979). The model plant *Arabidopsis thaliana* (L.) Heynh also serves as a host, which has facilitated some research activities into clubroot development (Dixon, 2009a).

Non-host crops and resting spore germination

Interestingly, in some non-hosts of *P. brassicae*, primary infection by the pathogen can occur, but secondary (cortical) infection is observed only rarely, and even when it is, no resting spores are produced (McFarlane, 1952; Ludwig-Müller *et al*., 1999). These non-host species assumedly release root exudates similar to host species, thereby stimulating resting spore germination (Friberg *et al*., 2006). However, since the germinated spores cannot complete the infection cycle on the non-host, the resting spore bank in the soil is depleted without the formation of new resting spores (McFarlane, 1952; Ludwig-Müller *et al*., 1999). McFarlane (1952) observed primary infection in the non-hosts *Lolium perenne* L. (perennial ryegrass), *Reseda odorata* L. and *Tropaeolum majus* L. with no evidence of secondary infection. However, in another study secondary infection was observed in a cultivar of perennial ryegrass, and the secondary zoospores produced on that cultivar of ryegrass were pathogenic to other host and

non-host root hairs (Feng *et al*., 2012). It was hypothesized that since the ryegrass plants used in Feng *et al*. (2012) study were inoculated with secondary zoospores, *P. brassicae* was able to bypass the primary infection stage, which is believed to induce host resistance to the secondary infection stage. Indeed, the ryegrass itself did not show any evidence of the development of galls or resting spores (Feng *et al*., 2014). Other non-host species that showed evidence of secondary infection include *T. majus* L*., Carica papaya* L., *R. alba* L. and *Beta vulgaris* L. (Ludwig-Müller *et al*., 1999).

1.2.3 Pathotypes of *P. brassicae* in Canada

Physiologic specialization is known to occur in *P. brassicae*, meaning that there are multiple, morphologically identical strains of the pathogen that differ in their ability to infect different host genotypes. In the clubroot system, these strains are often referred to as 'races' or 'pathotypes', which are defined by their virulence on a host differential set. A number of differential sets have been developed to identify pathotypes of *P. brassicae*. The best known of these include the differentials of Williams (1966), Somé *et al*. (1996) and the European Clubroot Differential (ECD) (Buczacki *et al*., 1975). The hosts of Williams (1966) consist of two genotypes of *B. napus* var. *rapifera* and two *B. oleracea* var. *capitate*, while those of Somé *et al*. (1996) consist of three genotypes of *B. napus*. The ECD set is comprised of three subsets consisting of five genotypes each of *B. rapa, B. napus*, and *B. oleracea* (Buczacki *et al*., 1975). While these differentials, especially those of Williams (1966), have been used extensively to identify pathotypes of *P. brassicae* in Canada, they have some limitations. In particular, they could not distinguish between strains of the pathogen that can overcome the resistance in CR canola. As such, a new differential system, termed the Canadian Clubroot Differential (CCD) set (Strelkov *et al*., 2018), was established recently.

The CCD set consists of 13 hosts including those of Williams (1966) and Somé *et al*. (1996), selected hosts of the ECD, plus several *B. napus* cultivars relevant to canola breeding and/or production (Strelkov *et al*., 2018). The CCD set has a greater differentiating capacity than the earlier differentials, and allows identification of pathotypes that can overcome resistance in *B. napus*. Since the hosts of Williams and Some *et al*. also are included, testing of isolates of *P. brassicae* on the CCD set also permits classification according to those systems, providing continuity. Unique virulence patterns on the differentials represent distinct pathotypes, and are assigned a letter for identification (A, B, C, etc.). Since a pathotype designation according to Williams (1966) also is obtained (2, 3, 5, etc.), the pathotype designations from both systems can be combined. For example, pathotype A on the CCD set is a variant of pathotype 3 on the differentials of Williams (Strelkov *et al*., 2018), and hence can be referred to as pathotype 3A. This is the most common of the 'new', resistance-overcoming pathotypes of *P. brassicae*. A clear understanding of the pathotype composition of the clubroot pathogen is important in helping to guide resistance-breeding activities (Strelkov and Hwang, 2014).

1.3 Detection and quantification of *P. brassicae*

1.3.1 Bioassays

As an obligate parasite, *P. brassicae* cannot be cultured. Therefore, other methods have been used to detect and quantify the presence of the pathogen in soil, water and plant tissue. The oldest of these methods involves the use of bioassays, in which susceptible bait plants are grown in test soil to determine the presence or absence of viable *P. brassicae* inoculum. An ideal host for use in bioassays is the Chinese cabbage (*Brassica rapa* var. *pekinensis*) cv. 'Granaat', since it is susceptible to all pathotypes of *P. brassicae* (Strelkov *et al*., 2018). Seeds or small seedlings are planted in the test soil and grown under greenhouse conditions for 6-8 weeks in order to

provide enough time for symptom development. At this point, the plants are carefully harvested and the roots washed and evaluated for clubroot development (Faggian and Strelkov, 2009). The presence of symptoms indicates the presence of viable inoculum (resting spores). The amount of inoculum in the test soil also can be estimated, by comparison with clubroot development on plants grown in similar but 'clean' soil inoculated with known amounts of *P. brassicae* resting spores (Melville and Hawken, 1967).

Although reliable, bioassays require a significant investment of time, space and materials; therefore, they are not ideal for the evaluation of large numbers of samples (Cao *et al*., 2007). Soil samples require a minimum of 1,000 spores per gram of soil (depending on soil type and environmental conditions) in order for clubroot symptoms to develop and be visible to the naked eye, and as such bioassays may not detect low levels of infestation (Faggian and Strelkov, 2009). Moreover, the amount of soil sample available can be limited and not sufficient for a bioassay, and bioassays are not suitable for testing of plant tissue samples (which requires microscopy, as discussed below).

1.3.2 Microscopy and fluorescent microscopy

Detection of *P. brassicae* may also be carried out by examining root tissues under the microscope for signs of *P. brassicae* infection (MacFarlane, 1952). Observations of advanced infections of the root cortex may be observed in stained free-hand sections, but reliable confirmation of root hair infection may require more careful preparation of samples. For example, the root samples can be subjected to a series of ethanol washes and fixed in wax so that cross sections can be taken (reviewed in Becnel, 1997). The root cross sections are then stained with hematoxylin and eosin before they can be examined under the microscope for the presence or absence of *P. brassicae* plasmodia (reviewed in Becnel, 1997). In addition, tissue samples and

solutions may be examined for the presence of resting spores by fluorescence microscopy, subject to staining with the appropriate fluorochromes (Takahashi *et al*., 1988; Takahashi *et al*., 1989). This approach improves the ability to detect spores and can be adapted to determine spore viability by differential staining in viable spores fluoresce blue and nonviable spores fluoresce red (Faggian and Strelkov, 2009). Nonetheless, although they can be reliable, microscopy based methods for detection of *P. brassicae* can be time-consuming and costly, require specific equipment and user skills, and introduce a high level of risk for human error (Cao *et al*., 2007; Faggian and Strelkov, 2009).

1.3.4 Polymerase chain reaction (PCR) and quantitative PCR (qPCR)

Detection techniques based on the polymerase chain reaction (PCR), which selectively amplify target DNA, are fast, sensitive and can produce highly reliable results (Cao *et al*., 2007). The first protocols for PCR-based detection of *P. brassicae* were published in the late 1990s. Ito *et al*. (1999) developed a nested PCR assay based on amplification of a pentyltransferase gene from the pathogen. Other protocols have targeted the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) (Faggian *et al*., 1999; Wallenhammer and Arwidsoon, 2001; Cao *et al*., 2007). The method of Cao *et al*. (2007), which represented the first single-tube, non-nested PCR assay for detection of *P. brassicae*, has served as the foundation for much of the molecular testing carried out in Canada. This method amplifies a fragment of the 18S ITS 1 region of the rDNA repeat, is highly specific and can consistently detect a few as 1×10^3 resting spores per gram of soil (Cao *et al*., 2007). Nonetheless, these earlier PCR-based protocols were not quantitative, and as such could not be used to measure inoculum levels in soil or plant tissue.

Rennie *et al*. (2011) developed one of the first quantitative PCR (qPCR)-based assays for measuring *P. brassicae*. This was an intercalating dye-based (SYBR Green) protocol initially

used to quantify the presence of resting spores present in dust and soil. Additional protocols have followed (e.g., Sundelin *et al*., 2010; Cao *et al*., 2014). For example, the qPCR method of Cao *et al*. (2014) uses a hydrolysis probe and has been applied to the measurement of *P. brassicae* in infected root tissues. One concern associated with qPCR-based approaches to measuring the clubroot pathogen is their potential inability to distinguish between viable and non-viable (dead) resting spores, since these are based on detection of DNA. Al-Daoud *et al*. (2017) recently published a qPCR protocol that includes the addition of propidium monoazide (PMA), a chemical that prevents amplification of DNA from non-viable resting spores. This PMA-PCR analysis further improved the quantification of viable *P. brassicae* resting spores.

While molecular methods have allowed for faster, more reliable detection and quantification of *P. brassicae* in soil and plant material, work continues in this area, making use of novel and emerging technologies such as whole-genome sequencing.

1.4 Clubroot management strategies in Canada

Clubroot management can be based on cultural, biological and chemical control strategies, as well as on the deployment of genetic resistance. A single or combination of strategies in these categories may be employed to control the disease. An integrated crop management plan is recommended, since clubroot development can be highly dependent on environmental conditions and other factors. Indeed, when conditions are favorable for disease development and when inoculum levels are high, clubroot may still be severe despite management attempts by the grower. In the next section of this chapter, various existing and potential clubroot management options will be discussed. Throughout this discussion, it should be kept in mind that Prairie canola fields are generally very large in size (~65 ha), which may reduce the feasibility of some control measures (Ahmed *et al*., 2011).

1.4.1 Biological strategies

Biological control refers to the use of living organisms to reduce the survival or activity of a pest or pathogen (Garrett, 1956, reviewed in Dixon, 2014). *Heteroconium chaetospira* (Grove) M. B. Ellis, *Phoma glomerata* (Corda) Wollenq and Hochapfel, *Bacillus spp.* and *Pseudomonas* spp. are just some of the organisms that have been found to reduce clubroot disease and have potential as biological control agents. The root endophytic fungus *H. chaetospira* has been found to successfully suppress *P. brassicae* in Chinese cabbage at inoculum densities of 1 x 10^5 spores g^{-1} soil or lower (Narisawa *et al*., 2005). A specific culture of *P. glomerata* that produced epoxydon was discovered to completely suppress clubroot symptoms on Chinese cabbage at 250 mg mL^{-1} when 30 mL of the solution was poured over 180 g of the infested soil (Arie *et al*., 1998). Bacteria including *Bacillus* spp. and *Pseudomonas* spp. were found to reduce the survival of *P. brassicae* (Einhorn *et al*., 1991). Under controlled conditions, a formulation of *B. subtilis* (Serenade), *Gliocladium catenulatum* (syn. *Clonostachy rosea f. catenulate*) and *Streptomyces griseoviridis* (Mycostop) reduced the clubroot disease severity index (DSI) by 91–61% under low to moderate inoculum pressure, respectively, with similar results observed in the field when disease pressure was lower (Peng *et al*., 2011a). Extracts from a species of seagrass, *Posidonia australi* Hook. F., have demonstrated the ability of the species to stimulate germination of *P. brassicae* (Hata *et al*., 2002) and may have potential for depleting resting spore levels in the soil.

Despite promising results, biological control has not yet proven to be a practical option for clubroot management in Canada. Challenges associated with the mass production of the biocontrol agents, their application and winter survival are just some of the issues faced. Some species also grow very slowly and take a long time to colonize the target area, which is not ideal

in Canada where the growing season is very short (Gossen *et al*., 2013). Furthermore, much of the research conducted so far has produced inconsistent results, highlighting the impact of soil moisture, pH, pathogen density and temperature on the effectiveness of biocontrol agents (Narisawa *et al*., 2005).

1.4.2 Chemical strategies

The nature of *P. brassicae* as a soilborne pathogen that reproduces only inside the host roots makes its control via fungicide application difficult. Many fungicides have been tested for their effectiveness in clubroot management. In Canada, fluazinam (Allegro®, Omega®) and cyazfamid (Ranman) have garnered the most research attention (Peng *et al*., 2015). Fluzinam is registered for the control of *P. brassicae* on vegetable crops and cyazfamid is only registered for the control of other diseases. Band-incorporation of fluazinam into transplant rows of broccoli or cauliflower at 3 L ha^{-1} increased yields up to 80% relative to spot drenches or sprays (Donald *et al*., 2001). In studies with canola, Ahmed *et al*. (2011) found that fluzinam and cyazofamid were effective only where soil inoculum levels were low. Hwang *et al*. (2011b) documented that quintozene (Terraclor) was consistently effective at reducing clubroot severity, but its use in Canada is highly restricted. Other products tested including cyazofamid (Ranman) and fluazinam (Allegro) reduced clubroot disease severity at the higher rates tested, but the results were not consistent over site and year (Hwang *et al*., 2011b). In canola cropping systems, it seems that the impact of fungicides on clubroot severity and yields is not always consistent, and the benefit obtained does not compensate for the cost and effort involved in their application. Fungicide application appears to be economic and effective only on vegetable crops when the soil inoculum level is low.

Fungicidal seed treatments also have been explored for their efficacy in controlling clubroot. Seed treatments, however, are most effective as the seed is germinating, if the pathogen is not active in the soil (i.e., when soil temperature or moisture are low) at the time of sowing, the fungicide will break down and any benefit will be lost before the pathogen becomes active (Hwang *et al*., 2011b; Gossen *et al*., 2012). Since primary infection by *P. brassicae* can continue for several weeks after germination, if the seed treatments are to have any effect, they must persist at high enough concentrations for several weeks as the soil warms up (Sharma *et al*., 2011a). Nonetheless, seed treatments may help to reduce the risk of dissemination of *P. brassicae* in seedborne contaminants such as dust and plant debris (Rennie *et al*., 2011; Hwang *et al*., 2012a). All fungicides tested as a seed treatment in Hwang *et al*. (2012a) greenhouse study reduced disease severity index relative to the control with the greatest reductions from the application of azoxystrobin (Dynasty 100 FS) and flusulfamide (Nebijin 5SC). Beyond this possible contribution to seed sanitization, it is difficult to provide producers with fungicide solutions to this pathogen. At present, no fungicides are registered in Canada for clubroot management on canola.

1.4.3 Genetic resistance

Genetic resistance is the most cost-effective and environmentally friendly approach to manage clubroot (Peng *et al*., 2015). Resistance breeding efforts in Canada began shortly after the identification of *P. brassicae* on canola in Alberta. In 2009, the first CR canola cultivar, '45H29', was released on the market, followed by a suite of other CR cultivars from various suppliers in 2010. Currently, there are 28 registered CR canola cultivars available in Canada (Canola Council of Canada, 2018). The CR canola cultivars on the Canadian market today are derived from ECD 04 or Mendel which has demonstrated complete resistance against five old

and all 15 of the new virulent *P. brassicae* pathotypes (Fredua-Agyeman *et al*., 2018). The longevity of the resistance in CR cultivars depends largely on the diversity of the pathogen population and resistance deployment practices in the field (Cao *et al*., 2009). In Canada, the pathotype composition of *P. brassicae* appears to be fairly diverse (Xue *et al*., 2008; Strelkov *et al*., 2018), suggesting greater risk for resistance breakdown. Moreover, most resistance appears to be based on single, major genes (Gossen *et al*., 2013; Fredua-Agyeman *et al*., 2018), making it vulnerable to pathotype shifts. In greenhouse experiments, clubroot severity was found to increase significantly on a resistant canola cultivar after just two cycles of exposure to the same *P. brassicae* population or single-spore isolate (LeBoldus *et al*., 2012). Despite the loss in the effectiveness of clubroot resistance in the 170 fields identified to date, ECD 04 is still a valuable resistance source, but the selection pressures imposed by short rotations of these cultivars warrant for multi-gene resistance and the implementation of other clubroot management strategies (S. E. Strelkov, *personal communication*; Fredua-Agyeman *et al*., 2018).

