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Contribution of non-host crops of *Plasmodiophora brassicae* to clubroot management and inoculum potential

by

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Abstract

Clubroot, caused by *Plasmodiophora brassicae* Woronin, is a major soilborne disease of canola in Alberta. Root exudates from host (Chinese cabbage (*Brassica rapa* var. *pekinensis*, cv. Granaat), canola (*Brassica rapa* L.)) and non-host (perennial ryegrass (*Lolium perenne* L.)) plants were found to significantly stimulate germination of pathogen resting spores, suggesting that these plants could be used as bait crops to manage clubroot. In greenhouse studies, cropping of *B. rapa* L. canola and perennial ryegrass significantly reduced clubroot severity in a subsequent *B. napus* L. canola crop, but more research is needed before bait crops can be recommended for clubroot management. Secondary zoospores produced on ryegrass could infect canola, resulting in disease development and indicating that ryegrass could contribute to *P. brassicae* inoculum in the soil. The manipulation of seeding dates and cropping of resistant cultivars were also assessed as clubroot management tools and found to reduce the severity of disease. Sustainable management of clubroot of canola will likely require an integrated approach, incorporating various strategies.

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1. Introduction and literature review

1.1 Clubroot disease

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is an economically important disease of the family Brassicaceae (Howard et al. 2010). The first record of clubroot and its transmission was by Palladius in Italy in the 4th Century AD, who observed spongy roots on rape, turnips and radishes after soil was fertilized with manure from livestock fed infected roots (Watson and Baker 1969). Clubroot became widely distributed in Europe, and in 1736 was first reported in England on turnip (Howard et al. 2010). It is likely that clubroot was introduced to the United States, Canada, Australia and New Zealand in infected fodder turnips used to feed the livestock brought over by early European settlers (Howard et al. 2010).

1.1.1 Distribution of clubroot on canola

In Canada, clubroot has historically occurred in the eastern parts of the country and British Columbia, where it can be an important problem in vegetable cole crop production (Rimmer et al. 2003). The first report of clubroot on non-vegetable cruciferous crops in Canada was on rape in Newfoundland in 1965 (Howard et al. 2010). However, despite extensive cropping of canola and mustard throughout the prairies for many decades, it was not until 2003 when the first cases of clubroot were identified on *Brassica napus* L. canola (Tewari et al. 2005). That year, 12 out of a total of 70 commercial canola fields surveyed near St. Albert, AB, were found to be clubroot-

infested (Strelkov et al. 2005). In one severely infested field, about 94% of the plants were infected, with approximately 30% yield loss (Strelkov et al. 2005). A larger disease survey in 2005 revealed the occurrence of clubroot in a total of 41 commercial canola fields, mainly in the Edmonton, AB, region (Strelkov et al. 2006a). By the fall of 2008, Strelkov et al. (2009) indicated that there were a total of 405 fields with confirmed clubroot in Alberta, distributed over 14 counties and a rural area of northeast Edmonton. In 2009, a total of 51 new cases of the disease were found in canola crops throughout central Alberta, despite generally dry conditions that were unfavorable for disease development (Strelkov et al. 2010). This brought the total number of fields with confirmed clubroot to at least 456 by the fall of 2009, indicating that clubroot of canola has become endemic in central Alberta and is likely spreading. Complete losses were observed in several canola crops with severe infestations of the disease (Howard et al. 2010).

1.1.2 Symptoms

The most typical characteristic of clubroot disease is the development of swollen clubs or galls on the roots of susceptible host plants. On canola, clubroot galls may be confused with small, round hybridization nodules that sometimes form on the roots, although these are rare (Hartman 2007). The spongy interior texture of a clubroot gall can be used to distinguish the gall from the hybridization nodules, which have a uniformly dense texture inside. Moreover, hybridization nodules will not decay rapidly to a peaty appearance as do clubroot galls (Hartman 2007).

The formation of root galls can hinder water and nutrient uptake by infected plants, leading to the development of above-ground symptoms that include stunting, wilting, yellowing and premature plant death. The extent of the above-ground symptoms is a function of both the severity of the root galling, as well as the timing of the initial infection of the host. For instance, while seedling infection can result in above-ground wilting, stunting and yellowing, an infection that occurs at later stages in the host life cycle may result only in premature ripening and the shriveling of the seeds (Howard et al. 2010). Patches of prematurely ripened canola caused by *P. brassicae* infection may sometimes be confused with Sclerotinia stem rot, blackleg or Fusarium wilt, all of which may also cause similar above-ground symptoms. In such cases, proper diagnosis should include digging up the affected plants to check for formation of galls on the roots (Hartman 2007).

1.2 *Plasmodiophora brassicae*: the causal agent of clubroot

1.2.1 Life cycle of *P. brassicae*

There are three main stages in the life cycle of *P. brassicae*: survival in the soil, root hair infection, and cortical infection (Kageyama and Asano 2009). A diagrammatic representation of the life cycle of *P. brassicae* is shown in Figure 1-1. In the first stage, it is the resting spores that allow the pathogen to survive for many years in the absence of a susceptible host and which also serve as the primary inoculum. When a resting spore germinates, it produces a primary zoospore with two flagellae. The zoospores can swim in the free water in the soil, and cause infections by penetrating the cell walls of root hairs. This stage is termed the root hair infection or primary infection stage (Kageyama

and Asano 2009). These primary infections do not cause symptoms visible to the naked eye, and do not result in significant yield or quality losses (Howard et al. 2010). In the root hairs, the pathogen produces primary plasmodia. After a series of nuclear divisions, the primary plasmodia develop into zoosporangia, which form clusters in the root hair. Afterwards, the secondary zoospores are released from the zoosporangia, which can penetrate the host and invade the cortical tissues of the main roots (Howard et al. 2010), causing the cortical infection or secondary infection. Inside the infected cells, the pathogen develops into secondary plasmodia which proliferate and are associated with cellular hypertrophy, resulting in gall formation in the root tissues (Kageyama and Asano 2009). Eventually, the secondary plasmodia are cleaved into millions of resting spores in the galls. As the root tissue decays, the resting spores are released into the soil to complete one life cycle. The pathogen usually completes one cycle per season and cannot spread rapidly in the soil, as zoospore motility is quite limited (Howard et al. 2010).

1.2.2 Resting spore germination

P. brassicae resting spores can survive in the soil for long periods. The half-life of inoculum in heavily infested fields was reported to be 3–6 years, with the level of infestation declining to below detectable levels only after 17.3 years (Wallenhammar. 1996). When the resting spores germinate, they produce zoospores and initiate the life cycle. However, the resting spores are influenced by the surrounding soil environment. Temperature, moisture content, and position in the soil profile will influence spore longevity, and acidic soils can increase the rate of production of primary zoospores

relative to alkaline soils, but do not have a large impact on resting spore germination itself (Dixon 2009b).

Not only can the surrounding environment affect spore germination, but so can the age and storage conditions of the clubroot galls. Resting spores from old, decaying galls germinated sooner than from younger galls (Macfarlane 1970). Scanning electron microscopy revealed that while mature resting spores are covered with spine-like structures, the young spores are covered in a fibrous material. Mature spores also germinate regardless of the presence of calcium ions, whereas young spores require the presence of calcium ions (Kageyama and Asano 2009). Therefore, resting spore populations are not uniformly mature and their ability to germinate is associated with the level of maturation of individual spores (Kageyama and Asano 2009).

Surface-disinfected resting spores showed higher germination than non-disinfected resting spores (Asano et al. 2000). *P. brassicae* resting spores were surface-disinfected with 2% (w/v) cloramine-T solution for 20 min and then incubated in an antibiotic solution (1 *u*g/ml of colistin sulfate, 1 *u*g/ml of vancomycin hydrochloride, and 6 *u*g /ml of cefotaxime sodium) for 1 day (Asano et al. 2000). The germination of the surface-disinfected resting spores increased steadily for 6 days, but then stopped almost completely in the following days. The non-disinfected resting spores germinated slowly until 10 days. At the end of the experiment, the total germination rate of surface-disinfected resting spores (12%) was significantly higher than that of the non-disinfected resting spores (6.7%). Therefore, it seems that the germination of resting spores can be influenced by the surrounding microflora.

It has also been observed that root exudates from host and non-host species can stimulate *P. brassicae* resting spore germination (Kageyama and Asano 2009). Germination was hastened and the proportion of spores that germinated increased in the presence of cabbage plants or of diffusate from cabbage roots (Macfarlane 1970). In an aqueous solution in a laboratory study, the exudates from the roots of perennial ryegrass (*Lolium perenne* L.) stimulated the germination of *P. brassicae* resting spores (Friberg et al. 2005). Therefore, the cropping of bait plants, which can induce germination of resting spores and thereby decrease the soil inoculum load, might be a useful tool for the management of clubroot, as discussed in more detail below.

1.2.3 Host range

All 330 genera and 3700 species of the family Brassicaceae are predicted to be potential hosts for *P. brassicae* (Howard et al. 2010). Cultivated crops seem to be particularly susceptible to this pathogen. This includes all varieties of *Brassica oleracea* L., the occidental cole vegetables (Brussels sprouts, cabbage, calabrese/green broccoli, cauliflower, culinary and fodder kale, kohlrabi); *Brassica rapa* L., including turnip, turnip rape, sarson, and an enormous range of Asian variants which provide leaf and root vegetables such as *B. rapa* var. *pekinensis* (Chinese cabbage) and *B. rapa* var. *chinensis* (pak choi); *B. napus*, including swede (rutabaga), oil seed rape, and fodder rape; and seed, condiment (mustard) and vegetable crops derived from *B. carinata, B. nigra,* and *B. juncea* (Dixon 2009a). Related genera such as radish (*Raphanus*), cruciferous weeds, for example *Sinapis*, and decorative ornamentals including stocks (*Matthiola* spp.) and wallflower (*Cheiranthus cheiri* L.) can also be infected (Dixon 2009a).

1.2.4 The terminology related to the study of pathogenic diversity in *P. brassicae*

A "population" of *P. brassicae* refers to a collection of resting spores recovered from infested soil or galls of an infected plant and used to inoculate a set of differential hosts (Buczacki et al. 1975). A "single-spore isolate" is a population derived from a club inoculated with a single resting spore and maintained in isolation (Voorips 1995). The terms "pathotype" and "race" are often used interchangeably in the literature to discuss physiologic specialization in *P. brassicae*. However, in recent Canadian studies, the term pathotype has been more commonly used (see for example Xue et al. 2008), since neither the populations of the pathogen nor the differential hosts possess the genetic uniformity necessary to apply the concept of races to the clubroot pathosystem (Parlevliet 1985).

1.2.5 Pathotypes of *P. brassicae* in Canada

There have been various systems proposed for the pathotype classification of *P. brassicae* populations and single-spore isolates. In studies of the pathogenic diversity of field populations of *P. brassicae* from Canada, the differential set of Williams (1966), which consists of two cabbage and two rutabaga cultivars, has been commonly used. In Europe, the European Clubroot Differential (ECD) set, which consists of 15 host genotypes representing three subsets (a *B. rapa*., a *B. napus* and a *B. olearacea* subset) (Buczacki et al. 1975), has been more widely employed to analyze pathogenic diversity in *P. brassicae*. More recently, the differential set of Somé et al. (1996), which consists of three *B. napus* genotypes, has also been employed in some studies.

In a study by Hildebrand and Delbridge (1995), a total of 10 populations of *P. brassicae* from Nova Scotia were examined, eight of which were designated as pathotype

3, one as pathotype 2 and another as pathotype 1 based on the differentials of Williams (1966). Field populations of *P. brassicae* from Alberta were classified as pathotypes 3 and 5 on the differential set of Williams (1966), or as ECD 16/15/12 and ECD 16/15/0 on the ECD set, respectively, if an index of disease of <50% was taken as a resistant reaction (Strelkov et al. 2006b). Pathotype 3 or ECD 16/15/12 was predominant on canola. Similarly, in another study conducted by Strelkov et al. (2007), all *P. brassicae* field populations tested from Alberta were classified as ECD 16/15/12 on the hosts of the ECD set, or as pathotype 3 or P_2 on the differentials of Williams (1966) or Somé et al. (1996), respectively. In contrast, a population from British Columbia was classified as pathotype 6 or ECD 16/2/12, respectively, on the differentials of Williams (1966) or the ECD set, while a population from Ontario was classified as pathotype 6 or ECD 16/0/14.

It is important to note that field populations may consist of a mixture of pathotypes, and rare pathotypes within that mixture may not be detected, particularly if they occur at very low frequencies (Strelkov et al. 2007). Therefore, the diversity of the pathogen may not be fully evaluated when tests are conducted using *P. brassicae* populations.

Xue et al. (2008) used single resting spore-derived isolates instead of field populations of *P. brassicae* to assess the diversity of this pathogen. They found that the pathotype composition of *P. brassicae* in Canada was more diverse when single-spore isolates were examined instead of populations. In Alberta, at least three and possibly four pathotypes were identified among 14 single-spore isolates tested, whereas a maximum of only two pathotypes had been reported by Strelkov et al. (2006b) and Strelkov et al. (2007) when populations of the pathogen were examined. Thus far, pathotypes 2, 3, 5, 6,

and 8 (as classified on the differentials of Williams (1966)) have all been reported from Alberta, with pathotype 3 being the predominant strain (Howard et al. 2010).

1.2.6 Ecological roles of *P. brassicae* (Phytomyxea)

Taxonomically, *P. brassicae* has most recently been placed in the Class Phytomyxea within the Phylum Cercozoa (Bass et al. 2009). Organisms within this class can play three ecological roles in food webs, namely: (1) serving as a food source, (2) facilitating the transfer of energy derived from primary producers or from saprobes and other parasites, and (3) indirectly affecting carbon flow by altering the growth and chemical composition of their host (Neuhauser et al. 2011).

The walls of *P. brassicae* resting spores contain high amounts of energy rich substances, consisting of 25% chitin, 38% protein, 17.5% lipid and 2.5% carbohydrate (Buczacki and Moxham, 1983). Therefore, these thick-walled resting spores possess high nutritional energy and can serve as an excellent source of energy for large protists and animals in the soil ecosystem. Moreover, since the thick walls are resistant to degradation in the environment, the resting spores may be consumed sequentially by more than one individual before being completely digested. In this manner, the resting spores have the potential to transfer energy from primary producers to consumers (Neuhauser et al. 2011). Indeed, Neuhauser et al. (2011) indicated that in aquatic ecosystems, the zoospores of Phytomyxea facilitate a significant transfer of energy from primary producers (photosynthetic organisms) and primary consumers (heterotrophic stramenopiles) to secondary and tertiary consumers such as grazing zooplankton or filter-feeding metazoa.

Upon infection, *P. brassicae* can cause hypertrophy and change the metabolic status of its host, resulting in the accumulation of nutritients (glucose, sucrose, starch, amino acids, sterols and fatty acids) in the clubbed roots (Neuhauser et al. 2011). These rich sources of nutrition make the clubbed roots an attractive food for various micro- and macro-organisms, and are quickly colonised by bacteria and fungi after the resting spores are formed (Neuhauser et al. 2011). Therefore, *P. brassicae* also has a significant potential to affect carbon flow from plants, particularly in agricultural systems where large amounts of galled root tissue may be formed.

1.3 Detection of *P. brassicae*

Detection of the clubroot pathogen in the soil before planting of a susceptible crop is a good method to avoid the disease, but requires a sensitive and reliable technique to measure or quantify the pathogen. The detection and quantification of *P. brassicae* has been hindered by the fact that this is an obligate parasite, and as such cannot be cultured on axenic medium. There have been four main approaches to detect *P. brassicae* in soil and other samples: the use of bait plants, fluorescence microscopy, serology, and the use of polymerase chain reaction (PCR)-based techniques. These are discussed in detail below.

1.3.1 Bait plants (Bioassays)

The use of bait plants is the most reliable method to detect *P. brassicae* in the soil. Samuel and Garrett (1945) developed a method to estimate the relative amounts of

germinated spores in different soils, by counting infected root hairs. They grew cabbage seedlings for 1 week and stained the tap root with aceto-carmine, counting the number of root hairs containing zoosporangia of *P. brassicae* along 2 cm of root. However, this method was affected by soil alkalinity, degree of infection and soil-moisture content. To avoid the influence of various environmental factors on infection, Colhoun (1957) grew the plants in pots under controlled conditions favourable for infection for a minimum period of 5 weeks. If careful sampling of the naturally infested soil was conducted and contamination of one soil sample from another was prevented, satisfactory results could be obtained (Colhoun 1957). Testing methods were adapted and refined by researchers. More recently, Wallenhammar (1996) sampled the soil at 50 points along a diagonal line in a field and avoided contamination by changing gloves to handle each soil sample. Good plant germination, favourable growing conditions, adequate nutrition and a reasonable disease assessment protocol provided reliable bioassay results in this study.

Bait plant techniques are useful, however, only when inoculum reaches levels greater than 1000 spores per gram dry soil, which is the generally accepted threshold for symptom development (Faggian and Strelkov 2009). These methods are also time-consuming and can require large amounts of greenhouse space. Therefore, they cannot become the basis of a routine diagnostic test for large numbers of samples for practical and economic reasons (Faggian and Strelkov 2009).

1.3.2 Fluorescence Microscopy

Takahashi and Yamaguchi (1987) developed a fluorescence assay to detect *P. brassicae* in the soil. They mixed the infested soil suspension with a single fluorochrome,

examined the mixture solution and counted the number of spores showing strong fluorescence under a fluorescence microscope. However, the fluorescence staining method using a single fluorochrome did not give a satisfactory result. Takahashi and Yamaguchi (1988) improved the assay by using two fluorochromes to evaluate spore viability. The spores that reacted with calcofluor white M2R (a fluorochrome that binds to the chitin of the spore cell walls) and exhibited an intense blue fluorescence were classified as viable. The spores that could be stained with ethidium bromide (a fluorochrome that penetrates the damaged spores) and exhibited a red fluoresence were classified as non-viable. Both Takahashi and Yamaguchi (1988) and Takahashi and Yamaguchi (1989) found a significant correlation between the percentage of blue (viable) spores and disease severity on susceptible plants. However, Donald et al. (2002) indicated that there was no correlation between spore viability and the development of disease symptoms as predicted by the fluorescent-staining method.

As indicated by Faggian and Strelkov (2009), fluorescing soil particles and other artefacts can make it difficult to identify *P. brassicae* resting spores under a microscope, and sample throughput is restricted by operator skill. Therefore, fluorescence microscopy cannot be the basis for a routine diagnostic assay for the detection of *P. brassicae* resting spores in the soil.

1.3.3 Serology

The first serology detection method for *P. brassicae* was developed by Arie et al. (1988). They used the fluorescent-antibody technique with the IgG and FITC-conjugated antirabbit IgG-sheep IgG to stain clubroot infested soil and root samples. Although this

method could detect resting spores effectively, the cross-reactivity of the antiserum with other microorganisms was not evaluated and detection limits were not discussed (Faggian and Strelkov 2009).

Lang et al. (1989) developed a dot immunobinding assay to detect *P. brassicae*. The antiserum of *P. brassicae* did not react with surface antigens of resting spores of *Polynyxa*, and did not cross react with *Pythium ultimun*. Trow, *Rhizoctonia solani.* J.G. Kühn and *Fusarium oxysporum* (Schlecht.) Snyder & Hansen (Lang et al. 1989). Wakeham and White (1996) assessed western blotting, dip-stick, dot blot, indirect enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence for the potential to detect resting spores of *P. brassicae*. The dip-stick assay, indirect ELISA and indirect immunofluorescence were the most sensitive techniques, with a detection limit of 1×10^2 spores/ g soil (Wakeham and White 1996). The polyclonal antisera showed low cross-reactivity with the plasmodiophorid pathogen *Spongonspora subterranea* (Wallr.) Lagerheim.

Serologic assays can be considered as a routine test for the detection for the resting spores of *P. brassicae*. However, Faggian and Strelkov (2009) noted that since polyclonal antisera cannot be reproduced and are limited in quantity, their usefulness as a diagnostic tool is limited. Serologic assays based on monoclonal antibodies may improve specificity and provide an infinite source of antibodies (Faggian and Strelkov 2009), and hence may represent a desirable alternative.

