University of Alberta

Spheroidal gall formation and seedborne infestation by *Plasmodiophora brassicae* as overlooked aspects of clubroot biology and epidemiology

by

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Dedication

My family

My friends

Emily, I will not dedicate this to dogs... that is absurd

Abstract

Plasmodiophora brassicae, the causal agent of clubroot, can infect many species of the Brassicaceae. Infection results in the formation of galls on the roots of susceptible plants. Most galls are spindle-shaped, but spheroid galls can also develop and are regarded as resistance structures. Histopathological characterization of spheroid galls revealed that they result from a proliferation of extrastelar tissues, and although pathogen spread is contained within these tissues, resting spores are still produced. A quantitative PCR protocol was also developed to estimate the numbers of resting spores present as external contaminants of seeds and tubers harvested from clubroot-infested fields. Quantifiable levels of infestation were found on 7 of 46 samples tested, suggesting that farmers should avoid the planting of common, untreated seeds from clubroot-infested fields. Collectively, the results presented in this thesis advance our understanding of *P. brassicae* biology and epidemiology, and provide the foundation for future studies.

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Chapter 1: Introduction and Literature Review

1.1. General Introduction

Woronin, in 1877, confirmed that *Plasmodiophora brassicae* is the causal agent of clubroot, although accounts of the disease existed long before then (Karling, 1968). There have been other diseases correctly and incorrectly attributed to species of the genus *Plasmodiophora,* and Karling (1968) gives six probable species of *Plasmodiophora* and their respective hosts. These species along with 26 others comprise the monophyletic taxon Plasmodiophoramycota (Dick, 2001). The Plasmodiophoramycota also includes *Spongospora subterranea* var. *subterranea* (powdery scab of potato), *S. subterranea* var. *nasturtii* (Crookroot of watercress), *Polymyxa graminis* and *P. betae* amongst others (Dick, 2001).

Clubroot has recently emerged as a major constraint to canola (*Brassica napus*) production in the province of Alberta, Canada (Howard *et al.*, 2010). While only 12 clubroot-infested fields were found when the disease was first identified on canola in central Alberta in 2003 (Tewari *et al.*, 2005), more than 450 infested fields were reported by 2009 (Strelkov *et al.*, 2010), with the outbreak spreading to southern and eastern regions of the province (Fig. 1.1).

P. brassicae research has been focused on disease management and epidemiology, pathosystem biology, and taxonomy. Since clubroot is regarded as an agricultural pathogen, it has been largely excluded from ecological discourse.

The following work was developed in light of epidemiological and biological concerns stemming from anecdotal and historical observations of seedborne infestation and spheroid gall formation.

There have been eight principle tracts of research regarding *Plasmodiophora brassicae*, which are: the microscale interaction of resting spores/zoospores with roots, the phylogenetics of *P. brassicae*, the anatomy of gall development, the physiological and molecular aspects of infection, lifecycle construction and elucidation, resistance breeding, epidemiology, and agricultural management. This literature review will touch on most of these topics, with emphasis on the lifecycle of *P. brassicae*, the anatomy and molecular aspects of infection, and sampling and detection techniques for assessing the epidemiology of the disease. The research presented in chapters two and three will advance our understanding of the anatomy of gall development and epidemiology of this pathogen, respectively. The conclusions and future work will provide possible trajectories for some of the previously mentioned research topics and a synthesis of our findings.

1.2. Research

1.2.1. The available literature and research progress

The literature on clubroot and *Plasmodiophora brassicae* is hardly saturated. Many meaningful contributions to our understanding of the biology of this organism are still to be made, but not without considerable effort and persistence.

Historical efforts in clubroot science have focused on developing resistant varieties and researching stages of the lifecycle. More detailed analysis of *P. brassicae* biology has become possible with the advent of molecular techniques and tools, including proteomic analysis (Cao et al., 2008; Devos et al., 2006; Feng et al., 2010), molecular-based phylogenetic analysis (Archibald and Keeling, 2004; Down et al., 2002), molecular detection and quantification (Cao et al., 2007; Faggian et al., 1999; Ito et al., 1999; Kageyama et al., 2003; Rennie et al., 2011; Sundelin et al., 2010; Wallenhammar and Ardwisson, 2001), studies of pathogen development (Siemens et al., 2009; Siemens et al., 2009a), and resistance breeding (Diederichsen et al., 2009; Nagaoka et al., 2010; Piao et al., 2009; Sakamoto et al., 2008). Research progress in the clubroot pathosystem will continue to advance as more molecular insights are presented, especially if the genome of *P. brassicae* is sequenced.