Resistant cultivars are believed to release root exudates that enhance resting spore germination, since primary infection is often observed (Kageyama and Asano, 2009; LeBoldus *et al*., 2012). Moreover, while clubroot symptom expression in CR canola is highly reduced, it is usually not completely eliminated (Hwang *et al.*, 2011a). An analysis of the impact of CR canola cropping systems on *P. brassicae* resting spore levels in the soil detected increased spore concentrations in the spring following a CR canola crop (Ernst *et al*., 2019). These results indicate some proliferation of *P. brassicae* pathotypes in the roots of resistant hosts, and these pathotypes likely will increase with every exposure (Xue *et al*., 2008; Ernst *et al*., 2019), eventually resulting in pathotype shifts and noticeable clubroot symptoms (LeBoldus *et al*., 2012; Strelkov *et al*., 2016). Furthermore, even the planting pf CR canola with effective

resistance can be associated with, off-types in seed lots, gene segregation, incomplete resistance, and weed hosts, which can also increase inoculum loads (Howard *et al*., 2010; Hwang *et al*., 2011a). This highlights the importance of using resistant cultivars as part of an integrated clubroot management plan, in order to maintain the durability and longevity of the resistance (Diederichsen *et al*. 2003; Peng *et al*., 2015).

1.4.4 Cultural strategies

Cultural management strategies have been used historically to control many plant diseases, including clubroot. While in recent decades farmers have relied more heavily on genetic resistance and chemical control, many cultural techniques are still effective. The goal of cultural control is to manipulate the plant growth environment to make it unfavorable for disease. A number of different cultural strategies that have been used or considered for clubroot management are discussed in this section.

Decoy/bait crops

The use of decoy or bait crops is based on the idea that the enhanced germination of *P. brassicae* resting spores are observed with some non-host species and can be exploited to reduce the soil inoculum loads. Crops such as, leek (*Allium porrum* L.), winter rye (*Secale cereale* L.), perennial ryegrass (*Lolium perenne* L.) and Japanese leafy daikon (*Raphanus sativus* L.) have been shown to enhance resting spore germination and primary root hair colonization without subsequent secondary cortical infection or completion of pathogen life cycle (Frigberg *et al*., 2006; Murakami *et al*., 2004). It is also possible to plant a susceptible crop that becomes infected, which is then destroyed (by ploughing or herbicide application) before *P. brassicae* has

the time to complete its life cycle. As such, resting spore levels are depleted while no new inoculum is produced.

Despite the theoretical promise associated with bait crops, their efficacy in field trials has been mixed at best. A bait crop study in northern Europe looked at four potential bait crops, red clover (*T. pretense* cv. Sara), leek (*A. porrum* cv. Regius), perennial ryegrass (*L. perenne* cv. Helmer) and winter rye (*S. cereal* cv. Amilo) and found no effect on clubroot disease incidence or severity after 10 months of plant growth (Friberg *et al*., 2006). Fallow treatments appear to yield greater reductions in disease severity compared to bait crops. An earlier study conducted by Ikegami (1985) showed that a 5 year fallow period resulted in a substantial decrease in resting spores. Similarly, Ahmed *et al*. (2011) found that clubroot incidence and severity were lower following a fallow treatment than after a cruciferous bait crop, the latter having only a small impact on resting spore concentration and no effect on disease severity. It is possible that if soil resting spore concentrations are too high, the reductions associated with planting of bait crops may not be sufficient enough to have a major effect; however, this approach may prove more successful when spore concentration are low to moderate (Ahmed *et al*., 2011; Frigberg *et al*., 2006).

Manipulating seeding date

P. brassicae germination and infection ability is substantially affected by soil temperature. By planting canola earlier, into colder soil, the onset of infection could be delayed to a later growth stage (Horiuchi and Hori 1980; Hwang *et al*., 2011a). The optimal temperature for spore germination ranges from 20 to 26°C; seeding when soil temperatures are below the optimum could result in a reduction in clubroot severity (McDonald and Westerveld, 2008). Hwang *et al*. (2011a) tested three seeding dates for canola, and found that plants seeded at the

earliest date had lower clubroot severity and increased plant height and yield compared with plants seeded at the later dates. Although manipulation of seeding date appears to hold promise for clubroot management, opportunities for earlier seeding are limited, given the short growing season in Canada (Gossen *et al*., 2014). Indeed, canola usually is already seeded early in the Prairies, to maximize yield potential and ensure sufficient time for crop maturity.

Crop rotation

Adopting sufficiently long crop rotations can be challenging for clubroot management, because *P. brassicae* can survive in the soil for up to 20 years (Wallenhammer, 1996). Moreover, the number of crops other than canola that can be included in rotations in much of the Prairies is minimal (barley, wheat and peas). A rotation break of four or more years is often recommended between susceptible crops to significantly reduce soil inoculum load (Strelkov *et al*., 2006), which is unacceptably long for many growers. Nonetheless, some recent studies suggest that significant decreases in resting spore concentrations occur after just a 2-year break away from susceptible hosts (Peng *et al*., 2015; Ernst *et al.*, 2019). In field experiments conducted in Quebec, *P. brassicae* resting spores levels fell from 2.1×10^5 spores per gram of soil to 7×10^4 spores per gram of soil, after two years without growing canola (Peng *et al*., 2015). Similarly, in an assessment of soils from commercial cropping systems in Alberta, Ernst *et al*. (2019) found that the concentration of *P. brassicae* resting spores two years after a CR canola crop was not significantly different from the concentration two years after a fallow period or non-host crop. These findings from Alberta agree with those of Peng *et al*. (2015) from Quebec that a ≥2 year break away from a CR canola crop results in a significant decline in resting spore concentration. Nonetheless, even a large decline in resting spore levels can still leave more than enough spores in the soil to cause significant clubroot on a susceptible host, particularly if the initial inoculum

load was very high (Hwang *et al.*, 2010). It has been estimated that each infected canola plant has the potential to return up to 8 x 10^8 resting spores to the soil (Hwang e*t al.*, 2014).

Dixon (2014) calculated that if *P. brassicae* resting spores have a half-life of 3.6 years, as reported by Wallenhammer (1996), it would take 18 years in the absence of a susceptible host for a field population of the pathogen to decrease to 3% from the original. This indicates that while crop rotations may help in reducing soil inoculum load they may not be sufficient on their own to provide adequate clubroot management.

1.4.5 Soil amendments

Soil amendments refer to the application of nutrients or other compounds to the soil to alter its properties, creating a less favorable environment for *P. brassicae* and clubroot development. Various amendments, including lime, boron, nitrate-nitrogen and other nutrients, applied alone or in combination, have been evaluated over the years. The results of some of these assessments are discussed below.

Nutrient amendments

Boron is a nutrient amendment that has been documented to delay clubroot development by interfering with primary root hair infection and the secondary cortical infection stage (Webster and Dixon, 1991). Karamanos *et al*. (2002) found a strong positive correlation between a reduction in clubroot severity and an increase rate of boron, which began at rates >2 kg ha^{-1}. Phytotoxic effects from boron application started at 5.1 kg ha^{-1} (Karamanos *et al*., 2002). Another study by Deora *et al*. (2014) revealed that soil type is an important factor in the ability of boron applications to control clubroot, with organic soils absorbing more boron than mineral soil, since high pH increases boron absorption (Huettl, 1976). In Canada, however, most clubroot

susceptible crops are restricted to mineral soils which quickly leach out boron (Parks and White, 1952; Yermiyahu *et al*., 1988).

Although boron application may yield positive results, precautions should be taken when considering its use. The amount of boron needed must be calculated as precisely as possible, since there is a narrow range between boron deficiency and toxicity in the soil (Tanaka and Fujiwara, 2008). Boron phytotoxicity has also been observed at moderate rates of application on canola seedlings (S. F. Hwang, *personal communication*). Also, high rates of boron in the soil may help control clubroot in the current year, but hinder subsequent crops included in a rotation (Nable *et al*., 1997).

Nitrogen and combinations of lime and nitrogen are other amendments applied to soil that have been studied for clubroot management. Calcium cyanimide ($CaCN_2$) is reported to inhibit resting spore germination (Naiki and Dixon, 1987). The nitrogen is a slow release source with herbicidal and fungitoxic effects, with the calcium raising the soil pH (Klasse, 1999). A one week interval is required between application and planting to allow the conversion of the cyanamide into well-tolerated forms of nitrogen (Tremblay *et al*., 2005). Hwang *et al*. (2011b) found no effect on clubroot severity with calcium cyanamide applied at high rates. In contrast, Tremblay *et al*. (2005) recorded a significant decrease in clubroot severity and a 14 fold increase in cauliflower yield when $CaCN_2$ was applied in the spring following an intensive fall application of calcitic lime ($CaCO_3$) versus when $CaCN_2$ was applied on its own.

Liming

Agricultural lime is produced in many forms, including limestone ($CaCO_3$), quicklime (CaO), and hydrated lime ($Ca(OH)_2$) products. These are used primarily to neutralize soil acidity

(Kenny and Oates, 2000). Limestone is the raw mineral deposited throughout the world and is used to create all other forms of lime (Kenny and Oates, 2000). Quicklime is produced by the thermal decomposition of limestone and hydrated lime is produced by reacting quicklime with water (Kenny and Oates, 2000). Lime can come in many forms and the amount of calcium supplied, the neutralizing value, rate of release, and particle size have a significant influence on how effectively and quickly the lime will work in the soil and against clubroot (Campbell and Greathead, 1989; Donald and Porter, 2009). In addition to those factors, soil type is an important consideration as well, since different soils will respond differently to lime application. It may be difficult to adjust the pH of soils with a high buffering capacity (Welch *et al*., 1976). "Lime nonresponsive" soils also have been documented and are described as high pH soils that fail to control clubroot (Myers *et al*., 1981).

The application of lime as a soil amendment to control clubroot is an ancient practice that is still commonly used in some cropping systems (Fletcher *et al*., 1982). *Plasmodiphora brassicae* is known to favour acidic soils; at a pH ≥ 7.2, clubroot severity is reduced (Donald and Porter, 2009). Therefore, the application of lime to increase soil pH may result in a reduction in clubroot development. In addition to raising the soil pH, the application of lime results in a calcium influx to the soil, resulting in suppressed resting spore germination and primary infection (Niwa *et al*., 2008). The timing of seeding or first exposure to pathogen inoculum after lime application is critical. Webster and Dixon (1991) found that most of the reductions in root hair infection and clubroot incidence and severity occurred 0-14 days after inoculation following exposure to elevated calcium levels and a pH of 7.2; the greatest reductions occurred in the first 7 days post-inoculation. Increasing pH at > 9 days post-inoculation failed to suppress clubbing symptoms (Webster and Dixon, 1991). Similarly, Murakami *et al*. (2002) tested several lime

products at different sowing dates post-application, and found that sowing 2 weeks after lime application resulted in a more significant decrease in disease (severity) index than sowing after 4 weeks. Webster (1986) found that root hair infections in Chinese cabbage seedling transplants were minimized by exposure to alkaline pH environments within 3-7 days of inoculation; prolonged exposure gave no further reduction in root hair infection but did suppress disease severity.

The application of calcium carbonate ($CaCO_3$)-based limes to increase soil pH has been examined in several studies. In one report, increasing the soil pH from 6.2 to 7.1 with calcium carbonate significantly reduced the percentage of un-germinated *P. brassicae* spores in the rhizosphere from 37.0% to 15.8% showing that spore germination is inhibited under neutral conditions created by lime application (Niwa *et al*., 2008). In a study conducted with canola in Alberta, Hwang *et al.* (2011b) found that limestone significantly reduced clubroot severity when applied at 5.0 and 7.5 t ha^{-1}, but the results were not consistent over sites or years. Tremblay *et al.* (2005) reported a decrease in clubroot severity from 70% to 20-30% on cruciferous vegetables with the application of hydrated lime, which were similar to the reductions achieved with calcium cyanamide. Lime has also been reported to decrease resting spore density in the soil by 17-31% following treatment with calcium cyanamide, by 12-29% following treatment with dolomite, and by 20-39% following treatment with calcium carbonate; however, no significant differences were observed when evaluating the percentage of root hair infection (Murakami *et al*., 2002).

Liming appears to be the most useful soil amendment method. The reduction of clubroot development achieved by liming affects all parts of the pathogen lifecycle. Due to the nature of *P. brassicae* as a soilborne pathogen, soil type and properties will play a vital role on how

effective each product and rate may work (reviewed in Dixon, 2009; Gossen *et al*., 2016). Much of the research conducted on the effects of liming was carried out under greenhouse or lab conditions without regard to the many uncontrollable variables observed in the field. Many of the methods used to determine root hair infection were based on microscopy techniques that are subject to user skill. Although there are many promising studies on the use of lime to inhibit disease and reduce spore density, there are possible risks associated with its application in the field. For example, lime reduces the availability of trace elements (such as boron, iron and manganese) important for plant growth and does not control clubroot when the inoculum is level high and the environment is highly conducive (Anon, 1979; Colhoun, 1953).

1.5 Research objectives

As *P. brassicae* continues to spread across the Prairies, and the number of fields with resistance issues continues to climb, the need for effective management options to supplement CR canola in the field is crucial. Treatments that delay infection for several weeks after seeding are likely to be the most effective at reducing clubroot severity, increase yield and prolong the durability of resistance (Hwang *et al.*, 2011b). The research in this thesis was focused on the evaluation of two lime products that might have potential for the management of clubroot, and consisted of three main objectives:

1) To determine whether the application of hydrated lime can help to reduce clubroot incidence and severity under field conditions and increase yields.
2) To evaluate the effectiveness of hydrated lime and limestone across multiple inoculum concentrations.
3) To determine if the application of lime products affect the quantity of root hair colonization under the varying rates of lime and inoculum applied. Reduction or

elimination of root hair colonization by the pathogen would be vital to supplement resistant cultivars and suppress infection of susceptible host species in order to alleviate the pressure for pathotype shifting and reproduction.

Chapter 2: Evaluation of hydrated lime as a clubroot management tool in canola in Alberta, Canada.

2.1 Introduction

Clubroot, caused by *Plasmodiophora brassicae* Woronin, is a soilborne disease that can infect all members of the Brassicaceae family. Infected plants are recognized by the formation of distinct club-shaped galls on the tap and lateral roots. The galled roots interfere with the plant's ability to take up water and nutrients, leading to the expression of above-ground symptoms such as wilting, stunting and premature ripening. In severe cases, clubroot can cause up to 100% yield loss and decrease the seed oil content by 5-6% (Pageau *et al*., 2006; Strelkov *et al*., 2007). *P. brassicae* survives in the soil as extremely durable resting spores that can remain viable for up to 20 years (Wallenhammar, 1996).