1.3.4 Polymerase Chain Reaction

PCR-based techniques require only DNA and are ideally suited to detection of *P. brassicae* from soil and tissue samples, and are also relatively fast and convenient (Faggian and Strelkov 2009). Buhariwalla et al. (1995) designed PCR primers for the amplification of polymorphic DNA from *P. brassicae*. Although these primers produced a small number of polymorphic and highly amplified fragments of DNA, these fragments were difficult to distinguish from a large number of fragments produced by arbitrary primers, and from the amplification products from host DNA (Buhariwalla et al. 1995). Therefore, this particular protocol could not be used for diagnostic purposes.

A single-tube nested (STN) PCR protocol was developed by Ito et al. (1999) to detect *P. brassicae* in the soil. This STN PCR protocol utilized outer primers to amplify a 1457 bp-fragment from *P. brassicae* DNA and nested primers to amplify a 398 bp-fragment within the 1457 bp-fragment. This protocol could detect as little as one resting spore per g of inoculated soil. However, to improve the detection of *P. brassicae* in naturally infested soil, the STN PCR products were subjected to another round of PCR amplification (double PCR), using the nested primers (Ito et al.1999).

Faggian et al. (1999) also developed a nested PCR protocol for detection of *P. brassicae*, using primers designed from the ribosomal repeat and ITS regions of rDNA. The specificity of the primers was tested against more than 40 common soil organisms, host plants and spore suspension contaminants, as well as *P. brassicae* isolates. Sensitivity was determined to be 0.1 fg of *P. brassicae* DNA when it was used as a pure template, corresponding to as few as 1000 spores per g of potting mix (Faggian et al.1999). However, this protocol still required a two-step PCR.

Another two-step nested PCR protocol was reported by Wallenhammar and Arwidsson (2001). This protocol could detect the DNA in various soil types with an inoculum level of *P. brassicae* corresponding to a disease severity index higher than 21% in a greenhouse bioassay (Wallenhammar and Arwidsson. 2001). Finally, a simple, one-step PCR protocol was developed by Cao et al. (2007) to detect *P. brassicae* in plant and soil samples. As described by Cao et al. (2007), these primers did not amplify any DNA fragment from non-infected plant hosts, non-infested soil or common soil fungi and bacteria tested. Quantities of 100 fg or less of total *P. brassicae* DNA, or 1×10^3 resting spores per gram of soil, could be detected consistently using the primers and PCR protocol, corresponding to an index of disease of 11% or lower when the soil was bioassayed (Cao et al. 2007). Most recently, quantitative PCR-based techniques have been under development by various research groups, and could serve to enhance efforts to detect and quantify *P. brassicae* in soil and plant samples.

1.4 Disease management

Given the persistence of *P. brassicae* resting spores in the soil, and the significant negative impact of clubroot on the yield and quality of susceptible host crops, appropriate management of the disease is very important. As such, a number of different management strategies have been developed to control clubroot of crucifers.

1.4.1 Host resistance

Host resistance can provide the cheapest and most effective control measure for clubroot disease. However, susceptibility to clubroot varies widely between different types of cruciferous crops. As reported by Howard et al. (2010), Asian vegetables (Chinese cabbage, bok choy, suey choy and park choy), cabbage and Brussels sprouts are amongst the most susceptible. Broccoli, cauliflower, collards, kale, kohlrabi, rutabaga and turnip are considered moderately resistant, while radish is perhaps the most tolerant to clubroot. In Canada, some resistant varieties cruciferous vegetables have been developed, including the rutabagas 'Kingston' and 'York' and the cabbage 'Richelain'. Prairie Farm and Ranch (2010) indicated that in spring 2009, Pioneer Hi-Bred launched the first clubroot-resistant canola hybrid, '45H29'. Two more clubroot-resistant canola hybrids, '73-77RR' and '73-67RR', became available in 2010, and Canterra will be introducing another resistant hybrid in 2011. In Australia, resistant commercial vegetable cultivars, including the cabbage cv. Maxfield and the cauliflower cv. Highfield, were released in 2007 (Donald and Porter 2009). In the UK and mainland Europe, clubroot-resistant white cabbage cvs. Tekila, Kilaton, and Kilaxy, and the cauliflower cv. Clapton, were introduced in 2005 (Donald and Porter. 2009). The resistant oilseed rape cv. Mendel has been available to growers in UK since 2003, but a serious disease outbreak occurred because of a short rotation of this cultivar on clubroot-infested land (Oxley 2007). Indeed, the overuse of resistant varieties under conditions of high disease pressures may lead to a breakdown of resistance (Howard et al. 2010). For example, in Australia, continuous cropping of older resistant cultivars of Chinese cabbage (cv. Yuki) led to control failures (Donald and Porter 2009).

At present, resistance genes from stubble turnips (*B. rapa*) are the most effective and most widely used for resistance breeding in different *Brassica* crops (Diederichsen et al. 2009). The resistance to *P. brassicae* in turnips was introduced into Chinese cabbage, oilseed rape, and *B. oleracea* in various breeding programs (Diederichsen et al. 2009).

1.4.2 Liming of the soil

Colhoun (1958) indicated that incidence and severity of clubroot was reduced at soil pH 7.2 or higher. Therefore, lime may be applied on a field to raise the soil alkalinity and create unfavourable conditions for clubroot infection and development. This strategy is most effective where inoculum levels are relatively low, and its use should be preceded by a soil test to determine if treatment is agronomically desirable (Howard et al. 2010). As indicated by Howard et al. (2010), lime amendments are available in various forms, including agricultural lime (calcium carbonate and calcitic lime), dolomitic lime (calcium and magnesium carbonate), hydrated lime (calcium hydroxide), and quicklime (calcium oxide).

The particle size of the lime, timing of application, and soil type can all influence the effectiveness of lime application (Donald and Porter 2009). Soil limed with fine limestone can significantly reduce the incidence of clubroot relative to soil limed with a coarser fraction (Dobson et al. 1983). Dobson et al. (1983) suggested that the surface area of the lime particles was more important than the source of lime. With respect to timing of the lime application, most significant reductions in disease severity in greenhouse experiments were observed when treatments were applied over a 3-7 day period after inoculation (Donald and Porter. 2009). Finally, Donald and Porter (2009) indicated that

it is difficult to adjust the pH of soils with a high buffering capacity. Lime also failed to control clubroot in a "lime nonresponsive" soil, even after the soil pH was changed to 7.7 (Donald and Porter 2009). Indeed, many control failures have been reported at or above pH 7.2 (Myers et al. 1981), so increasing the soil pH may not be a sufficient clubroot control strategy on its own.

1.4.3 Fungicides

A number of fungicidal treatments are available for clubroot control. Among the most effective and widely used chemicals is mercurous chlorid (Calomel ™). However, the high mammalian toxicity of mercury and the persistence of this chemical in the environment has led to an almost complete withdrawal of this chemical from the world market (Donald and Porter 2009). In a greenhouse evaluation of some systemic fungicides, Buczacki (1973) found that benomyl, thiophanate, thiophanate methyl and NF 48 showed promise for control of clubroot by incorporation into the soil. These compounds are all precursors of the fungitoxic substances methyl benzimidazol-2-ylcarbamate (MBC) or ethyl benzimidazol-2-ylcarbamate (EBC) (Buczacki 1973). Other chemicals, such as alkylene bisdithiocarbamate, pentachloronitrobenzene, flusulfamide (MTF651, Nebijin™), Fluazinam (Shirlan™ or Omega™), are also used for the management of clubroot on vegetables (Donald and Porter 2009). Recently, cyazofamid (Ranman ™) was used by Mitani et al. (2003) against *P. brassicae* on Chinese cabbage. These researchers found that cyazofamid at 0.3 mg/l inhibited resting spore germination by about 80%, and at 3–10 mg/1, cyazofamid exhibited fungicidal activity against resting spores of *P. brassicae* (Mitani et al. 2003). When cyazofamid was applied to infested

soil, both root hair infection and club formation caused by *P. brassicae* were strongly inhibited at 1–3 mg/kg dry soil (Mitani et al. 2003).

In Canada, there are several fungicides registered for control of clubroot on cruciferous vegetables: pentachloronitrobenzene (Adobe® 75WP, Crusoe® 75WP and Quintozene 75WP) and fluazinam (Allegro 500F), and these can be applied as pre- or post-planting drenches on transplants (Howard et al. 2010). However, no fungicides are registered in Canada for the control of clubroot on canola. In Australia, only quintozene (PCNB) and fluazinam are registered for the control of clubroot on vegetable Brassicas (Donald and Porter 2009). No pesticides are approved for use against clubroot in Europe (Oxley 2007).

1.4.4 Crop rotation

Rotating susceptible cruciferous vegetables with non-hosts has the potential to reduce *P. brassicae* inoculum levels in the soil (Howard et al. 2010). Cereal grains, alfalfa, onions, peas, beans and carrots are some examples of non-host crops that could be included in a rotation (Howard et al. 2010). Wallenhammar (1996) indicated that in a field with 100% infestation, the level of infestation declined below a detectable level after 17.3 years, and that the half-life of spore inoculum was 3.6 years. Therefore, when the soil inoculum concentration is sufficiently high, long rotations are needed to reduce soil inoculum levels below a disease-causing threshold (Donald and Porter 2009).

1.4.5 Bait crops

As noted above, root exudates of host and non-host plants have been reported to induce *P. brassicae* resting spore germination. Friberg et al. (2005) indicated that in a laboratory study, an aqueous solution of root exudates from a non-host plant species (perennial ryegrass) stimulated spore germination more than those from other plant species, including the susceptible host species Chinese cabbage.

Given their influence on spore germination, the use of bait crops has been proposed as a clubroot management strategy. In this approach, a bait crop is sown and becomes infected by *P. brassicae*, after which it is ploughed under before the pathogen completes its life cycle. In this way, the number of clubroot spores in the soil is reduced (Donald and Porter 2009). Several studies have been conducted to analyze the effectiveness of bait crops as a tool to control clubroot. Murakami et al. (2000) indicated that leafy daikon can reduce disease severity on Chinese cabbage and can also reduce the number of *P. brassicae* resting spores in pots. In a field experiment, the numbers of resting spores were reduced when leafy daikon was grown in advance of Chinese cabbage. However, there was no reduction in disease severity in the Chinese cabbage. Another study conducted by Friberg et al. (2006) found that disease severity was lower in all bait crop treatments relative to a plant-free control, following incorporation and decomposition of plant roots in a greenhouse experiment. However, none of the bait crops assessed could reduce the persistence of clubroot resting spores in the soil under field conditions (Friberg et al. 2006). Therefore, the utility of bait crops still needs to be examined, particularly with respect to a canola/*P. brassicae* pathosystem.

1.5 Research objectives

Clubroot has become a major disease of canola in Alberta, reducing both crop yields and seed oil content. Bait crops, as noted above, have the potential to reduce clubroot severity. Given the seriousness of the clubroot outbreak, and the need for an integrated disease management system, all potential approaches should be considered in the formulation of a disease management plan. As such, the focus of this dissertation is on the evaluation of bait crops as a general clubroot management strategy in a canola cropping system, and on increasing understanding of how bait crops could impact resting spores of *P. brassicae*. The research has four specific objectives: (1) to evaluate the effectiveness of bait crops in reducing clubroot severity in subsequent crops of canola, (2) to examine the effect of root exudates from different plant species on the germination of *P. brassicae* resting spores under laboratory conditions, (3) to better understand *P. brassicae* pathogenicity by investigating the infection of canola by secondary zoospores produced on a non-host (ryegrass), and (4) to assess the effect of seeding date, seedling age and canola cultivars on the severity of clubroot of canola.

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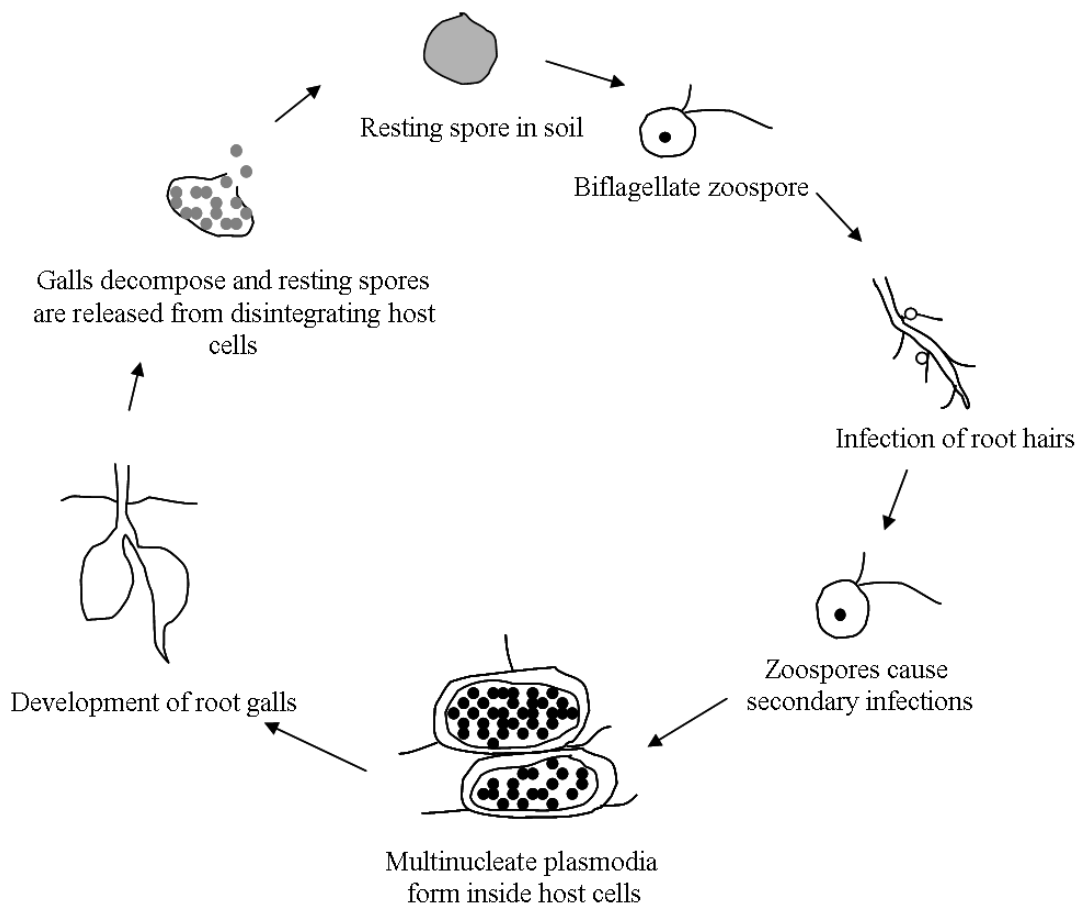

Figure 1-1. Diagrammatic representation of the life cycle of *Plasmodiophora brassicae*, causal agent of clubroot of crucifers (S.E. Strelkov).

2. Effect of host and non-host root exudates on *Plasmodiophora brassicae* resting spore germination

2.1 Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is one of the most serious soilborne diseases of crucifers throughout the world. In 2003, a total of 12 out of 70 commercial canola (*Brassica napus* L.) fields surveyed near St. Albert, Alberta, Canada, were found to be clubroot-infested, in the first report of this disease in the prairie canola crop (Strelkov et al. 2005). Subsequent surveys have revealed an increasing number of clubroot-infested fields, with more than 500 confirmed cases by 2010 (Strelkov et al. 2011), mostly in central Alberta. Although the levels of infestation of most canola crops have been low to moderate, yield losses as high as 100% have been observed in severely infested fields (Strelkov et al. 2007). Therefore, clubroot appears to have become established as a pathogen of canola in Alberta, and may be spreading (Cao et al. 2009).

Management of clubroot is complicated by the longevity of *P. brassicae* resting spores, which can survive in the soil for many years (Wallenhammar 1996). However, this longevity can be variable and is influenced by the surrounding soil and environment. Temperature, moisture content, and position in the soil profile will all have an impact on resting spore survival (Dixon 2009). Resting spore germination is also influenced by the surrounding environment, as well as the age of the spores. For instance, young resting spores of *P. brassicae* require the presence of calcium ions for germination, whilst mature spores germinate regardless of whether calcium is present or not (Kageyama and

Asano 2009). It was also reported that resting spores from old, decaying galls germinated more rapidly than spores from young galls (Macfarlane 1970).

The presence of host and non-host root exudates can also induce resting spore germination. In a laboratory study, Friberg et al. (2005) found that exudates from roots of perennial ryegrass (*Lolium perenne* L.) stimulated the germination of *P. brassicae* resting spores in an aqueous solution. Macfarlane (1970) also found that the germination of *P. brassicae* resting spores was hastened and the germination rate was increased in the presence of cabbage (*Brassica oleracea* L. var. *capitata* cv. ' King of Hearts ') plants or of diffusate from cabbage roots.

Given these earlier reports on the effect of root exudates on *P. brassicae* resting spore germination, the current study was conducted to examine the effect of root exudates from host (Chinese cabbage (*Brassica rapa* var. *pekinensis*, cv. Granaat) and *B. napus* canola) and non-host plants (perennial ryegrass) on resting spore germination in Canadian populations of the pathogen. An understanding of the effect of root exudates from these plant species on spore germination in regional populations of *P. brassicae* will help determine whether or not the cropping of 'bait crops' that induce resting spore germination represents a viable clubroot management strategy in a canola cropping system.

2.2 Materials and methods

2.2.1 Production of root exudates

Six hundred seeds of canola, Chinese cabbage and perennial ryegrass were surface sterilized with 1% sodium hypochlorite (NaOCl) for 2 min and rinsed with sterile

distilled water (sdH_2O). The seeds were incubated for 1 week on moistened, sterilized filter paper in 9-cm diameter Petri dishes (at a density of 20 seeds per dish) to allow them to germinate. The roots of the seedlings were surface sterilized with streptomycin sulphate solution (50 ppm), and rinsed in sterile distilled water three times. Sixty seedlings of each plant species were transferred to 9-cm diameter Petri dishes containing 15 mL of a 1/5 dilution of modified Hoagland's solution (5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2mM $MgS0_4$, 2 mM KH_2PO_4) (Macfarlane 1970), and arranged so that the seedlings remained in an upright position (Figure 2-1). The Petri dishes were gently transferred into 11.4 liter clear plastic containers (41.9 cm $\times$ 29.2 cm $\times$ 15.9 cm), which contained a water-soaked sterilized paper towel at the bottom to keep the environment humid. After two weeks, the culture solution with root exudates from each crop was collected with a syringe and filter-sterilized (0.2 μm). The concentration of root exudates solution was standardized on an mL per root fresh weight (g) basis. The standardized solution was stored in a 50 mL centrifuge tube at 4 ℃ and used the next day.

2.2.2 Surface sterilization of *P. brassicae* resting spores

Frozen galls on canola roots collected from a field near Leduc, Alberta, were washed under running tap water to remove soil particles. The outer layer of the gall was removed with a knife, and the remaining part of the gall was surface-sterilized with 70% ethanol for 1 min, and then with 1% sodium hypochlorite for 20 min. Five grams of the surface-sterilized gall tissue was rinsed three times with sdH_2O, the galls were aseptically cut into small pieces, and homogenized in a 50 mL volume of sdH_2O with a blender. The homogenate was filtered through eight layers of cheese cloth, and the filtrate centrifuged

at 4000 ×g for 10 min. The resulting spore pellets were washed five times with sdH_2O and the spores were then surface-sterilized according to the procedure of Asano et al. (2000). The spore suspension was adjusted to a final concentration of 6×10^7 spores/mL with sdH_2O.

2.2.3 Treatment with root exudates and evaluation of spore germination

Germination of resting spores after treatment with root exudates of canola, Chinese cabbage and perennial ryegrass was evaluated by incubating 0.2 mL of spore suspension with 1.8 mL of each root exudates solution in 2 mL centrifuge tubes at room temperature under darkness. Resting spore germination was assessed every 24 hours over a 6 day period. Control treatments consisted of 0.2 mL of spore suspension incubated in 1.8 mL of 1/5 dilution of modified Hoagland's solution or 1.8 mL sdH_2O. Eight replicate tubes were prepared for each treatment (exudates and controls) for each time-point in the experiment. The entire experiment was repeated three times, with the repetitions referred to as Experiments 1, 2 and 3.

