Evaluation of *Plasmodiophora brassicae* for the occurrence of pH insensitive isolates

by

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Abstract

Clubroot (*Plasmodiophora brassicae*) is a serious soilborne disease of canola (*Brassica napus*), negatively affecting the Canadian agricultural sector. Since clubroot development is favored in acidic soils, the application of lime to increase soil pH to ≥ 7.2 is recommended as a disease management strategy. It is unknown, however, whether there is differential sensitivity to pH in *P. brassicae* isolates. A replicated greenhouse experiment was conducted in which a clubroot-susceptible canola genotype was grown at pH 6.3, 7.2 and 8.0 in a soil mix inoculated with each of five field isolates representing various pathotypes of *P. brassicae*. While clubroot symptoms were severe across isolates at pH 6.3 under both medium and high inoculum densities, results at pH 7.2 were more variable, with milder disease severity occasionally observed. One isolate (L-G2) appeared particularly sensitive to pH 7.2, causing lower levels of disease relative to the other isolates. Only trace symptoms of clubroot developed at pH 8.0, and only at the high inoculum density. In a follow-up experiment, three of the previous five isolates were tested on the same clubroot-susceptible canola genotype at pH 6.3, 7.0, 7.3, 7.6 and 7.9. Clubroot was severe at pH 6.3, 7.0 and 7.3, dropping significantly at pH 7.6 and again at pH 7.9, regardless of the isolate. An *in vitro* study of *P. brassicae* resting spore germination at pH 6.0, 6.5, 7.0, 7.5 and 8.0 indicated that germination rates were generally similar at pH 6.0 to 7.5, but very low at pH 8.0, suggesting that some of the reduction in clubroot observed in the greenhouse studies at pH 8.0 could reflect reduced germination. Collectively, the results indicate that there is some variability in the pH sensitivity of *P. brassicae* isolates, and that targeting a pH ≥ 7.2 may not always be sufficient for clubroot management.

Preface

This thesis is an original work by me, Marla Roth. I conducted all of the experiments included in the dissertation and wrote the first drafts of each chapter; these were edited by Dr. Stephen Strelkov who provided revisions and other suggestions to improve the quality and clarity of each section. Dr. Strelkov and Dr. Sheau-Fang Hwang provided advice and direction throughout every aspect of this project. I received support from Dr. Victor Manolii, a technician in the Plant Pathology Lab, who helped to sow seeds, inoculate plants, evaluate disease severity, and autoclave biohazardous material upon finishing the greenhouse experiments. Along with Dr. Manolii, many undergraduate and graduate students from the University of Alberta Plant Pathology Lab assisted with these same tasks. Ileana Strelkov provided training on wet lab tasks that allowed me to execute the PCR testing and *in vitro* experiments successfully. Longfei Wu provided statistical support during the analyses of the experimental results. No part of this thesis has yet been published, but preliminary results were presented at various scientific conferences and student events throughout my graduate program.

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Table of Contents

Chapter 1: Introduction 1

1.1 Clubroot disease 1

1.2 Clubroot in Canada and Alberta 2

1.3 Soil liming and pH 3

1.4 Hypothesis and objectives 4

Chapter 2: Literature review 5

2.1 Biology of *Plasmodiophora brassicae* 5

2.1.1 Taxonomy 5

2.1.2 Life Cycle 5

2.1.3 Host range 7

2.1.4 Pathotypes of *P. brassicae* in Canada 8

2.1.5 Influence of pH on *P. brassicae* and clubroot development 9

2.2 Clubroot management 10

2.2.1 Manipulation of environmental conditions 10

2.2.2 Sanitizing equipment 11

2.2.3 Biological control 12

2.2.4 Manipulating the seeding date 14

2.2.5 Crop rotation 15

2.2.6 Bait cropping 16

2.2.7 Fumigants and fungicides 18

2.2.8 Resistant cultivars 19

2.2.9 Soil liming 21

2.3 Conclusions 24

Chapter 3: Evaluation of *Plasmodiophora brassicae* isolates for pH sensitivity 25

3.1 Introduction 25

3.2 Materials and methods 27

3.2.1 Pathogen material 27

3.2.2 Greenhouse experiments 28

3.2.3 Disease assessment and plant growth parameters 30
3.2.4 Resting spore germination *in vitro* 30
3.2.5 Statistical analysis 32
3.3 Results 33
3.3.1 Experiment 1 33
3.3.2 Experiment 2 37
3.3.3 Resting spore germination 39
3.4 Discussion 49

Chapter 4: Conclusions and future research 63
4.1 Conclusions 63
4.2 Future research 65

Literature cited 67

Appendix 83

List of tables

Table 3.1 Summary of treatments included in Experiment 1, assessing the pH sensitivity of *Plasmodiophora brassicae* isolates representing pathotypes 3A, 3D, 3H, 5G and 5X 46

Table 3.2 Summary of treatments included in Experiment 2, characterizing the pH sensitivity of *Plasmodiophora brassicae* isolates representing pathotypes 3A, 3D, and 5G more precisely 47

Table 3.3 Linear mixed effects model for Experiment 1 Trial 2 to evaluate the effects of three pH treatments on mean clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of five isolates of *Plasmodiophora brassicae* .48

Table 3.4 Clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of five isolates of *Plasmodiophora brassicae* at two resting spore densities and three different pH values for Experiment 1, Trial 2 49

Table 3.5 Linear mixed effects model for Experiment 1, Trial 3 to evaluate the effects of three pH treatments on mean clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of five isolates of *Plasmodiophora brassicae* .52

Table 3.6 Clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of five isolates of *Plasmodiophora brassicae* at two resting spore densities and three different pH values for Experiment 1, Trial 3 53

Table 3.7 Linear mixed effects model for Experiment 2 Trial 1 to evaluate the effects of five pH levels on mean clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of three isolates of *Plasmodiophora brassicae*....56

Table 3.8 Clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of three isolates of *Plasmodiophora brassicae* at five different pH values for Experiment 2, Trial 1...57

Table 3.9 Linear mixed effects model for Experiment 2 Trial 2 to evaluate the effects of five pH levels on mean clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of three isolates of *Plasmodiophora brassicae*....60

Table 3.10 Clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of three isolates of *Plasmodiophora brassicae* at five different pH values for Experiment 2, Trial 2 61

Table 3.11 Two-way ANOVA for an *in vitro* assessment of the effects of pH and day on percentage resting spore germination for each of five isolates of *Plasmodiophora brassicae* (Trial 1) 63

Table 3.12 Two-way ANOVA for an *in vitro* assessment of the effects of pH and day on percentage resting spore germination for each of five isolates of *Plasmodiophora brassicae* (Trial 2) 63

List of figures

Figure 3.1 Clubroot disease severity index (DSI) of canola plants following inoculation with each of five isolates of *Plasmodiophora brassicae* at two resting spore densities (‘med’ = 5×10^5 resting spores/mL; ‘high’ = 1×10^7 resting spores/mL) and grown in a soil mix at pH 6.3 or pH 7.0 for Experiment 1, Trial 2 64

Figure 3.2 Clubroot disease severity index (DSI) of canola plants following inoculation with each of five isolates of *Plasmodiophora brassicae* at two resting spore densities (‘med’ = 5×10^5 resting spores/mL; ‘high’ = 1×10^7 resting spores/mL) and grown in a soil mix at pH 6.3 or pH 7.0 for Experiment 1, Trial 3 65

Figure 3.3 Percentage germinated resting spores of each of five isolates of *Plasmodiophora brassicae* over a six-day period in a modified Hoagland’s solution with pH adjusted to 6.0, 6.5, 7.0, 7.5, or 8.0 with Tris-base (Trial 1). Germination is expressed as the proportion of germinated resting spores out of all resting spores visually assessed microscopically on each day 66

Figure 3.4 Percentage germinated resting spores of each of five isolates of *Plasmodiophora brassicae* over a six-day period in a modified Hoagland’s solution with pH adjusted to 6.0, 6.5, 7.0, 7.5, or 8.0 with Tris-base (Trial 2). Germination is expressed as the proportion of germinated resting spores out of all resting spores visually assessed microscopically on each day 67

Chapter 1: Introduction

1.1 Clubroot disease

Clubroot [*Plasmodiophora brassicae* Woronin] is a serious soilborne disease of the Brassicaceae family found in many countries where cruciferous crops are grown (Dixon 2009a; Rahman et al. 2014). Symptoms associated with clubroot development include stunted growth, wilting, premature ripening, reduced seed yield and quality and, as its name suggests, hyperplasic and hypertrophic club-shaped root galls (Howard et al. 2010). These characteristic clubbed roots hinder water and nutrient uptake to aboveground plant tissue and are largely responsible for the reduced yield and quality associated with infected crops. Pathogen inoculum is released from the clubbed roots upon their decay, allowing for infection of subsequent crops grown in the same soil (Hwang et al. 2012).

Clubroot originated as an Old-World disease, with its occurrence on crops noted during the 13th century, and possibly even earlier in the Roman era (Dixon 2009b). In the 1800s, the Russian biologist M.S. Woronin (1878) studied *P. brassicae* in St. Petersburg, Russia, and determined it to be the cause of clubroot disease. In Europe, crucifers such as cabbage, cauliflower, and turnips were all known to be affected by *P. brassicae*, and the pathogen was eventually transferred to the New World through animal fodder containing infected swedes and turnips (Dixon 2009b; Howard et al. 2010). It is believed that *P. brassicae* reached China and Japan when oilseed rape was transferred from Europe (Dixon 2009b). Clubroot becomes problematic in agricultural settings in which the intense cultivation of *Brassica* spp. and their close relatives provides the appropriate conditions for the pathogen to thrive in the absence of predation, hence its label as a “disease of cultivation” (Dixon 2009b; Feng et al. 2014).

1.2 Clubroot in Canada and Alberta

European settlers likely introduced clubroot of crucifers to Canada through infested fodder brought overseas for livestock (Dixon 2009b; Howard et al. 2010). The first published records of clubroot occurring in Canada are from the 1920s, beginning in British Columbia, Quebec, and the Atlantic provinces (Howard et al. 2010). By the late 20th century, clubroot was endemic to many areas of Canada, specifically those with moist, compacted, acidic soils low in organic matter (McDonald et al. 2004; Howard et al. 2010; Gossen et al. 2016). However, despite the widespread cultivation of canola (*Brassica napu*s L.) in the Prairie Provinces, reports of clubroot in this region were sporadic and the disease was usually restricted to home or market gardens (Howard et al. 2010). In 2003, however, clubroot was identified for the first time on the Prairie canola crop, when a dozen infested fields were recorded in central Alberta, near Edmonton (Tewari et al. 2005). This finding caused concern for growers and the canola industry in general, as the crop contributes $7.13 billion CAD annually to the provincial economy (Canola Council of Canada 2017).

Given the potential impact of clubroot on canola production, annual surveys were initiated to monitor the occurrence and spread of the disease across Alberta. These surveillance activities have led to confirmation of the disease in 3353 fields in the province (Strelkov et al. 2020). While the clubroot outbreak is still most severe in central Alberta, the disease is now found in 44 of 66 counties or municipal districts across the province where canola is grown (Strelkov et al. 2021). Clubroot is also found with increasing frequency in Saskatchewan, Manitoba and North Dakota (Dokken-Bouchard et al. 2012; Chittem et al. 2014; Gossen et al. 2015; Froese et al. 2019), suggesting its spread across the Northern Great Plains. This increased distribution reflects the movement of *P. brassicae*-infested soil and water (Donald and Porter 2009; Rennie et al. 2015). Attempts to slow down this spread such by making *P. brassicae* a Declared Pest under the Alberta

Agricultural Pests Act (Government of Alberta 2022) have met with limited success. Clubroot-resistant (CR) canola cultivars, which first became available in 2009, collectively represent one of the most important clubroot management tools (Strelkov et al. 2018). When effective, genetic resistance provides excellent control in an environmentally friendly and affordable manner. However, the deployment of CR cultivars also can exert significant selection pressure on pathogen populations (Strelkov et al. 2011), resulting in shifts in virulence and the emergence of *P. brassicae* strains capable of overcoming resistance (Rahman et al. 2014; Peng 2019; Hollman et al. 2021). As such, additional clubroot management strategies should be considered and used together as part of an integrated disease management plan.

1.3 Soil liming and pH

One of the oldest methods to manage clubroot is to apply lime to the soil to increase its pH (Colhoun 1953; Palm 1957; Dixon 2009a). The development of clubroot is favored in acidic soils, and hence increasing the soil pH can reduce symptom severity (Samuel and Garrett 1945; Macfarlane 1958). For example, a recent study in Alberta found that hydrated lime could be an effective tool for managing clubroot in canola, when optimal timing and rainfall are achieved (Fox et al. 2022). Liming and the general approach of increasing soil alkalinity for mitigating clubroot is based on the assumption that all strains of *P. brassicae* are sensitive to higher pH and respond in a similar way to pH changes. However, the continued evolution of *P. brassicae* in various environments warrants further study, to determine whether particular isolates or strains of the pathogen are less sensitive to alkalinity than others are. There is some evidence to suggest that this might be the case. For example, severe symptoms of clubroot were identified in a canola crop in Newell County, in southern Alberta (Strelkov et al. 2008), in a field with a soil pH of 7.8; such a high pH would generally be regarded as unfavorable for disease development. Similarly, the shifts

in the virulence of *P. brassicae* populations, which have been well-documented in western Canada (Strelkov et al. 2016) and other regions, also suggest adaptive potential in the pathogen, which may also apply to pH sensitivity.

1.4 Hypothesis and objectives

This aim of this research was to test the hypothesis that there is differential sensitivity to pH among field isolates of *P. brassicae* representing different strains and originating from different fields. The specific objectives were two-fold: (1) to compare the pH sensitivity of five isolates of *P. brassicae*, including one originating from a field with higher pH, under greenhouse conditions, and (2) to examine the effect of pH on *P. brassicae* resting spore germination rates *in vitro*, to determine whether there is any direct impact of pH on the spores. An understanding of the response to pH in *P. brassicae* will help to determine the effectiveness of soil liming as a tool in an integrated clubroot management plan.

Chapter 2: Literature review

2.1 Biology of *Plasmodiophora brassicae*

2.1.1 Taxonomy

Early researchers classified *Plasmodiophora brassicae* as a fungus (Kunkel 1918; Ayers 1944; Bryngelsson et al. 1988), but current scientific work and expertise has evolved the knowledge of *P. brassicae* to place it in its correct taxon, the eukaryotic subgroup Rhizaria. More specifically, it is a biotrophic protist within the phylum Plasmodiophoromycota (Braselton 1995; Schwelm et al. 2015). Plasmodiophorids commonly parasitize plants and some can serve as vectors of serious plant viruses, such as the potato mop top virus vectored by *Spongospora subterranea*, a close relative of *P. brassicae* that causes powdery scab of potato(Braselton 1995; Schwelm et al. 2015). Characteristics of the Plasmodiophorids include: (1) biflagellate zoospores released from haploid resting spores; (2) multinucleate plasmodia; (3) resting spores highly resistant to harsh environments; and (4) intracellular parasitism (Braselton 1995; Schwelm et al. 2015). The resting spores can survive in the soil for long periods; for *P. brassicae* spores, Wallenhammar (1996) reported a maximum life of 17 years in soil, with a predicted half-life of 3.6 years.

2.1.2 Life cycle

The life cycle of *P. brassicae* includes three main stages: (1) survival in the soil as resting spores, (2) germination of the resting spores to produce zoospores followed by infection of host root hairs, and (3) cortical root infection, which is associated with gall development and the formation of a new generation of resting spores (Dixon 2009b; Howard et al. 2010). These resting spores are released into the soil as the root galls decay, serving as inoculum for future infections (Macfarlane 1970; Kageyama and Asano 2009). Each gall potentially releases billions of viable

resting spores (Hwang et al. 2015), each ~3 to 4 μm in diameter (Macfarlane 1970; Buczacki and Cadd 1976). When environmental conditions conducive to clubroot development are in place, such as adequate soil moisture and air temperatures > 10°C (Colhoun 1953; Macfarlane 1970; Gossen et al. 2014), the resting spores germinate to initiate a new cycle of infection. Host root exudates have been suggested to increase resting spore germination rates (Macfarlane 1970; Friberg et al. 2005). Soil inoculum loads dictate the severity of infection, since there is a positive correlation between soil resting spore concentrations and disease severity index (Colhoun 1953; Naiki et al. 1978; Hwang et al. 2011a).

During germination, a biflagellate primary zoospore with two flagella of unequal lengths is released from individual resting spores (Ayers 1944; Kageyama and Asano 2009). The zoospore swims through water films in the soil to reach the cell wall of a root hair, upon which it will first encyst, and then penetrate; this stage is known as primary infection (Kageyama and Asano 2009; Howard et al. 2010; Dixon 2014). The primary infection stage occurs in all hosts and some non-host species; in the latter, the disease will not progress further, thereby potentially helping to deplete soil inoculum loads (Ren et al. 2016). Zoospores cannot survive long in the soil and therefore require penetration of a host root quickly. Once inside the root hairs, primary plasmodia develop and eventually form zoosporangia, from which secondary zoospores are released back into the soil. The secondary zoospores, which are morphologically identical to the primary zoospores, re-infect the host, entering the root cortical issue; this is known as secondary infection (Kageyama and Asano 2009; Howard et al. 2010). Inside the cortex, secondary plasmodia develop intracellularly, accompanied by hypertrophy and hyperplasia of the host root tissues, resulting in the typical 'clubs' or galls associated with clubroot disease. Eventually, the multi-nucleate secondary plasmodia cleave to form millions of resting spores, which are released back into the

soil as the infected roots decompose (Dixon 2009b; Kageyama and Asano 2009; Howard et al. 2010).