In Alberta, clubroot was first found on canola (*Brassica napus* L.) in 12 fields near Edmonton in 2003 (Tewari *et al*., 2005). The number of infested fields has increased greatly since then, mainly via the dissemination of *P. brassicae* on contaminated farm equipment (Cao *et al.*, 2009), and a total of 3,044 field infestations had been confirmed in the province by 2018 (Strelkov *et al.,* 2019). Over the past few years, clubroot also has been detected with increasing frequency in Saskatchewan (Strelkov and Hwang, 2014), Manitoba (Strelkov *et al*., 2018), Ontario (Al-Daoud *et al*., 2018) and North Dakota, (Chittem *et al*., 2014) indicating that the disease may become a problem over a much wider area of canola production. In addition to the movement of *P. brassicae* on machinery, resting spores of the pathogen also have been documented in windblown dust, water, and as an external contaminant on potato tubers and seeds of various crops (Rennie *et al*., 2011; Rennie *et al*., 2015; reviewed in Dixon, 2009).

At first, few tools were available for the effective management of clubroot on canola, but this changed in 2009/2010, when the first clubroot resistant cultivars become available to growers (Strelkov *et al*., 2011). Resistant canola has been grown widely throughout Alberta, but unfortunately, an erosion or loss of resistance has been observed in an increasing number of fields since 2013 (Strelkov *et al.*, 2016; Strelkov *et al.*, 2018). By the end of 2018, clubroot resistance appeared to be comprised in at least 190 fields in Alberta (S.E. Strelkov, *unpublished).* This loss of resistance has been attributed to pathotype shifts in *P. brassicae* populations, resulting from the selection pressure imposed by the cultivation of resistant hosts (Strelkov *et al.*, 2016; Strelkov *et al.*, 2018). Strategies that reduce inoculum levels and/or disease pressure may contribute to resistance stewardship, wherein the durability of resistance is maintained and prolonged (Peng *et al*., 2015).

Management practices such as the application of fungicides, biological control and multiple cultural strategies may be useful in supplementing cultivar resistance to clubroot (Peng *et al*., 2015; Narisawa *et al*., 2005; Ernst *et al*., 2019). Fungicides are used to control many plant diseases in Canadian cropping systems, and fluazinam (Allegro, Omega) and cyazofamid (Ranman) have gained the most attention in clubroot research due to their efficacy in controlling the disease in vegetable crops. These products, however, do not always provide satisfactory levels of clubroot control under field conditions, and they are not cost-effective over the large areas under canola cultivation (Peng *et al*., 2015). Similarly, despite promising results in greenhouse studies, biological control in Canada has yet to be proven practical in the field. Biocontrol agents require mass production and once applied, they take a long time to colonize the soil and likely will not survive winter conditions or extended dry periods (Gossen *et al*., 2013). Therefore, this management approach does not appear to be an effective option in the near

future (Gossen *et al*., 2013). Cultural strategies for clubroot management are very diverse and, historically, have been the most widely used control methods. Longer rotations out of host crops are recommended to prevent or slow down inoculum build-up and to extend the longevity of resistance (Strelkov *et al*., 2006; Peng *et al*., 2015). However, given the high value of canola and the limited cropping options available in much of western Canada, many producers maintain tight rotations with frequent inclusion of canola (Kutcher *et al*., 2013).

Soil amendments, including lime, have long been used in the management of clubroot on vegetable Brassicas. Liming increases soil pH and calcium levels, making conditions less favorable levels for the development of the disease (Murakami *et al*., 2002; Webster, 1986). Higher soil pH and calcium reduce the ability of *P. brassicae* to complete the primary stage of its life cycle, affecting resting spore germination, the production of primary zoospores, and root hair infection (Bochow, 1961; Niwa *et al*., 2008). An increase in soil pH is required 0-7 days after inoculation for a visible reduction in clubroot symptoms (reviewed in Dixon, 2009b). Treatments that delay infection for several weeks after seeding are also likely to reduce disease severity and have a positive impact on seed yield in clubroot susceptible canola grown in *P. brassicae*-infested soil (Hwang *et al*., 2011b). Lime formulations differ in the amount of calcium supplied and its subsequent rate of release (Campbell and Grethead, 1989). Limestone ($CaCO_3$) is the raw mineral deposited worldwide and is used as the main source for agricultural application to neutralize soil pH and improve plant growth (Oates, 2000; Wellman, 1930). Hwang *et al*. (2011b) found that a rate of 5.0 and 7.5 T ha^{-1} of limestone reduced clubroot disease severity at several sites in Alberta, but the results were inconsistent over site and year, perhaps because higher application rates were required.

High calcium hydrated lime is a dry powder produced by combining quicklime (CaO) with water, resulting in the product $Ca(OH)_2$ containing approximately 75% CaO and 25% H_2O (Graymont, Richmond, BC). Once mixed with water, hydrated lime quickly dissolves, resulting in a highly alkaline solution (pH 12.4) (Graymont). In a series of laboratory and pot tests conducted by Wellman (1930) on transplanted cabbage, hydrated lime completely inhibited clubroot infection, while there was no effect from the use of limestone.

With approximately 2.55 million hectares of soils with a pH $\leq$ 6.0 in western Canada (Lickacz, 2002), liming could be an appropriate strategy to manage clubroot and improve soil health. Limestone traditionally has been used for agricultural application due to its low cost and because for most uses, an immediate change in soil pH is not required (Wellman, 1930). Although research with hydrated lime has been limited due to its higher cost, this cost may be justified given the increasing occurrence of clubroot and the low pH soils found in many fields. Rapid increases in soil pH and calcium content prior to seeding appear to be vital for effective clubroot management, and the quicker activity of hydrated lime may be helpful in this respect (Webster, 1986; Webster and Dixon, 1991).

The objectives of this study were to (1) evaluate the efficacy of hydrated lime in reducing clubroot severity under field conditions at a site that was highly infested with *P. brassicae*, (2) compare the efficacy for clubroot control of varying rates of hydrated lime and limestone at different inoculum levels under greenhouse conditions, and (3) measure the effect of different lime treatments on *P. brassicae* proliferation in host root tissues.

2.2 Materials and Methods

2.2.1 Field trials

Replicated field trials were conducted in 2017 and 2018 to study the effects of multiple rates of hydrated lime on clubroot disease severity, yield and various other plant growth parameters. The trials were located at the Crop Diversification Center North (CDC-N), Edmonton, Alberta, in a clubroot nursery (53 38' 48"N, 113 22' 33"W) that is naturally infested with *P. brassicae*. The soil in this nursery is a black Chernozemic loam (Soil Classification Working Group, 1998). In each experimental year, the trial was replicated within the nursery. Site 1 was located on the east side of the 6 ha nursery, and site 2 was located on the west side of the nursery, about 400 m from site 1. The plots in the second year of the study were placed adjacent to the previous year's plots, to avoid any possible residual effects of the lime treatment. Treatments were arranged in a randomized complete block design with four replicates. Each plot was 1.5 m × 6 m with a 0.5 m buffer between plots and a 2 m buffer between replicates. Both trial locations soil was prepared by cultivation with a rototiller.

Hydrated lime ($Ca(OH)_2$, Graymont) treatments rates were calculated based on the targeted pH against the starting pH of the overall plot in the first year (2017). A suggested rate of 3.36 T ha^{-1} to increase the soil pH by 0.5 (Government of Alberta, 2002) was used for these calculations. In 2017, the pre-treatment pH values of sites 1 and 2 were 6.3 and 5.1, respectively. The first site treatments were calculated to target a pH of 7.0 (required 4250 g lime $plot^{-1}$ = 4.7 T ha^{-1}), pH of 7.5 (7250 g lime $plot^{-1}$ = 8.0 T ha^{-1}) and a pH of 8.0 (10,300 g lime $plot^{-1}$ = 11.4 T ha^{-1}). The second site treatments were calculated to target a pH of 6.0 (required 5440 g lime $plot^{-1}$ = 6.0 T ha^{-1}), pH of 6.5 (8470 g lime $plot^{-1}$ = 9.4 T ha^{-1}) and pH 7 (11,490 g lime $plot^{-1}$ = 12.7 T ha^{-1}). The same amount of lime was applied to the trials in 2018 to maintain consistency,

although the plot location changed and the starting pH differed. In 2018, the average pre-treatment pH values at sites 1 and 2 were 6.6 and 5.0, respectively, with the most variation between replicates. An attempt to measure soil pH after lime application was conducted at seeding time to confirm the targeted pH value; however, not all of the product was dissolved in the soil, thus giving a false reading of the actual pH at that point in time. Even in the following growing season, the product still had not completely dissolved. The lime treatments were spread across the plots manually, as evenly as possible, and incorporated immediately afterwards to a depth of 8-10 cm using a 2 m tiller attached to a tractor. One week after lime application and incorporation, the trials were seeded with the clubroot susceptible canola variety '45H31' (DuPont Pioneer, Mississauga, ON, Canada).

In 2017, the lime was applied and incorporated on June 8 at both sites. Canola was seeded 1 week later on June 16 and during that week, the plots received approximately 19 mm of natural rainfall. In 2018, the lime was applied and incorporated on May 22, followed by irrigation with 30 L of water over each 9 m^2 treatment plot. The trials were seeded on May 29; however, due to cutworm damage and low moisture, germination was extremely poor. The trials were sprayed for cutworms using Decis 5EC (Bayer, Germany) on June 14, and re-seeded on June 18. Over the 4 weeks between the lime application and the second seeding date, the trials received approximately 34 mm of rain in addition to the water applied on May 22. A second insecticidal spray with Decis 5EC + Lorsban NT (Corteva, USA) was applied to control cutworms on June 28.

Seedling emergence was recorded 7, 14 and 21 days after seeding, while plants were rated for clubroot symptom development 8 weeks after seeding. Briefly, 10 plants per plot were gently dug out of the soil, the roots were washed with water, and each root was rated on a 0 to 3

scale where: 0 = no galling, 1 = a few small galls, 2 = moderate galling, and 3 = severe galling (Kuginuki *et al*. 1999). The individual ratings were used to calculate an index of disease (ID) for each plot, according to the formula of Horiuchi and Hori (1980) as modified by Strelkov *et al*. (2006):

$$ID\ (\%) = \frac{\sum(n\times0+n\times1+n\times2+n\times3)}{N\times3} \times 100\%$$

Where: n = number of plants in a class; N = total number of plants in an experimental unit; and 0, 1, 2 and 3 = symptom severity classes.

Individual plant height, aboveground biomass and root weight were recorded for 10 plants per plot 8 weeks after seeding. Sites 1 and 2 were harvested with a small plot combine on October 6 in 2017 and on October 19 and 18, respectively, in 2018. The seeds were dried and cleaned before weighing and calculating yield.

2.2.2 Greenhouse trials

The effects of multiple rates of hydrated lime and limestone on clubroot development were compared at various inoculum levels on two canola genotypes. A susceptible clubroot cultivar '45H31' (DuPont Pioneer, Mississauga, ON, Canada) and a moderately resistant clubroot cultivar '9558C' (DuPont Pioneer, Mississauga, ON, Canada) treated with Prosper FX (Bayer Crop Science, Calgary, AB) were grown in a potting medium consisting of a mixture of 10% peat moss and 90% soilless mix (Sungro Professional Growing Mix, Sungro Horticulture, Seba Beach, AB) to create an initial soil pH of 5.3. Soil aliquots of 25 L were prepared and represented one treatment of inoculum, lime product and rate, for both cultivars.

The potting medium was inoculated with different concentrations of *P. brassicae* resting spores. The resting spores were extracted from root galls collected from a previous experiment (stored at -20°C) and infected with a field isolate classified as pathotype A on the Canadian Clubroot Differential (CCD) Set (Strelkov *et al*., 2018), or as pathotype 3 or P_2 on the systems of Williams (1966) and Somé *et al*. (1996), respectively. To extract the *P. brassicae* resting spores, galls were ground in a blender with water, then filtered through six layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, NC). A stock concentration of resting spores was estimated with a haemocytometer (VWR, Mississauga, Ontario). The volume of stock concentration used for inoculation was calculated based on targeting a final potting medium concentration of 1×10^6 resting spores g^{-1} medium for a 25 L aliquot of medium. Serially dilutions were performed with the stock concentration to target the three remaining final potting medium concentrations: 1×10^3, 1×10^4, and 1×10^5 resting spores g^{-1} potting medium. The resting spore suspensions were added to the potting medium and thoroughly mixed. Following soil inoculation with *P. brassicae*, the potting medium was treated with limestone (Zero Grind, Graymont, BC) or hydrated lime (Graymont, BC), at rates equivalent to 4.7, 8.1, 11.4 or 14.8 T ha^{-1} of lime, to adjust the pH to 6.0, 6.5, 7.0 or 7.5, respectively. Rates were calculated as per Alberta Agriculture and Forestry guidelines (Government of Alberta, 2002) as described earlier for the field trials. Following the application of lime and inoculation with the corresponding concentration of *P. brassicae* resting spores, each 25 L aliquot of potting medium was mixed thoroughly with 1.8 L of water and left in the greenhouse for 1 week prior to seeding.

The canola genotypes were sown 2 cm deep at a density of 16 seeds per 12 cm × 12 cm × 12 cm pot filled with 2.14 L of potting medium; treatments were thinned to five plants per pot 10 days after planting. The pots were maintained in a greenhouse at ca. 24°C with 30% relative

humidity under natural light supplemented with artificial lighting (16 h day/8 h night). The pots were placed on water-filled trays for the first 2 weeks after seeding to ensure sufficient moisture for clubroot development, following which holes were poked in the trays and the plants were top-watered as needed with watering cans. Pots were fertilized with a 0.1% solution of 20:20:20 (N:P:K) once a week until harvest.

In summary, a total of 80 treatments were examined. These included all combinations of the four tested inoculum concentrations (1×10^3, 1×10^4, 1×10^5 and 1×10^6 resting spores g^{-1} medium), five rates of each lime product (limestone or hydrated lime at rates of 0.0, 4.7, 8.1, 11.4 or 14.8 T ha^{-1} of lime) and two canola cultivars (45H31 and 9558C). There were two control treatments for each inoculum concentration (example: 1×10^3 resting spores g^{-1} medium + no limestone and 1×10^3 resting spores g^{-1} medium + no hydrated lime) where the medium was inoculated, but did not receive a lime treatment. Treatments were replicated five times, with one pot per replicate. The entire experiment was repeated, with the first trial conducted in 2017 and the second in 2018. In 2017, inoculation and liming mixing took place on August 22, followed by seeding on August 30 and harvest on October 31. In 2018, soil inoculation and liming occurred on May 11, followed by seeding on May 18 and harvest on July 10. At harvest, the plants were dug out from the potting medium, the roots were washed and scored for clubroot symptom severity on a 0 to 3 scale (Kuginuki *et al*., 1999). The individual severity ratings were used to calculate an ID for each replicate as described above for the field trials. Plant height was recorded for each plant and averaged per replicate. Additionally, dry-weights were recorded after the harvested plants were allowed to dry in the greenhouse for 1 week.

2.2.3 PCR analysis

Soil samples were collected two weeks before lime application in the field trials to determine the starting level of *P. brassicae* inoculum. In 2017, approximately 500 g of soil was collected across the trial replicates and mixed together, while in 2018 the soil samples were collected and processed separately for the individual plots. The soil samples were air-dried at room temperature and then ground to a powder in a mortar with a pestle. Total genomic DNA was extracted from the soil samples following Cao *et al.* (2007), and stored at 4°C if they were to be analyzed within 1 week, or at -20°C for longer term storage. In the greenhouse trials, root samples were collected to quantify colonization by *P. brassicae*. Briefly, 10-day old seedlings were removed carefully from the potting medium and washed in standing water; the root system was excised with a scalpel and stored in a small bag in a -20°C freezer until further processing. Total genomic DNA was extracted from these root samples with a NucleoSpin® Plant II DNA Isolation Kit (Macherey-Nagel GmbH & Co. KG, Germany) as per the manufacturer's instructions. The DNA samples were stored at 4°C if they were to be analyzed within 1 week, or stored at -20°C for longer periods.