At each time-point, 60 μL of each spore/root exudates suspension was fixed with aceto-orecin red stain, and three slides were prepared from each replicate of each treatment. On each slide, 100 resting spores were examined along a transect under the light microscope. Resting spores were considered to have germinated if they were empty and remained unstained by aceto-orecin, and could be readily distinguished from red ungerminated spores (Naiki et al.1987) (Figure 2-2). After 6 days of incubation, aggregation of resting spores and the growth of other microbes made spore counting inaccurate. Therefore, the experiment was conducted over a 6 day time-course.

2.2.4 Chemical analysis

Seven days after the completion of Experiment 3, the root exudate solution in each treatment as well as the controls was chemically characterized. Nitrogen (N), potassium (P), sulphur (S), calcium (Ca), magnesium (Mg) and boron (B) concentrations, as well as the pH value, were determined via the Inductively Coupled Plasma (ICP) Method, 3120 B, and the Electrometric Method, 4500-H+ B. All chemical analyses were performed by Exova in Edmonton, Alberta.

2.2.5 Data analysis

The resting spore germination data were subjected to analysis of variance (ANOVA) using PROC GLM of the SAS statistical package (version 9.1.3, SAS Institute, Cary, NC), and differences among treatments at each time-point were assessed using Duncan's multiple range test ($P \leq 0.05$).

2.3 Results

2.3.1 Experiment 1

At the beginning of Experiment 1 (0 days), the resting spore germination rate was close to zero in each treatment. After one day of incubation, the resting spores had begun to germinate in all treatments, but the percent germination was significantly higher ($P \leq 0.05$) for those spores incubated with root exudates versus sdH_2O or nutrient solution alone (Figure 2-3a). This trend continued throughout the 6 day time-course of the experiment, and root exudates from all of the crops tested significantly increased the

percent germination relative to the nutrient solution or water alone. The percent germination was lowest in the sdH_2O control and highest for those spores incubated in the perennial ryegrass root exudates solution. After 6 days, the percent germination was 17% for resting spores incubated with root exudates from perennial ryegrass, 15% for spores incubated in exudates from Chinese cabbage, and 13% for spores incubated in exudates from canola (Figure 2-3a). In contrast, it was only 4.6% and 2.6% for spores incubated in nutrient solution or sdH_2O, respectively.

2.3.2 Experiment 2

The trends observed in the second repetition of the root exudates experiment (Experiment 2) (Figure 2-3b) were similar to those observed in Experiment 1. Germination of the resting spores commenced at day 1 and was significantly ($P \leq 0.05$) higher for spores in the root exudates solutions versus spores in the nutrient solution or sdH_2O over the entire time-course of the experiment. As in Experiment 1, the percent germination was highest for those spores incubated with exudates from perennial ryegrass (12% on day 6), followed by spores incubated with exudates from Chinese cabbage (9%), canola (8.5%), nutrient solution (4.8%) or sdH_2O (4.5%).

2.3.3 Experiment 3

The same trends observed in Experiments 1 and 2 were also observed in Experiment 3, with the percent germination significantly ($P \leq 0.05$) higher for those spores incubated with root exudates as opposed to nutrient solution or sdH_2O alone

(Figure 2-3c). As was also the case in Experiments 1 and 2, the percent germination was highest for resting spores incubated with root exudates from perennial ryegrass (13% at 6 days), followed by spores incubated with exudates from Chinese cabbage (12.5%), canola (11.4%), nutrient solution (4.2%) or sdH_2O (3.2%).

The chemical composition differed between the various root exudates treatments and controls (Table 2-1). The treatment with exudates from Chinese cabbage had a higher concentration of N, K, S, Ca and Mg relative to the other two root exudates treatments (perennial ryegrass and canola). The highest concentrations of N, K, S, Ca and Mg were detected in the nutrient solution control. The treatment with exudates from perennial ryegrass had the lowest concentrations of boron and N. Most of the chemical components were weakly correlated with resting spore germination. The pH of the solution was 6.34 for Chinese cabbage, 6.92 for canola, 7.05 for perennial ryegrass, 6.28 for the nutrient solution and 7.39 for the sdH_2O control.

2.4 Discussion

Plant roots can synthesize, accumulate and secrete a diverse array of compounds, such as amino acids, organic acids, polysaccharides and proteins, and these compounds are broadly referred to as root exudates (Flores et al. 1999). These compounds serve important roles as chemical attractants and repellents in the rhizosphere, the narrow zone of soil immediately surrounding the root system (Walker et al. 2003). Under natural conditions, dormant resting spores require external stimulants for germination (Feng et al. 2010). Macfarlane (1970) concluded that resting spore germination was stimulated by material released from host roots. This result was later confirmed by Suzuki et al. (1992)

and Friberg et al. (2005). Similarly, in the current study, root exudates from Chinese cabbage, canola and perennial ryegrass were found to significantly stimulate spore germination relative to nutrient solution or sdH_2O alone. Interestingly, the *P. brassicae* non-host species, perennial ryegrass, appeared to have a greater stimulatory effect than the hosts Chinese cabbage and canola (Figure 2-3). Friberg et al. (2005) also found that perennial ryegrass stimulated resting spore germination more than Chinese cabbage. While the reason for this is not clear, it is likely due to the nature of the root exudates from the different plants, particularly with respect any components of the exudates that may be particularly important in inducing germination.

Suzuki et al. (1992) identified a putative germination-stimulating factor (GSF), which was described as a heat stable, fairly polar and low molecular mass compound. The GSF was not considered to be related to clubroot resistance or host recognition, since it was not specific to the root exudates of clubroot susceptible crucifers, but instead was also found in exudates of a resistant crucifer and the non-host lettuce (Suzuki et al. 1992). Recently, Feng et al. (2010) confirmed the stimulatory effect of host root exudates on *P. brassicae* resting spore germination, and found that treatment of these exudates with a serine protease (Pro1) from the pathogen further increased germination rates. Feng et al. (2010) hypothesized that the germination stimulating factors consist of two components: (i) compounds innate to the root exudates containing caffeic acid, coumalic acid and corilagin, and (ii) hydrolytes produced by the enzyme activities of Pro1 and/or other enzymes. In the case of component (ii), the involvement of hydrolytic enzymes not only generates stimulatory chemicals, but also provides a potential avenue for the pathogen to recognize its host (Feng et al. 2010).

Chemical components present in spore suspensions/root exudates may play a role in resting spore germination, or at least be influenced by the spore germination process. In the current study, although the root exudates from perennial ryegrass contained the lowest concentration of boron and N (with the exception of the sdH_2O control), treatment with exudates from this crop resulted in the highest germination percentage. Dixon (2009) indicated that in field and controlled laboratory studies, boron had a substantial effect on the ability of *P. brassicae* to invade root hairs and colonize host plants. By raising the boron content of the rhizosphere, it may be possible to limit the ability of *P. brassicae* zoospores to infect the host roots and cause symptoms (Dixon 2009). To our knowledge, the impact of boron on spore germination has not been studied. It is possible, however, that increasing the concentration of boron could decrease the resting spore germination rate, and thereby serve to control clubroot.

As with boron, N may also serve to decrease *P. brassicae* resting spore germination, thereby reducing clubroot disease development. High concentrations of nitrate consistently suppress symptoms of many plant diseases, but there have been few investigations into its effect on clubroot (Dixon 2009). In greenhouse and laboratory studies, Page (2001) found that calcium nitrate was associated with a decrease in *P. brassicae* infection and a subsequent reduction in the severity of symptom expression. However, whether this association was related to the effect of the nitrogen or the calcium is not clear.

Calcium plays a very important role in clubroot disease development. The germination of resting spores and the motility of zoospores can be affected by the amount of calcium in the soil (Dixon and Page 1998). High concentrations of calcium could

possibly reduce zoospore flagellar action and have a great impact in the period between spore germination and penetration of the root hairs (Dixon 2009). Hydrogen ion content (pH) is also an important factor affecting clubroot disease. Webster and Dixon (1991) demonstrated that alkaline pH reduced total root hair infection rates and retarded the maturation of plasmodia, sporangia and zoosporangia. In fact, the effect of calcium on clubroot disease is usually discussed in association with the pH of the soil. Dixon (2009) indicated that high concentrations of calcium at pH 6.2 or 7.2 reduce the total number of root hair infections and pathogen maturation rates relative to control treatments. In the current study, the percent germination of resting spores incubated with the nutrient solution alone, which contained the highest concentration of Ca and had a pH of 6.26, was lower than all of the treatments containing root exudates (Table 2-1). Nonetheless, spores incubated with root exudates from Chinese cabbage, which also had a relatively high concentration of Ca and a pH of 6.32, exhibited the second highest germination percentage of any of the treatments. This suggests a complex relationship between pH, Ca content and the composition of the root exudates themselves.

The finding, in the present study, that root exudates from host and non-host plants had a stimulatory effect on *P. brassicae* resting spore germination suggests that the deployment of bait crops, which could stimulate spore germination and thereby reduce soil inoculum loads, may be an effective strategy for managing clubroot of canola. The next chapter in this thesis is devoted to examining this possibility.

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Table 2-1. Chemical composition and pH of solutions representing root exudates treatments and controls in Experiment 3, as determined via the Inductively Coupled Plasma (ICP) Method, 3120 B and Electrometric Method, 4500-H+ B. All chemical analyses were performed by Exova in Edmonton, Alberta.

Treatment*	N (mg L^{-1})	K (mg L^{-1})	S (mg L^{-1})	Ca (mg L^{-1})	Mg (mg L^{-1})	Na (mg L^{-1})	B (mg L^{-1})	pH
Chinese cabbage	35.5	49	11	31	8	4	0.14	6.34
Canola	2.35	25	3	5	<1	5	0.17	6.92
Perennial ryegrass	0.05	7	8	13	3	<4	0.11	7.05
Nutrient solution	42.1	57	14	41	9	<4	0.12	6.26
Sterile distilled water	0.01	<4	<3	<2	<1	<4	0.08	7.39

*Each treatment consisted of 0.2 mL of a *Plasmodiophora brassicae* resting spore suspension (6×10^7 spores/mL) combined with 1.8 mL of each root exudates solution, 1.8 mL modified Hoagland's solution ("nutrient solution"), or 1.8 mL sdH_2O.

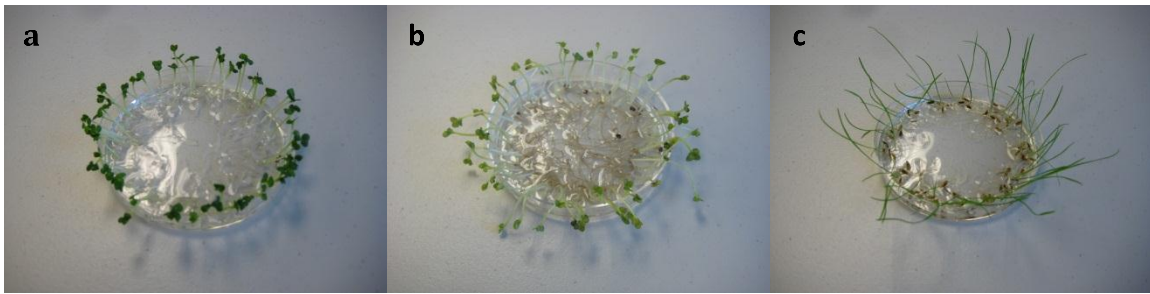


Figure 2-1. Production of root exudates of: a) Chinese cabbage, b) canola, and c) perennial ryegrass in 5 cm-diameter Petri dishes.

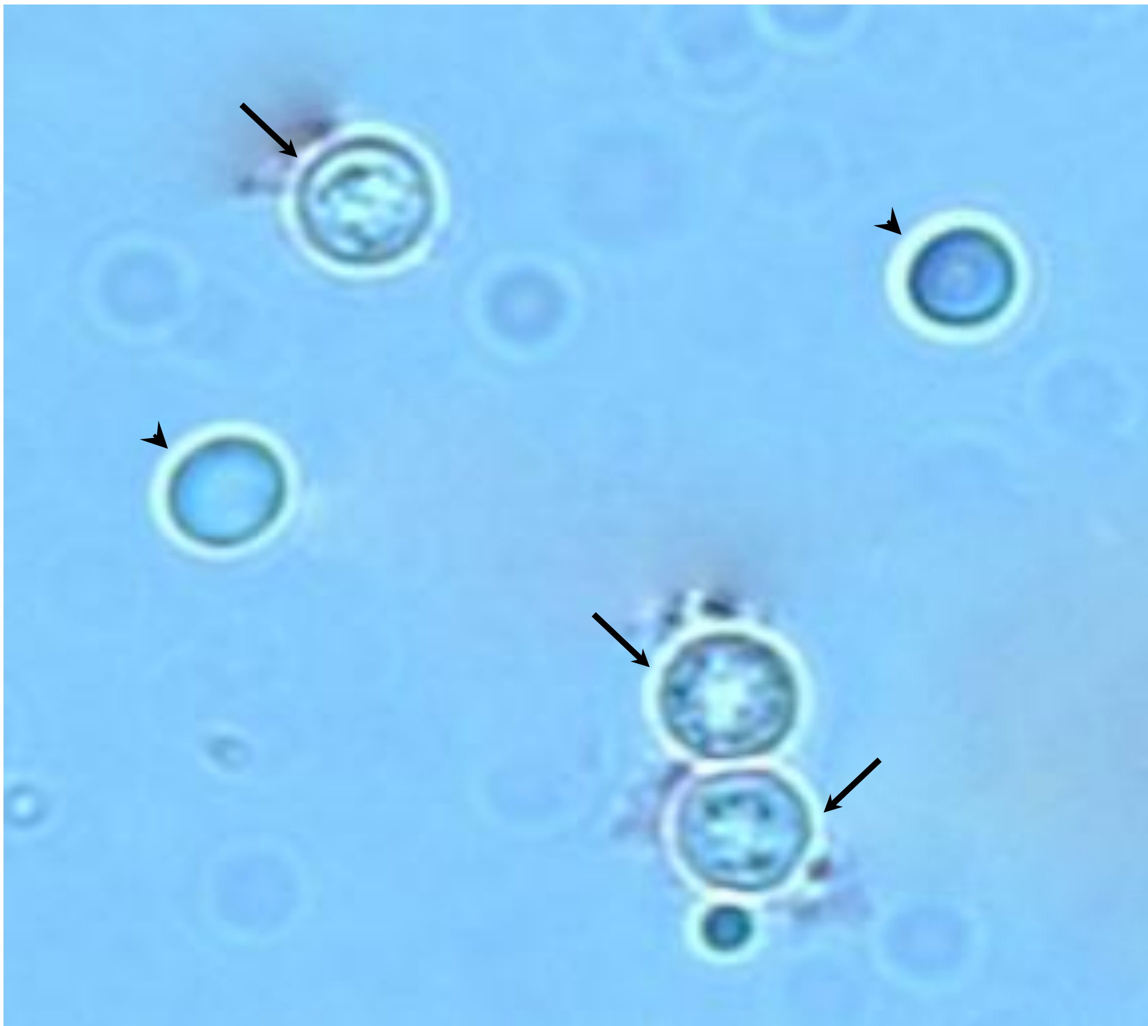
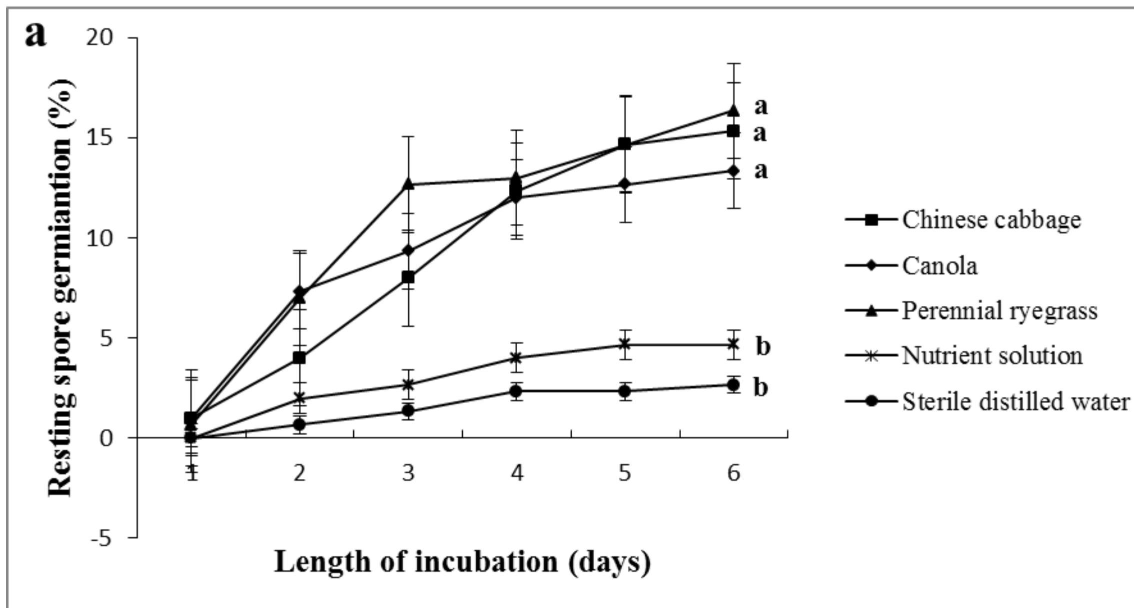
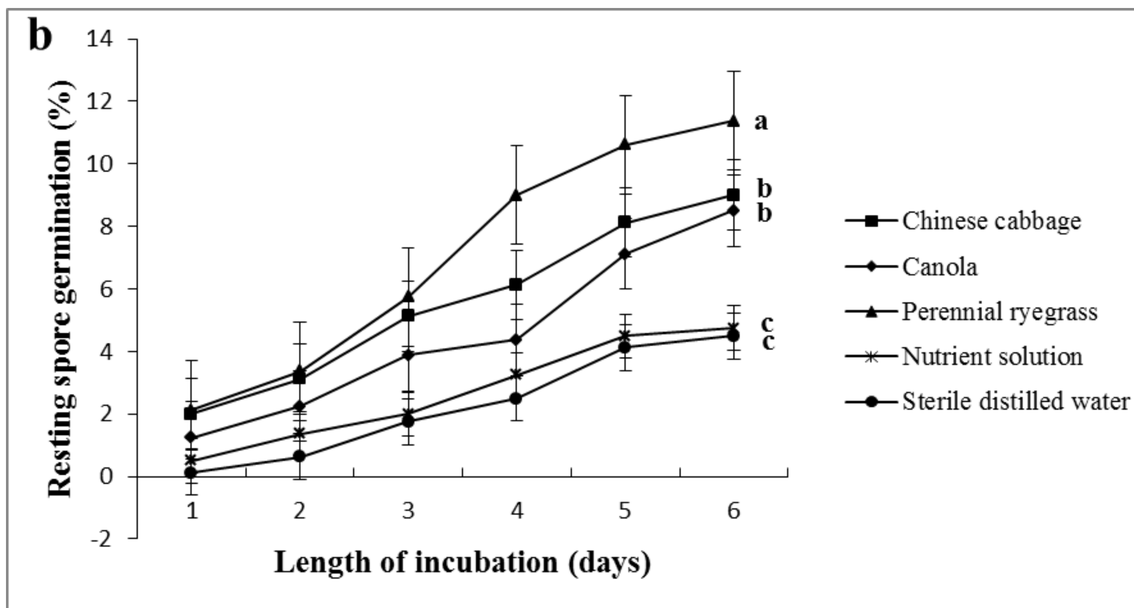


Figure 2-2. Resting spores of *Plasmodiophora brassicae* after staining with aceto-orecin. Germinated spores (arrowheads) are empty and are not stained by the aceto-orecin, while non-germinated spores (arrows) are not empty and are stained by the aceto-orecin.


a
Resting spore germiantion (%)
20
15
10
5
0
-5
1
2
3
4
5
6
Length of incubation (days)
a
a
a
b
b
Chinese cabbage
Canola
Perennial ryegrass
Nutrient solution
Sterile distilled water


b
Resting spore germination (%)
14
12
10
8
6
4
2
0
-2
1
2
3
4
5
6
Length of incubation (days)
a
b
b
c
c
Chinese cabbage
Canola
Perennial ryegrass
Nutrient solution
Sterile distilled water

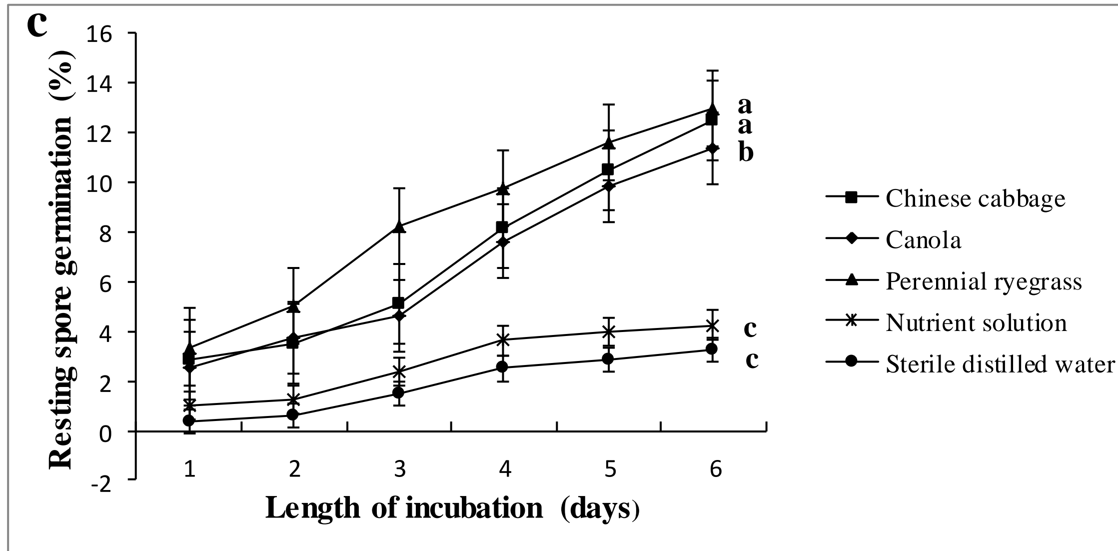


Figure 2-3. Resting spore germination (%) in root exudates solutions from canola, Chinese cabbage and perennial ryegrass. Nutrient solution (modified Hoagland's solution) alone and sterile distilled water served as controls. The same experiment was repeated three times: (a) Experiment 1, (b) Experiment 2, and (c) Experiment 3. The different letters beside each line indicate significant differences as assessed using Duncan's multiple range test ($P \leq 0.05$).