The continuous cultivation of susceptible hosts results in significant levels of resting spores in clubroot-infested fields (Hwang et al. 2011a). In contrast, rotations with non-host plants can significantly reduce resting spore loads (Friberg et al. 2006). Two to four years out of a susceptible host is generally believed to be sufficient for reducing the inoculum density below what is required for severe infestation (Wallenhammar 1996; Peng et al. 2014). However, the severity of the initial infestation will affect the length of break required, and in severely infested fields, breaks of 5-years or more may be needed (Howard et al. 2010).

2.1.3 Host range

Plasmodiophora brassicae infection is of primary concern for plants in the family Brassicaceae. While all members of this family are potential hosts of *P. brassicae*, most research has focused on the genera *Brassica*, *Raphanus*, and *Arabidopsis* (Dixon 2009b; Howard et al. 2010). Commercially important host species include Brussels spouts (*Brassica oleracea* var. *gemmifera*), cabbage (*B. oleracea* var. *capitata*), broccoli (*B. oleracea* var. *italica*), cauliflower (*B. oleracea* var. *botrytis*), kale (*B. oleracea* var. *acephala*), kohlrabi (*B. oleracea* var. *gongylodes*), canola or oilseed rape (*B. napus*), rutabaga (*B. napus* var. *rapifera*), turnips (*B. rapa* var. *rapa*), Chinese cabbage (*B. rapa* var. *pekinensis*), and mustards such as *Sinapis* spp. (Dixon 2009b; Howard et al. 2010). Cruciferous weeds endemic to the Canadian prairies are also potential hosts and may help to maintain *P. brassicae* inoculum levels in the soil (Hennig et al. 2022); as such, the removal of these plants in and around fields is recommended (Donald and Porter 2009). Common weeds that may be susceptible to clubroot include wild mustard (*Brassica kaber*), shepherd's purse (*Capsella bursa-pastoris*), camelina/false flax (*Camelina sativa*), stinkweed

(*Thlaspi arvense*), and white mustard (*Brassica hirta*) (Howard et al. 2010). Nonetheless, the susceptibility of specific ecotypes of different weed species can vary (Buczacki and Ockendon 1979), suggesting that genetics play a large role in individual susceptibility to clubroot. Ren et al. (2016) also studied cruciferous weeds and ornamentals in China and reported that all cruciferous weeds studied developed disease, but one ornamental, hoary stock (*Matthiola incana*), provided interesting results. Hoary stock developed primary infection but not secondary infection, implying that it is resistant to *P. brassicae.* Primary infection alone has also been shown to take place in several non-crucifers, including Indian cress (*Tropaeolum majus*), papaya (*Carica papaya*) (Ludwig-Müller et al. 1999), red clover (*Trifolium pratense*) and perennial ryegrass (*Lolium perenne*) (Howard et al. 2010).

2.1.4 Pathotypes of *P. brassicae* in Canada

Plasmodiophora brassicae exhibits physiologic specialization (Honig 1931; Karling 1968) and includes many different races or pathotypes. These pathotypes are distinguished by their virulence patterns on groups of host genotypes, referred to as 'differential sets' in the field of plant pathology. Many differential sets have been used to identify pathotypes of *P. brassicae*. These include the differentials of Williams (1966), Somé et al. (1996), and the European Clubroot Differential (ECD) set (Buczacki et al. 1975). All three systems have been commonly used in Canada to characterize *P. brassicae* populations (Strelkov and Hwang 2014). Pathotype 3, as classified on the differentials of Williams (1966), is prevalent in canola in Alberta and Saskatchewan (Strelkov et al. 2006; Xue et al. 2008; Cao et al. 2009; Hollman et al. 2021), although multiple other pathotypes, including pathotypes 2, 5, 6 and 8, were also reported from Alberta and elsewhere. While effective, the differentials of Williams (1966) could not distinguish isolates of *P. brassicae* with differential virulence on clubroot resistant (CR) canola cultivars

(Strelkov et al. 2016). This led to the development of a new system, known as the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018), which could more accurately reflect the virulence profiles of the clubroot pathogen from Canadian canola. In the CCD system, pathotype designations include a number to indicate the Williams' pathotype, followed by an uppercase letter denoting the CCD classification (Strelkov et al. 2018; Askarian et al. 2021). This way, multiple variants (e.g., 3A, 3B, etc.) of a single Williams' pathotype may be described. As will be described below, some of these pathotypes are capable of overcoming the resistance in many CR canola cultivars, while others are not.

2.1.5 Influence of pH on *P. brassicae* and clubroot development

Increases in soil calcium and alkalinity are known to inhibit *P. brassicae* and reduce clubroot severity, but the mechanism(s) by which this occurs is not fully understood. Studies have examined the effect of calcium and alkalinity on different stages of the pathogen life cycle, including the resting spore stage as well as during primary and secondary infection. Murakami et al. (2011) and Webster and Dixon (1991) noted that both calcium and pH must be elevated for a reduction in root galling. Macfarlane (1952) found that liming soils inhibited resting spore germination but the resting spores persisted, resulting in later infection. Donald and Porter (2004) analyzed resting spore germination in the presence of host root exudates against varying calcium levels and pH conditions. They reported that the effect of calcium on root hair infection was dependent on alkalinity: as pH increased, root hair infection and the effect of calcium decreased. At pH 5.5, for example, clubroot was inhibited only in the presence of high calcium, while at a pH of 8.0 the majority of root hairs were free of infection regardless of the calcium level. Similarly, Myers and Campbell (1985) and Webster and Dixon (1991) reported that primary infection was reduced at $\geq$ pH 7.2, and that while there was an inverse relationship between clubroot severity

and soil pH, a high pH on its own in the absence of calcium ions did not hinder infection. Suzuki et al. (1992) also reported that at low inoculum concentrations, 1 M calcium was slightly toxic to *P. brassicae* resting spores and reduced their viability. No studies, however, have compared the pH sensitivity of a collection of isolates representing different pathotypes or originating from different fields.

2.2 Clubroot management

2.2.1 Manipulation of environmental conditions

As indicated by the concept of the 'Disease Triangle', an appropriate combination of a suitable host, a favorable environment, and a virulent pathogen are required for the occurrence of plant disease (Stevens 1960). As such, changes to the environment to make conditions less favorable for *P. brassicae* may represent an important line of defense against clubroot development. Given the soilborne nature of this disease, soil type and soil conditions are particularly important in this respect.

Wallenhammar (1996) found that clay soils were most favorable for *P. brassicae* infection, followed by silt, fine sand, and coarse sand. Clays have a higher water holding capacity than other soil types and, given the requirement for water for resting spore germination and zoospore motility, clay would be expected to favor clubroot development. Wallenhammar (1996) also reported that a high average infestation occurred in soils with low humus (< 6%), suggesting that increases in organic matter content could help to combat the disease. In contrast, Gossen et al. (2016) found that the bulk density of the soil had a greater effect on clubroot severity than soil type, organic matter content, or pathotype. Bulk density serves as an indicator of soil compaction, and compacted soil promotes water logging and subsequent clubroot development (Dixon 2009a)

because poor drainage and high soil moisture promote the movement of zoospores in water films (Gossen et al. 2013). Furthermore, the reduced pore spaces likely facilitate the ability of zoospores to locate host roots (Gossen et al. 2013). It is likely that numerous factors may be at play, and that both soil type and bulk density, along with other factors that favor water retention, can influence clubroot development.

Soil nutrient levels are another consideration for plant disease development (De Corato 2020). Calcium has been shown to be beneficial at mitigating clubroot. Webster and Dixon (1991) found that increasing soil calcium levels via liming reduced *P. brassicae* infection and symptom severity. The type of calcium product added matters, however, and those products that raised both calcium and pH were the most effective. Boron, in the form of borax (boric acid), can inhibit both primary and secondary infection by *P. brassicae*, with its effectiveness increased at higher soil pH (Webster and Dixon 1991b). Boron has been reported to strengthen plant cell walls (Pollard et al. 1977; Loomis and Durst 1992), presumably aiding in defense against the clubroot pathogen. Nevertheless, Dixon (1996) questioned whether the element actually strengthens *Brassica* root cells, or if it somehow limits *P. brassicae* inoculum directly. Clearly, attempts to alter soil bulk density whilst manipulating soil nutrient levels could be challenging for growers, and additional work is required to translate basic knowledge of soil environmental conditions into practical management recommendations.

2.2.2 Sanitizing equipment

Given its soilborne nature, *P. brassicae* can spread in soil carried as a contaminant on farm and other machinery. In Alberta, clubroot was found most commonly at the field entrances (Cao et al. 2009), confirming that this is an important mechanism for pathogen dissemination (Cao et al. 2009; Strelkov et al. 2011; Gossen et al. 2013). Therefore, the most basic efforts to prevent the

spread of clubroot include sanitizing any equipment that will be entering a field, including machines such as tractors and tillers, and even footwear and other tools. Sanitization includes several steps, starting with scraping or knocking off large chunks of soil from machinery, followed by power washing down to the metal or rubber, and finally, for risk-averse farmers, disinfecting with a non-corrosive agent such as Spray Nine (a water-based alkaline cleaner containing proprietary ingredients) (Government of Alberta 2022). Due to time constraints given the short growing season on the Canadian prairies, growers often work long hours during seeding and harvesting and may neglect the sanitization of equipment to save time (Gossen et al. 2013). Efforts to educate growers and the surrounding community about clubroot prevention and the importance of sanitization could promote greater awareness regarding this mechanism of transmission. Ultimately, due-diligence should be practiced by all individuals entering a field, including farm workers, oil and gas industry personnel, construction companies, and recreational users (Government of Alberta 2021).

2.2.3 Biological control

While the chemical control of phytopathogens is widely practiced, increasing environmental awareness revolving around pesticides has generated interest in biological disease control (Whipps 1997). Some biological controls agents have been noted for their ability to inhibit clubroot. Gossen et al. (2016) analyzed the effects of two biofungicides, *Bacillus subtilis* (Serenade) and *Clonostachys rosea* (Prestop), against *P. brassicae* pathotypes 3 and 6 in four soil types. The authors determined that the efficacy of each biofungicide was affected by soil type, with neither product effective in a peat mix or in a minimally compacted soil-less mix. Possibly, the products did not make contact with the inoculum if the bulk density was too low. Chet (1990), as cited in Cheah and Page (1997), reported that *Trichoderma* spp. are commonly used to combat

a variety of soilborne pathogens. Indeed, Antônio dos Santos et al. (2017) studied the effects of both limestone and the biofungicide *Trichnoderma harzianum* to control clubroot on cauliflower and found that the limestone benefitted plant growth and reduced disease severity, while *T. harzianum* had no effect with or without lime. On Chinese cabbage grown in a *P. brassicae*-infested soil under greenhouse conditions, Cheah and Page (1997) determined that 17 of 25 *Trichoderma* isolates tested significantly decreased disease severity. The authors then selected 10 of the most effective isolates and evaluated them under field conditions, where nine isolates reduced clubbing while increasing aboveground plant biomass.

In a Polish study, Kurowski et al. (2015) reported that EM-1, a biofungicide containing lactic acid bacteria, phototrophic bacteria, actinomycetes, and other fungi and yeasts showed variable efficacy against clubroot on four crucifers grown in microplots in a 3-year study. Narisawa et al. (2005) studied *Heteroconium chaetospira*, a dematiaceous hyphomycete, and its effect on *P. brassicae* on Chinese cabbage. *H. chaetospira* subsists throughout the host root cortical cells, persists long-term, and is believed to be largely unaffected by other microorganisms outside of the root. In experiments under a variety of soil moisture and pH conditions, *H. chaetospira* was found to be effective at suppressing disease until soil moisture and resting spore density ($\geq 1 \times 10^6$ resting spores/g soil) become too high (Narisawa et al. 2005). The biocontrol agent reduced clubroot severity by 90 to 100% in soils with a pH of 5.5, 6.3 and 7.2, but only at spore densities of 1×10^4 and 1×10^5 resting/g soil. In fields with a high inoculum density, including many found in Alberta, it appears that biological may not be a feasible option for clubroot control. Based on the results of these studies, many factors affect the efficacy of biofungicides, including the soil characteristics and biocontrol agent. Furthermore, none of these studies examined how the addition of microorganisms to limit *P. brassicae* could potentially influence beneficial soil microbes.

2.2.4 Manipulating the seeding date

In severely infested fields, a management option as simple as manipulating the seeding date may influence clubroot severity, since *P. brassicae* resting spore germination appears to be temperature dependent (Buczacki et al. 1978; Thuma et al. 1983; Sharma et al. 2011; Gossen et al. 2012). Buczacki et al. (1978) found that a minimum average temperature of 19.5 °C was required for severe clubroot development on many Brassicas. Other studies also noted a positive correlation between temperature and clubroot symptoms, with a minimal amount of disease at 14 °C (Thuma et al. 1983). These observations suggest that early seeding of Brassicas, when temperatures are cooler, could help in reducing clubroot. In field trials conducted in Ontario, Gossen et al. (2012) found that clubroot development on Shanghai pak choy and Chinese cabbage was significantly reduced when these crops were seeded in May or September, when temperatures were < 17 °C, even at relatively high spore concentrations. In contrast, seeding in July, when the highest average temperatures occurred, resulted in the greatest disease severity, followed by seeding in June and August.

Similarly, Sharma et al. (2011) showed that temperature has a clear and consistent effect on clubroot development. In their study, 10-day-old Shanghai pak-choy seedlings (*B. rapa* subsp. *chinensis*) were inoculated with *P. brassicae* and then maintained at 10, 15, 20, 25 or 30 °C. The roots were assessed for clubroot symptom development every four days until 42 days after inoculation. No symptoms developed at any time when plants were kept at 10 °C, while the most severe symptoms and highest rates of cortical infection occurred at 25 °C. Plants maintained at 20 or 30 °C developed intermediate clubroot symptoms, while those kept at 15 °C developed only mild symptoms that were visible only later in the time course. Resting spore production was greatest in the roots of plants maintained at 20, 25, or 30 °C (Sharma et al. 2011), suggesting that

cool weather could also provide benefits for subsequent crop seasons by reducing the amount of inoculum added to the soil.

In another study, McDonald and Westerveld (2008) found that clubroot incidence and severity on Shanghai pak choy and Chinese cabbage were greatest in July and August and lowest in October. Both air and soil temperatures were positively correlated with disease on both crops. Cool weather (< 12 °C) shortly before harvest resulted in minimal symptoms, while temperatures of 20 to 22 °C resulted in the most severe symptoms. McDonald and Westerveld (2008) suggested that early or late cropping is beneficial for reducing clubroot on Asian Brassica crops, and that there may be benefits to any short-season Brassica including canola. Nonetheless, one potential concern associated with early or late seeding is the possibility of extreme cold weather, which could damage or kill crops.

2.2.5 Crop rotation

Crop rotation with non-host plants is a highly beneficial practice for clubroot management and reducing disease severity in subsequent crops. While resting spores remain in the soil until environmental factors are suitable and hosts present, without a host, they eventually degrade, and inoculum density is reduced (Peng et al. 2015). Even a reduction in inoculum density can alleviate infection (Macfarlane 1952). Peng et al. (2015) reported that a two-year break from susceptible canola resulted in a significant decrease in resting spore loads compared with a one-year break, while a four-year break was not significantly different from a two-year break. Similarly, in a study of resting spore dynamics in CR canola-cropping systems in Alberta, Ernst et al. (2019) also found a rapid decrease in soil resting spore concentrations in the soil after a two-year break from canola. Collectively, the studies of Peng et al. (2015) and Ernst et al. (2019), both of which were conducted in Canada, indicated a rapid initial drop in inoculum levels, after which resting spore numbers

stabilize with a small proportion of the spores persisting for a much longer period. These Canadian studies appear to contrast with earlier work from Europe, which suggested a linear decline in spore numbers over time and a spore half-life of 3.6 years (Wallenhammar 1996). This could reflect different conditions in Canada vs. Europe, or the fact that the spore levels in the Canadian studies were tracked by quantitative PCR, while in the earlier European study, inoculum viability was estimated based on host symptom development. Regardless, there is undoubtedly a benefit from longer rotations out of canola or other host crops in *P. brassicae*-infested fields; unfortunately, given the greater economic returns associated with canola and limited alternatives in many parts of the Prairies, short rotations are favorable to growers (Strelkov et al. 2011).

2.2.6 Bait cropping

Bait or decoy cropping is a simple concept that aims to reduce soil inoculum by planting host or non-host plants but not allowing *P. brassicae* to complete its life cycle (Ahmed et al. 2011). Resting spores of the pathogen germinate in the presence of the bait crop and complete primary infection of the root hairs. Host bait crops are then killed mechanically or with herbicide before completion of the pathogen life cycle, while the non-hosts may become infected but will not allow the life cycle to come to completion (Friberg et al. 2005; Kageyama and Asano 2009). In this way, bait cropping can limit the severity of clubroot infestation, by promoting germination of the resting spores where they cannot locate a suitable host (non-host plants), by preventing secondary infection (non-host plants), or by preventing (through ploughing or herbicide application) the pathogen from reaching a stage where resting spores can be released back into the soil (host plants). The basis of this strategy is to utilize plants to reduce resting spore loads while preventing the release of new inoculum.