All samples were subjected to conventional PCR analysis to determine the presence or absence of *P. brassicae* DNA using primers TC1F and TC1R as described by Cao *et al*. (2007). Those samples which tested positive for the presence of *P. brassicae* DNA were analyzed further by quantitative-PCR (q-PCR) following Rennie *et al*. (2011), with resting spore concentrations estimated on a five-point standard curve generated with standards of 1×10^2, 1×10^3, 1×10^4, 1×10^5 and 1×10^6 resting spores g^{-1} soil (Rennie *et al*., 2011) or g^{-1} root tissue (Cao *et al.*, 2014). Pathogen levels in the field soil samples are reported as resting spores g^{-1}, while in the root tissue samples they are reported as resting spores g^{-1} root tissue.

2.2.4 Statistical analysis

All statistical analysis was performed using RStudio v. 1.1.463 (2009-2018 RStudio, Inc.). Regression analysis was conducted on field results from 2017 and 2018 when Pearson's correlation coefficient was significant. If correlation was not significant, the data were subjected to a one-way analysis of variance based on the Tukey test. The plant parameters analyzed included canola germination, plant height, shoot weight, root weight, yield and clubroot ID to evaluate the efficacy of hydrated lime vs. non-treated controls. A logarithmic transformation was applied to clubroot ID to normalize the data and a non-parametric test (Krustal-Wallis) was used to evaluate plant height. Non-transformed means are presented for consistency. For all analyses, differences were considered to be significant at $P < 0.05$ unless otherwise stated.

An analysis of variance using the General Linear Model was used to analyze which factors significantly affected each parameter measured following a gamma distribution. Effects of cultivar, inoculum concentration, lime product, and lime rate were assessed for the individual plant measurements of height and disease severity rating, and the net dry (5 plants per replicate) root and shoot weight were subjected to a mean separation based on least-squares mean, Tukey test. These same factors also were evaluated for their effect on the quantity of *P. brassicae* DNA in 10-day-old root samples as determined by qPCR analysis. In the greenhouse trials, "year" was considered as a block because the trials were conducted in separate greenhouses using different inoculum sources, and as a result, the two trials are presented and analyzed separately. The means of each parameter are presented in this study. The data represent the means of five replicates in the greenhouse study and three replicates in the q-PCR analysis. Differences were considered significant at $P < 0.05$ unless otherwise specified.

2.3 Results

2.3.1 Field trials

Disease severity

In 2017, all rates of lime reduced clubroot severity compared with the control treatment at both sites. The average ID for the control treatment (pH 6.3) at site 1 was 47%. The lowest rate of lime, 4.7 T ha^{-1}, decreased the ID to 37.5% (Table 2.1). The moderate rate of lime (8.0 T ha^{-1}) reduced ID to 6.7%, and the highest rate of lime (11.4 T ha^{-1}) reduced it to 4% (Table 2.1). The two highest rates of lime applied were significantly different from the control and the lowest rate of lime, but not from each other. The log-transformed ID and lime rate were positively correlated ($R^2 = 0.46$, $P = 0.0023$, $F_{(1,14)} = 13.78$). As determined by q-PCR, the average *P. brassicae* resting spore concentration in the soil at site 1 (2017) was 3.3×10^3 spores g^{-1} soil (range of 1.65×10^3 to 5.05×10^3 spores g^{-1} soil).

The second site in 2017 will only be discussed briefly due to an error in staking replicates 3 and 4, which resulted in only the first two replicates receiving the true rate of lime. The disease pressure seemed to be much higher at this site, with the control treatment (pH 5.1) developing an ID of 78%. The lowest rate of lime (6.0 T ha^{-1}) reduced the ID to 67%, while the moderate rate (9.4 T ha^{-1}) reduced it to 45% and the highest rate of lime (12.7 T ha^{-1}) reduced it to 43% (Table 2.1). Although the results from site 2 in 2017 were not analyzed statistically because of the staking error, they did suggest significant visual and measurable differences in plant health and yield with increasing lime rates. As determined by q-PCR, the average *P. brassicae* resting spore concentration in the soil at site 2 (2017) was 1.1×10^5 spores g^{-1} soil (range of 1.04×10^4 to 2.10×10^5 spores g^{-1} soil).

In 2018, there was no treatment effect observed at either site (Table 2.2). Site 1 developed no visible symptoms of clubroot in three of the replicates and only very mild symptoms in the fourth replicate. The ID in the control treatment was just 3% and IDs varied from 1 to 4% across the various lime treatments (Table 2.2). As was the case in 2017, clubroot disease pressure was higher at site 2. The average ID in the control treatment was 61%, which was not significantly different from the 67 to 68% observed across the various lime treatments (Table 2.2). There were no significant correlations observed in 2018 between clubroot severity and lime application. The average *P. brassicae* resting spore concentration was calculated via q-PCR analysis and was 1.22×10^4 spores g^{-1} soil (range of 2.59×10^3 to 4.34×10^4 spores g^{-1} soil) at site 1 and 8.55×10^5 spores g^{-1} soil (range of 1.39×10^3 to 8.27×10^6 spores g^{-1} soil) at site 2 in 2018.

Plant growth parameters

There were no significant differences observed in root or shoot weight, plant height or yield at site 1 in 2017. However, there was a correlation ($R^2 = 0.3513$, $P = 0.009$, $F_{(1,14)} = 9.124$) between the log-transformed ID and root weight. Above-ground biomass and lime rate also were correlated ($R^2 = 0.3319$, $P = 0.011$, $F_{(1,14)} = 8.45$). Similarly, germination was significantly affected by lime rate. The lowest rate of lime significantly reduced germination count by 16%, while the moderate to high rates of lime significantly reduced germination by 40% and 46%, respectively, compared with the control (Table 2.1). The germination count at the moderate and high rates of lime was significantly different from the control and the lowest rate of lime, but not from each other. Although there was a significant effect of lime rate on canola germination, yield remained unchanged or increased slightly while ID decreased (Table 2.1). Plant height increased

from 17% to 25% compared with the control. While numerical increases in yield were observed at the two highest rates of lime, these were not statistically significant.

At the second site in 2017, no statistical analysis was conducted as a result of the staking error mentioned above. Nevertheless, shoot weight appeared greater at the lowest (1855 g per 10 plants), moderate (2132.5 g per 10 plants) and highest (2625 g per 10 plants) rates of lime relative to the control (1490 g per 10 plants) (Table 2.1). Plants also were, on average, 21 to 44% taller in the lime treatments versus the control. These numerical increases were consistent with the visual appearance of the plants in each of the treatments, which were bigger with a denser canopy (Figure 2.1). As was observed in site 1, germination count also appeared to decrease with increasing lime rate; ID also declined and therefore, survival rate and yield were higher with increasing rates of lime. Numerically, yields also were greater in the treatments with low (536.7 kg ha^{-1}), moderate (1075.6 kg ha^{-1}) and high rates (1191.1 kg ha^{-1}) compared with the control (268.9 kg ha^{-1}) (Table 2.1).

The only significant differences observed in 2018 were with respect to germination counts at site 1, where the lowest germination was observed at the highest rate of lime (Table 2.2). No significant differences were detected between shoot weight, root weight, plant height or yield across any of the treatments (Table 2.2). Shoot weight ranged from 1827.0 g per 10 plants to 2188.0 g per 10 plants across the treatments compared with 1862.5 g per 10 plants in the control (Table 2.2). Plants in the lime treatments were on average 4 cm to 10 cm taller than the control treatment. Root weight ranged from 132.8 g per 10 roots to 150.0 g per 10 roots, while the control averaged 127.8 g per 10 roots (Table 2.2). Yield improved insignificantly from the control (1875.0 kg ha^{-1}) with low (1644.8 kg ha^{-1}), moderate (2032.2 kg ha^{-1}), and high (1982.8 kg ha^{-1}) rates of lime (Table 2.2). At site 2, shoot weight ranged from 899.0 g per 10 plants to

1541.8 g per 10 plants, compared to 1331.3 g per 10 plants in the control (Table 2.2). Plant height was on average 4.6 cm to 1.2 cm shorter than the control (122.5 cm). Although there was no decrease in ID at this site, there were some numerical declines in root weight associated with lime treatment, but these were not significant (Table 2.2). Similarly, no significant differences in yield were recorded at site 2 in 2018, and varied from 791.9 kg ha^{-1} for the low rate of lime to 861.7 kg ha^{-1} for the high rate, compared with 745.3 kg ha^{-1} for the control (Table 2.1).

2.3.2 Greenhouse trial

Disease severity

In the first greenhouse trial conducted in 2017, clubroot severity was significantly affected by the canola cultivar grown ($F_{(1,398)} = 446.9$, $P < 2.2 \times 10^{-16}$), lime product applied ($F_{(2,396)} = 376.6$, $P < 2.2 \times 10^{-16}$), inoculum concentration ($F_{(3,393)} = 8.6$, $P = 1.56 \times 10^{-05}$), and their interactions (cultivar*inoculum ($P = 0.000275$), cultivar*lime product ($P = 5.06 \times 10^{-05}$), lime*inoculum ($P < 2.2 \times 10^{-16}$), cultivar*lime*inoculum ($P = 6.69 \times 10^{-08}$)). The susceptible cultivar (45H31) developed an ID of 92 to 100% across all resting spore concentrations in the absence of lime amendments. The application of hydrated lime at any of the rates evaluated resulted in a decrease in ID to 0% across all spore concentrations, with the exception of the 8.1 T ha^{-1} rate at 1×10^6 spores g^{-1} medium, which developed an ID of 18% (Table 2.3). On the moderately resistant cultivar 9558C, ID ranged from 9 to 13% in the absence of lime amendments; this declined to 0% across all resting spore concentrations when hydrated lime was applied at any of the rates examined (Table 2.3). The reduction in ID appeared to be less pronounced with the application of limestone (Table 2.4). On the susceptible canola 45H31,

significant reductions in ID were observed only at the lower resting spore concentrations (1×10^3 and 1×10^4 spores g^{-1} medium), and limestone treatment at any rate seemed to have no effect on ID at spore concentrations of 1×10^5 and 1×10^6 spores g^{-1} medium. Similar trends were observed with the canola cultivar 9558C, except that ID values generally were lower given the moderately resistant nature of this cultivar (Table 2.4). As was observed with 45H31, limestone at any rate did not appear to have a consistent effect on ID on 9558C, relative to the control treatments, at 1×10^5 and 1×10^6 spores g^{-1} medium.

When the greenhouse trial was repeated in 2018, the IDs in the control treatments were generally less than in 2017 at the lower inoculum concentrations, but similar at the higher concentrations (Tables 2.5 and 2.6). Overall, the trends in 2018 were consistent with those observed in 2017, and ID was significantly affected by cultivar ($F_{(1,398)} = 356.2$, $P < 2.2 \times 10^{-16}$), lime product ($F_{(2,396)} = 247.7$, $P < 2.2 \times 10^{-16}$), inoculum concentration ($F_{(3,393)} = 46.0$, $P < 2.2 \times 10^{-16}$), and the interactions of cultivar*inoculum ($P = 0.00069$). On the clubroot susceptible canola 45H31, ID in the non-limed control treatments increased from 10% at 1×10^3 resting spores g^{-1} medium to 100% at 1×10^6 spores g^{-1} medium. The application of any rate of hydrated lime resulted in no visible symptoms of clubroot (ID of 0%) at any of the resting spore concentrations evaluated (Table 2.5). On the moderately resistant cultivar 9958C, ID values in the controls ranged from 0% at 1×10^3 resting spores g^{-1} medium to 8% at 1×10^6 spores g^{-1} medium, and treatment with any rate of hydrated lime resulted in an ID of 0% across inoculum levels (Table 2.5). As was observed in the first trial, the application of limestone seemed to be effective at reducing IDs mainly at the lower spore concentrations (Table 2.6). On 45H31, any rate of limestone reduced ID to 0% at the lowest inoculum concentration (1×10^3 spores g^{-1} medium); at 1×10^4 spores g^{-1} medium, all rates also significantly reduced ID, but it was 0% only

at the two highest rates (Table 2.6). Clubroot development also decreased significantly with application of limestone at 1 $\times 10^4$ spores g^{-1} medium to 1 $\times 10^6$ spores g^{-1} medium; however, the lowest ID achieved increased with increasing inoculum concentration. On the moderately resistant canola 9558C, no clubroot symptoms developed on the control or any of the treatments at 1 $\times$ 10^3 spores g^{-1} medium, and no clear trends or significant differences were observed with limestone treatment at any of the higher spore concentrations. Indeed, at spore concentrations from 1 $\times$ 10^4 to 1 $\times$ 10^6 spores g^{-1} medium, IDs fluctuated in a fairly narrow range from 0 to 12% across all limestone rates.

Plant growth parameters

In the first greenhouse trial conducted in 2017, plant height was affected by cultivar ($F_{(1,397)} = 324.4$, $P < 2.2 \times 10^{-16}$), lime product ($F_{(2,395)} = 77.6$, $P < 2.2 \times 10^{-16}$), inoculum concentration ($F_{(3,389)} = 153.1$, $P < 2.2 \times 10^{-16}$) and interactions (cultivar*lime ($P < 2.2 \times 10^{-16}$), inoculum*cultivar ($P = 55.0 \times 10^{-15}$), inoculum*lime ($P < 2.2 \times 10^{-16}$), rate*inoculum ($P = 5.3 \times 10^{-12}$), cultivar*inoculum*lime ($P = 1.2 \times 10^{-13}$), inoculum*rate*lime ($P = 6.6 \times 10^{-09}$), and cultivar*inoculum*rate*lime ($P = 0.02785$)). On the susceptible canola 45H31, the most pronounced effects on plant height were observed at the higher spore concentrations (1 $\times$ 10^5 to 1 $\times$ 10^6 spores g^{-1} medium), while they fluctuated near the height of the control treatments at the lower concentrations (Table 2.3). On the moderately resistant cultivar 9558C, no clear trend was discernible with respect to hydrated lime treatment and plant height. There were some rates that reduced plant height by 2 cm to 13 cm, with the largest reductions observed at the lower inoculum concentrations, and there were also rates that increased plant height by 2 cm to 8 cm with the tallest plants observed at the higher inoculum concentration; however, the differences were not significant compared to the control (Table 2.3). All rates of limestone appeared to

increase the height of 45H31, by as much as 22 cm relative to the control treatments, most differences were significant at inoculum concentrations of 1×10^5 spores g^{-1} medium or 1×10^6 spores g^{-1} medium (Table 2.4). On 9558C, significant increases in plant height were observed with increasing rates of limestone at concentrations of 1×10^3 spores g^{-1} medium or 1×10^5 spores g^{-1} medium, and declined at the highest inoculum, compared to the control (Table 2.4).