Molecular biology has provided valuable insights into the biology of *P. brassicae,* but it is important to maintain a comprehensive perspective in our research, by synthesizing broad tracts of work into condensed concepts, hypotheses and theories. This is especially true considering the multidisciplinary nature of plant pathology. The time has come, perhaps, for a new comprehensive review or reference text on the subject, considering the most contemporary treatises on the plasmodiophorids were published in 1968 (Karling, 1968) and 1983 (Buczacki, 1983). These are invaluable works, but diligence in compiling and synthesizing the current literature is essential for scientific growth in this facet of plant pathology. A series of comprehensive reviews addressing many

aspects of *P. brassicae* biology was published in volume 28 (2009) of the *Journal of Plant Growth Regulation,* and should be considered required reading for establishing the current status of knowledge on *P. brassicae* and clubroot.

1.2.2. Constraints to the study of *P. brassicae*

The biotrophic nature of *P. brassicae* is the most obvious constraint to its scientific study. As axenic culture of *P. brassicae* is not probable in the foreseeable future, special techniques and methods must be developed to work with this pathogen. The difficulty in studying *P. brassicae* is further compounded as the salient symptoms of clubroot occur below ground, under the soil. Taken together, these features lead to a formidable and difficult research program.

Despite these issues, *P. brassicae* lends itself to certain research topics. There are many scientific opportunities and projects regarding clubroot, as many fundamental aspects of its biology are unknown. Furthermore, the disease symptoms, once revealed from the soil environment are quite conspicuous, enabling quick field identification and data collection. The resting spores are very resilient, and do not require special consideration for collection or storage (short term storage at 4°C, long term storage at −20°C or storage as dried galls at room temperature). As resting spores are sessile and passively vectored by environmental factors, *P. brassicae* is suitable as a model for soil-borne disease pattern studies and the effects of these patterns on ecological processes (Campbell and Noe, 1985). This aspect of clubroot biology is notably understudied in *P. brassicae,* aside from preliminary work by Cao et al. (2009) and Faggian et al. (2001). As *P. brassicae* is listed as a controlled pest in Alberta (Agricultural Pests

Act, 2010), special considerations must be employed for research plots and handling of the pathogen. These measures are best practices applied to mitigate any risk that clubroot research may have on canola or cruciferous vegetable production in Alberta. While necessary, these practices do limit plot availability and generate the need for extra handling and sanitation steps. This may not be of concern in areas of limited canola production or widespread *P. brassicae* distribution, where governmental *P. brassicae* controls are not necessary. As evidenced in Fig. 1.1, *P. brassicae* spread in Alberta is extensive, and has been progressing on canola crops since its discovery in 2003 (Tewari et al., 2005).

1.3. Plasmodiophora brassicae

1.3.1. The life cycle of *P. brassicae*

The lifecycle of *P. brassicae,* as summarized in Fig. 1.2 has been compiled from the works of numerous authors, namely Braselton (2001), Buczacki (1983), Cook and Schwartz (1930), Ingram and Tommerup (1972), Kageyama and Asano (2009), Karling (1968), Ludwig-Müller (1999), and Tommerup and Ingram (1971), all of whom provided detailed synthesis and study of the lifecycle of *P. brassicae*. Highlighted in these works is the apparent consensus of a biphasic lifecycle, where the presence of a primary and a secondary phase exist in the root hairs and root secondary cortex, respectively (Buczacki, 1983). The term cortex will be used generally to refer to either the secondary or primary cortex as these tissues are anatomically and morphologically similar, both have the capacity to be

infected, but have different developmental orgins. For accuracy, however, the term secondary cortex will be used in place of primary cortex when referring to secondarily thickened roots while, the term primary cortex will be employed when describing roots prior to secondary tissue formation. Many members of the Brassicaceae form storage organs below ground, typified by the formation of a multiseriate layer of secondary cortex and extensive thickening due to the formation of vascular cambium derivatives (Peterson, 1972; Ting and Wren, 1980; Webster and Radin, 1972). Incidentally, this sort of development is comparable to that observed in clubroot galls and has been cursorily discussed by Gustafsson et al., (1986). Certainly, the distortion of auxin and cytokinin concentrations during clubroot disease and the importance of secondary growth are comparable between storage organs and clubroot galls.