The appropriate environmental conditions must be in place to induce germination of resting spores, including soil pH, temperature, humidity, and soil inorganic ions; however, it is also likely that compounds (root exudates) emitted from the roots further encourage germination (Macfarlane 1970; Suzuki et al. 1992). The reason that *P. brassicae* spore germination occurs in soils with non-host plants is that certain root exudates are likely present in species beyond crucifers (Suzuki et al. 1992; Hata et al. 2002); therefore, some non-hosts are potential bait crops. For example, Suzuki et al. (1992) characterized the same 'germination stimulating factor' in the root exudates of lettuce (*Lactuca sativa*) as in clubroot-susceptible and clubroot-resistant turnips. Moreover, Friberg et al. (2005) determined that perennial ryegrass (*Lolium perenne*) can induce resting spore germination in liquid culture, and Rashid et al. (2013) reported that the root exudate solution from perennial ryegrass stimulated resting spore germination more strongly than exudates from either Chinese cabbage or canola. Conversely, in a follow-up study by Friberg et al. (2006), the authors could not replicate their findings with the same bait crops, including perennial ryegrass, in field and greenhouse experiments. The authors suggested that the complexity of the soil environment (texture, chemistry, biotic interactions) might have hindered detection of the spore germination factor(s) by the pathogen. Ren et al. (2016) found that hoary stock (*Matthiola incana*), a crucifier, developed only primary infection in soil infested with *P. brassicae* and resting spores were not produced in the roots, suggesting its potential as a bait crop.

While the idea surrounding the efficacy of bait crops to manage clubroot seems plausible, experiments clearly demonstrate mixed success. One possible explanation for this is that in highly infested fields, the reduction in spore levels associated with bait cropping is not sufficient to cause a decline in disease levels, while in mildly infested soils, bait crops can reduce inoculum levels sufficiently (Naiki et al. 1978; Friberg et al. 2006; Ahmed et al. 2011). Further studies conducted

under field conditions with variable infestation rates may provide clarity regarding the full potential of bait cropping as a clubroot management strategy.

2.2.7 Fumigants and fungicides

In general, soil fumigation is costly and used only in localized areas to prevent the spread of resting spores or to eradicate spores in recently established areas, such as field entrances. An early study by Buczacki and White (1977) analyzed pots containing soil, *P. brassicae*, cabbage (*B. oleracea*) seedlings, and a variety of fumigants. Many fumigants greatly reduced the percentage of infection to 0 or 1%, namely Basamid, Chloropicrin, Ditrapex C.P, and Downfume M.C.z. The use of some fumigants, including formaldehyde (5%), Telone (2%), and Vapam (2%), still resulted in a mild level of disease, which was nonetheless considerably lower than in the controls. Hwang et al. (2014) studied the effects of varying levels of Vapam (metham sodium) in clubroot-infested soil and found that it greatly reduced primary and secondary infection in canola, leading to improved plant growth, so long as the application rate was not too high to cause phytotoxicity. An important consideration when working with Vapam, however, is that it is a non-selective toxin, potentially causing harm to the health of humans and other organisms if not used or applied properly (Hwang et al. 2014b). Some fumigants have been banned due to potential health and/or environmental damage (Donald and Porter 2009). While soil fumigants show some promise in reducing clubroot (Buczacki and White 1977; Hwang et al. 2014), their use over large areas to combat the disease is environmentally undesirable and not financially feasible.

Fungicide application is a management strategy that has occasionally been evaluated for clubroot management. Buczacki (1973) reported variable success with nine fungicides studied for clubroot control; the greatest reductions in percent infection and disease severity were obtained with benomyl, thiophanate, thiophanate-methyl and a formulation called NF 48. Furthermore,

some of these effective fungicides showed greater efficacy against populations of *P. brassicae* recovered from Brussels sprouts than from rape and *vice versa*, suggesting that fungicide application is not guaranteed to suppress all populations of the pathogen. Indeed, Tanaka et al. (1997) reported that the fungicides trichlamide, flusulfamide, and fluazinam were highly effective only against a weakly virulent population of *P. brassicae*. More recently, Hwang et al. (2012) studied five fungicides and their effects on clubroot disease severity in canola, including Dynasty 100 FS (azoxystrobin), Helix Xtra (thiamethoxam + difenoconazole + metataxyl + fludioxonil), Nebijin 5SC (flusulfamide), Prosper FX (clothianidin +), and Vitavax RS. The authors reported that all five fungicides were effective in reducing disease severity, but Dynasty 100 FS and Nebijin 5SC had the greatest efficacy in greenhouse experiments. The application of Cruiser resulted in the lowest disease severity and highest yields in a field with an inoculum load of 1×10^8 resting spores/g soil. The fungicides also increased seedling emergence, but only at one site. The mechanism(s) by which fungicides inhibit *P. brassicae* and subsequent gall development is unknown (Hwang et al. 2012), but Tanaka et al. (1999) postulated that flusulfamide prevents resting spore germination. Hwang et al. (2012) noted that resting spores of *P. brassicae* treated with Nebijin 5SC did not significantly suppress resting spore viability or disease severity. In summary, fungicide application for *P. brassicae* control has shown mixed success. Additionally, the cost of fungicides and the large acreages typically associated with canola production in western Canada likely preclude their widespread use (Hwang et al. 2014).

2.2.8 Resistant cultivars

The most effective and convenient (for growers) clubroot management strategy is the deployment of clubroot resistant (CR) canola cultivars (Rahman et al. 2014; Peng et al. 2015; Strelkov et al. 2018). The first CR cultivar on the Canadian market, '45H29', was released in 2009.

This was soon followed by other cultivars from various seed companies, and as of October 2021 there are eight companies offering 55 CR canola cultivars to choose from (Canola Council of Canada 2021). Clubroot resistance is highly effective against many pathotypes of *P. brassicae*, and resistance has allowed growers to continue planting canola even in heavily infested fields (Strelkov et al. 2018). Nonetheless, while the cropping of CR canola results in a significant reduction in resting spore loads compared with susceptible varieties, the latter still contribute to the release of resting spores (Hwang et al. 2011b; Ernst et al. 2019).

Since most, if not all, CR cultivars offer only single gene-based resistance (Diederichsen et al. 2009; Fredua-Agyeman et al. 2018), the loss of resistance may occur due to virulence shifts in *P. brassicae* populations (Hasan et al. 2012; Peng et al. 2014b; Strelkov et al. 2018). Increasing inoculum densities can also pose a risk to the effectiveness of resistance, particularly in plants with single gene resistance (Peng et al. 2019). Moreover, without knowledge of the existing pathotypes in a specific field, it may be difficult to select an appropriate CR variety (Cao et al. 2009). In greenhouse studies, repeated exposure to a pathogen population or single-spore isolate resulted in the rapid erosion of clubroot resistance in several host genotypes (Leboldus et al. 2012). Under field conditions, resistance-breakdown has been observed in a variety of CR Brassica crops, including canola or oilseed rape as well as cruciferous vegetables (Diederichsen et al. 2003; Piao et al. 2004; Strelkov et al. 2016). At present, clubroot resistance has been overcome in hundreds of fields across Alberta as well as at least one field in Manitoba, and the emergence of resistance-breaking pathotypes of *P. brassicae* represents one of the biggest challenges to sustainable canola production (Strelkov et al. 2018; Hollman et al. 2021). To improve the durability of CR canola cultivars, efforts must be made to incorporate multiple resistance genes in host genotypes rather than relying on a single major gene (Rahman et al. 2014).

Given the loss of resistance in many canola fields, it is clear that growers cannot rely on CR cultivars as their sole clubroot management strategy (Strelkov et al. 2016, 2018). The most sustainable control of clubroot is through the development of integrated disease management plans, where resistant varieties are coupled with other management strategies, such as the sanitization of equipment, longer rotations, changes in seeding date, and/or the application of lime (Donald and Porter 2009; Peng et al. 2014b; Strelkov et al. 2018).

2.2.9 Soil liming

The use of agricultural lime dates back to the Roman era, when it was applied to fields to support crop growth (Dix 1982). Today, lime is widely used to combat soil acidity, improve crop growth, improve soil quality, and increase crop tolerance to drought or wet conditions (Graymont 2019). In canola production systems, lime has been demonstrated to benefit soil structure and thereby plant productivity (Scott et al. 2003), and even to reduce soluble aluminum and manganese to levels below toxicity in acid soils (Canola Council of Canada 2020). Calcium, in the form of lime, can help to maintain the integrity of cell membranes and promote the uptake of various cations (Alam et al. 1999). In crops susceptible to clubroot, the most notable benefit of lime is its ability to suppress the disease by increasing soil alkalinity (Dobson et al. 1983; Myers and Campbell 1985).

While the application of lime to control clubroot has shown mixed success, lime can reduce resting spore density and primary root hair infection by zoospores, and thereby decrease disease severity (Myers and Campbell 1985; Murakami et al. 2002). Most research indicates that achieving a soil pH of ≥ 7.2 is ideal for minimizing clubroot development (Myers and Campbell 1985; Howard et al. 2010; Dobson et al. 1983; Webster and Dixon 1991a; Gossen et al. 2013), and that a pH of 8.0 can result in deformed pathogen structures and no clubbed roots (Myers and Campbell

1985). Myers and Campbell (1985) postulated that calcium and magnesium ions serve as the basis for limiting primary infection, since they found that alkaline soil in the absence of these ions did not provide protection against the disease. Murakami et al. (2011) suggested that alkalinity and calcium ions together suppress clubroot, while Suzuki et al. (1992) noted an inhibitory effect of ionic calcium specifically on resting spore germination. Macfarlane (1952) found that alkalinity (pH of 8.0) and low soil moisture prevented the spontaneous germination of resting spores, but only temporarily. Dobson et al. (1983) concluded that while it is unknown how lime works to limit clubroot, the interaction of calcium with other soil elements requires further study.

In a recent study conducted in Alberta, Fox et al. (2022) found that the application of hydrated lime to mitigate the impact of clubroot on canola under field conditions gave mixed results. In one of two years of their study, the application of hydrated lime reduced clubroot severity by 35-91%, while in the second year, no significant effect was observed (Fox et al. 2022). There are many potential reasons for the variable control of clubroot when using lime. Dobson et al. (1983) cited issues related to the amount of soil moisture, the length of the period between lime application and sowing, variation in soil types, and high inoculum loads. Liming of soils that are highly infested with resting spores can greatly limit its efficacy, especially under moisture and temperature regimes suitable for the disease (Colhoun 1953; Webster and Dixon 1991a). Soil type is another important consideration, since organic matter and cation exchange capacity can buffer lime, and water content is influenced by soil type. The particle size of lime and effectiveness of mixing are also important to consider in controlling disease (Dobson et al. 1983). Although a variety of lime products are available, generally speaking, the finer the particle size the more effective the product is at increasing pH and suppressing disease (Dobson et al. 1983; Howard et al. 2010; Fox et al. 2022). Small particles dissolve more easily, homogenize in the soil more

readily, and are better able to neutralize acidic cations than large-particle size products (Dobson et al. 1983). Furthermore, failure to mix lime evenly can create microsites of low pH, and as roots grow and transition through zones with high and low pH, they potentially contact acidic areas conducive to infection (Dobson et al. 1983). The timing of lime application and host exposure to the inoculum also matter. Webster and Dixon (1991a) found that the greatest reduction in clubroot symptoms occurred when the pH was 7.2 and host exposure to elevated calcium was within 0 to 14 days from contact with the inoculum. The best results occurred when hosts were exposed to the pathogen within 7 days under these conditions.

Soil pH ultimately affects many soil properties, including available nutrients and their uptake by plants (Alam et al. 1999). Adding lime to fields to combat clubroot may cause other issues, especially when the pH becomes very high. For example, high pH is known to cause ionic imbalances and nutrient deficiencies (Alam et al. 1999). A soil pH of 7.5 to 8.5 can limit phosphate uptake in plants (United States Department of Agriculture n.d.), and these high pH values may be necessary to control clubroot. In highly alkaline soils > 9.0, Rengasamy (n.d.) advised to plant tolerant species since nutrient toxicity and sodicity can occur. The addition of lime over an entire field can come at a high cost and require significant amounts of time and energy to incorporate properly into the soil (Ahmed et al. 2011). The cost of purchasing and incorporating lime in fields for clubroot control must be less than the cost associated with the yield losses caused by the disease itself if lime is to be a viable management tool.

Early research by Colhoun (1953) indicated that a variety of parameters affect the development of clubroot, such as temperature and moisture levels, and raising the pH to control clubroot is a complex process. Colhoun (1953) also suggested that given the diversity that could exist amongst *P. brassicae* populations, some isolates may differ in their pH sensitivity. Despite

these early suggestions, as of the 2020s (to our knowledge), no research had been conducted to compare the pH-sensitivity of *P. brassicae* isolates. If strains of the pathogen exist that exhibit a reduced sensitivity to higher pH, then liming of the soil may not be an effective clubroot management method in fields where such strains are present.

2.3 Conclusions

Given the rapid shifts in the virulence of *P. brassicae* populations in western Canada and the erosion of resistance in CR canola (Leboldus et al. 2012; Strelkov et al. 2018; Hollman et al. 2021), it is clear that other disease control methods need to be used in combination with genetic resistance as part of an integrated clubroot management plan. Given the interest in liming as another strategy for clubroot management, it is important to improve our knowledge of the pH sensitivity of *P. brassicae* populations from Alberta.

Chapter 3: Evaluation of *Plasmodiophora brassicae* isolates for pH sensitivity

3.1 Introduction

Plasmodiophora brassicae (Woronin) is a soilborne, obligate parasite causing clubroot of crucifers (Dixon 2009a). As its name suggests, disease symptoms include the development of club-shaped root galls, which interfere with water and nutrient uptake, resulting in stunted growth, wilting, premature ripening, and reduced seed yield and quality in affected plants (Howard et al. 2010). The pathogen survives in the soil as resting spores, which can persist for many years and serve as inoculum for future infections (Macfarlane 1970; Wallenhammar 1996). In western Canada, clubroot has emerged as one of the most important diseases of canola (oilseed rape; *Brassica napus* L.), a crop that contributes an estimated $30 billion CAD annually to the national economy (Canola Council of Canada 2021). The occurrence of *P. brassicae* has been documented in more than 3300 fields in the province of Alberta (Strelkov et al. 2020) and the pathogen occurs with increasing frequency on canola in Saskatchewan and Manitoba (Dokken-Bouchard et al. 2012; Gossen et al. 2015; Froese et al. 2019). In heavily infected canola crops, yield losses ranging from 30-100% have been reported (Strelkov and Hwang 2014).

In western Canada, clubroot is managed mainly by the deployment of clubroot-resistant (CR) canola cultivars (Peng et al. 2014a; Rahman et al. 2014), which first became available in 2009. These cultivars provide excellent resistance to many of the pathotypes of *P. brassicae* (Strelkov and Hwang 2014) and have enabled the continued cultivation of canola even in fields highly infested with the pathogen (Peng et al. 2014). Nonetheless, the virulence of *P. brassicae* populations can shift quickly in response to host selection pressure (Leboldus et al. 2012), and novel, resistance-breaking pathotypes of the pathogen were detected in canola fields in Alberta beginning in 2013 (Strelkov et al. 2016). Since then, pathotypes capable of overcoming resistance

have emerged in several hundred fields (Strelkov et al. 2018, 2021; Hollman et al. 2021), highlighting the need for integrated clubroot management plans that rely on multiple strategies for disease control. A number of such strategies have been suggested, including longer rotations out of canola in *P. brassicae*-infested fields, sanitization of farm and other machinery to remove pathogen inoculum, and liming of infested soil (Donald and Porter 2009; Strelkov et al. 2011).

Lime can suppress clubroot development by increasing soil pH, thereby creating an unfavorable environment for the pathogen (Macfarlane 1952; Dobson et al. 1983; Myers and Campbell 1985). A variety of lime products are available, including dolomite ($CaMg(CO_3)_2$), agricultural lime ($CaCO_3$), quick lime (CaO) and hydrated or slaked lime ($Ca(OH)_2$) (Murakami et al. 2002; Graymont 2019). Most research suggests that a soil pH of ≥ 7.2 is needed to achieve sufficient control of clubroot (Myers and Campbell 1985; Howard et al. 2010; Dobson et al. 1983; Webster and Dixon 1991a; Gossen et al. 2013), although the mechanism(s) by which lime reduces the disease are not fully established. Myers and Campbell (1985) found that alkaline soil in the absence of calcium and magnesium did not provide protection against clubroot, leading them to hypothesize that these ions are important for limiting primary infection. Suzuki et al. (1992) noted an inhibitory effect of ionic calcium specifically on resting spore germination, while Murakami et al. (2011) suggested that alkalinity and calcium ions together suppress clubroot. A recent study conducted in Alberta suggested that hydrated lime may be an effective tool for reducing clubroot in canola when optimal timing and rainfall are achieved (Fox et al. 2022), further increasing interest in lime as a management tool for clubroot.