Dry shoot weight was affected by cultivar ($F_{(1,398)} = 96.0$, $P < 2.2 \times 10^{-16}$), inoculum ($F_{(3,395)} = 26.8$, $P = 9.2 \times 10^{-16}$), lime product ($F_{(2,393)} = 6.0$, $P = 0.0027$), and lime rate ($F_{(1,392)} = 22.4$, $P = 3.1 \times 10^{-06}$) with significant interactions of lime inoculum*lime ($P = 1.0 \times 10^{-06}$), and inoculum*rate ($P = 5.1 \times 10^{-07}$). All rates of hydrated lime increased dry shoot weight on 45H31 relative to the control treatments, with these increases most significant at the higher inoculum levels (1×10^5 and 1×10^5 spores g^{-1} medium). In the case of the partially resistant cultivar 9558C, however, the effect of rate resulted in significant reductions in dry shoot weight of 21 to 94% with increasing rate of hydrated lime at all inoculum levels except for 1×10^5 spores g^{-1} medium (Table 2.3). Limestone treatment resulted in significant increases in shoot weight of 45H31 at all inoculum levels except 1×10^6 spores g^{-1} medium (Table 2.4); on 9558C, limestone increased dry shoot weight at the two lowest rates of limestone and two lowest inoculum concentrations. At the two higher inoculum concentrations, the effect of limestone on 9558C did not follow any trend (Table 2.4).

Dry root weight was affected by cultivar ($F_{(1,394)} = 642.7$, $P < 2.2 \times 10^{-16}$), lime product ($F_{(2,392)} = 79.6$, $P < 2.2 \times 10^{-16}$), inoculum concentration ($F_{(3,396)} = 57.4$, $P < 2.2 \times 10^{-16}$), rate ($F_{(1,395)} = 211.9$, $P < 2.2 \times 10^{-16}$) and their interactions (cultivar*lime ($P = 0.0027$), inoculum*cultivar ($P = 5.1 \times 10^{-05}$), inoculum*lime ($P < 2.2 \times 10^{-16}$), rate*cultivar ($P = 0.00095$), inoculum*rate ($P = 9.1 \times 10^{-13}$), inoculum*rate*cultivar ($P = 0.041$), and

inoculum*rate*lime ($P = 0.0021$)). Hydrated lime reduced the dry root weight of both 45H31 and 9558C, likely as a result of the reduction or elimination of root clubs. In the susceptible cultivar 45H31, the average root weight of the control treatments ranged from 3.15 g to 3.71 g, while in treatments receiving hydrated lime, the dry root weight significantly decreased and ranged from 0.26 to 0.90 g due to the elimination of galling (Table 2.3). In the moderately resistant cultivar 9558C, the average root weight of the control treatments was 0.70 g to 0.93 g, but any rate of hydrated lime reduced this weight further at all inoculum concentrations. Similar trends were observed with the application of limestone, in a manner consistent with the decreases in clubroot severity observed for those treatments (Table 2.4).

The patterns observed when the greenhouse trial was repeated in 2018 resembled those in 2017. Plant height was significantly affected by cultivar ($F_{(3,396)} = 59.5$, $P < 2.2 \times 10^{-16}$), lime product ($F_{(1,395)} = 59.3$, $P = 1.2 \times 10^{-13}$) and inoculum concentration ($F_{(2,393)} = 26.7$, $P = 1.2 \times 10^{-11}$) and interactions including, cultivar*lime ($P < 2.2 \times 10^{-16}$), inoculum*lime ($P = 1.3 \times 10^{-09}$) and inoculum*cultivar*lime ($P < 2.2 \times 10^{-16}$). In 45H31, plant height increased significantly by 0.4 to 24.1 cm when subjected to hydrated lime treatments, with the largest changes occurring at the higher inoculum concentrations (Table 2.5). In the partially resistant cultivar 9558C, the application of hydrated lime resulted in no significant changes in plant height at the three lower inoculum concentrations, regardless of rate, while a significant reduction in plant height was observed at some rates at the highest inoculum concentration (Table 2.5). The application of limestone significantly increased the height of 45H31 by 2.2 cm to 33.9 cm across all inoculum concentrations (Table 2.6). In case the of cultivar 9558C, plant height following limestone application varied by -29.5 cm to +12.7 cm compared with the control across all inoculum

concentrations, generating significantly taller plants at some rates at the three lowest inoculum concentrations and significantly shorter plants at the highest inoculum concentration (Table 2.6).

Dry shoot weight in the 2018 trial was again affected by cultivar ($F_{(1,394)} = 41.1$, $P = 4.5 \times 10^{-10}$), inoculum concentration ($F_{(3,395)} = 24.2$, $P = 2.8 \times 10^{-14}$) and lime product ($F_{(2,392)} = 7.8$, $P = 0.00048$), plus the interactions of inoculum*lime ($P = 9.1 \times 10^{-15}$), inoculum*cultivar ($P = 6.0 \times 10^{-15}$), cultivar*lime ($P = 8.9 \times 10^{-13}$), inoculum*lime*rate ($P = 0.043$), inoculum*cultivar*lime ($P < 2.2 \times 10^{-16}$) and cultivar*lime*rate ($P = 0.021$). On 45H31, hydrated lime generally increased dry shoot weight significantly at all inoculum levels except 1×10^3 spores g^{-1} medium, relative to the control treatments; the largest increases were observed at the highest inoculum level (Table 2.5). On 9558C, treatment with hydrated produced no clear significant differences at the two lowest inoculum concentrations. At 1×10^6 spores g^{-1} medium, however, almost all rates of hydrated lime significantly reduced dry shoot weight. Limestone application resulted in increased dry shoot weight in 45H31 at the three highest inoculum levels, but was associated with small decreases (1 to 6%) in dry shoot weight at the lowest inoculum concentration (Table 2.6). On 9558C, the limestone treatments increased dry shoot weight only at 1×10^5 spores g^{-1} medium and decreased it at 1×10^6 spores g^{-1} medium, compared with the control, regardless of rate.

Dry root weight was affected by cultivar ($F_{(1,394)} = 400.7$, $P < 2.2 \times 10^{-16}$), inoculum concentration ($F_{(3,395)} = 107.4$, $P < 2.2 \times 10^{-16}$) and lime product ($F_{(2,392)} = 206.2$, $P < 2.2 \times 10^{-16}$), as well as the interactions of inoculum*lime ($P < 2.2 \times 10^{-16}$), inoculum*cultivar ($P = 0.022$), cultivar*lime ($P = 6.0 \times 10^{-08}$), inoculum*cultivar*rate ($P = 0.0015$), inoculum*cultivar*lime ($P = 9.4 \times 10^{-07}$) and inoculum*cultivar*lime*rate ($P = 0.012$). Hydrated lime significantly reduced dry shoot weight at the three highest inoculum concentrations for 45H31, and at 1×10^6 spores g^{-1}

medium for 9558C (Table 2.5). This is likely due to the elimination or reduction of root galling as noted earlier. Similarly, the application of limestone reduced the dry root weight in 45H31 relative to the control at the three highest inoculum concentrations, but not to the same extent as hydrated lime. A significant reduction in root weight was observed for 9558C following limestone application at an inoculum concentration of 1×10^6 spores g^{-1} medium (Table 2.6).

2.3.3 Quantification of *P. brassicae* in host roots

In the first greenhouse trial conducted in 2017, the ability of *P. brassicae* to colonize host root tissues was significantly affected by lime product ($F_{(2,231)} = 99.4$, $P < 2.2 \times 10^{-16}$), rate ($F_{(3,228)} = 9.0$, $P = 1.5 \times 10^{-05}$) and inoculum concentration ($F_{(3,225)} = 301.1$, $P < 2.2 \times 10^{-16}$). Although cultivar did not appear to be a significant factor for this parameter, all interactions including those with cultivar were significant (significance ranging from P 0.001 to $P < 2.2 \times 10^{-16}$). The amount of pathogen DNA in the roots of the seedlings, expressed as resting spores g^{-1} root tissue, decreased with increasing rate of hydrated lime in both the susceptible canola 45H31 and the moderately resistant 9558C (Fig. 2.2). No pathogen was detected in the roots of either cultivar at the lowest inoculum concentration (1×10^3 spores g^{-1} medium) following treatment with any rate of hydrated lime (Figure 2.2). When the inoculum concentration increased to 1×10^4 spores g^{-1} medium, reduced levels of *P. brassicae* were quantified in the roots of both cultivars at 4.7 T ha^{-1} hydrated lime, but rates of ≥ 8.1 T ha^{-1} were sufficient to eliminate any detectable infection. Similarly, at inoculum concentrations of 1×10^5 and 1×10^6 spores g^{-1} medium, rates of ≥ 11.4 or 14.8 T ha^{-1} hydrated lime, respectively, were required to eliminate any detectable levels of *P. brassicae* in the roots, although lower rates of lime did decrease the amount of the pathogen relative to the controls (Fig. 2.2). Significant differences in the amount of pathogen were detected only at the highest inoculum concentration for hydrated lime when the

cultivar 45H31 was grown. The application of limestone generally resulted in one to two-fold reductions in the amount of *P. brassicae* in the roots of both 45H31 and 9558C, but significant differences in the amount of pathogen present were only observed at the highest inoculum concentration when limestone was applied and the cultivar 45H31 was grown (Fig. 2.3).

In the second greenhouse trial conducted in 2018, all factors, cultivar ($F_{(1,234)} = 21.7$, $P = 6.6 \times 10^{-06}$), lime product ($F_{(2,232)} = 105.9$, $P < 2.2 \times 10^{-16}$), lime rate ($F_{(3,229)} = 3.7$, $P = 0.013$) and inoculum concentration ($F_{(3,226)} = 84.3$, $P < 2.2 \times 10^{-16}$), as well as all interactions significantly (ranging from $P = 0.012$ to $P < 2.2 \times 10^{-16}$) affected the amount of *P. brassicae* detected in the roots. The pathogen was not detectable in roots of the cultivar 45H31 following the application of hydrated lime to soil that had been inoculated with 1×10^3, 1×10^4 and 1×10^5 spores g^{-1} medium (Fig. 2.4). At the highest inoculum level (1×10^6 spores g^{-1} medium), a rate of ≥ 8.1 T ha^{-1} hydrated lime was required to completely eliminate pathogen infection (Fig. 2.4). In 9558C, any rate of hydrated lime was sufficient to prevent detection of the *P. brassicae* in the roots of seedlings grown in inoculum concentrations of 1×10^3 and 1×10^5 spores g^{-1} medium [1]. The lowest rate of hydrated lime reduced the amount of pathogen detected in the tissues, when grown at inoculum concentrations 1×10^4 and 1×10^6 spores g^{-1} medium, but a rate ≥ 8.1 T ha^{-1} was needed to completely eliminate infection (Fig. 2.4). However, significant differences in the amount of pathogen present were established only at the highest inoculum concentration for both cultivars when hydrated lime was applied. When limestone was incorporated in the soil, detectable *P. brassicae* in the roots was eliminated only at the lowest inoculum concentration. In 45H31, detectable infection was eliminated at limestone rates of 4.7, 11.4 and 14.8 T ha^{-1}, but was only reduced at 8.1 T ha^{-1} (Fig. 2.5). In 9558C, all rates of limestone eliminated detectable infection by the pathogen at the lowest inoculum concentration, but no significant differences

were observed compared with the control at any of the other rates (Fig. 2.5). Significant differences in the amount of pathogen detected in the roots were observed only at inoculum concentrations of 1×10^4 and 1×10^6 spores g^{-1} medium in both cultivars when limestone was applied.

2.4 Discussion

Based on the results of the field trials, there may be some promise for the use of hydrated lime as a management tool for clubroot on canola. In 2017, all rates of hydrated lime reduced clubroot severity compared with the control treatment. At site 1, ID was reduced by about 90% at the highest (11.4 T ha^{-1}) and moderate (6.7%; 8.0 T ha^{-1}) rates of lime. Despite these significant decreases in clubroot severity, however, the yields remained similar. This may reflect the fact that the number of emerged canola seedlings decreased significantly at the two highest rates of lime, so that any gains in yield resulting from reduced clubroot may have been offset by decreased plant density. Nonetheless, a reduction in clubroot severity could contribute to inoculum management, by reducing the number of new *P. brassicae* resting spores added to the soil at the end of the growing season (Shinoda *et al*., 2005). Murakami *et al*. (2002) reported a reduction in resting spores resulting from the application of lime. In the current study, however, post-harvest resting spore concentrations were not obtained in the field experiments, and further experimentation would be required to evaluate this hypothesis under Alberta conditions.

Results similar to those at site 1 were found at site 2 in 2017, but unfortunately, two replicates were lost due to a staking error. The two replicates that remained were not sufficient to conduct a robust statistical analysis of this site, but visually the plants appeared much healthier in the lime treatments versus the controls, and numerical increases in plant height and yield were recorded with increasing rates of lime. At this site, the ID was 78.3% in the control, and 45% and

43% in plots treated with the moderate and high rates of lime, respectively. Although these values were relatively high, they nonetheless represented a substantial decrease in clubroot severity and likely accounted for the improved yield at site 2. The soil at site 2 (pH 5.1) was more acidic than at site 1 (pH 6.3), which may have made conditions more favorable for clubroot development in the former. Moreover, at this level of acidity, micronutrient availability is reduced and concentrations of Al^{+3} become toxic (Bhering *et al*., 2017), which could have caused deleterious effects on plant health in the controls and enhanced the effect of liming in the treated plots. Li *et al*. (2018) reported that the effects of liming were greater on those soils with the lowest initial pH.

In contrast to the results in 2017, hydrated lime did not reduce clubroot severity when the field trial was repeated in 2018. In part, this may reflect the much lower disease pressure at site 1 in 2018. Nonetheless, mean shoot weight, plant height and yield improved slightly with increasing lime rate, while the germination rate decreased significantly only at the highest rate of lime. The improvement in plant growth can likely be attributed to the increase in pH to a level where soil conditions are more ideal for plant growth (Bhering *et al*., 2007; Li *et al*., 2018). At site 2 in 2018, clubroot was severe in all plots (mean IDs of 60-67%), yet there was no effect of liming on clubroot severity. This may have been due to the length of the interval between liming and sowing in 2018 vs. 2017. In 2017, canola was seeded 1 week after lime application, and the plots received a large amount of rainfall 2 days after application. In contrast, in 2018, canola had to be reseeded 3 weeks after lime application due to cutworm damage. Murakami *et al*. (2002) found that the interval between liming and sowing is critical for the efficacy of lime treatments, with greater decreases in clubroot severity observed when soils were limed 2 weeks instead of 4 weeks prior to sowing of the crop. Other studies have found similar results, which led Dixon

(2009b) in a review of the literature to conclude that a high pH and calcium influx must be present within the first 3-7 days of inoculation for observable declines in clubroot. In an evaluation of limestone and wood ash treatment for clubroot management in canola, Hwang *et al*. (2011b) also found that results were not consistent over sites or years. Many soil and environmental factors can affect the efficacy of soil amendments such as hydrated lime (Li *et al*., 2018). Although the experiments in this thesis were not designed to evaluate the effect of timing on the efficacy of hydrated lime for clubroot control, the results seem to confirm earlier studies indicating the importance of appropriate timing. The results from 2017 suggest that when the timing between sowing and lime application is optimized and conditions are conducive for clubroot development, the severity of the disease can be significantly decreased.

Multiple rates of hydrated lime were evaluated in this study to find an optimal application rate that could be used to help manage clubroot on canola in the field. The only phytotoxic effect observed was on canola germination, but this appeared to be compensated for by improved plant health and increased yields on the individual plants. Webster and Dixon (1991) eliminated clubroot development under high pH and calcium concentrations, but also found phytotoxic effects on the host plants. Among the rates tested in the current trials, the moderate (8.0 T ha^{-1}) and high (11.4 T ha^{-1}) rates of lime appeared most effective. In many cases, the clubroot severity obtained with the moderate rate of lime was significantly lower than in the control and lowest treatment rate, but not significantly different from the highest rate. Therefore, it may be more cost-effective to apply the moderate rate of lime, at least in soils in which pH > 6.0. Specific recommendations regarding application rate are difficult, however, since these would vary depending on the initial soil pH. Indeed, the efficacy of hydrated lime for clubroot management

appears to be influenced by many factors, as discussed above, as well as by the initial resting spore concentration in the field.