3. Effect of host and non-host bait crops on the severity of clubroot on canola (*Brassica napus* L.)

3.1 Introduction

In recent years, clubroot has emerged as an important disease of canola (*Brassica napus* L.) in Alberta, Canada (Howard et al. 2010). One of the major challenges associated with managing this disease is the production, by the pathogen, of resting spores that can remain viable in the soil for many years, where they serve as the inoculum for future clubroot epidemics.

Root exudates of host and non-host plants have been reported to induce *P. brassicae* resting spore germination. In a laboratory study, Friberg et al. (2005) reported that root exudates from the non-host plant species perennial ryegrass (*Lolium perenne* L.) stimulated spore germination more than those from other plant species, including the susceptible host Chinese cabbage (*Brassica rapa* var. *pekinensis*). Macfarlane (1970) also found that the germination of *P. brassicae* resting spores was hastened, and the germination rates were increased, in the presence of cabbage (*Brassica oleracea* L. var. *capitata* cv. ' King of Hearts ') plants or of diffusate from cabbage roots.

Plants that induce or enhance germination of *P. brassicae* resting spores have been suggested as a potential tool for the management of clubroot (Murakami et al. 2000; Friberg et al. 2006). In such an approach, these plants, which function as a "bait crop", would be sown prior to the crop of interest, in order to induce resting spore germination. The root hairs of the bait crop would become infected by *P. brassicae*, but prior to the pathogen completing its life cycle the bait crop would be destroyed. In this manner, the

soil resting spore load would be decreased, and the crop of interest would suffer less severe clubroot symptoms. Several studies have been conducted to test the utility of bait crops as a tool to manage clubroot. Murakami et al. (2000) found that leafy daikon (*Raphanus sativus* L.) could reduce the severity of clubroot symptoms on Chinese cabbage and could also reduce the number of *P. brassicae* resting spores in pots. Similarly, in a field experiment, the number of resting spores was reduced when leafy daikon was grown in advance of the Chinese cabbage. However, there was no reduction in clubroot symptom severity under field conditions. In another study, it was reported that clubroot severity was reduced when perennial ryegrass was used as a bait crop under greenhouse conditions (Friberg et al. 2006). In field experiments, however, this non-host did not have a significant impact on the persistence of *P. brassicae* resting spores in the soil or on clubroot severity in the test plants (Friberg et al. 2006).

A proactive clubroot management strategy should employ all available tools. Given the recent emergence of clubroot as a major disease of canola in Alberta, there is interest in evaluating the efficacy of various disease management approaches, including the use of bait crops. To this end, the principal aim of this research was to assess the efficacy of host and non-host bait crops in reducing the severity of clubroot in subsequent crops of canola.

3.2 Materials and methods

3.2.1 Naturally infested soil

A black chernozemic soil from a field near Leduc, Alberta, with a heavy, natural infestation of clubroot was collected and brought to the laboratory. The soil was allowed

to air dry, passed through a sieve (with 2 mm pores) and homogenized with a blender. The concentration of resting spores in the naturally infested soil was estimated in a hemocytometer (the resting spore suspension was prepared as described below), and determined to be 6.3×10^6 spores/g soil. Aliquots (6 L) of the soil were then placed in 11.4 liter plastic containers (41.9cm $\times$ 29.2cm $\times$ 15.9cm) and used in a set of bait cropping experiments.

3.2.2 Inoculated potting mix

In a second set of experiments, the effect of bait crops on clubroot severity was assessed in a potting mix (Sunshine mix #4, Sun Gro Horticulture, Vancouver. BC. Canada) that had been inoculated with known quantities of *P. brassicae* resting spores. Briefly, a resting spore suspension was obtained by homogenizing *P. brassicae*-infected canola roots as per the protocol of Tewari et al. (2005), with the spore suspension filtered through eight layers of cheesecloth and adjusted to a concentration of 9.33×10^5 resting spores/mL. An 800 mL aliquot of this suspension was thoroughly mixed with 800g of potting mix to get a final resting spore concentration of 9.33×10^5 resting spores/g potting mixture. Eight hundred grams of this inoculated potting mix was placed in each 11.4 L plastic container and used in the bait cropping experiments.

3.2.3 Bait crop treatments

Two bait crops, *Brassica rapa* L. (Polish) canola cv. Reward and perennial ryegrass, were evaluated for their effectiveness in inducing *P. brassicae* resting spore

germination and reducing clubroot symptoms on *B. napus* L. (Argentine) canola. One hundred 1 week-old seedlings (produced as described in Chapter 2) of each bait crop were transplanted into plastic containers (41.9cm × 29.2cm × 15.9cm) filled with inoculated potting mix as four distinct rows (each consisting of 25 seedlings) per container. All of the containers were placed in a greenhouse and maintained at 20 ± 2°C/18 ± 2°C (day/night) with a 16-hour photoperiod. The containers were placed on trays and were watered daily from the bottom with tap water, the pH of which had been adjusted to 6.4 with HCl (six holes had been previously drilled on the bottom of each container to prevent waterlogging). The trial was arranged as a randomized complete block design (RCBD) with four replicates (containers) per treatment. The treatments were as follows: ryegrass (2 wk) - fallow (2 wk) - *B. napus* (6 wk) (treatment 1); *B. rapa* (2 wk) - fallow (2 wk) - *B. napus* (6 wk) (treatment 2); ryegrass (2 wk) - *B. rapa* (2 wk) - *B. napus* (6 wk) (treatment 3); *B. rapa* (2 wk) - ryegrass (2 wk) - *B. napus* (6 wk) (treatment 4); and fallow (4 wk) - *B. napus* (6 wk) (treatment 5).

At the end of each 2-wk cycle, the bait crops were uprooted and removed, and 100 seedlings of *B. napus* canola were transplanted into each container in the same manner as the bait crops. The canola plants were watered from the bottom for the first 2 wk after transplanting, and thereafter were watered daily from the top with a sprinkler. After 6 weeks, the plants were assessed for clubroot severity, plant height and dry biomass. The results for each treatment were compared to a fallow treatment in which no bait crops had been planted.

3.2.4 Evaluation of clubroot symptom severity

Clubroot disease severity was assessed on a 0-3 scale, where 0 = no galling; 1 = a few small galls; 2 = moderate galling; and 3 = severe galling (Kuginuki et al. 1999; Strelkov et al. 2006). An index of disease (ID) was then calculated using the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006):

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

Where ∑ is the sum total; *n* is the number of plants in a class; *N* is the total number of plants; and 0, 1, 2, and 3 are the disease severity classes. In each container, 50 *B. napus* canola plants were examined for the severity of clubroot.

3.2.5 Microscopic analysis of resting spores from inoculated potting mix

At the end of each bait crop cycle, the soil sample was collected from each replicate (container) and air dried. For each replicate, three subsamples of air-dried soil (500 mg) were assessed for resting spore concentration using a haemocytometer (Hausser Scientific, Horsham. PA. U.S.A) under a light microscope (400×). The isolation of resting spores from the soil was performed following the protocol of Castlebury et al. (1994) with some modifications (Q. X. Zhou, Crop Development Centre – North, Edmonton, AB, *personal communication*). For each subsample, 20 mL of sdH_2O was added to the 500 mg of air-dried soil and mixed evenly with a blender for 1 min. The mixture was filtered through 8-layers of cheese cloth, with the filtrate transferred to a 15 mL centrifuge tube and centrifuged at 3900 g for 15 min. The supernatant was discarded and 6 mL of a 50% (w/v) sucrose solution was added to the pellet. After 2 min of

agitation, the suspension was centrifuged at 1700 g for 5 min. The supernatant was transferred into a 50 mL centrifuge tube, mixed thoroughly with 45 mL of sdH_2O, and centrifuged at 3900 g for 15 min. The supernatant was discarded and 5 mL of sdH_2O added to the pellet and centrifuged as above. The final resting spore pellet was then mixed with 2 mL of sdH_2O and analyzed under the microscope.

3.2.6 DNA extraction from inoculated potting mix and quantitative PCR

Resting spores were isolated from the soil (as described above) and total genomic DNA was extracted from the resting spore pellet using a FastDNA®Spin kit for Soil (MP Biomedicals, Solon. Ohio. U.S.A) as per the manufacturer's instructions. The extracted genomic DNA was then analyzed by conventional and quantitative PCR (qPCR) to establish the presence and concentration of *P. brassicae* DNA in the soil.

Conventional PCR analysis was conducted as described by Cao et al. (2007) using the *P. brassicae* specific primers TC2F (5′-AAACAACGAGTCAGCTTGAATGCTAGTGTG-3′) and TC2R (5′-CTTTAGTTGTGTTTCGGCTAGGATGGTTCG-3′), with reaction conditions consisting of an initial heat denaturation step at 94 ℃ for 2 min; followed by 45 cycles of 94 ℃ for 30 s, 65 ℃ for 1 min, and 72 ℃ for 1 min; and a final extension at 72 ℃ for 10 min. The PCR products were resolved on ethidium bromide-stained 1% (w/v) agarose gels in 1× Trisacetate-EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5.), and visualized with a UV transilluminator (Bio-Rad, Mississauga. ON. Canada).

Those samples for which the presence of *P. brassicae* was confirmed by standard PCR were subjected to qPCR analysis. Standard curves for the qPCR analysis were

generated with DNA extracted (as described above) from *P. brassicae* resting spores. The DNA concentration was measured with a NANODROP™1000 Spectrophotometer (Thermo Scientific, Waltham, MA) at λ = 260 nm and adjusted to 1×10^3 ng/μL. The DNA was then serially diluted with sdH_2O at 10-fold intervals down to concentrations of 1×10^2 ng/μL, 1×10^1 ng/μL, 1×10^0 ng/μL, 1×10^{-1} ng/μL and 1×10^{-2} ng/μL. The DNA from each dilution was used as the template in qPCR at the aforementioned conditions.

The qPCR analysis was performed with the *P. brassicae*-specific primers RP2F (5'-TAATCGCCCTGGGTATGGTA-3') and RP2R (5'-CGCGATTCGACGTAGGACT-3'), as previously described by Hwang et al. (2011). All qPCR amplifications were conducted in a StepOne ™ (48-well) Real-time PCR System (Applied Biosystems, Foster. California. U.S.A) in a 20 μL reaction volume containing 1.0 μL of DNA template solution, 1.0 μL of each forward and backward primers (RP2F and RP2R) at 10 μM, 2.0 μL of MGB TaqMan® Probe RP2P at 1 μM (Applied Biosystems), 10 μL of TaqMan® Fast Universal PCR Master Mix (2×) (Applied Biosystems) and 5 μL of sdH_2O. All reactions were run at 95°C for 20s, followed by 40 cycles at 95°C for 1s and at 60°C for 20s.

3.2.7 Data analysis

ID, plant height, shoot dry weight and resting spore concentration were subjected to analysis of variance (ANOVA) using PROC GLM of the SAS statistical package (version 9.1.3, SAS Institute, Cary, NC), and differences among treatments at each time-point were assessed using Duncan's multiple range test ($P \leq 0.05$).

3.3 Results

3.3.1 Index of disease, plant height and shoot dry weight of *B. napus* canola after bait crop treatments

3.3.1.1 Naturally infested soil

The ID, plant height and shoot dry mass of *B. napus* canola grown following various cycles of bait crops are presented in Figure 3-1. In most cases, the ID obtained after the cropping of bait plants was lower than in the fallow treatment. Bait crop treatments (C-F-C, R-C-C and C-R-C) that included *B. rapa* canola resulted in a lower ID than those (R-F-C) consisting of only ryegrass. With the exception of treatment R-F-C, other bait crop treatments showed a significantly lower ($P \leq 0.05$) ID than the fallow treatment. Nonetheless, shoot dry mass and height were greatest in the fallow treatment. Plant height and dry mass were lowest in the bait crop treatments (R-C-C and C-R-C) that included both canola (host) and ryegrass (non-host).

3.3.1.2 Inoculated potting mix

Similar patterns to those observed in the naturally infested soil were obtained with respect to ID, plant height and dry mass of *B. napus* canola grown in the inoculated potting mix (Figure 3-2). The ID of the canola plants was significantly ($P \leq 0.05$) lower after the cropping of bait plants than after the fallow treatment. The bait crop treatments (C-F-C, R-C-C and C-R-C) that included *B. rapa* canola yielded a lower ID on *B. napus* canola than those (R-F-C) consisting of only ryegrass. Similarly, the ID obtained in the treatments C-F-C and C-R-C was significantly lower than in the treatment R-F-C. Plant

height and shoot dry mass were highest in the fallow treatment than in the bait crop treatments. The treatments (R-C-C and C-R-C) which consisted of two subsequent bait crops produced the lowest plant height and dry mass in the subsequent *B. napus* canola crop.

3.3.2 Quantification of *P. brassicae* resting spores from inoculated potting mix

The *P. brassicae* resting spore concentration in the soil decreased in all bait crop treatments relative to the original concentration (9.33×10^5 spores/g soil) after the first bait crop cycle (cycle 1) (Figure 3-3). The resting spore concentration was also lower in the fallow treatment. The spore concentration in each treatment continued to decrease after the second bait crop cycle (cycle 2). In general, the treatments that included *B. rapa* canola (C-F-C, R-C-C and C-R-C) as a bait crop had relatively lower spore concentrations than the other treatments after each cycle.

The genomic DNA extracted from each soil sample was tested by conventional PCR prior to qPCR analysis. Each DNA sample was positive for the presence of *P. brassicae*, as indicated by the amplification of a band of the 700 bp - 800 bp size when the amplicons were visualized by agarose gel electrophoresis (data not shown). In the qPCR analysis, all of the treatments showed a decrease in the quantity of *P. brassicae* DNA in the soil after cycle 1, and with the exception of R-C-C, the amount of pathogen DNA was lower in all bait crop treatments than in the fallow treatment (Figure 3-4). The amount of *P. brassicae* DNA in the soil declined further in all treatments after cycle 2.

The *P. brassicae* resting spore concentration (as reflected by the quantity of pathogen DNA) in the soil after cycle 2 affected the ID on the subsequent *B. napus*

canola crop (Table 3-1). Compared to the fallow treatment, the resting spore concentration, quantity of *P. brassicae* DNA, and ID in the subsequent *B. napus* canola crop were all lower after the bait crop treatments. Treatments (C-F-C, R-C-C and C-R-C), all of which included *B. rapa* as a bait crop, generally resulted in lower resting spore concentrations, amounts of pathogen DNA, and ID (although the amount of pathogen DNA was lower in R-F-C than in C-R-C). However, the treatment with the lowest resting spore concentration (C-R-C) was not the same as that producing the lowest quantity of *P. brassicae* DNA and ID (C-F-C).

3.4 Discussion

Hosts as well as some non-hosts of *P. brassicae* stimulate the germination of pathogen resting spores. Several previous studies (Macfarlane 1952, Murakami et al. 2000 and Friberg et al. 2006) have attempted to assess the utility of such plant species as bait crops that could be used to manage clubroot. After the root hairs of the bait crop become infected, the *P. brassicae* inoculum level in the soil could be reduced in two ways: (1) by destruction of the bait crop (via herbicide treatment or ploughing) prior to completion of the pathogen life cycle, and/or (2) by the removal of the infected bait crop, thereby directly removing a part of the *P. brassicae* biomass from the soil. In both cases, the concentration of resting spores in the soil would ultimately be reduced, and the crop of interest would suffer less severe clubroot symptoms.

In the current study, bait crop experiments with both naturally infested soil and artificially inoculated potting mix resulted in a lower ID in the subsequent *B. napus* canola crop (Figure 3-1 and Figure 3-2). These findings show that host and non-host bait

crops can indeed reduce clubroot symptom severity on canola under greenhouse conditions. Polish (*B. rapa*) canola as a host plant played a particularly important role in suppressing the development of clubroot in subsequent *B. napus* canola crops, but all bait crop treatments significantly reduced disease severity relative to the fallow treatment (Figure 3-1 and Figure 3-2). Perennial ryegrass as a non-host also reduced the ID on subsequent *B. napus* canola, but did not appear to be as effective as *B. rapa* canola in this respect. Several studies on the effects of non-hosts as bait crops for clubroot control have been conducted. Friberg et al. (2006) investigated four non-host plants species, leek (*Allium porrum* L.), winter rye (*Secale cereale* L.), perennial ryegrass and red clover (*Trifolium pretense* L.). These four bait crops all produced lower levels of disease on a subsequent Chinese cabbage crop under greenhouse conditions. Similarly, Murakami et al. (2000) found that under greenhouse conditions, leafy daikon could reduce the severity of clubroot symptoms on the subsequent Chinese cabbage crop and could also reduce the number of *P. brassicae* resting spores in pots. All of these studies suggest that there are a number of host and non-host crops that could be suitable as bait plants. While in the current experiments the host species *B. rapa* canola appeared to be a superior bait crop to the non-host ryegrass, the use of a non-host may be a less risky strategy, since the timing of the non-host removal/destruction would not be as critical (as long as the pathogen is shown to be unable to complete its life cycle on the non-host). In contrast, if removal of a host bait crop is not properly timed, infection of the bait plants could ultimately contribute to inoculum buildup in the soil.

Interestingly, while clubroot severity in *B. napus* canola was reduced in the bait crop versus fallow treatments, shoot dry mass and plant height were higher in the fallow

treatment (Figure 3-1 and Figure 3-2). This likely reflects depletion of nutrients in the containers, as no additional nutrients were applied during the course of the experiments. Therefore, while the bait crops used up the limited nutrients in the soil or potting mixture, these remained available for the *B. napus* canola when it was preceded by a fallow treatment. Similar results were obtained in an earlier study by Ahmed et al. (2011).