Nevertheless, while a weak (but significant) negative correlation was found between clubroot severity and soil pH in *P. brassicae*-infested fields in Alberta, the occurrence of the disease on canola was not restricted to fields with acidic soils (Strelkov et al. 2007); clubroot was

identified in fields with pH levels ranging from 4.8 to 7.6. Studies under controlled growth conditions also indicated that moderate levels of clubroot could develop at pH values well above the optimum for *P. brassicae*, at least when moisture and temperature were suitable (Gossen et al. 2013). Given the shifts in virulence that have been documented in *P. brassicae* (LeBoldus et al. 2012; Strelkov et al. 2016) and the diversity that can occur in pathogen populations (Strelkov et al. 2011; Hollman et al. 2021), it is possible that there could also be isolates or strains of the pathogen less sensitive to alkalinity. While this suggestion was first made nearly 70 years ago (Colhoun, 1953), to our knowledge no studies have yet been carried out to compare the pH sensitivity of *P. brassicae* isolates. The occurrence of pathogen strains with reduced sensitivity to pH would affect the effectiveness of liming as a clubroot management strategy. In this context, the objectives of this study were (1) to compare the pH sensitivity of five isolates of *P. brassicae*, including one originating from a field with higher pH, and (2) to examine the effect of pH on *P. brassicae* resting spore germination rates *in vitro*, to determine whether there is any direct impact of pH on the spores.

3.2 Materials and methods

3.2.1 Pathogen material

Five field isolates of *P. brassicae* (F3-14, F41-14, F1-14, CDCS, and L-G2) were examined in this study. Isolate CDCS, classified as pathotype 5G on the Canadian Clubroot Differential (CCD) set (Strelkov et al., 2018), was included because it was collected from a high pH (7.8) field in southern Alberta. Isolate F41-14 represented pathotype 3H (Strelkov et al. 2018), which was predominant in the province prior to the introduction of clubroot-resistant canola, while isolate L-G2 was classified as pathotype 5X, the first of the resistance-breaking pathotypes to be identified. Isolates F3-14 and F1-14 were classified as pathotypes 3A and 3D, respectively, which are the

most common among pathotypes capable of overcoming resistance (Strelkov et al. 2018; Hollman et al. 2021). All the isolates were maintained as frozen galls on the roots of susceptible canola plants. To prepare inoculum, the resting spores were extracted from infected root tissue following Strelkov et al. (2006), quantified with a haemocytometer (VWR, Mississauga, Ontario), and adjusted to a concentration of 5×10^5 or 1×10^7 resting spores/mL with distilled water for use as described below.

3.2.2 Greenhouse experiments

Experiment 1. Greenhouse Trials were conducted in 2020 and 2021 to evaluate the effect of different pH values on clubroot severity caused by isolates F3-14, F41-14, F1-14, CDCS, and L-G2 of *P. brassicae*. The clubroot-susceptible canola cultivar '45H31' was grown in 38 L bins filled with a 4:1 (vol:vol) mix of Sungro Soilless Sunshine Mix (#4) (Sun Gro Horticulture, Seba Beach, AB) and field soil. The field soil was a clay loam with high (13-14%) organic matter, collected from a field at the University of Alberta South Campus, Edmonton, AB, which had no history of clubroot. The soil was confirmed to be free of *P. brassicae* infestation by PCR testing with primers specific to the pathogen (Cao et al. 2007). The pH of the soil/Sungro mixture was ~6.3 and was adjusted to the target values of 7.2 and 8.0 via addition of hydrated lime ($Ca(OH)_2$) (Graymont, Exshaw, AB), based on recommendations from the Government of Alberta (Alberta Agriculture Food and Rural Development 1996). The lime was incorporated into the soil/Sungro mixture in a cement mixer, and the final pH was confirmed with an Orion Ross Sure-Flow 8165BNWP pH electrode (Thermo Scientific, Waltham, MA). The pH was also monitored weekly throughout the course of the experiment using 20 g aliquots of the soil/Sungro mixture collected from each bin. No lime was added to the control pH treatments (~6.3).

Once the soil/Sungro mixture of appropriate pH was in the bins, it was gently watered and five equally spaced, 0.60-cm-deep rows were made, with approximately 15 seeds of clubroot-susceptible canola '45H31' sown into each row. The seeds were covered with ~2.5 cm of the soil/Sungro mixture and thinned to approximately 10 seeds per row after 7 days. At this time, the seedlings were also inoculated with *P. brassicae* by pipetting 1 mL of a resting spore suspension (5×10^5 or 1×10^7 resting spores/mL) around the base of each plant. The bins were maintained in a greenhouse at 24°C with a 16-h photoperiod (natural light supplemented by artificial lighting) and assessed for clubroot development and various growth parameters, as described below, at 6 weeks following inoculation. The plants were bottom-watered through six holes made at the bottom of each bin, with the bins kept on water-filled trays to ensure high moisture conditions favorable for clubroot development. A total of 30 treatments (five pathotypes tested at three pH levels and two inoculum concentrations each) and three controls (three pH levels but no inoculum) were evaluated (Table 3.1). All treatments and controls were replicated four times (1 bin = 1 replicate) and the entire experiment was repeated independently a total of three times in 2020 and 2021.

Experiment 2. To characterize the pH sensitivity of *P. brassicae* more precisely, a second set of greenhouse Trials was conducted where clubroot development was compared at pH 6.3 (no lime applied), 7.0, 7.3, 7.6 and 7.9. This experiment was conducted as described above for Experiment 1, except that only isolates F3-14, F1-14 and CDCS were included and tested at a single inoculum concentration (1×10^7 resting spores/mL). These isolates were selected because they appeared to show some tolerance to higher pH conditions in Experiment 1. Treatments were replicated four times (1 bin = 1 replicate), and the entire experiment was repeated. Clubroot

severity and other plant growth parameters were evaluated as described below 6 weeks after inoculation.

3.2.3 Disease assessment and plant growth parameters

Plants were carefully removed from the soil/Sungro mixture, washed in tap water, and the roots were rated for clubroot severity 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 a disease severity index (DSI) for all plants in a bin (replicate) following Strelkov et al. (2006):

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

Where: n = number of plants in each rating; N = total number of plants in each experimental unit; and 0, 1, 2 and 3 = symptom severity ratings.

Following disease assessment, the shoot weight, root weight, and shoot height were also recorded for all plants in each treatment.

3.2.4. Resting spore germination *in vitro*

The effect of different pH values on *P. brassicae* resting spore germination rates was compared *in vitro* following Xiao (2012) and Rashid et al. (2013).

Preparation of host root exudates. Five-hundred seeds of the clubroot-susceptible canola ‘45H31’ were surface-sterilized with 1% sodium hypochlorite for 2 min, rinsed 3× with sterile distilled water (sdH_2O), and placed on moistened, sterilized filter paper in 9-cm diameter Petri dishes (~50 seeds/dish) under natural light to allow germination. After 7 days, the roots of the seedlings were washed with a 50-ppm streptomycin sulphate solution for1 min and rinsed 3× with

sdH_2O. The seedlings were then transferred to 15 mL conical tubes (Frogga Bio, Concord, ON) (5 seedlings per tube) filled with modified Hoagland's solution (5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2mM $MgS0_4$, 2 mM KH_2PO_4; MacFarlane 1970), the pH of which had been adjusted to 6.0, 6.5, 7.0, 7.5, and 8.0 via addition of Tris base (Tris(hydroxymethyl)aminomethane). The top of each conical tube was partially covered with parafilm to reduce evaporation, with the tubes placed on racks in plastic trays that were lined with water-soaked paper towels and covered with plastic wrap to ensure a humid environment. The root exudate solution was collected after 14 days and filter-sterilized (0.2 μm) using a syringe, with the concentration standardized on an mL per fresh root weight basis (Rashid et al. 2013). Root exudate solutions were stored at 4°C and used within 5 h of preparation.

Surface-sterilization of resting spores. Resting spores of each of the *P. brassicae* isolates F3-14, F41-14, F1-14, CDCS, and L-G2 were extracted from galled root tissue as described above. The outer surface of each root gall was peeled with a knife, and the remaining portion was surface-sterilized with 70% ethanol for 1 min followed by 1% sodium hypochlorite for 20 min (Xiao 2012; Rashid et al. 2013). The galled roots were then rinsed 3× with sdH_2O, and ~5 g of the material was homogenized in a blender in 100 mL of sdH_2O, filtered through 8-layers of cheesecloth and centrifuged at 4000×*g*. The resting spore pellets were washed 4× with sdH_2O and the spores surface-sterilized following Asano et al. (1999). The spore concentration was estimated with a hemocytometer and adjusted to a final concentration of 1×10^6 resting spores/mL as described above.

Treatment of spores with root exudates. Resting spores of *P. brassicae* were treated with the canola root exudates by placing 0.2 mL of the spore suspension in 1.5 mL of the root exudate solution in 2 mL microcentrifuge tubes. The antibiotic cefotaxime was also included at a

final concentration of 0.1 μg/mL (Asano et al. 1999; Rashid et al. 2013) to prevent bacterial growth. The microcentrifuge tubes were vortexed and the resting spore/root exudates suspensions incubated in darkness at 25°C, with resting spore germination assessed every 24 h over 6 days. Briefly, 35 μL of resting spore/root exudates solution was fixed in aceto-orcein stain (glacial acetic acid, Fisher Scientific, Markham, ON; orcein powder, Alfa Aesar, Tewksbury, MA) and three slides for each replicate of each treatment were examined by light microscopy following Rashid et al. (2013). Ungerminated spores stained purple (having absorbed the aceto-orcein), while germinated spores appeared clear, since they did not absorb the stain (Naiki et al. 1987; Rashid et al. 2013). The *in vitro* assessments of *P. brassicae* resting spore germination included 25 treatments (5 pH treatments × 5 pathogen isolates) replicated 5 times.

3.2.5. Statistical analysis

All data were recorded in Microsoft Excel (2016) and statistical analyses were conducted using RStudio (R v. 3.6.3 2020-02-29). Data from the greenhouse Trials were analyzed using a split-split plot for Experiment 1 and a split-plot for Experiment 2, with bin number as the blocking factor. In analysis of DSI in Experiment 1, pH 8.0 and 'no clubroot' were eliminated from statistical comparisons to obtain a balanced design and prevent a heavy skew in the distribution, since these treatments had a mean DSI of ~0%. In Experiment 2, 'no clubroot' was eliminated for the same reason. The data from the resting spore germination experiment was analyzed using a two-way ANOVA. Pairwise comparisons were computed for each experiment as a post-hoc analysis. Each replicate experiment was analyzed separately. Results were considered significant if the p-value was < 0.05.

3.3. Results

3.3.1. Experiment 1

Experiment 1 consisted of three greenhouse Trials (sequentially numbered 1, 2 and 3 and representing independent runs of the experiment). In Trial 1, clubroot severity resulting from inoculation by most isolates (F3-14, F41-14 and L-G2) was very low (DSI = 7% to 29%), even at the high spore density (1×10^7 resting spores/mL) and favorable pH (6.3), likely due to poor viability of the inoculum used. As such, the results of Trial 1 are not presented and were excluded from further analysis. When Experiment 1 was run again in Trials 2 and 3, new galled root material was used as the inoculum source for all of the isolates. The results of Trials 2 and 3 are presented separately, due to the significance of treatment interactions and differences.

Clubroot severity. In Trial 2 of Experiment 1, significant treatment effects on DSI included pH ($F = 45.16$, $p = 0.0067$), isolate ($F = 90.55$, $p = 4.06 \times 10^{-14}$), spore density ($F = 131.56$, $p = 1.71 \times 10^{-12}$), pH:isolate ($F = 47.56$, $p = 4.55 \times 10^{-11}$), and pH:isolate:spore density ($F = 6.06$, $p = 0.001$), while the interactions between pH:spore density ($F = 2.16$; $p = 0.15$) and isolate:spore density ($F = 1.50$; $p = 0.23$) were not significant (Table 3.3). In the case of isolates F3-14, F41-14, and CDCS, there were no significant differences in DSI between pH 6.3 and pH 7.2 under the high resting spore concentration (1×10^7 spores/mL), with DSI ranging from 90-97% across these treatments (Table 3.4; Fig. 3.1). Under the low spore concentration (5×10^5 spores/mL), however, DSI was significantly higher for F3-14 at pH 6.3 vs. 7.2 (84% vs. 69%) and for CDCS at pH 7.2 vs. 6.3 (86% vs. 66%). In the case of isolate L-G2, DSI was much lower at pH 7.2 (10-44%) vs. 6.3 (69-76%) under both the low (10% vs. 69%) and high (44% vs. 76%) spore densities. Similarly, isolate F1-14 also caused significantly lower DSI at pH 7.2 vs. 6.3 (83% vs. 97%), but only under the high spore density; under the low spore density, the DSI for F1-14

was similar at both pH values (65-68%) (Table 3.4; Fig. 3.1). Trace or no symptoms of clubroot (DSIs ranging from 0-1%) developed at pH 8.0. As expected, no symptoms were observed at all in the no inoculum controls.

In Trial 3 of Experiment 1, the following treatments and their interaction effects were significant on DSI: pH ($F = 5213.0$; $p = 5.86 \times 10^{-6}$), isolate ($F = 68.52$; $p = 8.93 \times 10^{-13}$), spore density ($F = 164.44$; $p = 1.04 \times 10^{-13}$), pH:isolate ($F = 5.78$; $p = 0.002$), isolate:spore density ($F = 4.13$; $p = 0.009$), pH:isolate:spore density ($F = 14.41$; 1.13×10^{-6}), but pH:spore density was not ($F = 1.71$, $p = 0.20$) (Table 3.5). LG-2 caused the lowest DSI, which was significantly lower than for the other four isolates ($p < 0.05$) (Table 3.5). The differences in DSI at pH 6.3 vs. 7.2 in Trial 3 were significant for all isolates under both the low and high inoculum concentrations (Table 3.6; Figure 3.2). In the case of F3-14, DSIs at pH 6.3 were 74% and 85%, respectively, at 5×10^5 spores/mL and 1×10^7 spores/mL, but declined to 8% and 15% at pH 7.2. Likewise, following inoculation with F41-14, DSIs declined from 75-94% at pH 6.3 to 8-25% at pH 7.2. Similar results were observed for isolates F1-14, CDCS and L-G2, with DSIs ranging from 78-100% at pH 6.3, but declining to 6-57% at pH 7.2 (Table 3.6; Figure 3.2). Like Trial 2, the lowest DSIs at pH 7.2 in Trial 3 were observed for isolate L-G2. At pH 8.0, no clubroot was identified following inoculation with any of the isolates, with the exception of trace symptoms (DSI = 0.3%) caused by F1-14 under the high spore density. The DSI in the no-inoculum controls was 0% regardless of pH.

Plant growth parameters. In Trial 2 of Experiment 1, significant effects on mean root mass resulted from pH treatment ($F = 158.4$, $p = 6.42 \times 10^{-6}$), *P. brassicae* isolate ($F = 9.32$, $p = 2.82 \times 10^{-5}$), spore density ($F = 45.23$, $p = 1.61 \times 10^{-8}$), pH:isolate ($F = 4.98$, $p = 0.0003$), pH:spore density ($F = 12.23$, $p = 5.74 \times 10^{-5}$), and pH:isolate:spore density ($F = 2.40$, $p = 0.03$). In contrast,

isolate:spore density was not significant ($F = 0.92$, $p = 0.46$) (Table 3.3). The mean root mass recorded at pH 8.0 was significantly lower ($p < 0.05$) than in the other two pH treatments for each isolate, with the exception of LG-2, for which the root mass at pH 7.2 vs. 8.0 did not differ (Table 3.4). In the case of isolates F3-14, F41-14, F1-14, and CDCS, the mean root mass between the pH 6.3 and pH 7.2 treatments was not significantly different, except for CDCS under the low spore density and pH 7.2, which was similar to mean root mass at pH 8.0 (Table 3.4). The mean root mass in the pH 8.0 treatment, regardless of the isolate, was comparable to that of the inoculum-free controls (Table 3.4). Shoot mass was significantly affected by isolate ($F = 3.66$, $p = 0.01$), spore density ($F = 6.72$, $p = 0.01$), and the interaction between pH:isolate ($F = 3.82$, $p = 0.002$), but not pH ($F = 2.87$; $p = 0.13$), pH:spore density ($F = 1.12$; $p = 0.34$), isolate:spore density ($F = 1.68$; $p = 0.17$), or pH:isolate:spore density ($F = 1.09$; $p = 0.39$) (Table 3.3). With the exception of isolates F41-14 and F1-14, significant differences in shoot mass were not observed among pH treatments or spore densities. In the case of F41-14, shoot mass at pH 7.2 under low spore density was significantly greater than at pH 6.3 under high spore density. For F1-14, shoot mass at pH 6.3 and high spore density was significantly lower than in all other treatments (Table 3.4).

Shoot height was significantly affected by pH ($F = 19.54$, $p = 0.002$), isolate ($F = 10.48$, $p = 9.83 \times 10^{-6}$), spore density ($F = 33.27$, $p = 6.89 \times 10^{-7}$), and the interactions between pH:isolate ($F = 11.53$, $p = 6.19 \times 10^{-8}$) and pH:spore density ($F = 6.45$, $p = 0.0035$), but not the interactions between isolate:spore density ($F = 1.98$; $p = 0.11$) and pH:isolate:spore density ($F = 0.97$; $p = 0.47$) (Table 3.3). A significantly greater mean shoot height at pH 8.0 was recorded only for plants inoculated with isolate F3-14 (Table 3.4). In the case of isolates F41-14 and F1-14, the pH 6.3 treatment at high spore density had a significantly shorter mean shoot height compared with the other pH treatments and spore densities. Although not included in the statistical analysis, the mean

shoot height of plants in the *P. brassicae*-free controls were numerically similar to the inoculated plants at pH 8.0. Additionally, plants inoculated at both spore densities with isolate LG-2 at pH 7.2 had mean shoot heights similar or greater than those of the *P. brassicae*-free controls, and greater than the other isolates in the same pH treatment (Table 3.4).