In the greenhouse experiments, all rates of hydrated lime reduced ID to 0% on both the susceptible and moderately resistant canola cultivars. The small pot size and the controlled conditions may have improved the efficacy of the treatments relative to the field study. Similarly, Li *et al*. (2018) found that the effects of liming were more pronounced in pot conditions than in the field. Treatment with lime resulted in increased plant height in the susceptible canola cultivar 45H31, likely reflecting the elimination of root symptom development. In contrast, in the moderately resistant 9558C, plant height remained similar or decreased slightly, possibly because even control plants of this cultivar developed only mild clubroot (so the benefits of clubroot mitigation were limited), while the hydrated lime may have had some phytotoxic effects. As expected, trends with respect to root weight were similar to those observed for ID, since decreased clubroot severity is associated with fewer galls and hence lower root mass. As such, as clubroot severity decreased, so did root dry weight. Shoot dry weight increased in 45H31 with the application of hydrated lime, as galling was eliminated and the plants could grow normally. In the partially resistant 9558C, however, the gains obtained from clubroot symptom reduction again were minor (given the mild symptoms that developed even in the controls), so phytotoxic effects likely resulted in the decreases generally observed in shoot weight for this cultivar.

The effects of limestone (calcium carbonate) on clubroot reduction were less pronounced than those of hydrated lime. This may reflect, at least in part, that the same rates (T ha^{-1}) of the two products were compared; given that the calcium carbonate equivalent (CCE) of hydrated lime is 135%, it can neutralize 1.35× as much acid as the same amount of limestone (Havlin *et*

al., 2004). As such, the latter would have been less effective at reducing the acidity of the potting medium. Differences in the efficacy of lime products also reflect other factors, including the amount and rate of release of calcium, particle size and others (Donald and Porter, 2009). Clubroot severity declined with increasing rate of limestone only at the two lowest inoculum levels, while no disease suppression occurred above 1×10^5 spores g^{-1} medium. Consequently, the impact on other growth parameters, such as plant height, was affected similarly. For example, height was increased in cultivar 45H31 across treatments at low inoculum concentrations, but did not differ at higher inoculum concentrations compared with the control. Shoot weight increased following limestone application at the lower inoculum concentrations, but remained unchanged at the highest concentration. As expected, root weight decreased with decreasing clubroot symptom severity. In the moderately resistant 9558C, no general trend in height were observed following limestone treatment, while shoot weight increased only slightly at the lowest inoculum concentration and increased at the highest inoculum concentration compared with the control. The amount of clubroot control that limestone can provide appears to be limited and seems restricted to situations where the *P. brassicae* inoculum concentration is low ($< 1 \times 10^4$ spores g^{-1} medium). This may help to explain why little benefit was observed from limestone application in earlier studies conducted in Alberta (Hwang *et al.*, 2008), since *P. brassicae* infestations $> 1 \times 10^8$ spores g^{-1} soil have been reported in some fields in this province.

The extent to which liming affected the ability of *P. brassicae* to proliferate in the host roots was evaluated by q-PCR analysis. Under greenhouse conditions, treatment with lime generally reduced or completely eliminated the pathogen in 10-day-old canola seedlings. As was the case with reductions in clubroot symptom development, hydrated lime was more effective than limestone at reducing the amount of the pathogen in the roots, especially at higher resting

spore concentrations. Statistical differences were observed mostly at the highest inoculum concentration (1×10^6 spores g^{-1} medium). Root hair infection has been reported to be linearly proportional to inoculum concentration (Webster and Dixon, 1991), so it is not surprising that the amount of lime needed to eliminate root hair infection increased with increasing inoculum concentration. Moreover, resting spore age, maturity and the relative prevalence of non-viable spores can alter the slope of the linear relationship between root-hair infection and spore concentration (Macfarlane, 1952; Dixon, 2009; Peng *et al*., 2015). In the present study, proliferation of *P. brassicae* in the roots was expressed as resting spores per g root tissue, as calculated based on the amount of genomic DNA relative to standard curves generated with known amounts of resting spores. While this facilitated comparisons, it is likely that in 10-day-old seedlings, most of the pathogen biomass actually occurred in the form of primary and secondary plasmodia as opposed to mature resting spores (Ayers, 1944; Sharma *et al*., 2011).

It is clear that while lime treatments hold some promise for clubroot mitigation on canola, the type of lime selected is important, and the extent of control afforded may be influenced by the timing of application as well as numerous environmental and soil factors. Nonetheless, the application of lime treatments may, when done right, significantly reduce clubroot severity and the proliferation of *P. brassicae* in the root tissues. Such effects may not only serve to improve plant health, but may contribute to long-term clubroot management by reducing selection pressure on pathogen populations and controlling soil inoculum loads.

Table 2.1. Effects of hydrated lime ($Ca(OH)_2$) on clubroot index of disease and plant growth parameters under field conditions in Edmonton, AB, Canada, 2017.

Site 1: Initial pH 6.3						
Lime Rate (T ha^{-1})	**Germination count (plants/6 m)**	**Index of disease (%)**	**Fresh shoot weight (g) 10 plants^{-1}**	**Fresh root weight (g) 10 plants^{-1}**	**Plant height (cm)**	**Yield (kg ha^{-1})**
0.0	84 a	46.7 a	1936.3 a	201.3 a	95.4 a	2229.2 a
4.7	72.8 b	37.5 a	2137.5 a	246.3 a	113.9 a	2182.2 a
8.0	56.1 c	6.7 b	2391.3 a	147.5 a	123.2 a	2477.5 a
11.4	52.4 c	4.1 b	3058.8 a	176.3 a	122.4 a	2511.4 a
Site 2: Initial pH 5.1 (2 replicates)						
0.0	75.5	78.3	1490	210.0	58.5	268.9
6.0	65.9	66.7	1855	167.5	72.5	536.7
9.4	64.2	45.0	2132.5	175.0	91.8	1075.6
12.7	47.9	43.3	2625	240.0	87.6	1191.1

*Means followed by the same letter do not differ according to Tukey test at $P > 0.05$

**Site 2 was not analyzed statistically because there were only two true replicates

Table 2.2. Effects of hydrated lime ($Ca(OH)_2$) on clubroot index of disease and plant growth parameters under field conditions in Edmonton, AB, Canada, 2018.

Site 1: Initial pH 6.6						
Lime Rate (T ha^{-1})	**Germination count (plants/6 m)**	**Index of disease (%)**	**Fresh shoot weight (g) 10 plants^{-1}**	**Fresh root weight (g) 10 plants^{-1}**	**Plant height (cm)**	**Yield (kg ha^{-1})**
0.0	85.9 a	3.3 a	1862.5 a	127.8 a	130.9 a	1875.0 a
4.7	75.1 a	1.7 a	2188.0 a	150.0 a	138.8 a	1644.8 a
8.0	82.8 ab	1.7 a	1827.0 a	132.8 a	134.8 a	2032.2 a
11.4	45.3 b	4.1 a	2101.5 a	138.8 a	140.1 a	1982.8 a
Site 2: Initial pH 5.0						
0.0	98.3 a	60.8 a	1331.3 a	225.3 a	122.5 a	745.3 a
6.0	105.5 a	67.5 a	1308.0 a	176.8 a	121.3 a	791.9 a
9.4	97.8 a	66.7 a	1541.8 a	215.3 a	117.9 a	746.4 a
12.7	115.1 a	66.7 a	889.0 a	172.3 a	118.4 a	861.7 a

*Means followed by the same letter do not differ according to Tukey test at $P > 0.05$

Table 2.3. Effects of hydrated lime ($Ca(OH)_2$) on index of disease (ID), plant height, dry shoot weight and dry root weight under greenhouse conditions 8 weeks after planting, year 1.

			Index of Disease (%)				Plant Height (cm)				Shoot Weight (g)				Root Weight (g)			
			Resting spores g^{-1} medium				Resting spores g^{-1} medium				Resting spores g^{-1} medium				Resting spores g^{-1} medium			
Cultivar	**Lime (T/ha)**	**pH**	**10^3**	**10^4**	**10^5**	**10^6**	**10^3**	**10^4**	**10^5**	**10^6**	**10^3**	**10^4**	**10^5**	**10^6**	**10^3**	**10^4**	**10^5**	**10^6**
45H31	0	5.3	97 a	92 a	100 a	100 a	41.3 c	32.8 a	30.8 b	28.3 b	5.51 c	3.25 b	3.89 b	3.05 d	3.43 a	3.71 a	3.58 a	3.15 a
	4.7	6.0	0 b	0 b	0 b	18 b	51.7 bc	42.0 a	70.6 a	58.1 a	6.46 bc	6.34 a	11.12 a	10.46 a	0.58 b	0.46 b	0.80 b	0.90 b
	8.1	6.5	0 b	0 b	0 b	0 b	69.4 a	41.1 a	60.8 a	58.3 a	8.78 a	6.32 a	10.02 a	8.46 b	0.82 b	0.60 b	0.78 b	0.60 b
	11.4	7.0	0 b	0 b	0 b	0 b	60.3 ab	40.4 a	64.8 a	60.6 a	7.34 ab	6.64 a	9.72 a	8.32 b	0.46 b	0.84 b	1.04 b	0.74 b
	14.8	7.5	0 b	0 b	0 b	0 b	61.9 ab	41.9 a	73.8 a	60.5 a	7.30 ab	2.86 b	9.90 a	5.94 c	0.64 b	0.26 b	0.84 b	0.46 b
9558C	0	5.3	13 a	11 a	9 a	10 a	66.6 ab	42.6 a	68.1 a	68.5 a	9.10 a	7.29 a	10.91 a	10.43 a	0.73 a	0.84 a	0.93 a	0.83 a
	4.7	6.0	0 b	0 a	0 a	0 a	53.6 b	41.2 a	71.1 a	69.5 a	6.96 ab	6.82 b	9.34 a	9.12 ab	0.40 b	0.44 bc	0.76 ab	0.70 ab
	8.1	6.5	0 b	0 a	0 a	0 a	72.1 a	41.5 a	69.7 a	66.9 a	8.18 ab	5.82 b	9.42 a	6.74 bc	0.68 ab	0.52 b	0.62 b	0.38 b
	11.4	7.0	0 b	0 a	0 a	0 a	66.3 ab	44.6 a	64.8 a	72.2 a	8.24 ab	5.84 b	10.08 a	8.12 b	0.44 ab	0.42 bc	0.70 ab	0.50 b
	14.8	7.5	0 b	0 a	0 a	0 a	65.0 ab	31.8 a	76.5 a	65.0 a	5.90 b	2.66 b	8.86 a	5.62 c	0.34 b	0.16 c	0.84 ab	0.40 b

*Means followed by the same letter do not differ at $P < 0.05$ within each column

Table 2.4. Effects of limestone ($CaCO_3$) on index of disease (ID), plant height, dry shoot weight and dry root weight under greenhouse conditions 8 weeks after planting, year 1.

			Index of Disease (%)				Plant Height (cm)				Shoot Weight (g)				Root Weight (g)			
			Resting spores g^{-1} medium				Resting spores g^{-1} medium				Resting spores g^{-1} medium				Resting spores g^{-1} medium			
Cultivar	Lime (T/ha)	pH	10^3	10^4	10^5	10^6	10^3	10^4	10^5	10^6	10^3	10^4	10^5	10^6	10^3	10^4	10^5	10^6
45H31	0	5.3	97 a	92 a	100 a	100 a	41.3 b	32.8 b	30.8 a	28.3 b	5.51 b	3.25 d	3.89 b	3.05 a	3.43 a	3.71 a	3.58 a	3.15 a
	4.7	6.0	24 b	64 b	100 a	100 a	64.0 a	40.4 ab	36.0 a	39.6 a	9.56 a	7.92 a	4.72 ab	2.94 a	1.78 ab	2.76 ab	3.72 a	2.68 a
	8.1	6.5	13 b	56 bc	100 a	100 a	59.7 a	43.2 a	33.3 a	27.2 b	9.06 a	7.30 ab	5.46 ab	3.06 a	1.04 b	2.22 ab	4.58 a	2.98 a
	11.4	7.0	11 b	40 cd	98 a	100 a	57.2 a	41.5 ab	38.0 a	27.8 ab	9.52 a	5.34 bc	5.72 a	3.36 a	0.90 b	1.00 b	4.42 a	2.88 a
	14.8	7.5	11 b	20 d	96 a	100 a	58.4 a	35.8 ab	38.6 a	30.2 ab	8.38 a	4.26 cd	5.94 a	2.88 a	0.98 b	0.62 b	4.06 a	3.00 a
9558C	0	5.3	13 a	11 a	9 a	10 b	66.6 ab	42.6 a	68.1 a	68.5 a	9.10 bc	7.37 a	10.91 a	10.43 ab	0.70 a	0.84 a	0.93 b	0.83b
	4.7	6.0	0 a	5 a	16 a	8 b	75.2 a	49.2 a	40.2 c	67.6 a	11.30 a	9.00 a	9.76 a	12.02 a	0.80 a	0.84 a	1.56 a	0.84 b
	8.1	6.5	1 a	4 a	13 a	20 ab	81.4 a	46.2 a	50.5 bc	55.2 a	10.64 ab	8.18 a	9.78 a	9.02 b	0.72 a	0.82 a	0.98 b	1.08 ab
	11.4	7.0	4 a	3 a	7 a	36 a	54.6 b	44.8 a	73.5 a	58.3 a	7.54 c	7.84 a	11.02 a	8.28 b	0.54 a	0.50 ab	0.92 b	1.30 a
	14.8	7.5	0 a	0 a	16 a	4 b	67.4 ab	37.1 a	60.8 ab	67.8 a	8.98 abc	4.21 b	10.62 a	10.08 ab	0.58 a	0.16 b	0.96 b	0.88 ab

*Means followed by the same letter do not differ at $P < 0.05$ within each column

Table 2.5. Effects of hydrated lime ($Ca(OH)_2$) on index of disease (ID), plant height, dry shoot weight and dry root weight under greenhouse conditions 8 weeks after planting, year 2.