While bait crops have shown promise for clubroot management in greenhouse experiments, the results in the field have been far less clear. Friberg et al. (2006) found that in a 3-year field experiment, none of the chosen bait crops reduced the concentration of *P. brassicae* resting spores in soils when these were tested in a plant bioassay. Murakami et al. (2000) reported that while the number of resting spores was reduced when leafy daikon was grown in advance of Chinese cabbage, there was no reduction in clubroot symptom severity under field conditions. Ahmed et al. (2011) also indicated that bait crops had no effect on clubroot severity on canola under field conditions. The complexity of environmental conditions in a field situation may help to explain these results. Webster (1986) reported that light may influence clubroot expression via an effect on host photosynthetic efficiency, and hence on energy reserves available to fuel clubbing. Light may also regulate the balance between shoot and root growth (Dixon 2009). Indeed, low light intensities were found to result in a reduction in root growth (Rausch et al. 1981), resulting in a reduction in clubroot symptoms.

Moreover, in heavily infested soils, the ability of a bait crop to reduce the spore load may not be sufficient to cause a decline in disease development in a subsequent susceptible crop (Ahmed et al. 2011). Preliminary experiments with a potting mix inoculated to a final concentration of 1×10^7 resting spores/g soil suggested that bait

crops were not able to significantly reduce ID relative to the control treatment (*data not shown*). In a heavily infested field, inoculum loads are often in excess of 1×10^6 resting spores/g soil. As such, bait crops may be useful as a management tool only in those fields in which the clubroot infestation is low to moderate in severity.

Microscopic analysis, conventional and quantitative PCR may be regarded as complementary tools for the detection and quantification of *P. brassicae* resting spores in bait crop and other studies. While a decline in the resting spore concentration was expected in the bait crop treatments, the observed decrease in resting spores and pathogen DNA in the fallow control, although smaller in magnitude, was nonetheless surprising. It is likely that water flow out of the plastic containers, and/or the mobility of *P. brassicae* zoospores, may have contributed to this decline. Nonetheless, given the significantly greater declines observed in the bait crop treatments (Figure 3-3 and Figure 3-4), this loss of pathogen biomass in the fallow treatment did not obscure the results, particularly since similar losses through water flow may have been expected in all treatments.

While the cropping of bait plants reduced clubroot disease in subsequent *B. napus* crops under greenhouse conditions, the results from this and other studies suggest that additional research will be required before this strategy can be widely applied in commercial canola cropping systems. Issues with the reduced impact of bait crops in the field, nutrient depletion, and the timing of bait crop removal will all need to be resolved before the sowing of bait plants can be recommended to farmers. Nevertheless, the promising results obtained under greenhouse conditions suggest that bait crops may

eventually have a place in the integrated management of clubroot of canola and other crucifers.

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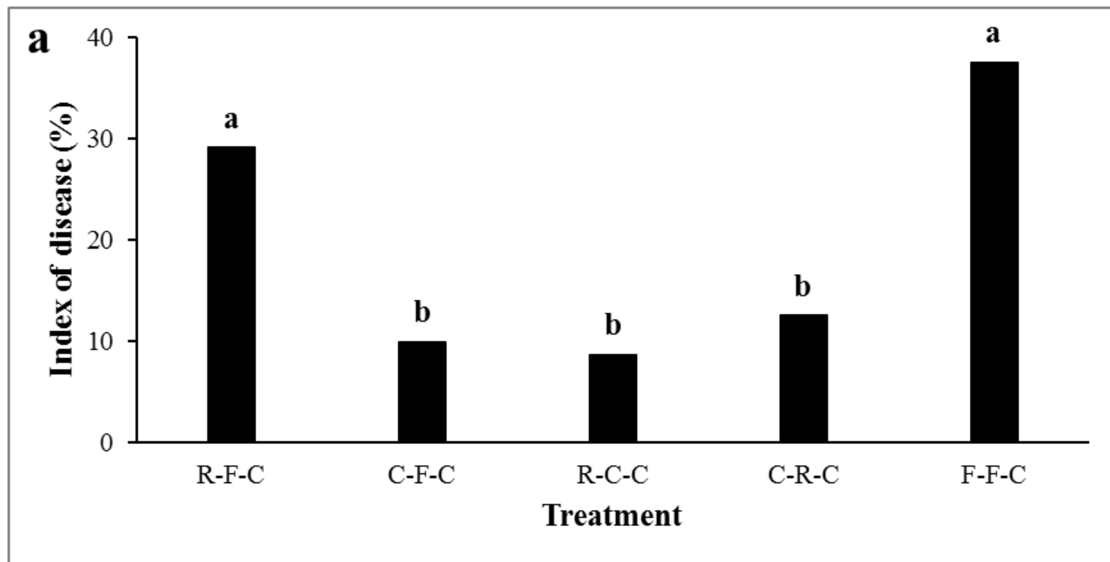
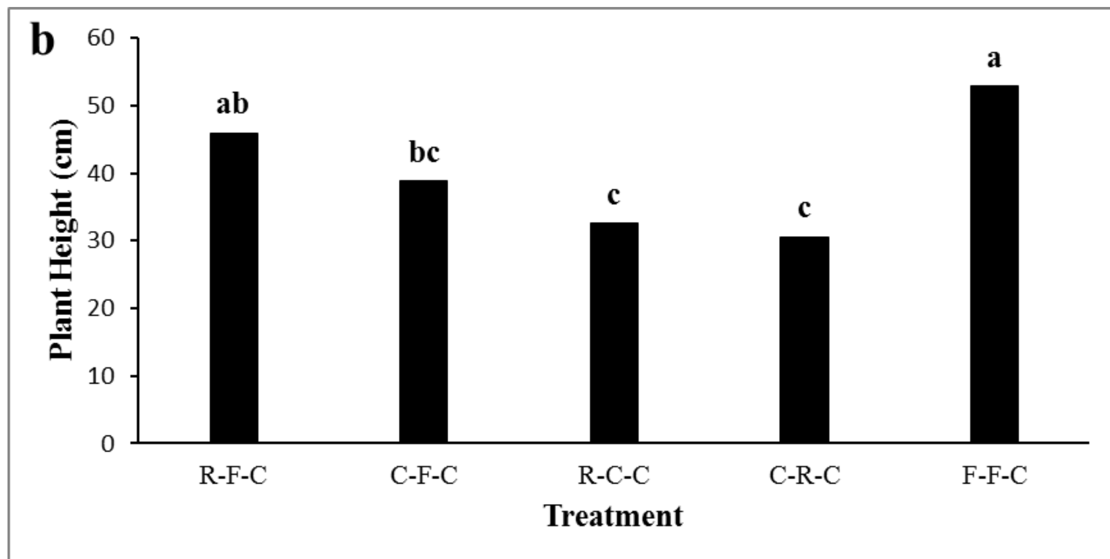
Table 3-1. Resting spore concentration and *P. brassicae* DNA content after two bait crop cycles in artificially inoculated potting mix, and index of disease (%) on a subsequent *Brassica napus* canola crop.

Treatment*	Resting spores (10^5/g soil)	DNA (10^{-2}ng/*u*L)	Index of Disease (%)
R-F-C	2.50ab**	0.62ab	84.33b
C-F-C	2.03abc	0.41b	51.89d
R-C-C	1.80bc	0.57b	79.49b
C-R-C	1.43c	0.65ab	72.56c
F-F-C	2.86a	0.96a	91.84a

*R-F-C = perennial ryegrass – fallow – *B. napus* canola rotation; C-F-C = *Brassica rapa* canola - fallow – *B. napus* canola rotation; R-C-C = perennial ryegrass – *B. rapa* canola – *B. napus* canola rotation; C-R-C = *B. rapa* canola – perennial ryegrass – *B. napus* canola rotation; F-F-C = fallow – fallow – *B. napus* canola rotation.

**Numbers followed in the same column followed by the same letter are not significantly different at $P \leq 0.05$, based on analysis of variance (ANOVA) using PROC GLM of the SAS.


a
Index of disease (%)
40
30
20
10
0
a
b
b
b
a
R-F-C
C-F-C
R-C-C
C-R-C
F-F-C
Treatment


b
Plant Height (cm)
60
50
40
30
20
10
0
ab
bc
c
c
a
R-F-C
C-F-C
R-C-C
C-R-C
F-F-C
Treatment

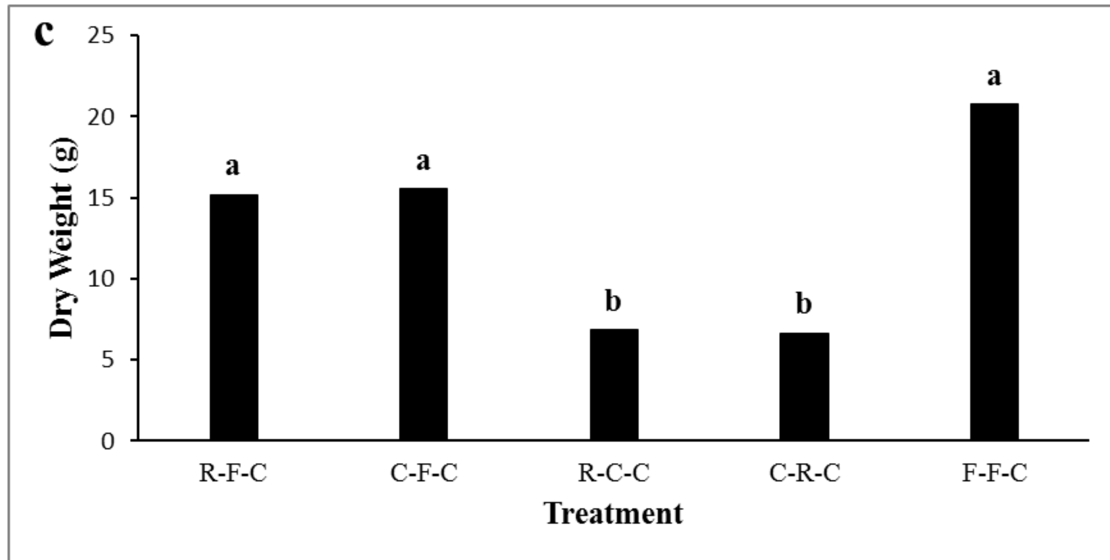
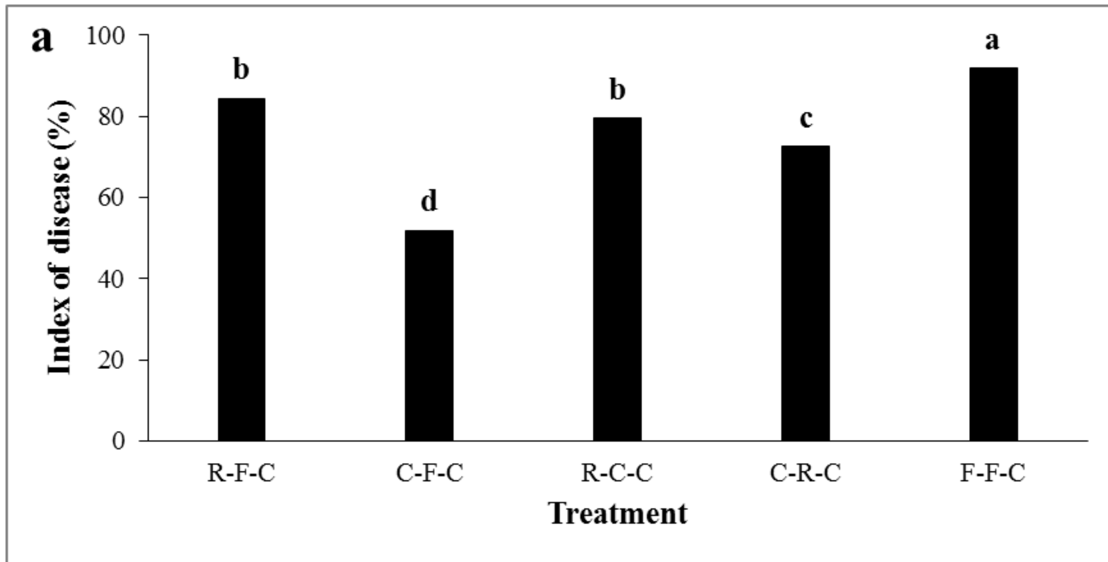
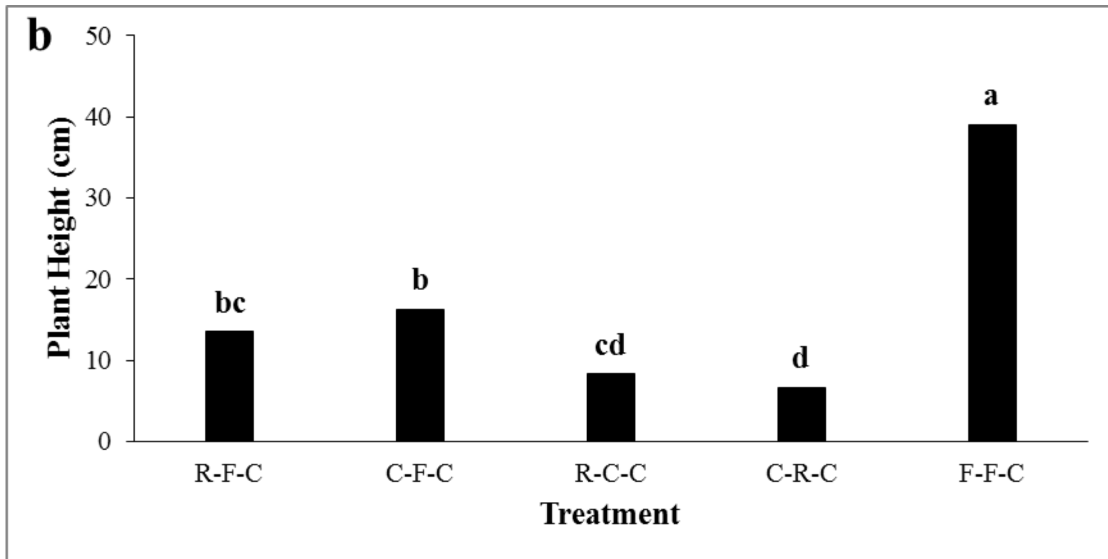


Figure 3-1. Index of disease (a), plant height (b) and dry mass (c) measured on *Brassica napus* canola grown on clubroot infested soil following various bait crop or fallow rotations. R-F-C = perennial ryegrass – fallow – *B. napus* canola rotation; C-F-C = *Brassica rapa* canola - fallow – *B. napus* canola rotation; R-C-C = perennial ryegrass – *B. rapa* canola – *B. napus* canola rotation; C-R-C = *B. rapa* canola – perennial ryegrass – *B. napus* canola rotation; F-F-C = fallow – fallow – *B. napus* canola rotation. Columns topped by the same letter do not differ at $P \leq 0.05$, based on analysis of variance (ANOVA) using PROC GLM of the SAS. Differences among treatments were assessed using Duncan's multiple range test.


a
100
80
60
40
20
0
Index of disease (%)
b
d
b
c
a
R-F-C
C-F-C
R-C-C
C-R-C
F-F-C
Treatment


b
50
40
30
20
10
0
Plant Height (cm)
bc
b
cd
d
a
R-F-C
C-F-C
R-C-C
C-R-C
F-F-C
Treatment

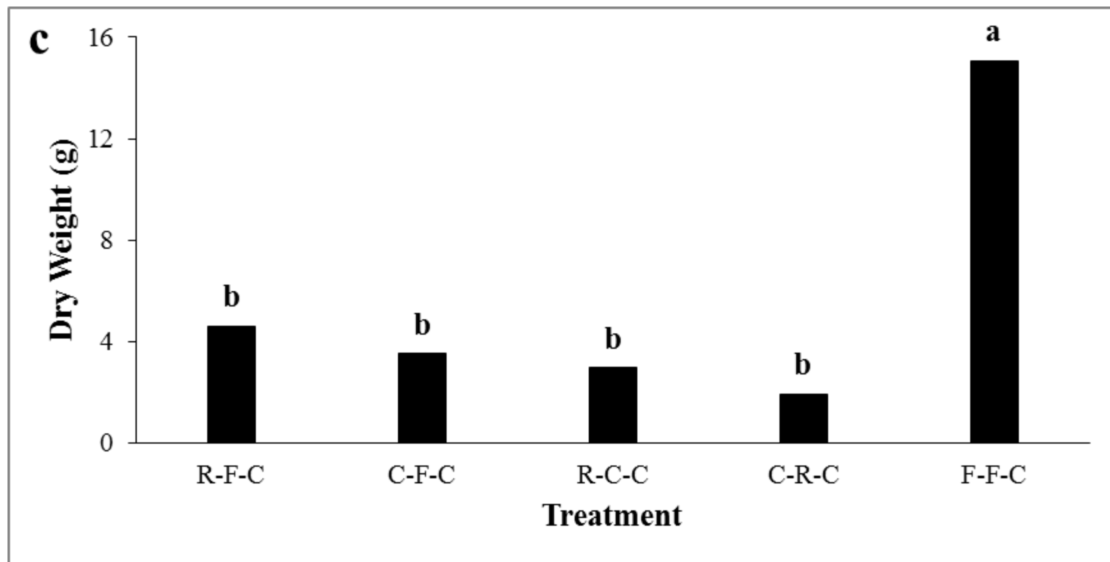


Figure 3-2. Index of disease (a), plant height (b) and dry mass (c) measured on *Brassica napus* canola grown on inoculated potting mixture following various bait crop or fallow rotations. R-F-C = perennial ryegrass – fallow – *B. napus* canola rotation; C-F-C = *Brassica rapa* canola - fallow – *B. napus* canola rotation; R-C-C = perennial ryegrass – *B. rapa* canola – *B. napus* canola rotation; C-R-C = *B. rapa* canola – perennial ryegrass – *B. napus* canola rotation; F-F-C = fallow – fallow – *B. napus* canola rotation. Columns topped by the same letter do not differ at $P \leq 0.05$, based on analysis of variance (ANOVA) using PROC GLM of the SAS. Differences among treatments were assessed using Duncan's multiple range test.

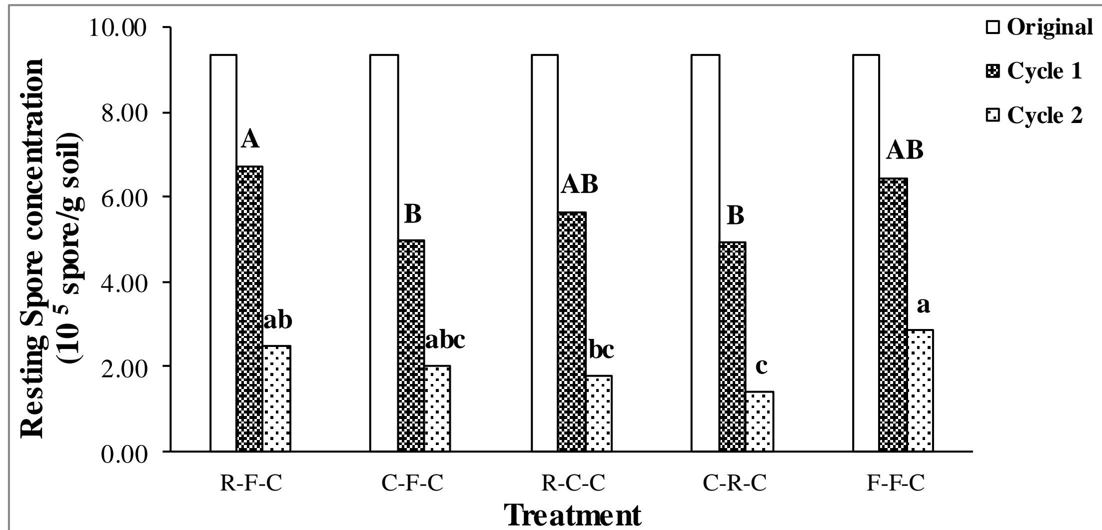


Figure 3-3. *Plasmodiophora brassicae* resting spore concentrations in inoculated potting mix before and after cycling of various bait crops or fallow periods. R-F-C = perennial ryegrass – fallow – *B. napus* canola rotation; C-F-C = *Brassica rapa* canola - fallow – *B. napus* canola rotation; R-C-C = perennial ryegrass – *B. rapa* canola – *B. napus* canola rotation; C-R-C = *B. rapa* canola – perennial ryegrass – *B. napus* canola rotation; F-F-C = fallow – fallow – *B. napus* canola rotation. Columns topped by the same letter do not differ at $P \leq 0.05$, based on analysis of variance (ANOVA) using PROC GLM of the SAS. Differences among treatments were assessed using Duncan's multiple range test.