In Trial 3 of Experiment 1, root mass was significantly affected by pH ($F = 161.51$, $p = 6.06 \times 10^{-6}$), *P. brassicae* isolate ($F= 6.65$, $p = 0.0004$), spore density ($F = 15.96$, $p = 0.0002$), and interaction effects including pH:isolate ($F = 2.59$, $p = 0.024$), and pH:spore density ($F = 9.89$, $p = 0.0002$). The interactions between isolate:spore density ($F = 0.75$, $p = 0.56$) and pH:isolate:spore density ($F = 0.88$, $p = 0.54$) were not significant (Table 3.5). At pH 6.3, mean root mass was significantly greater ($p < 0.05$) under high spore density compared with low spore density for plants inoculated with isolates F3-14, F41-14, or CDCS, but similar for plants inoculated with isolates F1-14 or LG-2 (Table 3.6). Regardless of isolate, mean root mass decreased as the pH increased, although differences were not always significant; this may have reflected generally more severe symptoms of clubroot at the lower pH value (Table 3.6). Shoot mass was affected by pH ($F = 27.98$, $p = 0.0009$), isolate ($F = 2.79$, $p = 0.04$), and the interaction between pH:isolate ($F = 3.74$, $p = 0.003$) (Table 3.5) but spore density ($F = 0.45$; $p = 0.50$), pH:spore density ($F = 1.74$; $p = 0.19$), isolate:spore density ($F = 2.17$; $p = 0.09$), and pH:strain:spore density ($F = 1.27$; $p = 0.28$) were not significant. The shoot mass of plants inoculated with isolates F3-14, CDCS or LG-2 was not significantly different, regardless of resting spore density or pH. In the case of plants inoculated with F41-14 and F1-14, shoot mass was lowest at pH 6.3 under high spore density (Table 3.6).

Treatments and their interactions significantly affected mean shoot height (pH: $F = 205.20$, $p = 0.0007$; isolate: $F = 9.24$, $p = 0.00012$; spore density: $F = 16.53$, $p = 0.0003$; pH:isolate: $F =$

11.64, p = 2.13 × 10^{-5}; pH:spore density: F = 9.90, p = 0.01; isolate:spore density: F = 3.59, p = 0.02) in Trial 3 of Experiment 1. In contrast, the interaction pH:isolate:spore density (F = 1.05, p = 0.40) was not significant (Table 3.5). Resting spore density and pH did not have a significant effect on the mean shoot height of plants inoculated with isolates F3-14 or LG-2 (Table 3.6). There were also no significant differences between the two spore densities for each pH treatment for any of the isolates with the exception of F1-14; plants inoculated with this isolate showed significantly reduced height at pH 6.3 at the high vs. low spore density. For all isolates except F3-14, plants in the pH 6.3 and 1 × 10^7 spores/mL treatment had the lowest mean shoot height. The clubroot-free controls had similar mean shoot heights compared with inoculated plants in the pH 7.2 and 8.0 treatments (Table 3.6).

3.3.2. Experiment 2

As for Experiment 1, the results for each Trial in Experiment 2 (sequentially numbered 1 and 2 and representing independent runs of the experiment) were analyzed separately due to differences in treatment interactions.

Clubroot severity. In Trial 1 of Experiment 2, DSI was affected by pH (F = 912.70, p = 8.63 × 10^{-15}), *P. brassicae* isolate (F = 30.18, p = 6.57 × 10^{-8}), and the interaction between pH:isolate (F = 11.15, p = 3.74 × 10^{-7}) (Table 3.7). Plants developed similar DSIs at pH 6.3, 7.0 and 7.3, regardless of isolate (Table 3.8). At pH 7.6, however, DSI declined significantly ($p < 0.05$) for all isolates (from an average of 97% at pH 7.3 to DSIs of 20-58% at pH 7.6). Further significant declines in DSIs were observed for all isolates at pH 7.9 (Table 3.8). Nonetheless, isolate F3-14 still caused a moderate level of disease (39%) at pH 7.9, compared with 6% and 3% for F1-14 and CDCS, respectively (Table 3.8). In Trial 2 of Experiment 2, DSI was significantly affected by pH (F = 1014.0, p = 4.60 × 10^{-15}), isolate (F = 122.25, p = 3.79 × 10^{-15}), and their interaction (F =

43.68, $p = 1.91\times 10^{-14}$) (Table 3.9). As in Trial 1, DSIs were similar for each isolate at pH 6.3, 7.0 and 7.3 (97-100%), dropping significantly at pH 7.6 (61-91%) and again at pH 7.9 (23-54%) (Table 3.10). No symptoms clubroot were observed in the *P. brassicae*-free controls in either Trial 1 or 2.

Plant growth parameters. In Trial 1 of Experiment 2, pH ($F = 64.54$, $p = 5.17 \times 10^{-8}$) and *P. brassicae* isolate ($F = 46.91$, $p = 6.69 \times 10^{-14}$), as well as their interaction ($F = 10.06$, $p = 3.60 \times 10^{-9}$), significantly affected mean root mass (Table 3.7). Across all isolates, root mass declined as pH increased, generally being significantly highest at pH 6.3 (F3-14) or pH 6.3 and 7.0 (F1-14, CDCS), and lowest at pH 7.9 (F3-14) or pH 7.6 and pH 7.9 (F1-14, CDCS) (Table 3.8). No significant differences in root mass were observed in the *P. brassicae*-free controls. In Trial 2 of Experiment 2, mean root mass was significantly affected by pH ($F = 53.10$, $p = 1.56 \times 10^{-7}$), isolate ($F = 107.69$, $p = 2.00 \times 10^{-16}$), and their interaction ($F = 8.89$, $p = 2.27 \times 10^{-8}$) (Table 3.9). Mean root mass was significantly ($p < 0.05$) lower at pH 6.3 vs. pH 7.0 for all *P. brassicae*-inoculated treatments, while root mass was similar for all inoculated treatments at pH 7.0 and pH 7.3 (Table 3.10). There were no significant differences between the mean root mass among any of the *P. brassicae*-free controls (Table 3.10).

In Trial 1 of Experiment 2, shoot mass was significantly affected by pH ($F = 41.03$, $p = 6.60 \times 10^{-7}$), isolate ($F = 21.06$, $p = 1.13 \times 10^{-8}$), and their interaction ($F = 3.32$, $p = 0.002$) (Table 3.7). Shoot mass was similar at pH 6.3, 7.0, and 7.3 for all isolates, and greatest at pH 7.6 and 7.9 (Table 3.8). In the *P. brassicae*-free controls, shoot mass fluctuated from 9.4 to 15.1g with no clear trends. In Trial 2, shoot mass was significantly affected by pH ($F = 9.71$, $p < 0.001$), isolate ($F = 55.18$, $p = 4.06 \times 10^{-15}$), and their interaction ($F = 6.42$, $p = 1.81 \times 10^{-6}$) (Table 3.9). In the case of isolates F1-14 and CDCS, shoot mass was highest at pH 7.9 (F1-14) or pH 7.6 and 7.9 (CDCS).

However, although there were numerical increases in shoot mass from the low to the high pH treatments for isolate F3-14, these were not significant (Table 3.10). In the *P. brassicae*-free controls, no general trends were observed, but shoot mass was highest at pH 7.0 and lowest at pH 7.9.

As with root and shoot mass, mean shoot height in Trial 1 of Experiment 2 was significantly affected by pH ($F = 35.74$, $p = 1.41 \times 10^{-6}$), isolate ($F = 52.44$, $p = 9.88 \times 10^{-15}$), and their interaction ($F = 6.65$, $p = 1.39 \times 10^{-6}$) (Table 3.7). Increases in pH and shoot height were positively correlated except in the *P. brassicae*-free control, where the pH treatment was insignificant ($p > 0.05$) (Table 3.8). No significant differences in height were detected for any of the isolates between pH 6.3 and 7.0, but differences did occur for height between pH 7.0 to 7.6 for F3-14, and between 7.3 to 7.6 for F1-14 and CDCS ($p < 0.05$) (Table 3.8). Shoot height in Trial 2 of Experiment 2 was also significantly affected by the pH treatment ($F = 138.10$, $p = 6.34 \times 10^{-10}$), isolate ($F = 225.30$, $p = 2.00 \times 10^{-16}$), and their interaction ($F = 14.80$, $p = 7.24 \times 10^{-12}$) (Table 3.9). In the case of isolates F3-14 and F1-14, significant increases in shoot height were observed at pH 7.6 and pH 7.6 and 7.9, respectively (Table 3.10). For plants inoculated with CDCS, shoot height at pH 7.3 was significantly greater than at pH 7.0, with large increases also observed at pH 7.6. There was no significant difference in mean shoot height in any of the *P. brassicae*-free controls (Table 3.10). All treatments in Trial 2 had lower mean shoot heights compared with the Trial 1 of Experiment 2 (Tables 3.8 & 3.10).

3.3.3. Resting spore germination

Resting spore germination was compared *in vitro* in two independent runs (Trials) of this experiment. The results are presented separately due to the significance of treatment interactions and differences.

In the first trial, the proportion of germinated resting spores was significantly affected by the *P. brassicae* isolate ($F = 4.95$, $p = 5.72 \times 10^{-4}$), pH treatment ($F = 28.20$, $p = 2.00 \times 10^{-16}$), day ($F= 50.29$, $p = 2.00 \times 10^{-16}$), isolate:pH ($F = 3.05$, $p = 4.17 \times 10^{-5}$), isolate:day ($F = 2.94$, $p = 1.43 \times 10^{-5}$), pH:day ($F = 7.52$, $p = 2.00 \times 10^{-16}$), and isolate:pH:day ($F = 1.58$, $p = 0.0011$) (Table 3.11). Germination rates were similar across most pH values with the exception of pH 8.0, at which it was significantly lower (Fig. 3.3). The percentage of germinated resting spores increased throughout most of the time-course, generally peaking at day 5 when germination ranged from 0% (at pH 8.0) to 44% (at pH 6.5) but decreased at day 6. In general, germination rates were greatest at pH 6.5, followed by pH 6.0 (Fig. 3.3). Nonetheless, there were exceptions; for instance, germination of the resting spores of isolate F3-14 at pH 6.5 were lower than at pH 6.0 or 7.0 at nearly all time-points (Figure 3.3). In the second Trial of the germination experiment, significant factors and their interactions included *P. brassicae* isolate ($F = 12.70$; $p = 3.31 \times 10^{-10}$), day ($F = 39.40$; $p = 2.00 \times 10^{-16}$), isolate:day ($F = 2.43$; $p = 0.0004$), pH:day ($F = 4.59$; $p = 7.06 \times 10^{-11}$), and isolate:pH:day ($F = 1.42$; $p = 0.01$), but pH ($F = 1.01$; $p = 0.40$), and the interaction between isolate:pH ($F = 1.66$; $p = 0.05$) were not (Table 3.12). The percentage of germinated resting spores generally increased over the time-course and was usually highest at day 6 for all isolates (and second highest at day 5) (Fig. 3.4). As in the first Trial, the lowest percentage of germinated spores for isolates F3-14 and F1-14 was observed at pH 6.5 and 7.5, respectively. Germination was also very low at pH 7.0 for the isolates F41-14 and CDCS (Figure 3.4). Unlike in in the first Trial, germination across different pH values was generally similar, and the percentage germination did not exceed 30% for any treatment.

3.4 Discussion

Given that the development of clubroot is generally favored by acidic soils (Dixon 2009a; Gossen et al. 2014), the application of soil amendments to increase soil pH represents a possible strategy to mitigate disease impact (Hwang et al. 2014). Lime, in particular, has been used for clubroot management in cruciferous vegetables for over a century and shown promise in canola, where it could complement genetic resistance (Fox et al. 2022). Nonetheless, pH amendments do not always produce consistent results, as indicated by a number of studies in recent years (Hwang et al. 2014; Gossen et al. 2014; Fox et al. 2022). The variable results associated with liming and other soil amendments may reflect the influence of environmental factors, such as moisture and temperature (McDonald and Westerveld 2008; Gossen et al. 2013, 2014), as well as soil inoculum levels (Macfarlane 1952; Hwang et al. 2011b, 2011a), on disease development. The length of time between the application of a soil amendment and seeding of the crop may also influence treatment efficacy; Fox et al. (2022) found that hydrated lime could significantly reduce clubroot on canola, but only when optimal timing and rainfall were achieved. Another long overlooked but potentially important factor that could also affect the efficacy of pH amendments is the pathogen itself. In this study, we compared the pH sensitivity of five isolates of *P. brassicae* collected in Alberta.

One of the isolates, CDCS, had originated from a high pH (7.8) field (S.E. Strelkov, pers. comm.), suggesting some adaptation to alkaline conditions. In the greenhouse, however, it did not show a markedly different response to higher pH than most of the other isolates. In Trial 2 of Experiment 1, CDCS did cause slightly higher disease severities than the other isolates at pH 7.2, but at pH 8.0, it caused only trace levels of the disease. In Trial 3, CDCS induced higher severity than most, but not all, of the other isolates at pH 7.2, and similar (i.e., none or trace) levels of disease at pH 8.0. In Experiment 2, in which the pH sensitivity of three isolates was examined on

a finer scale at pH 6.3, 7.0, 7.3, 7.6 and 7.9, the severity of clubroot caused by CDCS dropped significantly at pH 7.6, and again at 7.9, just like it did for the other isolates. There are several potential reasons why CDCS may not have appeared as tolerant to high pH as its origin from a high pH field would have suggested. First, CDCS is a field isolate, and not a single-spore isolate; it is well known that field isolates of *P. brassicae* may be heterogeneous (Jones et al. 1982; Manzanares-Dauleux et al. 2001; Xue et al. 2008). It is possible, therefore, that there were components in the original field population that were less sensitive to high pH, but which were not represented in the isolate we used in our experiments. Additional pathogen collections from the original field would need to be tested to evaluate this suggestion. Secondly, perhaps other factors in the field, such as weather, inoculum load or other soil properties, may have contributed to clubroot severity at higher pH in the original field.

While CDCS did not appear particularly insensitive to high pH, some differences in the general pH sensitivity of isolates were observed. Noteworthy was isolate L-G2, which consistently caused much milder disease severity at pH 7.2 than any of the other isolates examined. This suggests that this particular isolate could be controlled very well by liming or other strategies to increase soil pH. Isolate L-G2 is classified as pathotype 5X (Strelkov et al. 2016), the first of the resistance-breaking pathotypes to be identified from Canadian canola. Whether greater sensitivity to higher pH is a common characteristic among other isolates of this pathotype would have to be determined by testing of a wider collection of isolates. Indeed, while each of the isolates included in this study represented a distinct pathotype, it is not possible to make pathotype-wide generalizations based on our results because only a single isolate was tested per pathotype. Moreover, pathotypes are defined by their virulence on a host differential set, and there is no indication that virulence and pH sensitivity would be linked or even under similar genetic control.

Nevertheless, the evaluation of a larger collection of isolates, with multiple isolates tested per pathotype, may serve to determine whether any pathotype-specific conclusions can be made.

To achieve satisfactory clubroot control via the application of lime, most research indicates that treatments should target a soil pH of ≥ 7.2 (Myers and Campbell 1985; Howard et al. 2010; Dobson et al. 1983; Webster and Dixon 1991a; Gossen et al. 2013). The results of this study, however, suggest that a pH ≥ 7.2 may not always be sufficient. In Trial 2 of Experiment 1, severe symptoms of clubroot developed at pH 7.2 with most *P. brassicae* isolates. Moreover, in both trials of Experiment 2, where clubroot development was evaluated over a finer pH scale, statistically significant declines in clubroot severity were not observed until the pH increased to 7.6. At pH of 6.3, 7.0 and 7.3, DSI was always between 97-100%. The only exception to these trends was observed in Trial 3 of Experiment 1, where clubroot severity declined significantly at pH 7.2. Collectively, these observations would suggest that at a pH of 7.2-7.3, disease development may sometimes be severe, but minor variations in other conditions at this threshold could sometimes result in milder symptoms. It would seem, therefore, that achieving a pH ≥ 7.6 may provide more consistent control. Additional research, particularly under field conditions, will be needed to confirm such a recommendation.

Since canola is western Canada is a broad acre crop grown in very large fields (typically in 160 acre (~65 ha) 'quarter-sections'), the purchase and application of sufficient quantities of lime to increase soil pH to 7.2 or greater may be expensive or impractical in many situations (Hwang et al. 2014). Targeted application, for example in infested patches within a field or at the field entrances, where *P. brassicae* is most likely to be introduced (Cao et al. 2009), has sometimes been suggested (Fox et al. 2022; Hennig et al. 2022). A further consideration is the effect of soil pH on canola growth and nutrient availability. A suitable soil pH range for *B. napus* is 5.5 to 8.3,

whereas pH < 5.5 can contribute to toxic levels of certain nutrients, such as aluminum and manganese, and high pH soils can cause nutrient deficiencies, such as with zinc (Grant and Bailey 1993; Canadian Food Inspection Agency 2017; Canola Council of Canada 2020). In the current study, increases in pH were correlated with a decrease in root mass (reduced galling) and an increase in shoot mass, reflecting that increases in pH decreased disease severity, promoting the growth of plants. Based on the non-inoculated controls, there did not seem to be any effects of pH alone on canola growth and development, or any signs of phytotoxicity. Nonetheless, these plants were not grown until harvest, and the impact on yields, if any, was therefore not documented.