			Index of Disease (%)				Plant Height (cm)				Shoot Weight (g)				Root Weight (g)			
			Resting spores g^{-1} medium				Resting spores g^{-1} medium				Resting spores g^{-1} medium				Resting spores g^{-1} medium			
Cultivar	Lime (T/ha)	pH	10^3	10^4	10^5	10^6	10^3	10^4	10^5	10^6	10^3	10^4	10^5	10^6	10^3	10^4	10^5	10^6
45H31	0	5.3	10 a	81 a	83 a	100 a	71.0 b	70.4 b	68.1 b	45.7 b	7.29 a	5.33 c	4.37 b	2.40 b	0.47 a	1.44 b	1.42 b	2.16 b
	4.7	6.0	0 a	0 b	0 b	0 b	82.3 a	73.4 ab	66.2 b	73.5 a	7.56 a	5.18 bc	5.49 ab	6.60 a	0.42 a	0.24 a	0.26 a	0.38 a
	8.1	6.5	0 a	0 b	0 b	0 b	72.4 b	78.7 ab	82.3 a	74.8 a	6.99 a	7.93 a	7.69 a	6.80 a	0.34 a	0.46 a	0.40 a	0.35 a
	11.4	7.0	0 a	0 b	0 b	0 b	74.9 ab	76.0 ab	75.9 a	73.0 a	6.97 a	7.23 abc	7.36 a	6.13 a	0.40 a	0.31 a	0.40 a	0.35 a
	14.8	7.5	0 a	0 b	0 b	0 b	71.4 b	80.5 a	80.0 a	68.5 a	6.52 a	7.37 ab	6.36 ab	5.27 a	0.40 a	0.44 a	0.38 a	0.27 a
9558C	0	5.3	0 a	3 a	7 a	8 a	75.2 a	81.2 a	80.3 b	84.7 a	7.79 a	6.81 a	6.05 bc	9.20 a	0.38 a	0.35 a	0.34 a	0.62 a
	4.7	6.0	0 a	0 a	0 a	0 b	80.5 a	78.5 a	77.7 b	74.9 b	6.41 a	6.08 a	7.92 ab	6.30 b	0.29 a	0.26 a	0.36 a	0.30 b
	8.1	6.5	0 a	0 a	0 a	0 b	83.4 a	86.9 a	94.1 a	74.2 b	8.09 a	7.37 a	8.58 a	6.43 b	0.35 a	0.35 a	0.37 a	0.25 b
	11.4	7.0	0 a	0 a	0 a	0 b	75.6 a	81.5 a	81.4 b	79.4 ab	6.18 a	7.32 a	7.01 abc	6.37 b	0.24 a	0.38 a	0.39 a	0.34 b
	14.8	7.5	0 a	0 a	0 a	0 b	77.6 a	84.3 a	75.6 b	72.3 b	5.97 a	7.03 a	4.91 c	6.29 b	0.30 a	0.41 a	0.25 a	0.35 b

*Means followed by the same letter do not differ at $P < 0.05$ within each column

Table 2.6. Effects of limestone ($CaCO_3$) on index of disease (ID), plant height, dry shoot weight and dry root weight under greenhouse conditions 8 weeks after planting, year 2.

			Index of Disease (%)				Plant Height (cm)				Shoot Weight (g)				Root Weight (g)			
			Resting spores g^{-1} medium				Resting spores g^{-1} medium				Resting spores g^{-1} medium				Resting spores g^{-1} medium			
Cultivar	**Lime (T/ha)**	**pH**	**10^3**	**10^4**	**10^5**	**10^6**	**10^3**	**10^4**	**10^5**	**10^6**	**10^3**	**10^4**	**10^5**	**10^6**	**10^3**	**10^4**	**10^5**	**10^6**
45H31	0	5.3	10 a	81 a	83 a	100 a	71.0 b	70.4 b	68.1 c	45.7 c	7.29 a	5.33 ab	4.37 b	2.40 b	0.47 a	1.42 a	1.42 a	2.16 c
	4.7	6.0	0 a	23 b	53 b	100 a	71.7 ab	72.7 ab	90.6 a	70.6 b	6.88 a	6.34 ab	9.44 a	3.63 ab	0.41 a	0.51 b	1.09 ab	1.50 bc
	8.1	6.5	0 a	3 b	28 c	73 b	78.8 b	74.1 ab	78.6 b	81.6 a	7.18 a	4.89 b	6.10 b	4.72 b	0.53 a	0.40 b	0.41 c	1.20 c
	11.4	7.0	0 a	0 b	45 bc	96 ab	75.4 ab	79.7 a	84.4 bc	79.9 a	7.18a	7.38 a	9.29 a	3.33 ab	0.33 a	0.37 b	0.90 bc	1.73 abc
	14.8	7.5	0 a	0 b	37 bc	96 ab	73.2 ab	78.8 a	85.0 bc	77.6 ab	7.21 a	6.71 ab	8.79 a	3.97 ab	0.35 a	0.29 b	0.81 bc	1.94 ab
9558	0	5.3	0 a	3 a	7 a	8 a	75.2 b	81.2 ab	80.3 b	84.7 a	7.79 ab	6.81 a	6.05 c	9.20 a	0.38 a	0.35 a	0.34 a	0.62 a
	4.7	6.0	0 a	11 a	0 a	12 a	71.9 b	74.4 b	92.8 ab	55.8 c	5.60 c	6.65 a	8.70 ab	5.38 b	0.26 a	0.36 a	0.34 a	0.43 ab
	8.1	6.5	0 a	0 a	3 a	3 a	74.4 b	80.4 ab	86.4 a	68.8 b	6.53 bc	6.49 a	7.42 bc	7.43 ab	0.35 a	0.31 a	0.46 a	0.44 ab
	11.4	7.0	0 a	3 a	11 a	8 a	78.4 ab	81.4 ab	92.3 a	55.2 c	7.43 abc	6.17 a	10.32 a	7.38 ab	0.32 a	0.27 a	0.56 a	0.33 b
	14.8	7.5	0 a	0 a	0 a	12 a	87.9 a	84.6 a	92.8 a	63.2 bc	8.86 a	6.55 a	9.90 a	6.45 b	0.37 a	0.29 a	0.53 a	0.50 ab

*Means followed by the same letter do not differ at $P < 0.05$ within each column

Figure 2.1. The visual effects of adding decreasing amounts of hydrated lime (12.7 T ha^{-1}, 9.4 T ha^{-1}, 6.0 T ha^{-1}, and 0.0 T ha^{-1}) are shown on shoot weight (10 plants per plot) (panel A) and root weight (10 root per plot) (panel B) at field site 2, Edmonton, Alberta, in 2017.

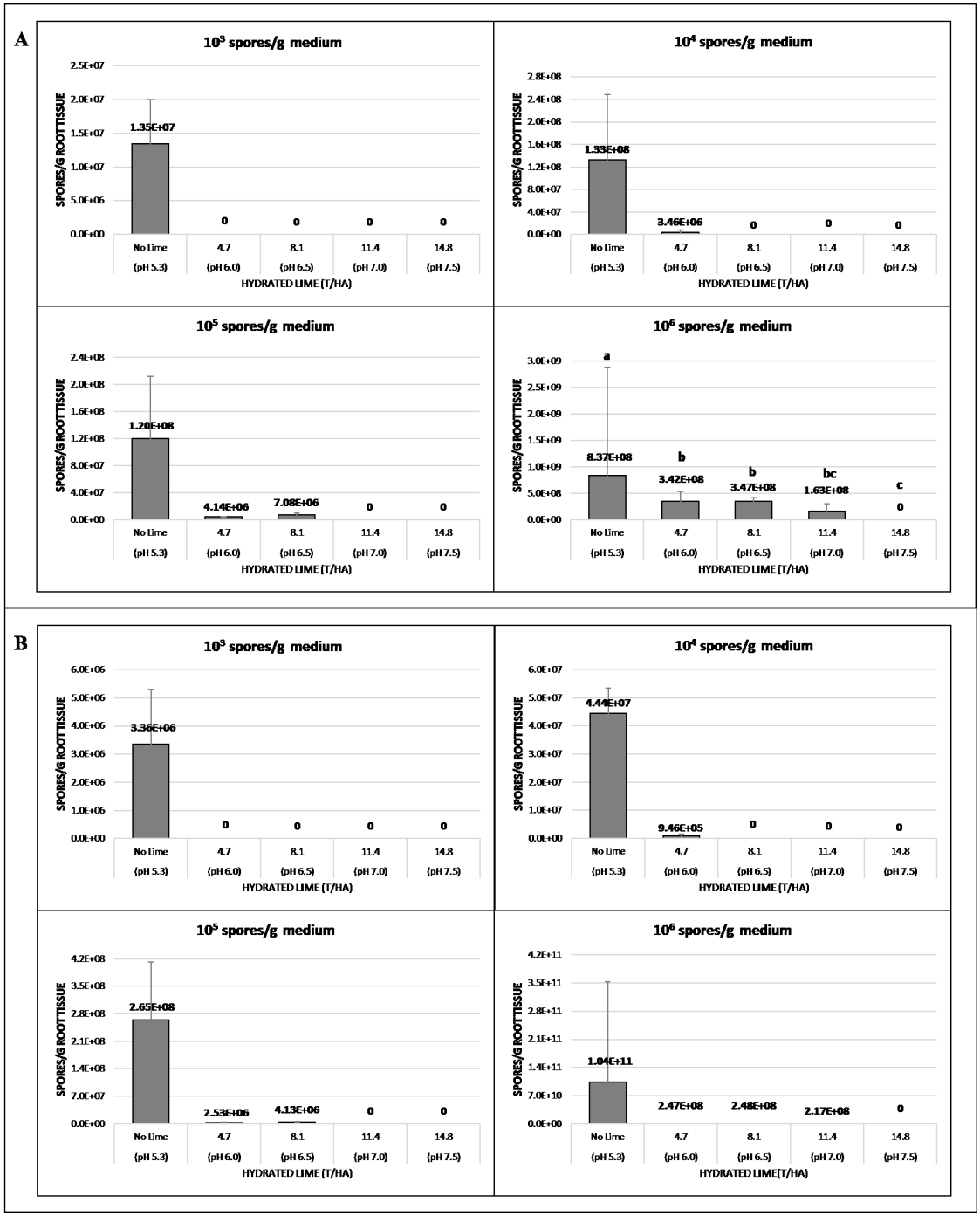

Figure 2.2. Amount of *Plasmodiophora brassicae* in the roots of 10-day-old seedlings of the canola cultivars 45H31 (panel A) and 9558C (panel B) as determined by quantitative PCR analysis. The seedlings were grown under greenhouse conditions in potting medium inoculated with 1×10^3, 1×10^4, 1×10^5 and 1×10^6 resting spores g^{-1} potting medium and treated with 4.7, 8.1, 11.4 or 14.8 T ha^{-1} of hydrated lime which targeted a pH of 6.0, 6.5, 7.0 or 7.5, respectively. Controls did not receive any lime (pH 5.3). These results are from the first run of this experiment in 2017. Means with no letters do not differ; means differences are denoted by different letters at $P < 0.05$.

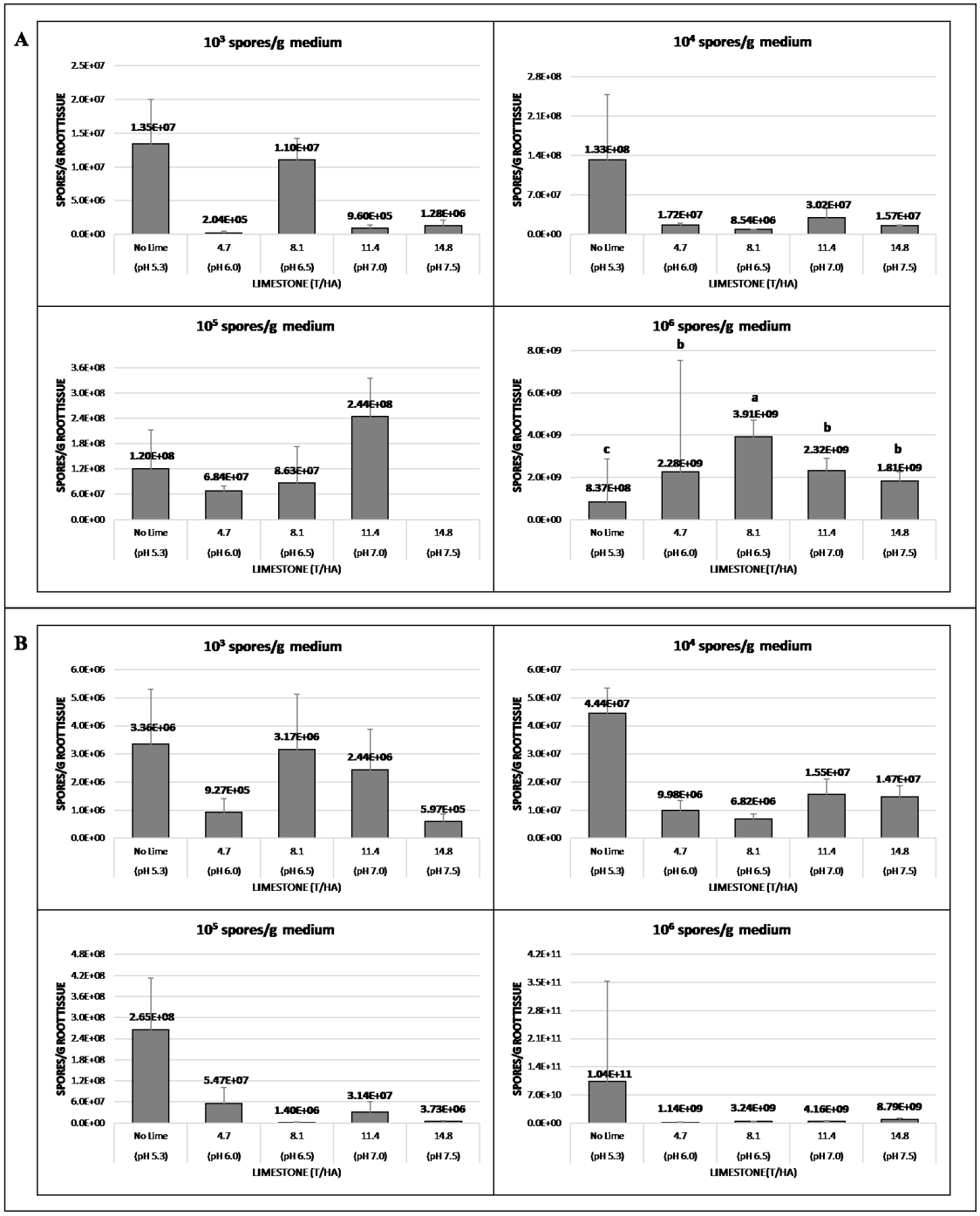

Figure 2.3. Amount of *Plasmodiophora brassicae* in the roots of 10-day-old seedlings of the canola cultivars 45H31 (panel A) and 9558C (panel B) as determined by quantitative PCR analysis. The seedlings were grown under greenhouse conditions in potting medium inoculated with 1×10^3, 1×10^4, 1×10^5 and 1×10^6 resting spores g^{-1} potting medium and treated with 4.7, 8.1, 11.4 or 14.8 T ha^{-1} of limestone which targeted a pH of 6.0, 6.5, 7.0 or 7.5, respectively. Controls did not receive any lime (pH 5.3). These results are from the first run of this experiment in 2017. Means with no letters do not differ; means differences are denoted by different letters at $P < 0.05$.