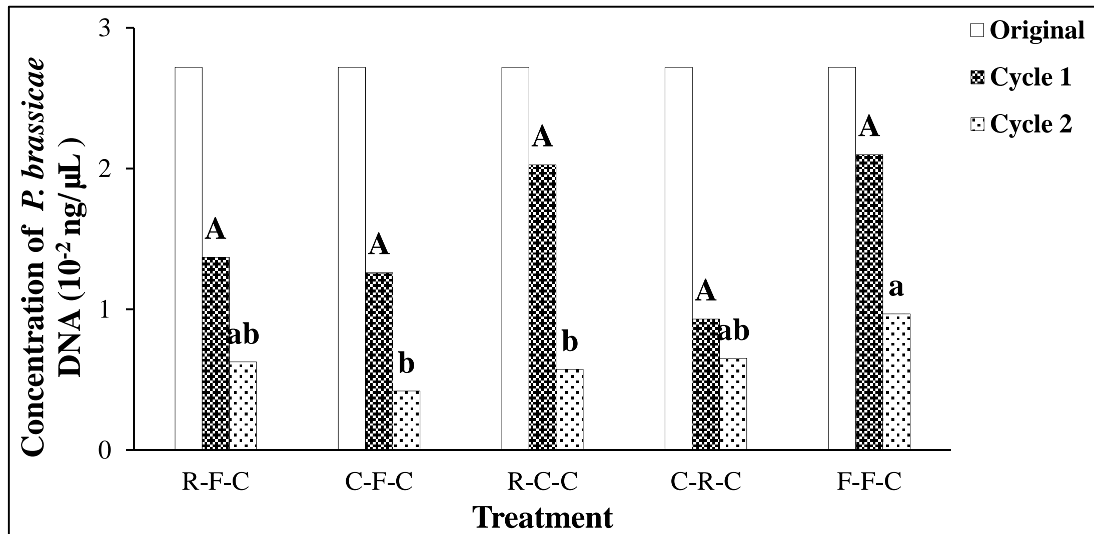


Figure 3-4. The concentration of *Plasmodiophora brassicae* DNA in inoculated potting mixture before and after cycling of various bait crops or fallow periods, as determined by quantitative PCR. R-F-C = perennial ryegrass – fallow – *B. napus* canola rotation; C-F-C = *Brassica rapa* canola - fallow – *B. napus* canola rotation; R-C-C = perennial ryegrass – *B. rapa* canola – *B. napus* canola rotation; C-R-C = *B. rapa* canola – perennial ryegrass – *B. napus* canola rotation; F-F-C = fallow – fallow – *B. napus* canola rotation. Columns topped by the same letter do not differ at $P \leq 0.05$, based on analysis of variance (ANOVA) using PROC GLM of the SAS. Differences among treatments were assessed using Duncan's multiple range test.

4. Infection of canola by secondary zoospores of *Plasmodiophora brassicae* produced on a nonhost (ryegrass)

4.1 Introduction

Plasmodiophora brassicae Woronin causes clubroot disease in cruciferous plants, and is an emerging threat to canola (*Brassica napus* L.) production in Canada (Hwang et al. 2011). The pathogen has a complex life cycle comprised of three stages: survival in the soil as resting spores, primary infection of root hairs, and secondary infection of and development within the root cortex (Ingram and Tommerup 1972; Naiki and Dixon 1987). Each resting spore germinates to release one oval-shaped or pyriform biflagellate motile spore known as a primary zoospore. These zoospores swim to and infect root hairs by penetrating the cell wall. Within the root hairs, primary plasmodia develop quickly when temperature conditions are optimum and cleave into zoosporangia, each containing 4–16 secondary zoospores, which are released into the soil. Primary infections do not produce macroscopic symptoms (Howard et al. 2010). The secondary zoospores penetrate the root epidermis of the host and invade the cortical tissues of the main roots. Secondary zoospores cannot be differentiated visually from primary zoospores. Secondary infection is followed by the development of secondary plasmodia within the root cortex, which results in the production of clubroot symptoms, i.e., club-shaped malformations of the roots (Kageyama and Asano 2009). Each secondary plasmodium will eventually be cleaved into large numbers of resting spores within the clubbed root. As the root tissues disintegrate, the resting spores are released into the soil to complete the disease cycle.

Many species in the Brassicaceae family, including crops and weeds such as *Capsella bursa-pastoris* (L.) Medik. and *Thlaspi arvense* L., are hosts of *P. brassicae* (Buczacki and Ockendon 1979; Dixon 2009). MacFarlane (1952) observed primary infection in nonhost plants such as *Lolium perenne* L. (perennial ryegrass), *Reseda odorata* L. and *Tropaeolum majus* L., but no cortical infection. In another study, secondary infection was demonstrated on the non-host species *T. majus*, *Carica papaya*, *R. alba* and *Beta vulgaris* (Ludwig-Müller et al. 1999).

Many aspects of *P. brassicae* pathogenesis are not fully understood (Hwang et al. 2011), but the elucidation of the mechanisms for pathogenesis could contribute to development of novel strategies in resistance breeding or clubroot management. One aspect of pathogenesis that is not well defined is the role of root hair infection in non-hosts on clubroot development in susceptible crops. Therefore, cross infection of canola and ryegrass by secondary zoospores produced on these two crop species was examined in the current study. The objectives of this study were to determine if there are differences in susceptibility to primary infection between host and nonhost species, if secondary zoospores can re-infect the root hairs, and if the pathogenicity of secondary zoospores produced in host and nonhost tissues differs.

4.2 Materials and methods

4.2.1 Plant material and *Plasmodiophora brassicae* field isolate

The canola cultivar (cv.) Westar and perennial ryegrass cv. Amazing were used as host and nonhost of *P. brassicae*, respectively. Galls that had developed on plants of cv. Westar after infection by *P. brassicae* were collected from experimental field plots in

Edmonton, AB, Canada (Google map coordinates: 53.647, -113.378). Resting spores isolated from these clubs were used as the initial inoculum.

4.2.2 Preparation of seedlings

Seeds of canola and ryegrass were surface-sterilized in 1% sodium hypochlorite for 5 min, washed with distilled water, and germinated on moistened filter paper for 7 days. The resulting seedlings were used in resting spore or secondary zoospore inoculations.

4.2.3 Inoculation with resting spores

The resting spore inoculum was prepared by homogenizing small portions of clubs in distilled water in a blender and passing the resultant slurry through eight layers of cheesecloth to produce a suspension of resting spores (Feng et al. 2010). The resting spore suspension was used to inoculate the soil-less mix (Sunshine mix #4, Sun Gro Horticulture, Vancouver, BC, Canada).

Seedlings were transplanted into inoculated soil-less mix in 40 cm × 25 cm × 15 cm plastic trays. The trays were kept in a growth chamber maintained at 24°C/18°C (day/night), 16-h photoperiod and 80% RH, and watered from the bottom every second day with tap water adjusted to pH 6.4 with HCl.

4.2.4 Inoculation with secondary zoospores

After 7 days growing in the inoculated soil-less mix containing 3.1×10^7 resting spores/g, about 200 plants each of canola and ryegrass were dug out and the roots were washed with tap water. The foliage was then cut off at the soil level, and the roots were rinsed three times by shaking at 150 rpm for 20 min in 200 mL of distilled water in a 500-mL flask. The roots were shaken in 50 mL distilled water at 100 rpm for 20 h to stimulate release of secondary zoospores. After removing the roots, 10 mL of the zoospore suspension was concentrated by centrifugation at 5000 $\times g$ for 5 min and adjusted to 2.5×10^6 spores/mL (Figure 4-1a). Ten samples of the concentrated suspension were examined microscopically to confirm the absence of resting spores. The original suspension was then adjusted to 1×10^4 spores/mL and used immediately for inoculation.

Thirty mL of zoospore suspension was transferred into a sterilized 96-hole, 200-μl pipet tip box. Seven-day old seedlings of canola or ryegrass were inserted through the tip rack into the box, making sure that the plants were upright and that the roots were immersed in the secondary zoospore suspension. The boxes were shaken at 30 rpm for 48 h, and then the seedlings were transplanted into 40 cm × 25 cm × 15 cm plastic trays containing autoclaved soil-less mix and maintained as described above. The experiments included four treatments termed C^C, C^R, R^C and R^R to indicate that plants of canola or ryegrass were inoculated with secondary zoospores produced on either plant species (Figure 4-1b). As a parallel control, ryegrass seedlings were transplanted into soil-less mix containing 1×10^5 resting spores/g and maintained under the same conditions.

4.2.5 Clubroot assessments

At 5 days after inoculation (dai), 30 root samples were collected from 10 seedlings per treatment and investigated using a Zeiss AX10 microscope (Carl Zeiss, Thornwood, NY). On each sample, 10 fields of view using the 10× objective lens were examined. In each field of view, three sets of data were collected: root hairs with primary infection and the total number of root hairs, the presence or absence of secondary infection, and the total number of secondary plasmodia. At 35 dai, all of the seedlings were harvested and the clubroot symptoms and root infection were investigated either visually or microscopically. The experiment was repeated.

4.2.6 Data analysis

The proportion (%) of primary and secondary infections and total number of secondary plasmodia were calculated for each root sample. The data were subjected to analysis of variance using PROC GLM of the SAS statistical package (version 9.1.3, SAS Institute, Cary, NC). There were no differences between repetitions of each experiment, so the data were combined for subsequent analysis. Differences among treatments were assessed using Duncan's multiple range test.

4.3 Results and Discussion

Seven days after inoculation by resting spores, secondary zoospores were produced in abundance from both canola and ryegrass. This confirms previous reports that the primary stage of infection continues to completion in at least some nonhost

species (MacFarlane 1952). When canola and ryegrass seedlings were inoculated with secondary zoospores from canola and ryegrass, more than 50% of root hairs in each treatment were infected at 5 dai (Figure 4-2a and Figure 4-3a). This demonstrates that secondary zoospores produced on ryegrass (a nonhost) can infect root hairs of both host and non-host plants. A previous study had demonstrated that secondary zoospores from root hairs of a host (Chinese cabbage) could infect root hairs of a host plant (Naiki et al. 1984), but this is the first demonstration that zoospores produced on a nonhost are also infectious. The observation that secondary zoospores can infect root hairs of both host and nonhost species provides strong evidence that *P. brassicae* can proliferate prior to secondary infection by cycling within root hairs. A rapid build-up of secondary zoospore populations through repetitive cycles in the root hairs likely increases the subsequent infection success of the pathogen in cortical tissues. There were no differences in the frequency of root hair infection among treatments (Figure 4-2a), suggesting limited or no nonhost resistance in the root hairs of ryegrass.

Inoculation of canola and ryegrass with secondary zoospores from either canola or ryegrass consistently resulted in secondary (cortical) infection in both species, with approximately 50% on canola and 40% on ryegrass (Figure 4-2b). The proportion of secondary infection and the number of secondary plasmodia per field of view were higher in canola inoculated with zoospores from canola (C^C) than in ryegrass inoculated with zoospores from ryegrass (R^R), with the other combinations intermediate (Figure 4-2b and c). This indicates that secondary infection by *P. brassicae* occurred slightly more readily on host than nonhost plants. There were no differences between the cross-inoculation treatments (C^R and R^C) for secondary infection or the number of secondary plasmodia.

This suggests that the nonhost resistance in ryegrass seedlings inoculated with zoospores from canola (R^C) was overcome by the high pathogenicity of secondary zoospores produced on a susceptible host.

The secondary plasmodia produced on ryegrass (Figure 4-3b) were visually similar to those produced on canola, irrespective of the origin of the secondary zoospores. Secondary infection of a nonhost species has been reported previously (Ludwig-Müller et al. 1999). In contrast, even though 20% of root hairs were infected in ryegrass plants grown in infested soil-less mix for 2 weeks (parallel control), no secondary infection was detected. This suggests that inoculation with secondary zoospores bypassed the nonhost-pathogen interaction that normally occurs during primary infection, so the ryegrass seedlings did not receive the stimuli from the pathogen that normally triggers the nonhost resistance response.

No clubs were found on roots from 55 R^C and 60 R^R ryegrass plants at 35 dai. Secondary plasmodia (Figure 4-4) were present in about one-third of the root samples from both treatments. There was no morphological difference among the plasmodia produced by the two treatments (R^C and R^R), but no vegetative plasmodia or resting spores were observed. These observations indicate that secondary plasmodia persisted on ryegrass roots for as long as 35 days, but infection did not progress beyond that stage of development.

Typical large clubs developed on 14% (10 out of 71) of canola plants inoculated with zoospores from canola (C^C) at 35 dai (Figure 4-5a). In contrast, tiny clubs (Figure 4-5b and c) were found on 16% (10 out of 64) of the canola root samples inoculated with zoospores from ryegrass (C^R). These clubs developed in clusters (5-10) in a bead-like

string on the upper parts of the roots. Within each club, resting spores were present in the infected cortical cells (Figure 4-5d). The difference in club size caused by inocula from canola and ryegrass indicates that there are differences in the ability of secondary zoospores derived from host and nonhost sources to induce normal club formation.

These data indicate that nonhost resistance to *P. brassicae* in ryegrass utilizes several mechanisms that are stimulated by different stages in the life cycle of *P. brassicae*. There may be little or no difference in primary infection (caused by primary zoospores) in canola and ryegrass. Also, primary infection in both canola and ryegrass was shown to result in the production and release of large numbers of secondary zoospores. Before this study was conducted, the rare observation of secondary infection on ryegrass inoculated with resting spores could have been explained in three ways: 1) secondary zoospores from ryegrass are not pathogenic, 2) ryegrass is completely resistant to secondary infection (innate nonhost resistance), or 3) resistance to secondary infection in ryegrass is induced by interaction with *P. brassicae* during primary infection. The first two explanations were ruled out by the results of the current study, which showed that secondary infection of ryegrass was possible (R^C and R^R) and that secondary zoospores from ryegrass were pathogenic on canola (C^R). The abnormal (small, bead-like) clubs that form on canola inoculated with zoospores from ryegrass indicate that secondary infection by zoospores from ryegrass does not proceed in precisely the same way as with zoospores from canola. This may indicate that differences in the secondary spores from canola and ryegrass play a role in the nonhost resistance to secondary infection that occurs in nature in ryegrass.

Nonhost resistance is often triggered in the early stages of infection (Heath 2000). For example, conidial germination, appressorium formation, and penetration of the plant epidermis occur normally for barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) on *Arabidopsis thaliana* (L.) Heynh**,** but hyphal growth ceases when hyphae penetrate plant cells (Ellis 2006). A previous study suggested that a resistance response might be triggered in nonhosts of *P. brassicae* such as *R. alba* (Ludwig-Müller et al. 1999). The current study indicates that there is an interaction between *P. brassicae* and ryegrass during root hair infection that affects subsequent secondary infection and club formation. Results from the current study indicate that resistance to secondary infection is induced during primary infection, and that ryegrass requires an extended period of contact for the development of nonhost resistance.

In addition to providing information on the pathogenesis of *P. brassicae*, the results of the current study lead to two recommendations for studies of clubroot. First, it is clear that secondary zoospores can cause primary infection, implying that studies of primary infection (Hwang et al. 2011) should generally be conducted using a low concentration of resting spores. If the concentration of resting spores is high, primary infection caused by secondary zoospores could quickly overwhelm potential differences in infection by primary zoospores. Second, nonhost plants can develop primary infection and produce secondary zoospores that can infect susceptible hosts. As a result, the presence of both host and nonhost weeds in greenhouse and field trials could influence the results of clubroot studies, and so needs to be carefully controlled.

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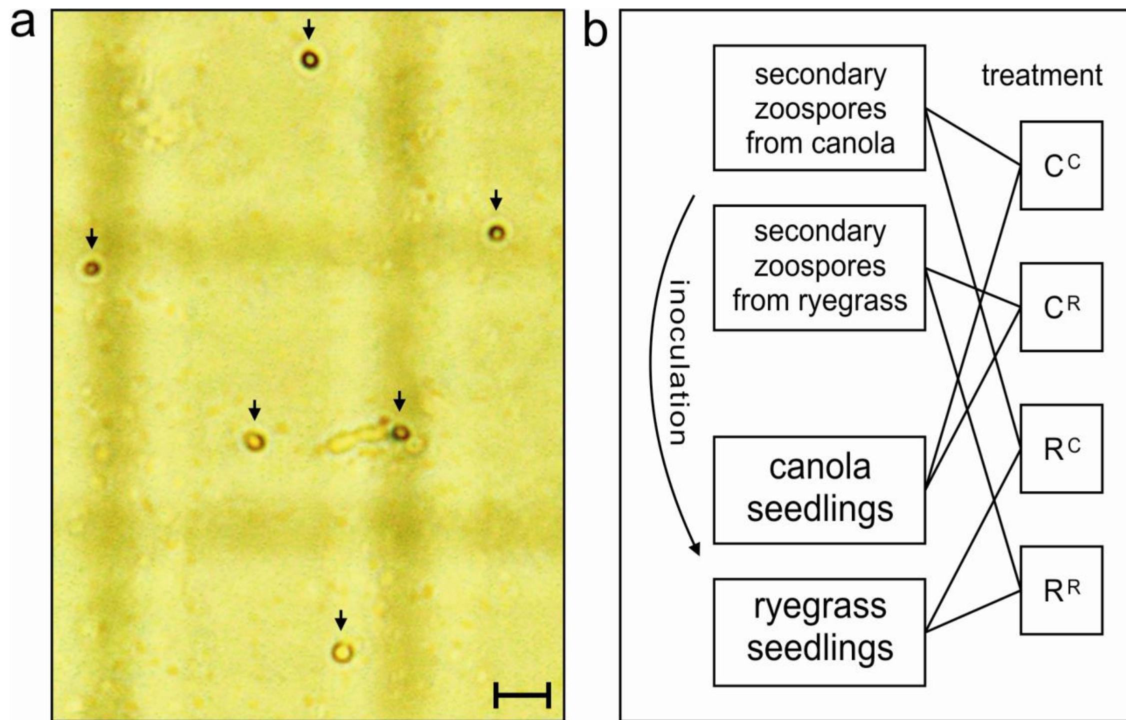

Figure 4-1. Inoculation of canola and ryegrass seedlings with secondary zoospores of *Plasmodiophora brassicae*. (a) Secondary zoospores released from canola roots after infection by resting spores. Arrows indicate the zoospores. Bar = 10 μm. (b) The four treatments (C^C, C^R, R^C and R^R) in the secondary zoospore infection study; C^C, C^R, R^C and R^R denote treatments in which plants of canola or ryegrass (indicated with capital letters C or R, respectively) were inoculated with secondary zoospores produced on either plant species (indicated by superscript letters).

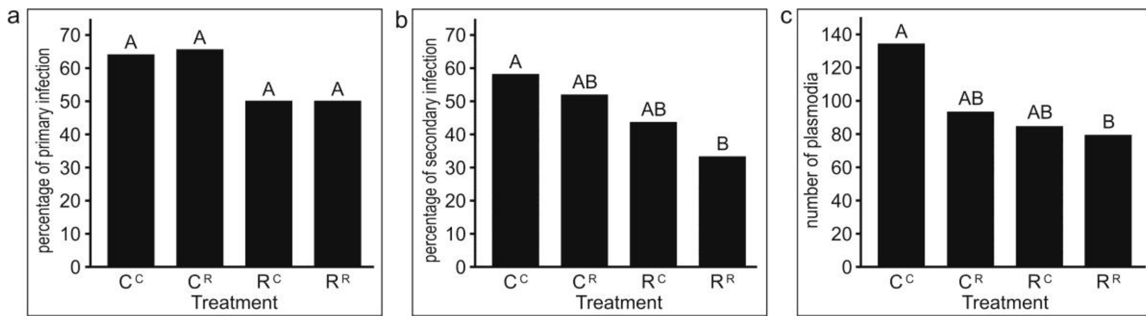

Figure 4-2. Percentages of primary (a) and secondary (b) infections, and numbers of secondary plasmodia (c) on canola and ryegrass at 5 days after infection with secondary zoospores collected from each of the two plant species. C^C, C^R, R^C and R^R denote treatments in which plants of canola or ryegrass (indicated with capital letters C or R, respectively) were inoculated with secondary zoospores produced on either plant species (indicated by superscript letters). Means in the columns followed by the same letter do not differ based on Duncan's multiple range test at $P \leq 0.05$.