The different types of lime ($CaMg(CO_3)_2$, $CaCO_3$, CaO, $Ca(OH)_2$) all include calcium, so when using lime to adjust pH, it is difficult to separate completely any effects that may result solely from pH changes versus the addition of calcium. Nonetheless, higher soil pH and calcium have been documented to reduce resting spore germination, root hair infection and the production of primary plasmodia (Naiki and Dixon 1987; Webster and Dixon 1991a; Donald and Porter 2004; Niwa et al. 2008; Fox et al. 2022). In this study, the effect of pH on resting spore germination was examined *in vitro* in modified Hoagland's solution (Macfarlane 1970), the pH of which was adjusted via the addition of Tris base ($C_4H_{11}NO_3$) (i.e., without calcium). In general, germination was highest at pH 6.5 for most isolates, and consistently lowest at pH 8.0. This suggests that high pH may directly reduce *P. brassicae* resting spore germination, which would contribute to the complete (or nearly complete) absence of symptoms observed at pH 8.0.

Clubroot represents one of the most significant challenges to canola production. While the deployment of resistant cultivars is the most important strategy for managing the disease, it is clear, given the emergence of resistance-breaking pathotypes of *P. brassicae*, that resistance cannot be used in isolation. Liming of the soil to increase pH is a potentially useful step towards a more

integrated clubroot management system. However, based on the current results, it appears that there is some variability in the pH sensitivity of pathogen isolates, and that targeting a minimum pH of 7.2 may not always provide sufficient clubroot control.

Tables and Figures

Table 3.1: *Summary of treatments included in Experiment 1, assessing the pH sensitivity of* Plasmodiophora brassicae *isolates representing pathotypes 3A, 3D, 3H, 5G and 5X.*

Treatment	*Isolate (pathotype)*	*pH*	*Spore density (resting spores/mL)*
1	F3-14 (3A)	6.3	5×10^5
2	F3-14 (3A)	6.3	1×10^7
3	F3-14 (3A)	7.2	5×10^5
4	F3-14 (3A)	7.2	1×10^7
5	F3-14 (3A)	8.0	5×10^5
6	F3-14 (3A)	8.0	1×10^7
7	F1-14 (3D)	6.3	5×10^5
8	F1-14 (3D)	6.3	1×10^7
9	F1-14 (3D)	7.2	5×10^5
10	F1-14 (3D)	7.2	1×10^7
11	F1-14 (3D)	8.0	5×10^5
12	F1-14 (3D)	8.0	1×10^7
13	F41-14 (3H)	6.3	5×10^5
14	F41-14 (3H)	6.3	1×10^7
15	F41-14 (3H)	7.2	5×10^5
16	F41-14 (3H)	7.2	1×10^7
17	F41-14 (3H)	8.0	5×10^5
18	F41-14 (3H)	8.0	1×10^7
19	CDCS (5G)	6.3	5×10^5
20	CDCS (5G)	6.3	1×10^7
21	CDCS (5G)	7.2	5×10^5
22	CDCS (5G)	7.2	1×10^7
23	CDCS (5G)	8.0	5×10^5
24	CDCS (5G)	8.0	1×10^7
25	L-G2 (5X)	6.3	5×10^5
26	L-G2 (5X)	6.3	1×10^7
27	L-G2 (5X)	7.2	5×10^5
28	L-G2 (5X)	7.2	1×10^7
29	L-G2 (5X)	8.0	5×10^5
30	L-G2 (5X)	8.0	1×10^7
Control	No inoculum	6.3	0
Control	No inoculum	7.2	0
Control	No inoculum	8.0	0

Table 3.2: *Summary of treatments included in Experiment 2, characterizing the pH sensitivity of* Plasmodiophora brassicae *isolates representing pathotypes 3A, 3D, and 5G more precisely.*

Treatment	*Isolate (pathotype)*	*pH*
1	F3-14 (3A)	6.3
2	F3-14 (3A)	7.0
3	F3-14 (3A)	7.3
4	F3-14 (3A)	7.6
5	F3-14 (3A)	7.9
6	F1-14 (3D)	6.3
7	F1-14 (3D)	7.0
8	F1-14 (3D)	7.3
9	F1-14 (3D)	7.6
10	F1-14 (3D)	7.9
11	CDCS (5G)	6.3
12	CDCS (5G)	7.0
13	CDCS (5G)	7.3
14	CDCS (5G)	7.6
15	CDCS (5G)	7.9
Control	No inoculum	6.3
Control	No inoculum	7.0
Control	No inoculum	7.3
Control	No inoculum	7.6
Control	No inoculum	7.9

Table 3.3: *Linear mixed effects model for Experiment 1 Trial 2 to evaluate the effects of three pH treatments on mean clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of five isolates of* Plasmodiophora brassicae; *p-values < 0.05 are bolded.*

	DSI (%)			*Root mass (g)*		
	denDF	F-value	P-value	denDF	F-value	P-value
Intercept	30	6022.019	**<.0001**	45	941.71	**<.0001**
pH	3	45.16	**0.007**	6	158.4	**6.42E-06**
isolate	24	90.55	**4.06E-14**	36	9.32	**2.82E-05**
sd	30	131.555	**1.71E-12**	45	45.2254	**1.61E-08**
pH:isolate	24	47.56	**4.55E-11**	36	4.977	**0.0003**
pH:sd	30	2.162	0.15	45	12.23	**5.74E-05**
isolate:sd	30	1.505	0.23	45	0.9233	0.46
pH:isolate:sd	30	6.055	**0.001**	45	2.3957	**0.03**

	Shoot mass (g)			*Shoot height (cm)*		
	denDF	F-value	P-value	denDF	F-value	P-value
Intercept	45	785.05	**<.0001**	45	3504.514	**<.0001**
pH	6	2.873	0.13	6	19.54	**0.002**
isolate	36	3.66	**0.01**	36	10.48	**9.83E-06**
sd	45	6.72	**0.01**	45	33.27	**6.89E-07**
pH:isolate	36	3.82	**0.002**	36	11.53	**6.19E-08**
pH:sd	45	1.12	0.34	45	6.45	**0.004**
isolate:sd	45	1.68	0.17	45	1.98	0.11
pH:isolate:sd	45	1.09	0.39	45	0.97	0.47

Table 3.4: *Clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of five isolates of* Plasmodiophora brassicae *at two resting spore densities and three different pH values for Experiment 1, Trial 2; letters denote significant differences between pH treatments and spore densities.*

Treatment			*Plant Biomass Measurements*			
Isolate	*pH*	*Spore density (spores/mL)*	*DSI (%)*[Δ]	*Root mass (g)*	*Shoot mass (g)*	*Shoot height (cm)*
F3-14 (3A)	No lime (pH 6.3)	5x10^{5}	84^{a}	3.8^{ab}	5.6^{ab}	51^{b}
F3-14 (3A)	No lime (pH 6.3)	1x10^{7}	90^{a}	3.5^{ab}	8.2^{b}	56^{b}
F3-14 (3A)	7.2	5x10^{5}	69^{b}	2.4^{b}	8.1^{ab}	66^{b}
F3-14 (3A)	7.2	1x10^{7}	90^{a}	4.3^{a}	8.9^{ab}	53^{b}
F3-14 (3A)	8.0	5x10^{5}	0*	0.5^{c}	13.9^{a}	94^{a}
F3-14 (3A)	8.0	1x10^{7}	0.5*	0.4^{c}	10.7^{ab}	89^{a}
F41-14 (3H)	No lime (pH 6.3)	5x10^{5}	80^{ab}	2.2^{b}	8.9^{a}	79^{ab}
F41-14 (3H)	No lime (pH 6.3)	1x10^{7}	93^{a}	4.5^{a}	6.6^{a}	58^{b}
F41-14 (3H)	7.2	5x10^{5}	75^{b}	3.2^{b}	8.4^{a}	74^{ab}
F41-14 (3H)	7.2	1x10^{7}	90^{a}	3.8^{ab}	8.3^{a}	69^{ab}
F41-14 (3H)	8.0	5x10^{5}	0*	0.3^{c}	9.0^{a}	86^{a}
F41-14 (3H)	8.0	1x10^{7}	0.5*	0.3^{c}	6.1^{a}	81^{a}
F1-14 (3D)	No lime (pH 6.3)	5x10^{5}	68^{c}	2.2^{b}	8.9^{ab}	79^{a}
F1-14 (3D)	No lime (pH 6.3)	1x10^{7}	97^{a}	5.4^{a}	7.4^{ab}	46^{b}
F1-14 (3D)	7.2	5x10^{5}	65^{c}	2.4^{b}	11.0^{ab}	89^{a}
F1-14 (3D)	7.2	1x10^{7}	83^{b}	3.1^{ab}	10.0^{b}	79^{a}
F1-14 (3D)	8.0	5x10^{5}	0*	0.3^{c}	9.9^{a}	86^{a}
F1-14 (3D)	8.0	1x10^{7}	1*	0.3^{c}	12.2^{ab}	84^{a}
CDCS (5G)	No lime (pH 6.3)	5x10^{5}	66^{b}	1.6^{b}	9.7^{ab}	91^{a}
CDCS (5G)	No lime (pH 6.3)	1x10^{7}	89^{a}	4.3^{a}	7.6^{ab}	66^{bc}
CDCS (5G)	7.2	5x10^{5}	86^{a}	4.0^{a}	11.8^{ab}	79^{ab}
CDCS (5G)	7.2	1x10^{7}	96^{a}	4.9^{a}	5.8^{b}	53^{c}
CDCS (5G)	8.0	5x10^{5}	0*	0.5^{b}	14.0^{a}	91^{a}
CDCS (5G)	8.0	1x10^{7}	0.5*	0.4^{b}	9.3^{ab}	89^{a}
L-G2 (5X)	No lime (pH 6.3)	5x10^{5}	69^{a}	2.3^{a}	11.6^{ab}	81^{bc}
L-G2 (5X)	No lime (pH 6.3)	1x10^{7}	76^{a}	2.6^{a}	8.7^{b}	74^{c}

L-G2 (5X)	7.2	5x10^{5}	10^{c}	0.7^{b}	15.7^{a}	109^{a}
L-G2 (5X)	7.2	1x10^{7}	44^{b}	1.9^{ab}	16.7^{a}	102^{ab}
L-G2 (5X)	8.0	5x10^{5}	0*	0.3^{b}	7.2^{b}	79^{c}
L-G2 (5X)	8.0	1x10^{7}	0*	0.4^{b}	10.8^{ab}	81^{bc}
No inoculum	No lime (pH 6.3)	-	0*	0.5*	12.1*	81*
No inoculum	7.2	-	0*	0.6*	12.9*	102*
No inoculum	8.0	-	0*	0.3*	7.5*	81*

*Removed from the statistical analysis [Δ]DSI values are also summarized visually in Figure 3.1

Plant biomass measurement values represent the average of 10 randomly selected plants of each treatment averaged across repetitions

Table 3.5: *Linear mixed effects model for Experiment 1 Trial 3 to evaluate the effects of three pH treatments on mean clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of five isolates of Plasmodiophora brassicae; p-values < 0.05 are bolded.*

	DSI (%)			*Root mass (g)*		
	denDF	F-value	P-value	denDF	F-value	P-value
Intercept	30	2865.48	**<.0001**	45	547.07	**<.0001**
pH	3	5213.0	**5.86E-06**	6	161.51	**6.06E-06**
isolate	24	68.52	**8.93E-11**	36	6.65	**4.00E-04**
sd	30	164.44	**1.04E-13**	45	15.96	**2.00E-04**
pH:isolate	24	5.78	**0.002**	36	2.59	**0.024**
pH:sd	30	1.71	0.20	45	9.89	**2.00E-04**
isolate:sd	30	4.13	**0.009**	45	0.75	0.56
pH:isolate:sd	30	14.41	**1.13E-06**	45	0.88	0.54

	Shoot mass (g)			*Shoot height (cm)*		
	denDF	F-value	P-value	denDF	F-value	P-value
Intercept	45	1899.33	**<.0001**	45	8954.22	**<.0001**
pH	6	27.98	**9.00E-04**	6	205.2	**7.37E-04**
isolate	36	2.79	**0.041**	36	9.243	**1.15E-04**
sd	45	0.45	0.50	45	16.53	**3.19E-04**
pH:isolate	36	3.74	**0.0028**	36	11.64	**2.13E-05**
pH:sd	45	1.74	0.19	45	9.90	**5.18E-03**
isolate:sd	45	2.17	0.09	45	3.60	**0.017**
pH:isolate:sd	45	1.27	0.28	45	1.05	0.40

Table 3.6: *Clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of five isolates of Plasmodiophora brassicae at two resting spore densities and three different pH values for Experiment 1, Trial 3; letters denote significant differences between pH treatments and spore densities.*

Treatment			*Plant Biomass Measurements*			
Isolate	*pH*	*Spore density (spores/mL)*	*DSI (%)*$^{\Delta}$	*Root mass (g)*	*Shoot mass (g)*	*Shoot height (cm)*
F3-14 (3A)	No lime (pH 6.3)	$5x10^{5}$	74^{a}	2.5^{b}	11.3^{a}	74^{a}
F3-14 (3A)	No lime (pH 6.3)	$1x10^{7}$	85^{a}	4.1^{a}	16.3^{a}	74^{a}
F3-14 (3A)	7.2	$5x10^{5}$	8^{b}	1.0^{c}	12.4^{a}	81^{a}
F3-14 (3A)	7.2	$1x10^{7}$	15^{b}	1.2^{bc}	14.9^{a}	86^{a}
F3-14 (3A)	8.0	$5x10^{5}$	0*	0.8^{c}	12.7^{a}	71^{a}
F3-14 (3A)	8.0	$1x10^{7}$	0*	0.7^{c}	13.4^{a}	81^{a}
F41-14 (3H)	No lime (pH 6.3)	$5x10^{5}$	75^{b}	2.8^{b}	11.6^{ab}	66^{bc}
F41-14 (3H)	No lime (pH 6.3)	$1x10^{7}$	94^{a}	4.7^{a}	8.2^{b}	58^{c}
F41-14 (3H)	7.2	$5x10^{5}$	8^{d}	1.2^{c}	15.2^{a}	86^{a}
F41-14 (3H)	7.2	$1x10^{7}$	25^{c}	1.3^{c}	13.0^{ab}	86^{a}
F41-14 (3H)	8.0	$5x10^{5}$	0*	0.5^{c}	9.5^{ab}	81^{ab}
F41-14 (3H)	8.0	$1x10^{7}$	0*	0.5^{c}	12.4^{ab}	74^{abc}
F1-14 (3D)	No lime (pH 6.3)	$5x10^{5}$	95^{a}	4.3^{a}	9.4^{ab}	61^{b}
F1-14 (3D)	No lime (pH 6.3)	$1x10^{7}$	100^{a}	5.4^{a}	4.3^{b}	36^{c}
F1-14 (3D)	7.2	$5x10^{5}$	14^{c}	1.3^{bc}	15.1^{a}	86^{a}
F1-14 (3D)	7.2	$1x10^{7}$	57^{b}	2.3^{b}	13.4^{a}	81^{a}
F1-14 (3D)	8.0	$5x10^{5}$	0*	0.7^{c}	12.0^{a}	79^{a}
F1-14 (3D)	8.0	$1x10^{7}$	0.3*	0.5^{c}	15.0^{a}	71^{ab}
CDCS (5G)	No lime (pH 6.3)	$5x10^{5}$	78^{b}	3.3^{b}	12.6^{a}	74^{ab}
CDCS (5G)	No lime (pH 6.3)	$1x10^{7}$	96^{a}	4.8^{a}	8.9^{a}	53^{b}
CDCS (5G)	7.2	$5x10^{5}$	18^{d}	1.4^{c}	11.6^{a}	81^{a}
CDCS (5G)	7.2	$1x10^{7}$	46^{c}	1.7^{c}	8.9^{a}	76^{a}
CDCS (5G)	8.0	$5x10^{5}$	0*	0.5^{c}	13.0^{a}	79^{a}
CDCS (5G)	8.0	$1x10^{7}$	0*	0.5^{c}	12.3^{a}	76^{a}
L-G2 (5X)	No lime (pH 6.3)	$5x10^{5}$	78^{b}	2.9^{a}	14.4^{a}	79^{a}
L-G2 (5X)	No lime (pH 6.3)	$1x10^{7}$	73^{a}	3.2^{a}	13.4^{a}	74^{a}

L-G2 (5X)	7.2	$5x10^{5}$	6^{c}	0.8^{b}	10.9^{a}	81^{a}
L-G2 (5X)	7.2	$1x10^{7}$	9^{c}	0.8^{b}	12.4^{a}	79^{a}
L-G2 (5X)	8.0	$5x10^{5}$	0*	0.6^{b}	15.0^{a}	81^{a}
L-G2 (5X)	8.0	$1x10^{7}$	0*	0.6^{b}	14.4^{a}	79^{a}
No inoculum	No lime (pH 6.3)	-	0*	0.5*	12.1*	76*
No inoculum	7.2	-	0*	0.6*	12.9*	84*
No inoculum	8.0	-	0*	0.3*	7.5*	79*