Primary zoospores are formed during zoosporogenesis in the resting sporangia, and germinate to release a heterokont, biflagellate, uninucleate primary zoospore (Braselton, 2001; Macfarlane, 1970; Tanaka et al., 2001). This zoospore contacts the host root, encysts and injects its cytoplasmic contents into the root hair lumen (Aist and Williams, 1971; Braselton, 2001). Multiple zoospores can infect the same root hair, but synchronic mitotic divisions occur within each respective primary plasmodium, leading to the formation of a continuous chain of zoosporangia, each forming multiple zoospores per zoosporangium (Buczacki and Clay, 1984; Ingram and Tommerup, 1972). Amoeboid movement between the continuous zoosporangia has been observed when zoospores are released into the rhizosphere (Buczacki and Clay, 1984). The abortion of the terminal zoosporangia

and dissolution of the appressed root hair wall enables the release of zoospores from the roothair (Buczacki and Clay, 1984). Zoospores have also been observed swimming in the root hair lumen, ostensibly released by a similar mechanism (Buczacki and Clay, 1984). The transition between primary and secondary stages (or using the terminology of Karling (1968), "sporangial and sporogenic phases") is largely unknown. Questions regarding the encystment of secondary zoospores on root hairs, in the root hair lumen, or on other epidermal cells have been similarly unanswered. How prevalent is zoospore plasmogamy in the rhizosphere and root hair lumen? Further, what is the basis for the observations of secondary plasmodial development prior to secondary zoospore formation, as recorded by Mithen and Magrath (1992)? The observations of Mithen and Magrath (1992) suggested that primary zoospores are capable of initiating secondary stages of the lifecycle. Without definite observations of these transitory stages, only circumstantial evidence exists that the secondary phases of the lifecycle are connected to the primary phases (Kunkel, 1918).

It is, perhaps, a meaningful observation that early secondary plasmodia bear many cytological features of fused secondary zoospores (bi-nucleate, anucleolate, devoid/reduced lipid droplets, spheroid morphology), but considering the similarity between primary and secondary zoospores, considerable investigative ability would be required to discern the two (Mithin and Magrath, 1992). It is worth mentioning, however, that Dobson and Gabrielson (1983) found exclusive roles for both primary and secondary zoospores with little or no

functional redundancy. The roles of primary and secondary zoospores in initiating the respective phases of the disease are, again, not conclusively established.

The stages of resting spore germination and encystment of primary zoospores on the root hairs, followed by the formation of primary plasmodia, precede the release of secondary zoospores that infect the root secondary cortex and stele (Mithin and Magrath, 1992). Re-infection of the root hairs by secondary zoospores, and the consequent production of more zoospores are probable, and may represent an inoculum-building microcycle (Donald et al., 2008; Karling, 1968; Kole and Gielink, 1963). Zoospore microcyling of sporangial phases on non-Brassicaceous hosts is also a likely but unconfirmed process for maintaining *P. brassicae* populations or building up field inoculum levels in the absence of typical hosts (Donald et al., 2008; Karling, 1968; Kole and Gielink, 1963; Kole and Philipsen, 1956). Questions regarding the mode of secondary zoospore encystment, and the probable necessity of secondary zoospore plasmogamy prior to encystment, are detailed on Fig. 1.2 and are regarded by Buczacki (1983) and Braselton (2001) as a transitory phase between the sporangial and sporogenic phases. This transitory phase is a point of misunderstanding, as much of the fundamental science regarding this phase has not been conducted.

Gall formation is a consequence of an initially actively motile myxamoeboid phase, which spreads throughout the root, progressing further into the secondary cortex, cambium and medullary rays in susceptible hosts (Dekhujien, 1976; Donald et al., 2008; Kunkel, 1918; Larson, 1934; Mithen and Magrath, 1992). The myxameoboid phase has been predicted to move rapidly

once it enters the cambium and spreads through the root in a mode described by Kunkel (1918). Conclusive evidence of pathogen spread through the vascular cambium is lacking, although concurrent infection of the stele and rays is certainly suggestive of such a pathway.

1.3.2. Resting spores of *P. brassicae*

Except for the resting spores, all of the other stages in the lifecycle of *P. brassicae* are ephemeral, existing and developing within the host tissues (sporangial and sporogenic stages) or in the rhizosphere/root lumen (primary and secondary zoospores). Therefore, the resting spore is of principal concern for clubroot management and sanitation. It is the stage that is disseminated on tools, machinery and, as will be presented later, seeds and propagative materials (Rennie et al., 2011).