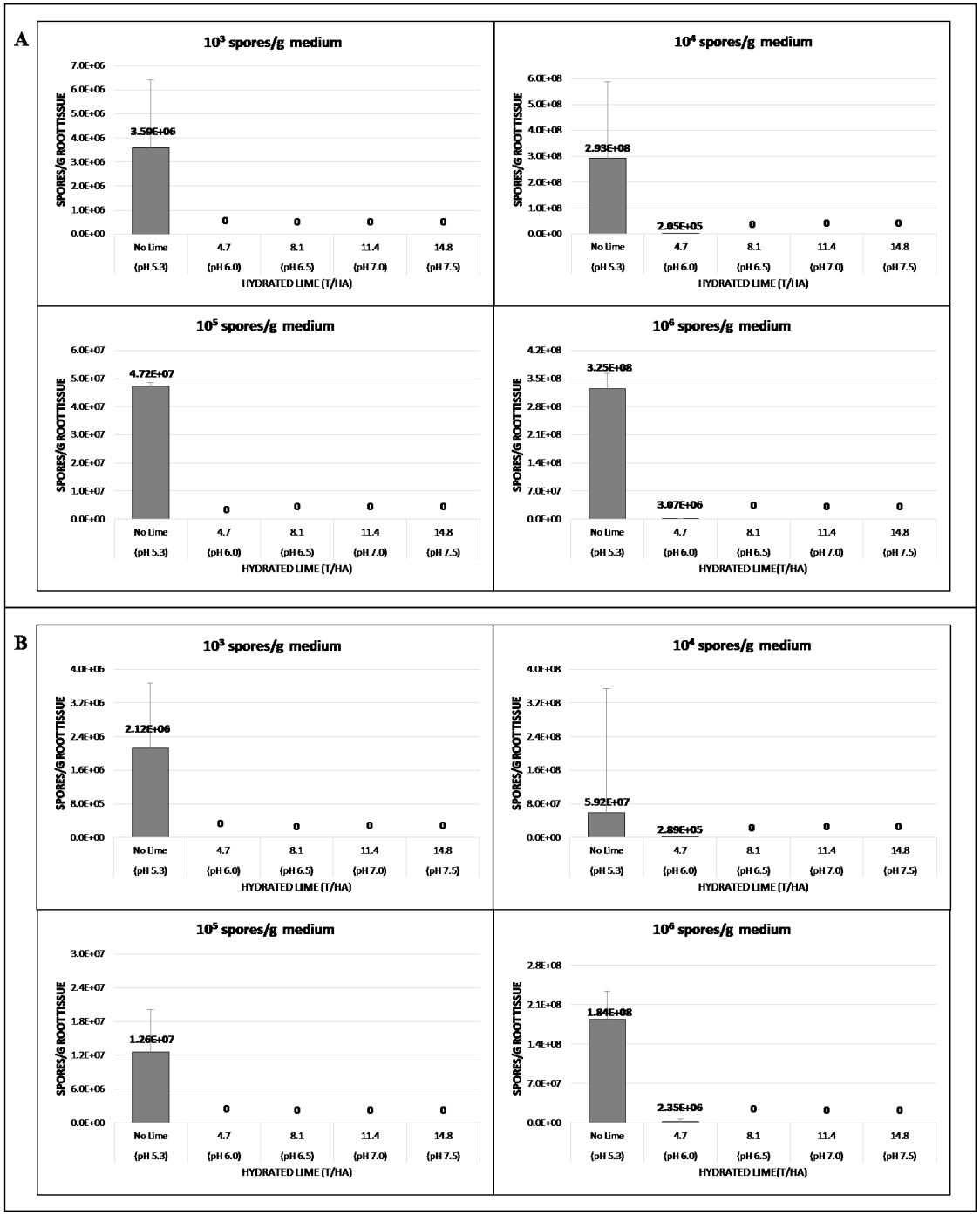

Figure 2.4. Amount of *Plasmodiophora brassicae* in the roots of 10-day-old seedlings of the canola cultivars 45H31 (panel A) and 9558C (panel B) as determined by quantitative PCR analysis. The seedlings were grown under greenhouse conditions in potting medium inoculated with 1×10^3, 1×10^4, 1×10^5 and 1×10^6 resting spores g^{-1} potting medium and treated with 4.7, 8.1, 11.4 or 14.8 T ha^{-1} of hydrated lime which targeted a pH of 6.0, 6.5, 7.0 or 7.5, respectively. Controls did not receive any lime (pH 5.3). These results are from the second run of this experiment in 2018. Means with no letters do not differ; means differences are denoted by different letters at $P < 0.05$.

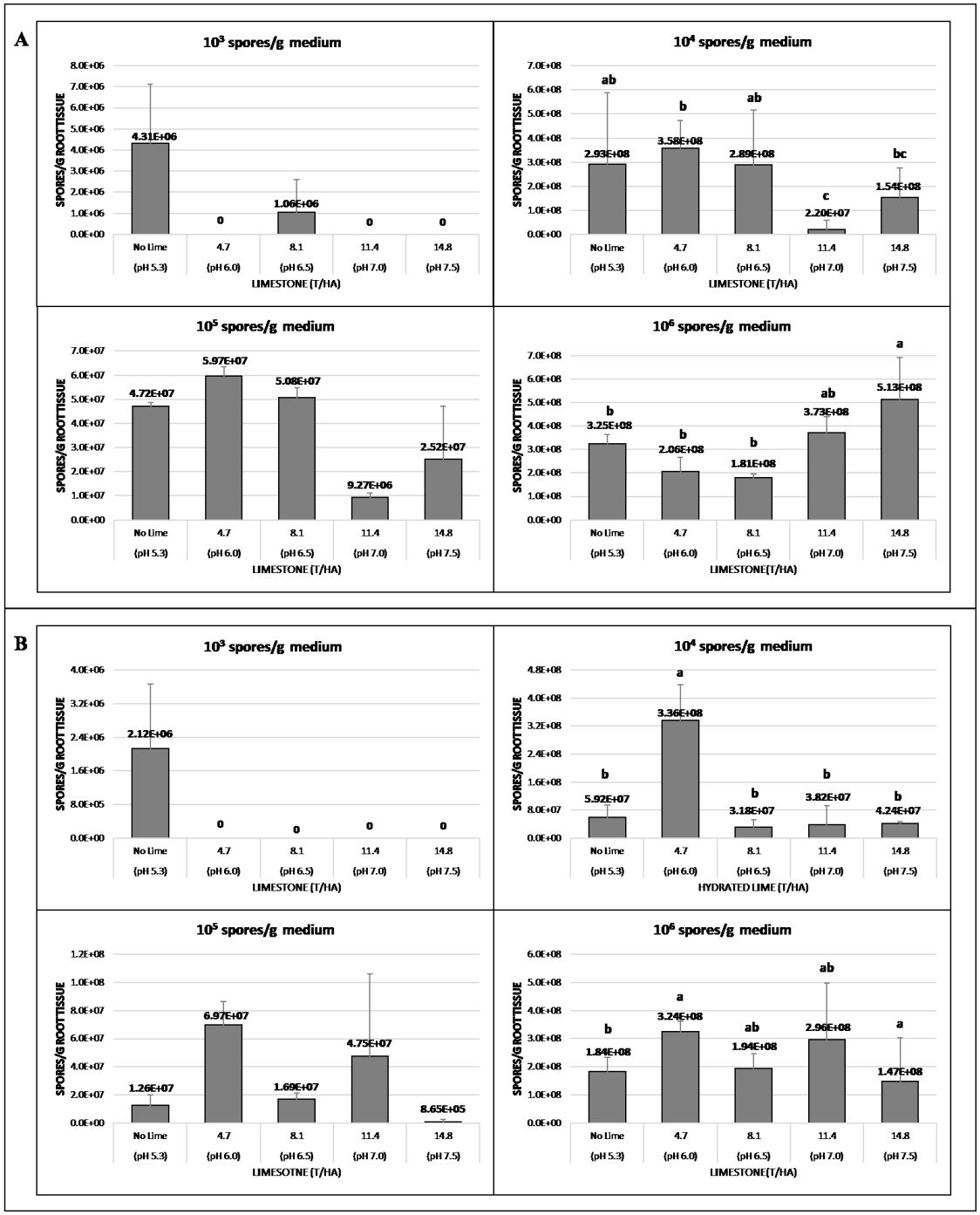

Figure 2.5. Amount of *Plasmodiophora brassicae* in the roots of 10-day-old seedlings of the canola cultivars 45H31 (panel A) and 9558C (panel B) as determined by quantitative PCR analysis. The seedlings were grown under greenhouse conditions in potting medium inoculated with 1×10^3, 1×10^4, 1×10^5 and 1×10^6 resting spores g^{-1} potting medium and treated with 4.7, 8.1, 11.4 or 14.8 T ha^{-1} of limestone which targeted a pH of 6.0, 6.5, 7.0 or 7.5, respectively. Controls did not receive any lime (pH 5.3). These results are from the second run of this experiment in 2018. Means with no letters do not differ; means differences are denoted by different letters at $P < 0.05$.

Chapter 3: Conclusions

3.1 General Conclusions

In Alberta, clubroot is managed primarily by the deployment of genetically resistant canola cultivars. Reliance solely on resistant cultivars, however, has increased the selection pressure exerted on *P. brassicae* populations, resulting in pathotype shifts and the erosion or loss of resistance in many fields (Strelkov *et al*., 2016; Strelkov *et al*., 2018). Indeed, repeated exposure of a resistance source to the same inoculum can lead to an erosion of the effectiveness of that resistance (LeBoldus *et al*., 2012). Many clubroot resistant (CR) cultivars also are not completely immune to clubroot, and may develop some symptoms of the disease at high *P. brassicae* inoculum concentrations (>10^7 spores g^{-1} soil) (Hwang *et al*., 2011a; Peng *et al*., 2011b); such concentrations are sometimes found in fields in central Alberta (Hwang *et al*., 2014). While proliferation of *P. brassicae* is reduced in CR cultivars, infection of these cultivars can contribute new resting spores to the soil, resulting in increases in the spore concentration (Hwang *et al*., 2011a; Ernst *et al*., 2019). Resting spores produced on a resistant host are presumably enriched for pathotypes capable of overcoming that resistance (Ernst *et al*., 2019). Furthermore, the presence of off-types, incomplete resistance, susceptible weeds and canola volunteers may offset the benefits of growing a resistant cultivar or completing a crop rotation (Hwang *et al*., 2011a). Therefore, an integrated management strategy is important for maintaining the effectiveness and longevity of genetic resistance. Clubroot management strategies that reduce or at least maintain resting spores at low levels will lessen inoculum pressure and infection, and hence also reduce the chances for pathotype shifts contributing to the stewardship of resistant cultivars (Diederichsen *et al*., 2003; Peng *et al*., 2011b, 2013, 2015).

Clubroot development is favored by acidic soils and high moisture (Karling, 1968). At a pH ≥ 7.2, the severity of the disease has been observed to be substantially reduced or eliminated (Donald and Porter, 2009). Webster and Dixon (1991) investigated the effects of pH on primary (root-hair) infection and clubroot development in the absence and presence of calcium, and observed both independent and synergistic effects of pH and calcium on the life-cycle of *P. brassicae* (Webster, 1986). An increase in soil pH and calcium has been hypothesized to inhibit resting spore germination, but effects on the ability of *P. brassicae* to respond to root exudates in the rhizosphere, or changes in the quality and quantity of the exudates themselves, have also been suggested (Niwa *et al*., 2008). The increase in soil pH may also affect the rate of production of primary zoospores in infected host tissue (Bochow, 1961), and suppress zoospore invasion, zoosporangial maturation and clubroot symptom formation (Niwa et al., 2008).

The importance of pH and calcium in clubroot development underscores the potential benefits of liming as a clubroot management strategy. With over 400 000 hectares of strongly acidic (pH of 5.1 to 5.5) soil and 1.8 million hectares of moderately acidic (pH of 5.6 to 6.0) soil farmed in Alberta (Government of Alberta, 2002), liming should already be incorporated into many farm management plans to improve plant growing conditions. Given the evidence suggesting that liming can also help manage clubroot, there is more reason to consider lime application in these acidic soils. In the field experiments conducted as part of this dissertation, clubroot severity on susceptible canola was reduced with increasing lime in one year, but no effect was observed in the second season. Inconsistent results also were reported previously by Murakami *et al*. (2002), who noted the importance of the time elapsed between the application of lime and the sowing of the crop. Rainfall during this interval also may be an important factor influencing disease development, since moisture is needed to break down the lime in order to

increase soil pH. Several studies have shown that the alkaline environment is required 0-7 days after inoculation in order to have an effect on root hair infection and subsequent clubroot symptom expression (Webster, 1986; Webster and Dixon, 1991; Donald and Porter, 2009). Nonetheless, when timing and application technique are correct and *P. brassicae* is present, excellent disease control can be achieved.

The greenhouse experiments presented in this thesis further underscored the importance of factors such as cultivar, lime product and inoculum concentration on the effectiveness of liming. While hydrated lime gave better control relative to limestone, phytotoxic effects may be observed on canola plants depending on the cultivar used, or if disease severity is not high enough to see the added benefits in above-ground plant health. However, the observed phytotoxic effect in this greenhouse study was likely due to small pot size creating a concentrate of lime in a small area, and is likely to not be observed under field conditions. In an earlier study to evaluate the effects of lime products beyond disease control, it was found that hydrated lime was the most effective at neutralizing soil acidity (Li *et al*., 2018). Nonetheless, in the present work, limestone did show some promise when inoculum concentrations or viability were lower. The rate of lime became a significant factor when root colonization by *P. brassicae* was measured by q-PCR analysis, with results generally consistent with observed symptom severity.

When applied correctly, liming can suppress the pathogenicity of inoculum, enhance host resistance and affect the longevity and germination of resting spores in the soil (Webster and Dixon, 1991; Dixon, 2014). This thesis provides important knowledge regarding the potential effectiveness of lime under field conditions in Alberta, differences in the efficacy of hydrated lime vs. limestone, and the influence of inoculum levels on the effects of lime. It is clear from this and earlier studies that the effectiveness of lime for clubroot management can vary and it is

not sufficient to base recommendations simply on lime product or initial soil pH. Inoculum concentration and viability must also be considered in order to achieve satisfactory levels of disease control. Moreover, while hydrated lime appears to provide superior clubroot control, at a cost of about \$320 tonne^{-1} it is considerably more expensive than limestone (\$54 tonne^{-1}). In fields that are only mildly infested with *P. brassicae*, the application of limestone may be sufficient as a tool to manage the pathogen. Liming an entire field may never be economical (Donald and Porter, 2009; Hwang *et al*., 2011b), but the application of lime to *P. brassicae*-infested patches in a field, such as hot spots and field entrances, could be an important strategy for clubroot management in canola. An integrated approach that includes liming may reduce disease pressure, pathotype selection pressure and resting spore density, which in turn will contribute to more sustainable clubroot management (Hwang *et al*., 2012b; Deora *et al*., 2011; Gossen *et al*., 2013, 2015).

3.2 Future studies and questions

Liming of soils to manage clubroot can be an effective strategy. This thesis provided examples of when liming is effective and not effective for clubroot control. Moreover, the results showed the significance of inoculum concentration and its viability when choosing a lime product and rate to apply. In the field, there are many factors that affect the efficacy of liming. Future studies need to consider multiple seeding dates after lime application, since the efficacy of treatments may vary depending on the lime product used and amount of rain received. The effect of lime on root colonization in the greenhouse could be used as a baseline for similar comparisons under field conditions. Monitoring of resting spore concentrations in the soil would also provide important information on the impact of liming on soil inoculum loads. Time-courses

examining proliferation of *P. brassicae* in infected roots at multiple points following lime treatment may also help to understand their impact on disease development.

The control of clubroot achieved in the greenhouse with limestone application at the two lowest inoculum concentrations needs further confirmation in the field. Limestone is a slower releasing product, so perhaps the interval between application and seeding would be different from that of hydrated lime. There was a slight phytotoxic effect of hydrated lime application observed on the moderately resistant cultivar under greenhouse conditions, so I would recommend repeating the trial in the field with a moderately resistant to resistant cultivar; this would help to determine if this was a true effect, or an artefact from the small pot sizes used in the greenhouse study. Given the variability observed when using different inoculum sources (i.e., less virulent collections), it may be worthwhile to directly evaluate the effect of resting spore viability/strength on the effectiveness of lime. This test could be as simple as using a blender to test the concentration of inoculum after adding 50 g of galls to 1 L of water and comparing it with standards tested in bioassays.

Multiple intervals between lime application and seeding date should be included in any assessments of new lime products. These products should also be tested at varying rates across multiple inoculum concentrations and, if possible, with different sources of inoculum. These parameters will all influence the efficacy of lime treatments in reducing clubroot severity in canola and other crucifers.

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