*Removed from the statistical analysis $^{\Delta}$DSI values are also summarized visually in Figure 3.2

Plant biomass measurement values represent the average of 10 randomly selected plants of each treatment averaged across repetitions

Table 3.7: *Linear mixed effects model for Experiment 2 Trial 1 to evaluate the effects of five pH levels on mean clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of three isolates of* Plasmodiophora brassicae; *p-values < 0.05 are bolded.*

	DSI (%)			*Root mass (g)*		
	denDF	F-value	P-value	denDF	F-value	P-value
Intercept	30	8400.663	**<.0001**	45	638.1445	**<.0001**
pH	12	912.7	**8.63E-15**	12	64.536	**5.17E-08**
isolate	30	30.18	**6.57E-08**	45	46.91	**6.69E-14**
pH:isolate	30	11.15	**3.74E-07**	45	10.06	**3.60E-09**

	Shoot mass (g)			*Shoot height (cm)*		
	denDF	F-value	P-value	denDF	F-value	P-value
Intercept	45	1036.42	**<.0001**	45	2710.21	**<.0001**
pH	12	41.03	**6.60E-07**	12	35.74	**1.41E-06**
isolate	45	21.06	**1.13E-08**	45	52.44	**9.88E-15**
pH:isolate	45	3.328	**0.002**	45	6.56	**1.39E-06**

Table 3.8: *Clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of three isolates of* Plasmodiophora brassicae *at five different pH values for Experiment 2 Trial 1; letters denote significant differences between pH treatments and spore densities.*

Treatment			*Plant Growth Parameters*		
Isolate	*pH*	*DSI (%)*	*Root mass (g)*	*Shoot mass (g)*	*Shoot height (cm)*
F3-14 (3A)	No lime (pH 6.3)	100[a]	8.7[a]	4.7[b]	46[c]
F3-14 (3A)	7.0	99[a]	4.8[b]	5.1[b]	71[bc]
F3-14 (3A)	7.3	97[a]	3.2[bc]	5.4[b]	86[ab]
F3-14 (3A)	7.6	58[b]	1.6[c]	8.5[ab]	102[a]
F3-14 (3A)	7.9	39[c]	1.1[d]	10.3[a]	107[a]
F1-14 (3D)	No lime (pH 6.3)	99[a]	6.2[a]	4.2[c]	46[c]
F1-14 (3D)	7.0	97[a]	4.2[a]	4.0[c]	53[bc]
F1-14 (3D)	7.3	97[a]	3.7[b]	5.7[bc]	74[b]
F1-14 (3D)	7.6	20[b]	0.8[c]	9.8[ab]	102[a]
F1-14 (3D)	7.9	6[c]	0.6[c]	12.6[a]	109[a]
CDCS (5G)	No lime (pH 6.3)	100[a]	5.6[a]	2.6[b]	36[c]
CDCS (5G)	7.0	100[a]	6.6[ab]	3.4[b]	53[bc]
CDCS (5G)	7.3	97[a]	4.1[b]	3.8[b]	69[b]
CDCS (5G)	7.6	31[b]	1.2[c]	12.1[a]	107[a]
CDCS (5G)	7.9	3[c]	0.6[c]	11.8[a]	122[a]
No inoculum	No lime (pH 6.3)	0*	0.7[a]	14.6[ab]	124[a]
No inoculum	7.0	0*	0.5[a]	9.6[c]	117[a]
No inoculum	7.3	0*	0.7[a]	9.4[c]	114[a]
No inoculum	7.6	0*	0.5[a]	10.4[bc]	124[a]
No inoculum	7.9	0*	0.7[a]	15.1[a]	119[a]

*Removed from the statistical analysis

Plant biomass measurement values represent the average of 10 randomly selected plants of each treatment averaged across repetitions

Table 3.9: *Linear mixed effects model for Experiment 2 Trial 2 to evaluate the effects of five pH levels on mean clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of three isolates of* Plasmodiophora brassicae*; p-values < 0.05 are bolded.*

	DSI (%)			*Root mass (g)*		
	denDF	F-value	P-value	denDF	F-value	P-value
Intercept	30	59809.37	**<.0001**	45	1287.45	**<.0001**
pH	12	1014	**4.60E-15**	12	53.1	**1.56E-07**
isolate	30	122.25	**3.79E-15**	45	107.69	**2.00E-16**
pH:isolate	30	43.68	**1.91E-14**	45	8.89	**2.27E-08**

	Shoot mass (g)			*Shoot height (cm)*		
	denDF	F-value	P-value	denDF	F-value	P-value
Intercept	45	699.9	**<.0001**	45	5316.43	**<.0001**
pH	12	9.71	**0.001**	12	138.1	**6.34E-10**
isolate	45	55.18	**4.06E-15**	45	225.3	**2.00E-16**
pH:isolate	45	6.42	**1.81E-06**	45	14.8	**7.24E-12**

Table 3.10: *Clubroot disease severity index (DSI), root mass, shoot mass, and shoot height of canola plants following inoculation with each of three isolates of* Plasmodiophora brassicae *at five different pH values for Experiment 2 Trial 2; letters denote significant differences between pH treatments and spore densities.*

Treatment			*Plant Growth Parameters*		
Isolate	*pH*	*DSI (%)*	*Root mass (g)*	*Shoot mass (g)*	*Shoot height (cm)*
F3-14 (3A)	No lime (pH 6.3)	99[a]	3.3[b]	1.6[a]	15[b]
F3-14 (3A)	7.0	100[a]	4.7[a]	2.4[a]	20[b]
F3-14 (3A)	7.3	99[a]	4.8[a]	4.0[a]	25[b]
F3-14 (3A)	7.6	82[b]	1.7[c]	4.8[a]	46[a]
F3-14 (3A)	7.9	50[c]	1.1[c]	4.9[a]	56[a]
F1-14 (3D)	No lime (pH 6.3)	99[a]	2.8[b]	1.5[b]	20[c]
F1-14 (3D)	7.0	100[a]	4.4[a]	2.2[b]	20[c]
F1-14 (3D)	7.3	99[a]	3.5[ab]	2.4[b]	23[c]
F1-14 (3D)	7.6	91[b]	2.4[b]	4.0[ab]	36[b]
F1-14 (3D)	7.9	54[c]	1.0[c]	5.8[a]	61[a]
CDCS (5G)	No lime (pH 6.3)	100[a]	3.1[b]	2.5[b]	18[c]
CDCS (5G)	7.0	100[a]	5.3[a]	2.2[b]	19[bc]
CDCS (5G)	7.3	97[a]	4.6[a]	3.8[b]	28[b]
CDCS (5G)	7.6	61[b]	1.8[c]	8.4[a]	61[a]
CDCS (5G)	7.9	23[c]	1.0[c]	10.4[a]	71[a]
No inoculum	No lime (pH 6.3)	0*	0.4[a]	8.8[ab]	69[a]
No inoculum	7.0	0*	0.5[a]	11.0[a]	71[a]
No inoculum	7.3	0*	0.5[a]	8.8[ab]	74[a]
No inoculum	7.6	0*	0.4[a]	8.9[ab]	74[a]
No inoculum	7.9	0*	0.4[a]	7.2[b]	71[a]

*Removed from the statistical analysis
Plant biomass measurement values represent the average of 10 randomly selected plants
each treatment averaged across repetitions

Table 3.11: *Two-way ANOVA for an* in vitro *assessment of the effects of pH and day on percentage resting spore germination for each of five isolates of* Plasmodiophora brassicae *(Trial 1); p-values < 0.05 are bolded.*

	% germinated		
	df	F-value	P-value
isolate	4	4.95	**5.72E-04**
pH	4	28.2	**2E-16**
day	5	50.29	**2E-16**
isolate:pH	16	3.05	**4.17E-05**
isolate:day	20	2.94	**1.43E-05**
ph:day	20	7.52	**2E-16**
isolate:pH:day	80	1.58	**0.0011**

Table 3.12: *Two-way ANOVA for an* in vitro *assessment of the effects of pH and day on percentage resting spore germination for each of five isolates of* Plasmodiophora brassicae *(Trial 2); p-values < 0.05 are bolded.*

	% germinated		
	df	F-value	P-value
isolate	4	12.7	**3.31E-10**
pH	4	1.006	0.4
day	5	39.4	**2E-16**
isolate:pH	16	1.658	0.05
isolate:day	20	2.432	**3.90E-04**
ph:day	20	4.59	**7.06E-11**
isolate:pH:day	80	1.42	**0.01**

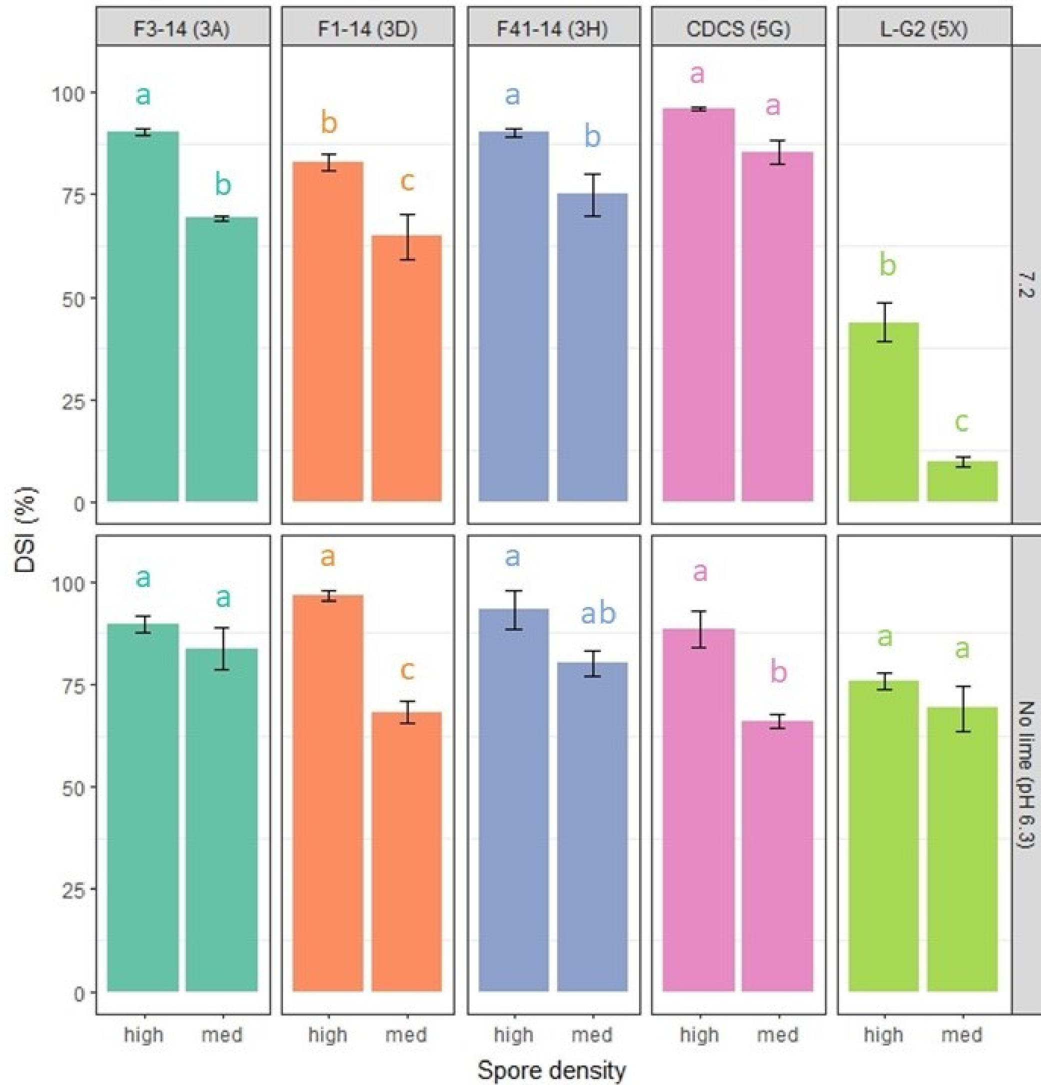

Figure 3.1: *Clubroot disease severity index (DSI) of canola plants following inoculation with each of five isolates of* Plasmodiophora brassicae *at two resting spore densities ('med' = 5 × 10^5 resting spores/mL; 'high' = 1 × 10^7 resting spores/mL) and grown in a soil mix at pH 6.3 or pH 7.0 for Experiment 1, Trial 2. Bars represent the standard error of the mean; significant differences (p < 0.05) in means for each isolate are denoted by different letters. The pathotype designations of each isolate are indicated in parentheses.*

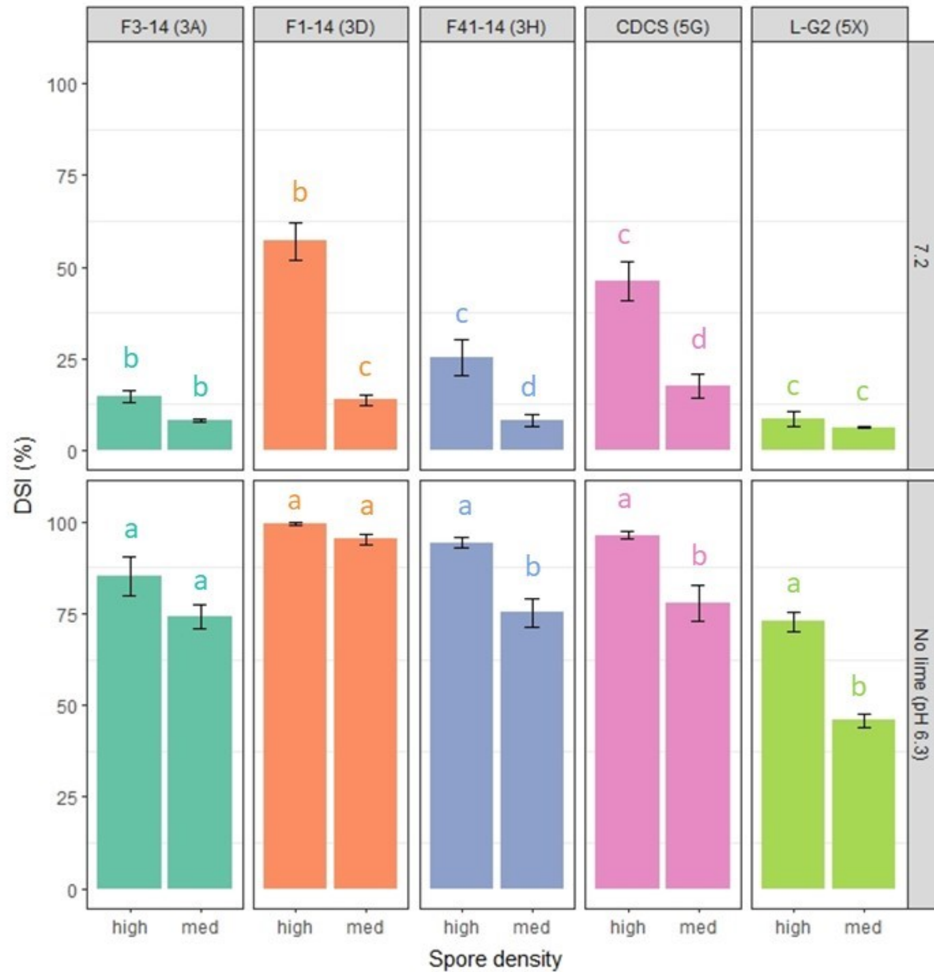

Figure 3.2: *Clubroot disease severity index (DSI) of canola plants following inoculation with each of five isolates of* Plasmodiophora brassicae *at two resting spore densities ('med' = 5 × 10^5 resting spores/mL; 'high' = 1 × 10^7 resting spores/mL) and grown in a soil mix at pH 6.3 or pH 7.0 for Experiment 1, Trial 3. Bars represent the standard error of the mean; significant differences ($p < 0.05$) in means for each isolate are denoted by different letters. The pathotype designations of each isolate are indicated in parentheses.*