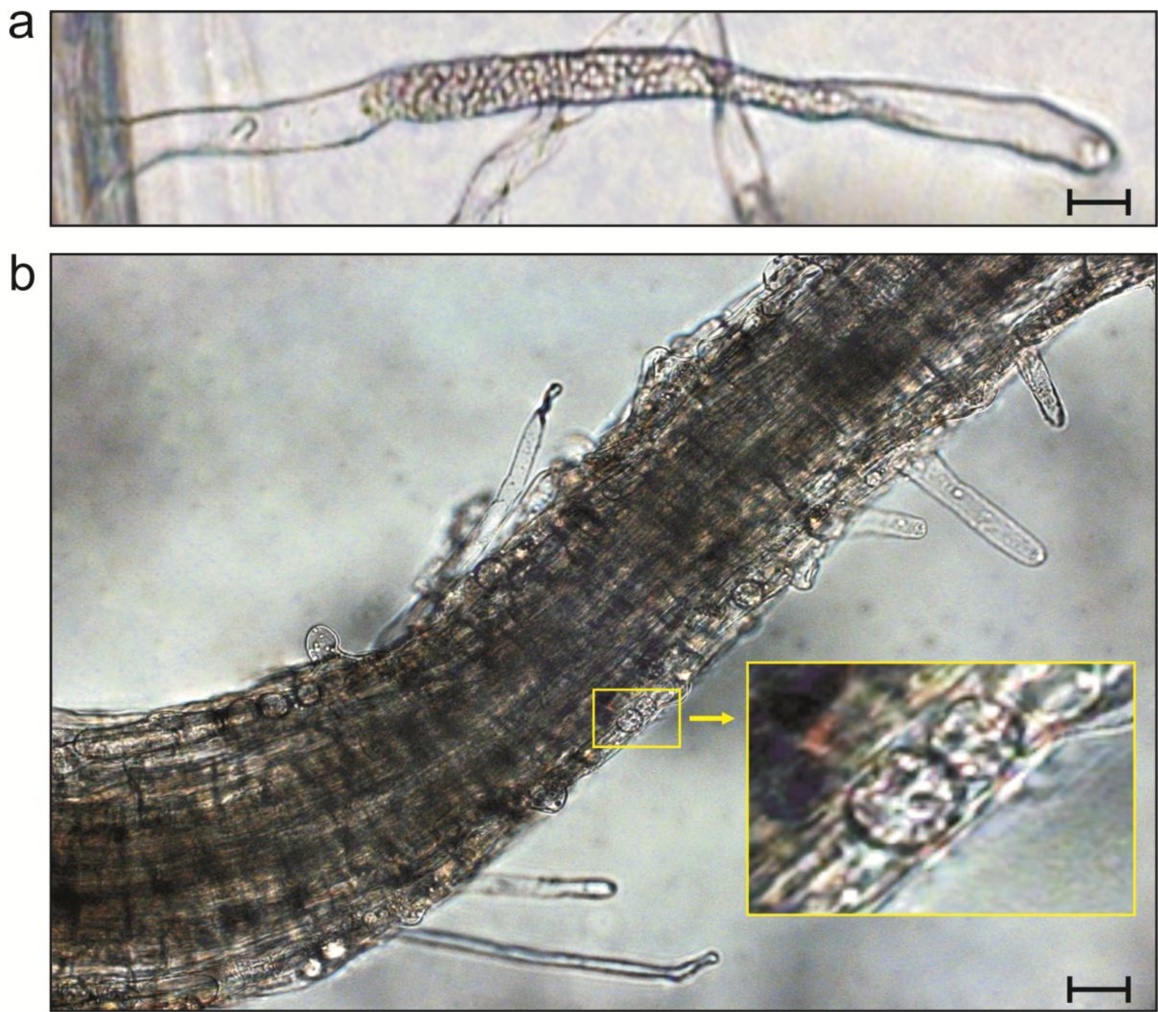

Figure 4-3. Primary (a) and secondary (b) infection of ryegrass 5 days after inoculation with secondary zoospores from canola. Bar = 10 μm.

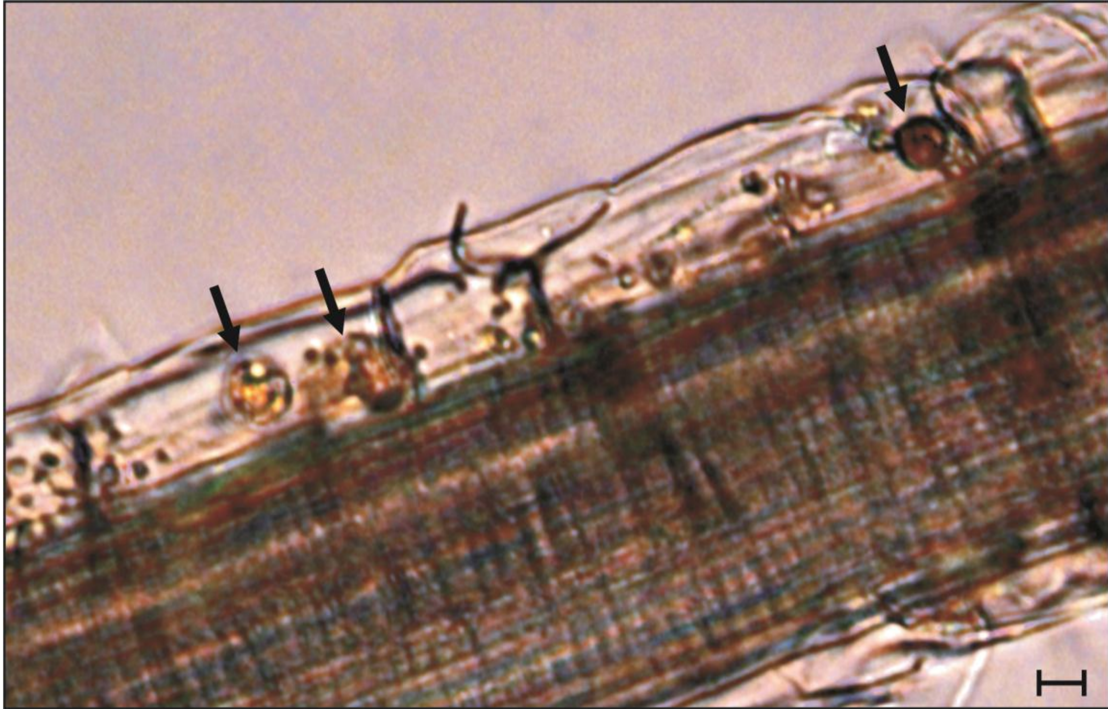

Figure 4-4. Secondary infection on ryegrass 35 days after inoculation with secondary zoospores from ryegrass. Arrows indicate the secondary plasmodia. Bar = 10 μm.

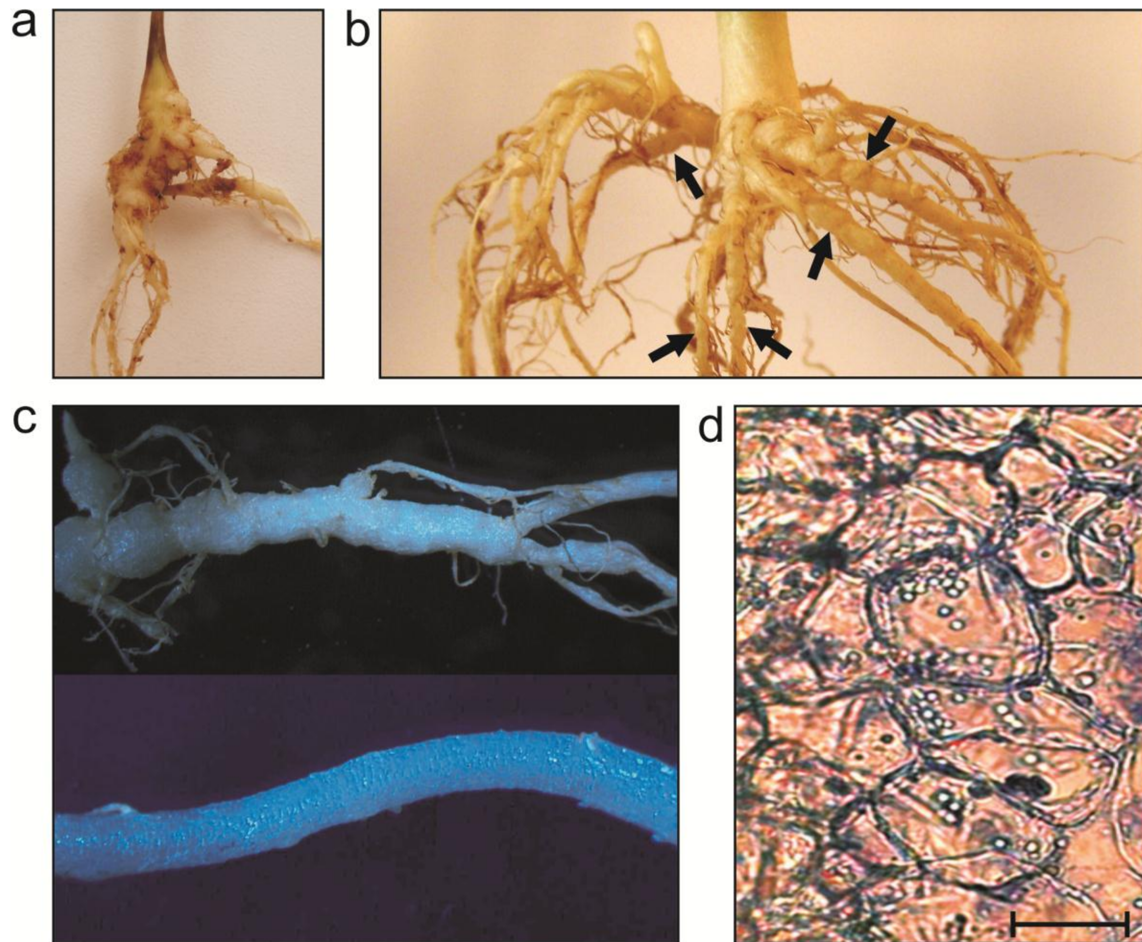

Figure 4-5. Clubs on canola roots at 35 days after inoculation with secondary zoospores from canola (a) or ryegrass (b-d). (b) Infected roots with tiny clubs, indicated by arrows. (c) Root with tiny clubs (top) and healthy root (bottom). (c) Cross-section of root with tiny clubs. Bar = 100 μm.

5. Impact of seeding date, seedling age and cultivar on clubroot severity, seedling emergence and yield of canola

5.1 Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is a devastating soilborne disease of cultivated crucifers worldwide. In recent years, *P. brassicae* has become a severe constraint to the production of canola (*Brassica napus* L., *B. rapa* L.) in central Alberta, Canada (Tewari et al. 2005; Strelkov et al. 2006). Symptoms of clubroot include swelling and abnormal growth (clubbing) of infected roots, wilting, stunting, premature ripening causing significant losses in biomass, grain yield, and oil content in canola (*Brassica napus* L. and *Brassica rapa*) (Dixon 2006; Pageau et al. 2006; Wallenhammar et al. 1999; Strelkov et al. 2006). Each large club contains millions of resting spores, so frequent cultivation of susceptible hosts favours the rapid build-up of resting spore populations.

A canola hybrid resistant to clubroot was released for commercial production in Canada in 2009, and immediately became one of the dominant cultivars grown in areas where clubroot is known to occur (S.E. Strelkov, *personal communication*). Several other resistant cultivars have since been released, but the number of clubroot resistance genes available for deployment is limited (Hirai 2006). Moreover, single gene resistance to clubroot has broken down quickly in winter canola and other crops (Kuginuki et al. 1999; Oxley 2007), particularly in areas where there is substantial genetic and pathotype variation in *P. brassicae*, such as western Canada (Strelkov et al. 2006; Xue et al. 2008; Cao et al. 2009). Production of lines with single gene resistance against a genetically

diverse pathogen on a large acreage will impose a strong selection pressure for pathogen genotypes that are able to overcome this resistance, and so eventual breakdown of resistance seems likely. Therefore, genetic resistance should be considered as just one component in the management of clubroot in canola on the Canadian prairies.

Several clubroot management strategies are used in the production of cruciferous vegetables, including drench application of fungicides and amendment with lime to increase the soil pH (Donald and Porter 2009). However, these options are not practical or cost effective for use in the production of field crops such as canola. For example, several tons of lime per hectare would be required to increase the soil pH to a level where clubroot severity would be reduced (Myers and Campbell 1985; Webster and Dixon 1991; Murakami et al. 2002). These large tonnages are not only too expensive to be cost-effective for a field crop, but are also physically impractical to source, apply and incorporate over hundreds of fields each year. Reduction in clubroot severity is also possible with the application of fungicides (Shimotori et al. 1996; Donald et al. 2001; Mitani et al. 2003) and soil amendment products (Hwang et al. 2008), but the rates used for horticultural crops are not cost-effective for canola. As a result, a range of alternative management strategies are being studied for their usefulness in canola production, including the timing of seeding (Gossen et al. 2009), biological control (Peng et al. 2009), and the use of bait crops (Kroll et al. 1984; Ikegami 1985; Murakami et al. 2001; Chapter 3).

Studies on the impact of inoculum density indicated increased clubroot severity and reduced plant height and seed yield of a susceptible canola cultivar with increasing inoculum density, and infection of younger seedlings resulted in higher clubroot severity,

shorter plants and lower yield than infection of older seedlings (Hwang et al., 2011a; Hwang et al., 2011b). Since clubroot has been detected, albeit at generally low levels, in fields sown to clubroot resistant canola cultivars (Strelkov et al. 2011), the effects of seedling age on disease development, plant growth parameters and yield in resistant cultivars grown in clubroot infested soil warrants investigation. The objectives of this study were to evaluate the effects of seeding date, seedling age and canola cultivar on the emergence, clubroot severity and yield of canola under greenhouse and field conditions.

5.2 Materials and methods

5.2.1 Field experiment (seeding date and cultivar)

Field trials to assess the impact of seeding date on disease severity, seedling emergence and yield of canola were conducted at two sites in naturally-infested soil. Small plots (6 m $\times$ 1.5 m) of canola were seeded on May 11, May 19 and May 28, 2008 at St. Albert and May 16, May 29 and June 4, 2008 at Leduc. Seeding dates as treatments were replicated four times in a randomized complete block (RCB) design. Additional seeding date trials were conducted on May 12, May 25 and June 13 in Leduc and May 11, May 25 and June 7 in Edmonton in 2010. Two canola cultivars, 45H29 and 45H26, resistant and susceptible to clubroot, respectively, were used at each location in the study. Data on seedling emergence was noted after three weeks of seeding and clubroot disease severity (0-3 scale; Chapter 3) and yield were recorded at crop maturity at both sites. Agronomic data, including plants/m^2, seedling emergence and seed yield, were subjected to ANOVA using the GLM procedure of SAS (SAS Inc., Cary, NC, USA). Mean separation was carried out following Fisher's LSD test.

5.2.2 Greenhouse experiment (seedling age and cultivar)

Experiments were conducted under greenhouse conditions with a 16-hour photoperiod (22 ^{o}C day/16 ^{o}C night) to test the effect of different canola cultivars and seedling age on clubroot disease severity, plant height and seed yield. The canola cultivars 45H29 and 45H26 that were used in the field experiment were also used in the greenhouse study. Both cultivars were seeded on five different dates: July 15, July 20, July 25, July 30 and August 4, 2009. Three seeds of each canola cultivar were sown into 12.7 cm-diameter disposable pots filled with soil-less potting mixture (Sunshine Mix 4; pH 6.5; SUN GRO® Horticulture Canada Ltd., Seba Beach, AB). After emergence, the plants were thinned to one plant per pot. The plants were inoculated with resting spores of *P. brassicae* (1.08×10^8 spores/ml) five days after the last seeding date (August 4th). The experiment was set up as an RCB design with 10 replications and one pot per replication. The pots were maintained on water-filled trays for the first two weeks after inoculation. After two weeks, all of the pots were moved onto a bench and watered daily from above to maintain a high level of soil moisture. At plant maturity, clubroot disease severity, plant height and seed yield were recorded. The disease severity was assessed as above. The experiment was conducted two times.

5.2.3 Data analysis

The data were tested for homogeneity of variance using normal probability plot. Analyses of variance for the plant height and seed yield data were performed following the General Linear Model procedure (Proc GLM) of SAS Statistical software (SAS Inc., Cary, NC, USA). Since the combined analysis of variance of the pooled data over the

trials indicated a significant trial effect, with trial × cultivar and trial × seedling age interactions being significant, the data were analyzed separately by trial. The mean separation was conducted using Fisher's LSD test ($P \leq 0.05$). Since there was no clubroot gall development in the resistant cultivar seeded on July 15th and July 20th, 2009, these seeding dates were excluded from the analysis of disease data using non-parametric data analysis.

5.3 Results

5.3.1 Field experiment

Analysis of variance indicated a significant effect of seeding date on seedling emergence and yield of canola in the St. Albert trial in 2008 (Table 5-1). Seedling emergence was significantly higher in the late seeding date (June 04) at Leduc and in the mid to late seeding (May 19 and May 28) at St. Albert. The canola yield at both sites was significantly higher in the early-seeded plots (Table 5-1). Clubroot severity was greater in the late seeding compared to the early seeding at St. Albert, since the median ranking and the relative effect of estimate were greater in the late seeding. Clubroot severity in the mid to late seeding was greater than in the early seeding in the Leduc trial (Table 5-2).

In the 2010 trial, seedling emergence of both the resistant and susceptible cultivars was greater in the later seeding than in the early seeding, while the seed yield was greater in the early or mid-seeding at the Edmonton site (Table 5-3). At the Leduc site, seedling emergence and seed yield were greater in the early seeding followed by the mid and late seeding (Table 5-3). No significant effect of seeding date on clubroot

severity was evident, since the median ranking and the estimated relative effect were not significantly different at either site (Table 5-4).

5.3.2 Greenhouse experiment

As expected, the resistant canola cultivar 45H29 developed little or no clubroot disease, regardless of seedling age. In the younger plants that were seeded later, disease severity ranged from 0 – 1, with a median disease rating of zero. There was also no significant difference in the estimated relative effect of the later seeding dates (Jul 25, 30 and Aug 4) on clubroot disease severity in the resistant cultivar (Table 5-5). In the susceptible cultivar 45H26, the median disease ratings for the older plants (seeded later) (July 15 and 20) was zero, and moreover, the median rank and estimated relative effects for older plants were significantly lower compared to the median rank and the estimated relative effects for the younger plants (July 25, 30 and August 4). Furthermore, the effect of seedling age (July 15 and 20) on clubroot was similar, and likewise there was no significant difference in clubroot severity in the plants seeded at the later dates (Table 5-5).

The height of the resistant cultivar was generally greater than that of the susceptible cultivar, and the height of cultivars gradually declined from the older to the younger plants (Figure 5-1). In the case of seed yield, a similar pattern of results was observed (Figure 5-2). The seed yield gradually declined over the seedling age. In the susceptible canola cultivar, however, the seed yield was greatly reduced in the plants seeded at the later three seeding dates.

5.4 Discussion

The intent of manipulating the seeding date to control clubroot is to choose the specific time when the weather and environmental conditions, particularly the soil conditions, are not favourable for disease development. Soil moisture level is regarded as a dominant environmental factor in determining clubroot disease severity, since *P. brassicae* resting spores require moisture for germination and the zoospores move through water films in the soil (Dixon 2009). Indeed, clubroot infection generally occurs only when there is abundant soil moisture, and the speed of disease development increases as the soil moisture content increases (Colhoun 1952; Colhoun 1953; Wellman 1930; Dixon 2009). The availability of free water may also influence host penetration and zoospore encystment at the root hair surface (Dixon 2009).