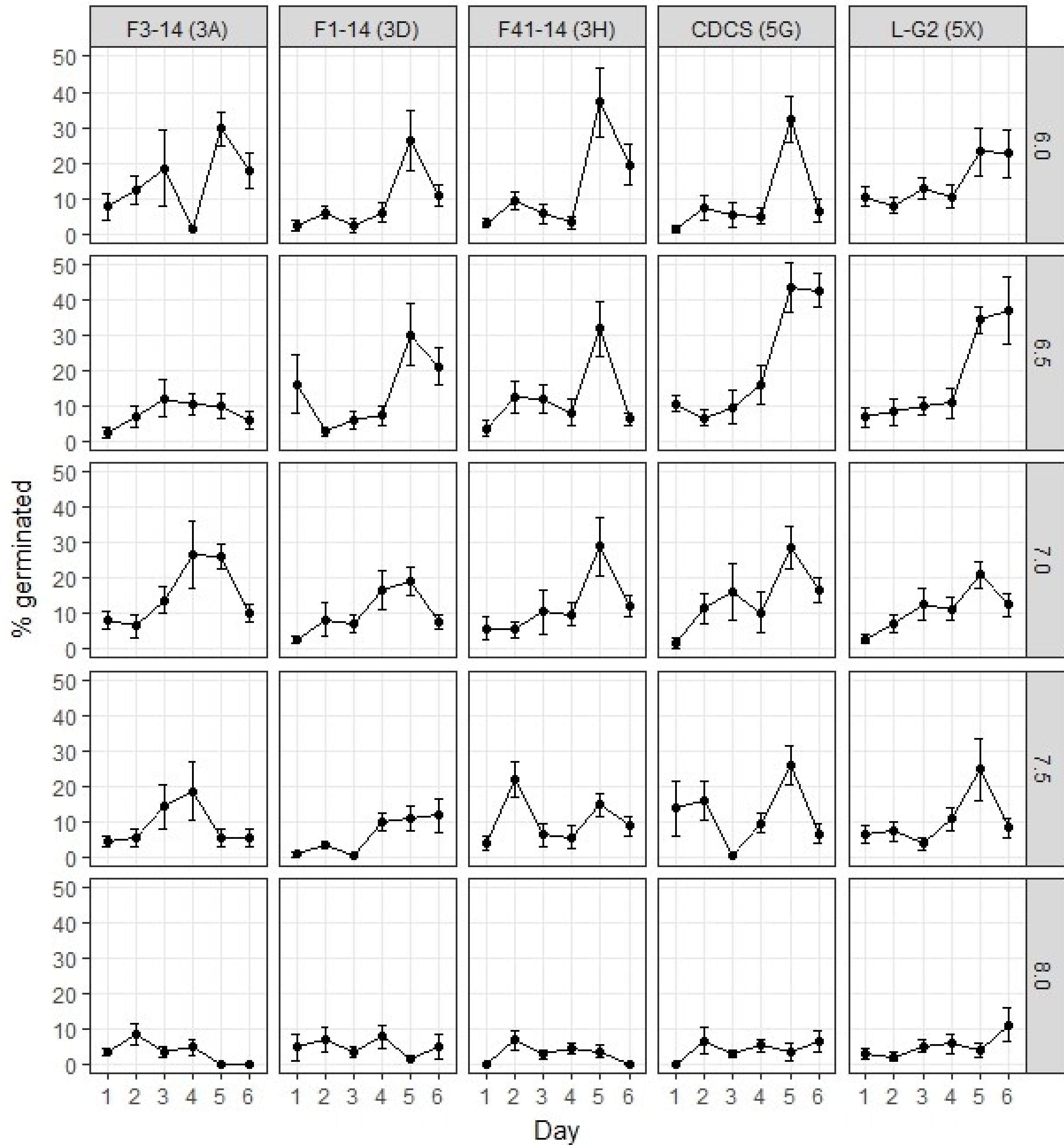

Figure 3.3: *Percentage germinated resting spores of each of five isolates of* Plasmodiophora brassicae *over a six-day period in a modified Hoagland's solution with pH adjusted to 6.0, 6.5, 7.0, 7.5, or 8.0 with Tris-base (Trial 1). Germination is expressed as the proportion of germinated resting spores out of all resting spores visually assessed microscopically on each day. Bars represent the standard error of the mean.*

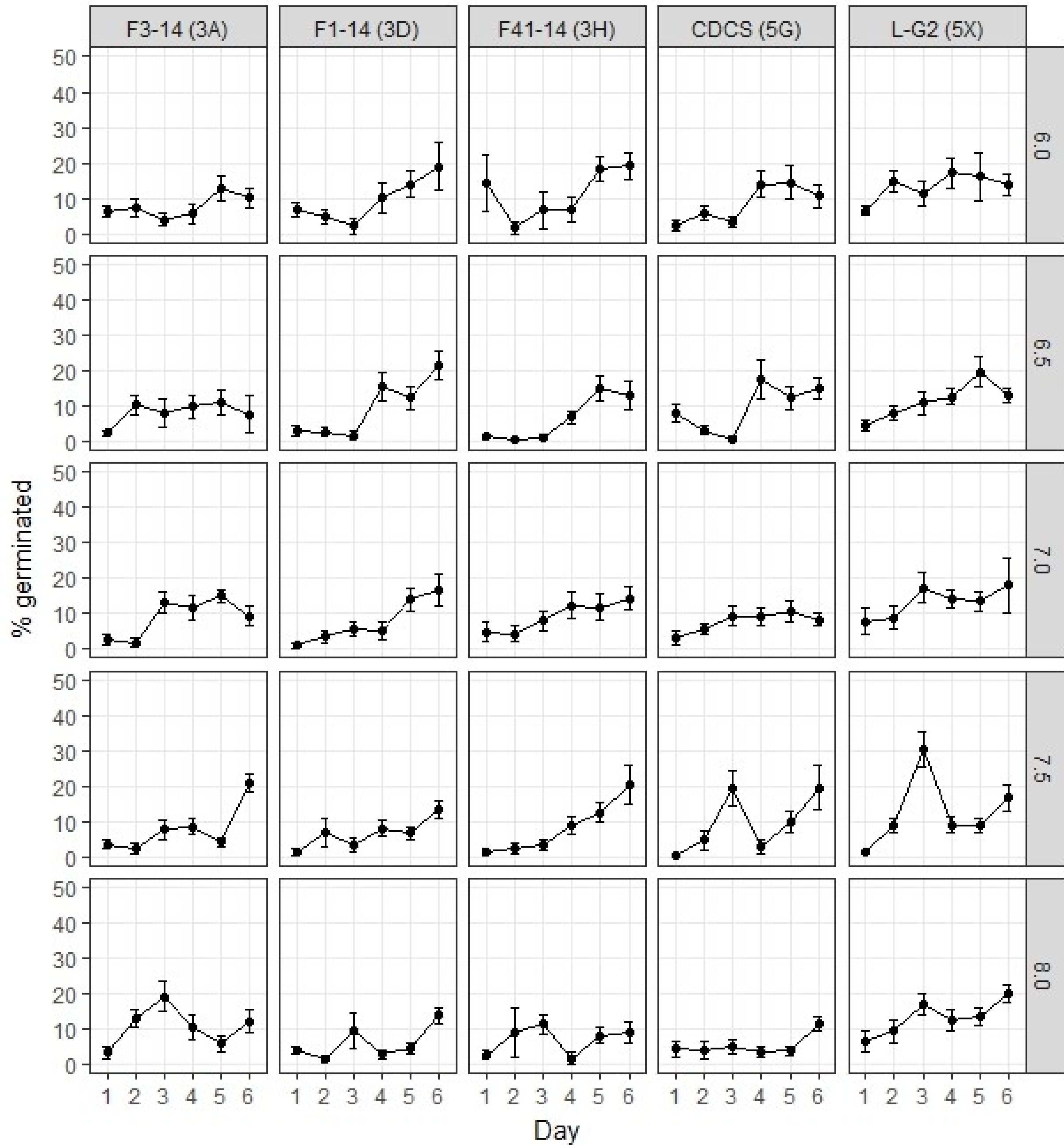

Figure 3.4: *Percentage germinated resting spores of each of five isolates of* Plasmodiophora brassicae *over a six-day period in a modified Hoagland's solution with pH adjusted to 6.0, 6.5, 7.0, 7.5, or 8.0 with Tris-base (Trial 2). Germination is expressed as the proportion of germinated resting spores out of all resting spores visually assessed microscopically on each day. Bars represent the standard error of the mean.*

Chapter 4: Conclusions and future research

4.1 Conclusions

Clubroot is an ongoing threat to the Canadian canola industry, requiring an integrated approach for its effective management. Deploying clubroot-resistant (CR) cultivars is the most promising strategy for preventing disease but, over time, these varieties exert selection pressure on *Plasmodiophora brassicae* populations, leading to the emergence of 'new' pathotypes that can overcome resistance (Leboldus et al. 2012; Strelkov et al. 2018). The tendency of growers to continue planting canola in short rotations in fields heavily infested with *P. brassicae* (Peng et al. 2014b) highlights the need for more comprehensive approaches to managing this pathogen, as well as the need for a stronger understanding of the diversity in responses across isolates.

Clubroot development is favored by acidic soils, which are common in Alberta (Myers and Campbell 1985; Alberta Agriculture Food and Rural Development 1996). Liming acidic soil to achieve a pH of ≥ 7.2 is often recommended as a strategy to reduce clubroot severity in cruciferous crops (Myers and Campbell 1985; Webster and Dixon 1991a; Murakami et al. 2002). Disease progression, however, is influenced by multiple factors, including soil inoculum concentration, temperature, soil moisture, and soil type (Colhoun 1953; Hwang et al. 2011a; Gossen et al. 2013, 2014). As discussed in this thesis, the pH sensitivity of individual isolates of *P. brassicae* may also differ. Therefore, the suggestion that raising the soil pH to a specific level can prevent disease does not fully account for the complexity of factors involved in clubroot development.

Clubroot is found in an increasing number of fields each year (Strelkov et al. 2021). A recent study examining pathotype shifts in the Canadian Prairies highlighted the increasing prevalence of resistance-breaking pathotypes of *P. brassicae*, underscoring the diversity in

virulence of *P. brassicae* populations (Hollman et al. 2021). The continued diversification in the virulence of the pathogen populations suggests that individual isolates belonging to a pathotype may respond differently to various management approaches. In fact, galled roots of infected plants contain multiple isolates representing a variety of pathotypes, and pathogen field populations are known to be genetically diverse (Jones et al. 1982; Manzanares-Dauleux et al. 2001). This means that the response of the pathogen to a management practice, such as liming of the soil, can be variable.

Hydrated lime has shown promise in limiting clubroot development under field conditions in Alberta (Fox et al. 2022), but it is unclear whether pH-insensitive *P. brassicae* isolates occur which could reduce the effectiveness of this treatment. In this thesis, disease development in a clubroot-susceptible canola cultivar was compared at pH 6.3, 7.2 and 8.0 following inoculation with each of five field isolates of *P. brassicae*, representing pathotypes 3A, 3H, 3D, 5G, and 5X. While clubroot symptoms at pH 6.3 were severe across isolates under medium and high inoculum densities, the results at pH 7.2 were more variable. One isolate appeared particularly sensitive to pH 7.2, causing very mild levels of disease relative to the other isolates. Little or no clubroot developed in response to any isolate at pH 8.0. In a second experiment, three of the isolates were tested on the same clubroot-susceptible canola at pH 6.3, 7.0, 7.3, 7.6 and 7.9. While clubroot was severe at pH 6.3, 7.0 and 7.3, disease declined significantly at pH 7.6 and again at pH 7.9. The results from these greenhouse trials suggested some variability in the pH sensitivity of the isolates. Considering that only five isolates were tested, it is likely that a wider range of responses would have been observed with a larger collection of isolates. In any case, the results seem to indicate that targeting a minimum pH of 7.2 may not always be enough for effective clubroot management.

In order to gain insights into the potential mechanisms by which clubroot development is inhibited at higher pH, an additional study was conducted where resting spore germination for the different isolates was compared *in vitro* at pH values ranging from 6.0 to 8.0. Germination rates were generally similar at pH 6.0 to 7.5, but very low at pH 8.0. This suggested that some of the reduction in clubroot severity observed at pH 8.0 under greenhouse conditions could reflect reduced spore germination.

4.2 Future research

While the effectiveness of lime to control clubroot in canola has shown inconsistencies, it has also shown potential in some studies and should, therefore, be considered as a management option. Lime works by raising the soil pH to create an environment more inhospitable to the pathogen. In a recent report, hydrated lime effectively reduced the clubroot disease severity index in canola and limited pathogen proliferation in host roots (Fox et al. 2022). To our knowledge, this thesis represents the first comparison of the pH sensitivity of a collection of *P. brassicae* isolates and should help to guide further efforts to improve clubroot management via soil amendments. This work also serves as a foundation for additional research, to continue to improve the understanding of the clubroot pathogen and its response to pH.

Future research on this topic should involve assessing the pH insensitivity of single-spore isolates of *P. brassicae*. A *P. brassicae*-infected root gall contains varying proportions of different isolates. Thus, using field galls for experimentation may introduce variability in the results and make it difficult to attribute an effect to one individual. Similarly, analysis of a larger collection of isolates may also provide a more accurate indication of the full range of pH sensitivity in *P. brassicae* populations. Recently, Askarian et al. (2021) established a large collection of *P.*

brassicae single-spore isolates. This could make an excellent resource for additional studies, since it includes well over 30 isolates that could be compared, all of them derived from single spores. In addition, more isolates could be recovered from fields with higher soil pH, such as those in southern Alberta. This could aid in clarifying the upper limits of tolerance for alkalinity in *P. brassicae*.

Assessing the pH insensitivity of a larger collection of *P. brassicae* isolates will enable stronger conclusions regarding the effectiveness of increasing the soil pH as part of an integrated clubroot management plan. Considering the high costs of lime, particularly hydrated lime, a better understanding of its potential at controlling this disease could help to justify its purchase and application, especially for localized patch management. Ultimately, comprehensive studies that investigate individual isolate responses to various management strategies will provide better direction for the management of clubroot.

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Appendix

Table A.1: *Weekly pH summary for each treatment in Experiment 1 Trial 2.*

pH Treatment	*Week of experiment*	*Average pH*
No lime (pH 6.3)	1	5.8
No lime (pH 6.3)	2	5.9
No lime (pH 6.3)	3	6.0
No lime (pH 6.3)	4	6.3
No lime (pH 6.3)	5	6.4
No lime (pH 6.3)	6	6.6
No lime (pH 6.3)	7	6.6
pH 7.2	1	7.1
pH 7.2	2	6.6
pH 7.2	3	6.8
pH 7.2	4	7.0
pH 7.2	5	7.1
pH 7.2	6	7.3
pH 7.2	7	7.4
pH 8.0	1	8.0
pH 8.0	2	7.9
pH 8.0	3	7.9
pH 8.0	4	8.1
pH 8.0	5	8.2
pH 8.0	6	8.1
pH 8.0	7	8.1

Table A.2: *Weekly pH summary for each treatment in Experiment 1 Trial 3.*

pH Treatment	*Week of experiment*	*Average pH*
No lime (pH 6.3)	1	5.6
No lime (pH 6.3)	2	6.1
No lime (pH 6.3)	3	6.1
No lime (pH 6.3)	4	6.3
No lime (pH 6.3)	5	6.7
No lime (pH 6.3)	6	7.0
No lime (pH 6.3)	7	-
No lime (pH 6.3)	8	7.0
pH 7.2	1	7.4
pH 7.2	2	7.4
pH 7.2	3	7.3
pH 7.2	4	7.4
pH 7.2	5	7.8
pH 7.2	6	8.0
pH 7.2	7	-
pH 7.2	8	8.0
pH 8.0	1	8.1
pH 8.0	2	7.9
pH 8.0	3	7.9
pH 8.0	4	7.8
pH 8.0	5	8.2
pH 8.0	6	8.3
pH 8.0	7	-
pH 8.0	8	8.3

Table A.3: *Weekly pH summary for each treatment in Experiment 2 Trial 1.*

pH Treatment	*Week of experiment*	*Average pH*
No lime (pH 6.3)	1	6.2
No lime (pH 6.3)	2	6.0
No lime (pH 6.3)	3	6.3
No lime (pH 6.3)	4	6.6
No lime (pH 6.3)	5	-
No lime (pH 6.3)	6	-
No lime (pH 6.3)	7	7.1
No lime (pH 6.3)	8	7.3
pH 7.0	1	6.6
pH 7.0	2	6.2
pH 7.0	3	6.5
pH 7.0	4	6.8
pH 7.0	5	-
pH 7.0	6	-
pH 7.0	7	7.3
pH 7.0	8	7.3
pH 7.3	1	7.2
pH 7.3	2	6.8
pH 7.3	3	7.0
pH 7.3	4	7.3
pH 7.3	5	-
pH 7.3	6	-
pH 7.3	7	7.6
pH 7.3	8	7.8
pH 7.6	1	7.7
pH 7.6	2	7.5
pH 7.6	3	7.7
pH 7.6	4	7.9
pH 7.6	5	8.1
pH 7.6	6	-
pH 7.6	7	-
pH 7.6	8	8.2
pH 7.9	1	7.7
pH 7.9	2	7.7
pH 7.9	3	7.9

pH 7.9	4	8.0
pH 7.9	5	8.3
pH 7.9	6	-
pH 7.9	7	-
pH 7.9	8	8.3

Table A.4: *Weekly pH summary for each treatment in Experiment 2 Trial 2.*

pH Treatment	*Week of experiment*	*Average pH*
No lime (pH 6.3)	1	6.1
No lime (pH 6.3)	2	6.3
No lime (pH 6.3)	3	-
No lime (pH 6.3)	4	-
No lime (pH 6.3)	5	-
No lime (pH 6.3)	6	-
No lime (pH 6.3)	7	6.9
pH 7.0	1	6.6
pH 7.0	2	6.6
pH 7.0	3	-
pH 7.0	4	-
pH 7.0	5	-
pH 7.0	6	-
pH 7.0	7	7.1
pH 7.3	1	7.2
pH 7.3	2	7.2
pH 7.3	3	-
pH 7.3	4	-
pH 7.3	5	-
pH 7.3	6	-
pH 7.3	7	7.6
pH 7.6	1	7.4
pH 7.6	2	7.5
pH 7.6	3	-
pH 7.6	4	-
pH 7.6	5	-
pH 7.6	6	-
pH 7.6	7	7.9
pH 7.9	1	7.8
pH 7.9	2	7.9
pH 7.9	3	-
pH 7.9	4	-
pH 7.9	5	-
pH 7.9	6	-
pH 7.9	7	8.2