With the exception of the field trials conducted in 2010, clubroot severity was higher at the later seeding date (Table 5-2; Table 5-4), while in all of the field trials in 2008 and 2010, seed yield was lower when canola was seeded later (Table 5-1; Table 5-3). The higher disease severity at the later seeding date may have resulted from high soil moisture levels, resulting from higher than normal precipitation rates in 2008. A wet May and June followed by a drier July and August provided relatively good growing conditions in central Alberta in 2008 (Rauhala and Turkington 2009). The higher clubroot severity in turn caused lower seed yields. Seedling emergence was also greater at the later seeding date in most experiments, except for the trials at Leduc in 2010 (Table 5-1; Table 5-3). The poorer seedling emergence at the early seeding dates may have resulted from lower soil moisture levels, implying that the early-seeded canola escaped infection at an early stage of the crop. In contrast, at the later seeding dates, seedling

emergence was greater because of improved moisture conditions, which also would have favoured clubroot development. Similar results were obtained by manipulating seeding date to minimize clubroot damage in *Brassica* vegetables (Gossen et al. 2009).

In the greenhouse experiments examining the impact of seedling age on clubroot development, disease severity was higher for the younger plants (i.e., those that had been seeded later) (Table 5-5). Previous research indicated that younger canola seedlings are more susceptible to infection by *P. brassicae* than older seedlings (Hwang et al. 2011a). In the older plants (earlier seeding), *P. brassicae* has a shorter period between infection and plant maturity to affect host development, which may reduce clubroot symptom expression (Hwang et al. 2011a). Moreover, the susceptibility of *Brassica* roots to infection by *P. brassicae* declines with increasing plant age, perhaps due to the thickening of cell walls (Mellano et al. 1970) and the formation of other barriers that limit pathogen ingress. The severity of clubroot symptoms has a large impact on the vegetative growth of canola, and seed yield and plant height were greater for older plants that had lower levels of disease (Figure 5-1; Figure 5-2). As reviewed in Chapter 1, the formation of root galls can hinder water and nutrient uptake by infected plants. In the older canola plants, on which galls developed galls later, root function may have been fairly normal during the earlier stages of the plant lifecycle (Hwang et al. 2011), resulting in higher yields and greater height.

In all field and greenhouse experiments, the resistant cultivar developed much fewer and less severe clubroot symptoms than the susceptible cultivar. Nonetheless, younger seedlings of the resistant cultivar could also be infected under greenhouse conditions (Table 5-5). Therefore, the canola cultivar 45H29 is not immune to clubroot,

since there was still minor symptom development on this genotype (Table 5-4; Table 5-5). Repeated cropping of this and other resistant cultivars likely would select for virulent *P. brassicae* pathotypes capable of overcoming the resistance, and hence the manipulation of seeding date (to further reduce symptom development) may be an effective strategy to prolong resistance. Likely because of the reduced symptom development, yield and plant height were greater in the resistant cultivar than the susceptible cultivar in all of the trials (Table 5-3; Figure 5-1; Figure 5-2). Ultimately, sustainable clubroot management will require an integrated approach, likely based on genetic resistance as a key pillar, but complemented with other strategies. The results obtained in this chapter suggest that the manipulation of seeding dates represent one such tool to complement clubroot resistance.

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Table 5-1. Effect of seeding date on emergence and yield of canola grown in clubroot-infested soils near Leduc and St. Albert, AB in 2008.

Treatment		Emergence (plants/m^2)*		Yield (t/ha)*	
		Leduc	St. Albert	Leduc	St. Albert
Early	May 11	-	58.6 b	-	1.18 a
Mid	May 19	-	167.8 a	-	0.78 b
Late	May 28	-	145.4 a	-	0.91 b
Early	May 16	50.0 b	-	0.81 a	-
Mid	May 29	78.1 b	-	0.42 b	-
Late	June 04	165.3 a	-	0.14 b	-

*Data are the means of four replications. Numbers followed by the same letter are not significantly different at $P \leq 0.05$, as determined by Fishers' protected Least Significant Difference test.

Table 5-2. Median rank and estimated relative effects of seeding date on clubroot severity of canola in Alberta in 2008.

Site	Seeding date	Median disease rating	Median rank ($R_{i.}$)	Estimated relative effect (p_i)*	Confidence interval (95%) for relative treatment effect	
					Lower	Upper
St. Albert	Early	0.5	4.5	0.33 (0.108)	0.219	0.543
	Late	1.5	9.3	0.72 (0.153)	0.528	0.807
	Mid	1	5.8	0.43 (0.108)	0.294	0.608
Leduc						
	Early	1.5	4.3	0.31 (0.120)	0.208	0.526
	Late	2	6.5	0.50 (0.108)	0.304	0.695
	Mid	2.5	8.8	0.68 (0.104)	0.473	0.791

*Standard errors (SE) are presented in brackets; the p_i estimates were calculated as SE($R_{i.}$)/N, where SE ($R_{i.}$) is the standard error of the mean rank for the ith treatment.

Table 5-3. Effect of seeding date on emergence and yield of canola grown in clubroot-infested soils near Edmonton and Leduc, AB 2010.

Site	Seeding date	Resistant cultivar*		Susceptible cultivar*	
		Emergence (plants/m^2)	Yield (g)	Emergence (plants/m^2)	Yield (g)
Edmonton	Early	22.13 b	2177.0 ab	33.50 b	837.0 a
	Mid	79.81 a	2506.0 a	73.50 a	769.8 a
	Late	80.25 a	1812.0 b	98.19 a	344.0 b
Leduc	Early	106.38 a	2679.0 a	115.06 a	2129.5 a
	Mid	90.44 b	1683.0 b	96.75 b	1203.0 b
	Late	52.75 c	1282.5 b	49.63 c	981.0 b

*Data are the means of four replications. Numbers followed by the same letter are not significantly different at $P \leq 0.05$, as determined by Fishers' protected Least Significant Difference test.

Table 5-4. Median rank and estimated relative effects of seeding date on clubroot severity on resistant and susceptible canola cultivars in Alberta in 2010.

Site	Seeding date	Median disease rating	Median rank ($Ri.$)	Estimated relative effect (*pi*)*	Confidence interval (95%) for relative treatment effect	
					Lower	Upper
Leduc						
Resistant cultivar	Early	0.5	6.5	0.50 (0.114)	0.3	0.699
	Late	0.5	6.5	0.50 (0.114)	0.3	0.699
	Mid	0.5	6.5	0.50 (0.114)	0.3	0.699
Susceptible cultivar	Early	1	6.9	0.53 (0.114)	0.341	0.702
	Late	1	6.9	0.53 (0.114)	0.341	0.702
	Mid	1	5.8	0.43 (0.170)	0.25	0.676
Edmonton						
Resistant cultivar	Early	0.5	5.8	0.43 (0.132)	0.267	0.649
	Late	0.5	5.8	0.43 (0.132)	0.267	0.649
	Mid	1	8	0.62 (0.154)	0.372	0.777
Susceptible cultivar	Early	2.5	5.6	0.42 (0.167)	0.245	0.668
	Late	2.5	6.3	0.47 (0.132)	0.292	0.679
	Mid	3	7.6	0.59 (0.114)	0.382	0.746

*Standard errors (SE) are presented in brackets; the *pi* estimates were calculated as SE($Ri.$)/N, where SE ($Ri.$) is the standard error of the mean rank for the *i*th treatment.

Table 5-5. Median rank and estimated relative effects of seedling age on clubroot severity on resistant and susceptible canola cultivars under greenhouse conditions.

Seeding dates	Median disease rating	Median Rank (*Ri*)	Estimated relative effect (*pi*)*	Confidence interval (95%) for relative treatment effect	
				Lower	Upper
Resistant cultivar					
July 25	0	29.5	0.48 (0.105)	0.41	0.558
July 30	0	29.5	0.48 (0.091)	0.41	0.558
August 4	0	32.5	0.53 (0.091)	0.452	0.61
Susceptible cultivar					
July 15	0	25.2	0.24 (0.074)	0.191	0.323
July 20	0	27.1	0.26 (0.094)	0.208	0.342
July 25	3	68.2	0.67 (0.085)	0.597	0.741
July 30	2.5	59.2	0.58 (0.081)	0.507	0.66
August 4	3	73	0.72 (0.076)	0.646	0.783

*Standard errors (SE) are presented in brackets; the *pi* estimates were calculated as SE(R*i*.)/N, where SE (R*i*.) is the standard error of the mean rank for the *i*th treatment.

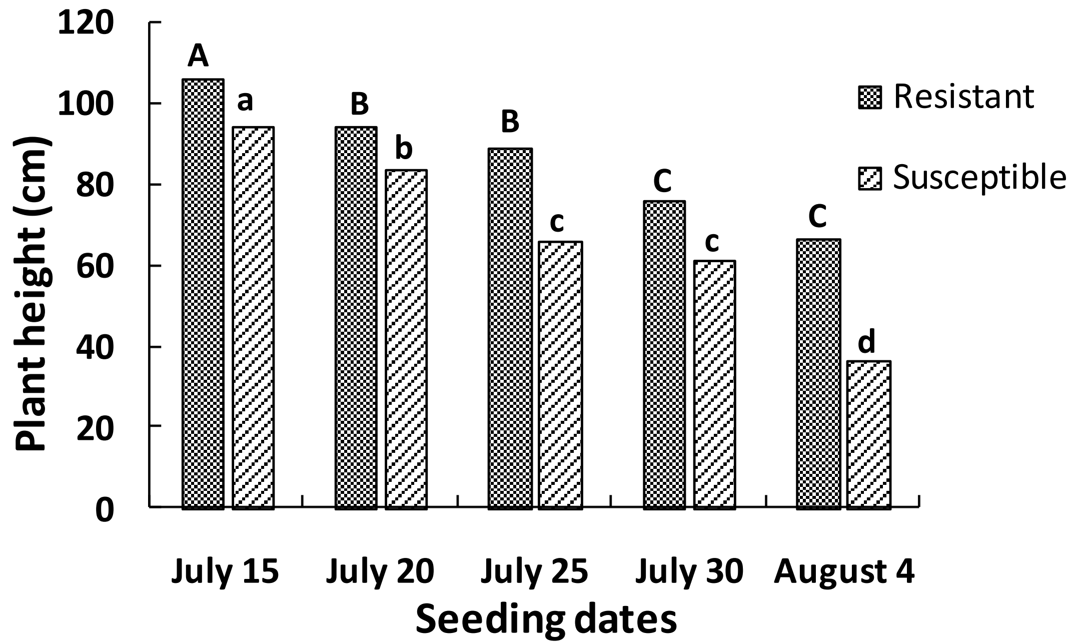

Figure 5-1. Impact of seedling age on plant height of the clubroot resistant canola cv. 45H29 and the clubroot susceptible canola cv. 45H26 (clubroot susceptible) when grown in *Plasmodiophora brassicae*-infested soilless mix under greenhouse conditions. Bars topped by the same letters are not significantly different at $P \leq 0.05$, as determined by Fisher's Protected Least Significant Difference.

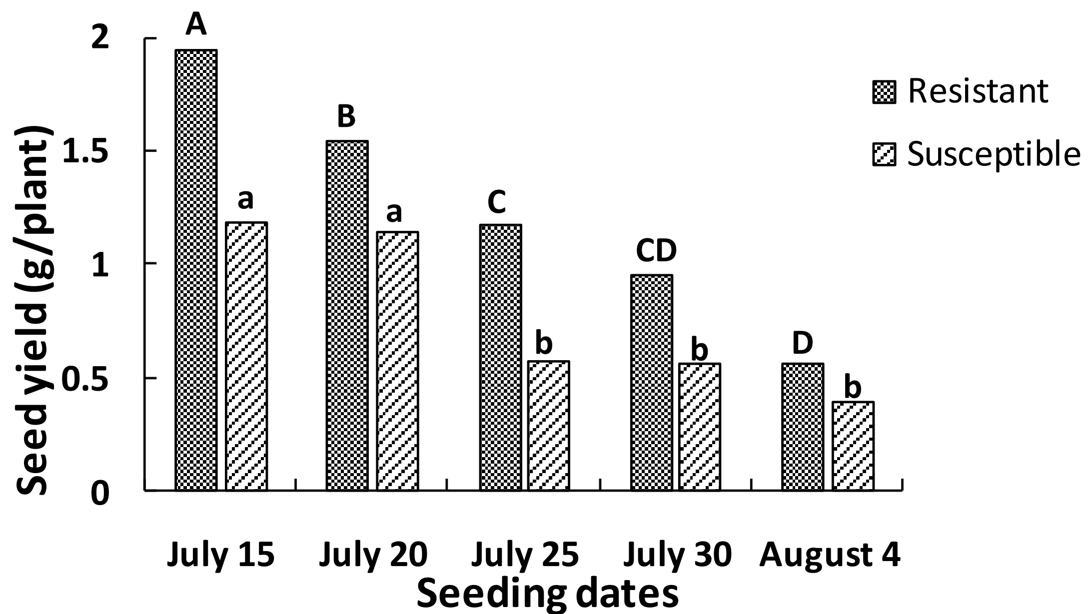

Figure 5-2. Impact of seedling age on yield of the clubroot resistant canola cv. 45H29 and the clubroot susceptible canola cv. 45H26 (clubroot susceptible) when grown in *Plasmodiophora brassicae*-infested soilless mix under greenhouse conditions. Bars topped by the same letters are not significantly different at $P \leq 0.05$, as determined by Fisher's Protected Least Significant Difference.

6. Summary and conclusions

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, has become a very serious soilborne disease of canola in central Alberta, Canada (Strelkov et al. 2005, 2006, 2009, 2010). The pathogen has a complex life cycle, with resting spore germination playing a particularly important role in disease development. Previous studies (Macfarlane 1970; Friberg et al. 2005) on the stimulation of resting spore germination by root exudates from host and non-host plants served as the foundation for the work presented in Chapter 2. In that chapter, root exudates from both host and non-host plants were found to significantly stimulate *P. brassicae* resting spore germination, when germination rates were compared with controls treated only with a nutrient solution or sterile distilled. Root exudates from perennial ryegrass, a non-host plant, were more effective for the induction of spore germination than the hosts Chinese cabbage or canola. The biological or biochemical basis for this effect, however, was beyond the scope of this study. Nonetheless, several mechanisms may be at play in stimulating resting spore germination. A putative germination-stimulating factor (GSF) (Suzuki et al. 1992), chemical components surrounding the rhizosphere, acidic or alkaline conditions, as well as the activity of other microorganisms, may all contribute to a complex environment in which the resting spores interact with external stimulant(s), resulting in germination.

Although the mechanism(s) involved in resting spore germination is not clear, the ability of both host and non-host plants to induce spore germination could be used as a tool in the management of clubroot disease, most notably via the cropping of bait plants.

In the greenhouse experiments conducted in Chapter 3, bait crops showed potential for reducing the severity of clubroot in a subsequent canola crop. Both *P. brassicae* host (canola) and non-host (ryegrass) species were effective in this regard, although the best results were obtained when host species were used as the bait crop. While the greenhouse studies presented in Chapter 3 showed that the use of bait crops holds some promise for the management of clubroot, numerous issues must be resolved before this approach can be recommended to farmers. Perhaps most significantly, previous field studies indicated that bait crops could not reduce clubroot severity in a subsequent crop of interest, even though they did reduce the resting spore concentration (Murakami et al. 2000). It is likely that in heavily infested soil, a small reduction in the resting spore load by the bait crop may not be sufficient to impact clubroot severity. Moreover, bait crops grown prior to the crop of interest may cause nutrient depletion in the soil, affecting growth of the crop of interest and necessitating additional inputs such as macro- and micro-nutrients.

To further our understanding of *P. brassicae* pathogenicity (Chapter 4), and to determine whether potential bait crops such as perennial ryegrass could actually contribute to the production of viable inoculum, secondary zoospores collected from infected root hairs of canola and ryegrass were inoculated onto healthy roots of both plant species. Five days after inoculation, the rates of root hair infection and formation of secondary plasmodia in the root cortex were similar on roots of all of the treatments. Thirty five days after inoculation, typical clubs developed on canola inoculated with secondary zoospores derived from canola, and tiny clubs also developed on canola inoculated with zoospores from ryegrass. In contrast, no clubs developed on ryegrass regardless of the inoculum source, although secondary infection of ryegrass roots

inoculated with secondary zoospores of *P. brassicae* could be observed microscopically. These results indicate that resistance to secondary infection in ryegrass is induced during primary infection. This study also revealed that secondary zoospores produced on a nonhost can infect a host species, and that secondary infection can occur in a nonhost plant species.

The limitations to the possible use of bait crops for clubroot management means that an integrated approach will be necessary to effectively control the disease. In this context, the manipulation of seeding date and the cropping of clubroot resistant canola cultivars were evaluated as additional tools in a clubroot management program (Chapter 5). Younger seedlings suffered greater disease severity and a greater reduction in plant height and yield than did older seedlings in both the resistant and susceptible canola cultivars; the studies conducted in Chapter 5 also revealed that the clubroot resistant canola cultivar 45H29 is not immune to the disease. It seems that while the manipulation of seeding date holds some promise as a clubroot management tool, it cannot be used in isolation.

The sustainable management of clubroot requires an integrated approach, which relies on the deployment of multiple tools and strategies for disease control. As a first step, obtaining background information on a particular field is very important for minimizing the possible impact of the disease. This information may be obtained from the site history (severity of clubroot in the most recent Brassica crop, rotational history, site preparation and treatments applied in previous crops, etc.) (Donald and Porter 2009), as well as through the use of tests, such as PCR analysis, to detect and quantify *P. brassicae*. If the pathogen does not appear to be present in a field, then management

strategies would be focused on preventing the introduction of inoculum, such as through the cleaning of farm machinery prior to entry into the field (Howard et al. 2010). However, if there is a history of clubroot in a particular field, then another set of strategies will be required.

One of the most important tools currently available for clubroot management is the deployment of genetically resistant canola cultivars (Strelkov et al. 2011). A number of companies have released cultivars that show excellent resistance to the predominant strains of *P. brassicae* in Canada. However, resistance cannot be used as a management tool in isolation, as *P. brassicae* can adapt quickly to the selection pressure imposed by the cropping of resistant cultivars (Strelkov et al. 2011). As such, genetically resistant canola cultivars should be rotated with other crops, or at the very minimum, with other canola cultivars carrying different sources of clubroot resistance. In addition, other approaches, including the liming of the soil to increase soil pH (Donald and Porter 2009), application of soil treatments and amendments (Hwang et al. 2011), and even biological control (Peng et al. 2011) may serve to reduce inoculum levels and therefore reduce disease pressure.

It is in this context of reducing inoculum levels that the work presented in this thesis may be most useful. Bait crops by themselves may not be sufficient to bring the level of clubroot down to an acceptable level, but they may serve to reduce the soil inoculum load, thereby reducing the disease pressure faced by genetically resistant cultivars. A lower inoculum load would also serve to reduce the size of the *P. brassicae* population subjected to the selection pressure imposed by a resistant cultivar, further serving to enhance the longevity of that resistance. Of course, any control measure must

be cost-effective to be adopted. Bait crops may cause nutrient depletion in the soil, and the need for additional fertilizer may not be financially offset by the benefit of a small reduction in the soil resting spore load (Chapter 3). Similarly, while the manipulation of seeding date holds some promise as a clubroot management tool (Chapter 5), it may not be practical or possible in all cases.

Nonetheless, the integration of some or all of these various approaches will eventually be necessary if clubroot is to be sustainably managed. While the exact nature of this integrated strategy is not yet clear and will likely continue to change over time, it will likely rely on genetic resistance as a cornerstone, with additional strategies used to supplement the durability of resistance and mitigate clubroot disease pressure (S.E. Strelkov, personal communication). Sanitation will likely also continue to play an important role in clubroot management, by helping to curtail spread of the disease. Collectively, these approaches may be successful in mitigating the long-term impact of clubroot on the Canadian prairies, although communication with growers and the industry, as well as continued research, will also be critical to staying ahead of this important disease.

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