The longevity of the resting spore relies on the anatomy and chemical composition of the spore wall. The spore wall consists of five discernible layers that differ in their chemical composition (Buczacki and Moxham, 1983). The innermost layer (layer five) is the spore membrane, derived from the inner plasmodial envelope (Williams and McNabola, 1967). The two middle layers (layers three and four) are also derived from the inner plasmodial envelope and are present along with layer five during resting sporangia formation (Williams and McNabola, 1967). The fibrous outer layer (layer one) and spiny proteinaceous layer two are deposited individually during development, partly from the host-derived intersporangial matrix (Williams and McNabola, 1967; Buczacki, 1983). The spiny layer two is deposited first, followed by the deposition of the fibrous

layer one; both are derived from host organelles in a unique form of holometabolism (Buckzaci, 1983). The five-layered resting spore wall is primarily composed of protein (33.6%) with high levels of chitin (25.1%) and lipids (17.5%) (Moxham and Buczacki, 1983). Much of this protein is contained in the matrix of layer two and is likely bound to the wall through charge interactions and through associations with lipids (Moxham et al., 1983). This five-layered arrangement is similar to that found in spores of the closely related protist *Polymyxa graminis*, but unlike in *P. graminis, P. brassicae* resting spores are not aggregated into a sporosorus (Chen et al., 1998).

Yukawa and Tanaka (1979) describe the resting sporangia of *P. brassicae* as a three layered structure. W1 constitutes the outer wall and the fibrous outer layer (referred to, respectively, as layers two and one by Buczacki and Moxham (1983)), W2 constitutes the electron opaque layer (referred to as layer three by Buczacki and Moxham (1983)), and W3 constitutes the electron-dense inner layer (referred to as layer four by Buczacki and Moxham (1983)) (Yukawa and Tanaka, 1979). Most of the disagreement between these two published reports on resting spore structure is fixated on the origin of layer one (as described by Buczacki and Moxham, 1983) and the inclusion of the plasma membrane as the fifth layer of the resting spore wall. The nomenclature of Buczacki and Moxham (1983) will be utilized in this thesis.

The presence of a germ pore is documented by Yukawa and Tanaka (1979) as a circular thickening of the electron opaque layer three delimited by a thickened layer four. It has been suggested that this thickened portion of layer

three functions as a plug, which is dissolved or degraded upon germination (Yukawa and Tanaka, 1979). Resting spores are quiescent as mature sporangia and when viewed *in situ*, share many of the same organelles as the primary plasmodia but without a nucleolus (Williams and McNabola, 1967). Zoospore maturation and germination occurs in the presence of environmental elicitors, after the dissolution of the germ pore (Macfarlane, 1970).

1.3.3. Primary zoospore and root hair encystment

In *P. brassicae*, there are two different zoosporic phases; the primary phase formed by the resting zoosporangia, and the secondary phase formed from the zoosporangia that develop from the primary plasmodia (Ingram and Tommerup, 1972; Fig. 1.2). Primary zoospore differentiation occurs within the resting sporangia and is initiated by the formation of two tightly constricted flagella within an invagination of the plasma membrane around the equator of the resting sporangium (Tanaka et al., 2001). Intracellular, early differentiated zoospores are comparable to resting sporangia, except for the presence of a mature endoplasmic reticulum and electron dense mitochondria, perhaps in preparation for further metabolic activity (Tanaka et al., 2001). At these early stages, the nucleus of the zoospore is anucleolate, comparable to the mature resting sporangia (Tanaka et al., 2001; Williams and McNabola, 1967).

Following early zoospore differentiation, the zoospore cytoplasm condenses and a periplastic space forms without flagella bound in an equatorial groove (although still aligned parallel to the equator) (Tanaka et al., 2001). The zoospore cell body becomes irregularly shaped, and contains a nucleolus in the

chromatin-rich nucleus (Tanaka et al., 2001). Small and large microbodies are regularly observed in mature zoospores (especially those with few lipid droplets), which bear similarity to plant glyoxysomes, perhaps because of a similar function (Tanaka et al., 2001). These structures are also observed in mature zoosporic cysts (Aist and Williams, 1971).

Mature primary zoospores are released from the resting sporangia through the dissolution or enzymatic degradation of the germ pore (Tanaka et al., 2001; Yukawa and Tanaka, 1979). This is referred to as the papilla stage by Macfarlane (1970), where the zoospore emerges from the zoosporangium. Zoosporangia regularly produce pseudopodia (Aist and Williams, 1970), and this papilla formation appears to be evidence of this ability. The process of germ pore formation is largely unknown, but chitinolytic enzymes and/or proteases are certainly involved (Tanaka et al., 2001). Recently, Feng et al. (2010) found that a serine protease enzyme produced by resting sporangia enhances zoospore germination, but the presence of other germination stimulation factors are required for germination to proceed. This is consistent with the constitution of the layers, as the outer resting spore wall contains a high proportion of proteins, the degradation of which would not release the zoospore unless the inner membranes were degraded (Moxham and Buczacki, 1983).

Zoospore movement in the rhizosphere is marginal, accounting for less than 13 cm of active horizontal movement where moist conditions permit, although it is uncertain if this is exclusively due to flagellar propulsion (Karling, 1968). Zoospore movement in the soil is likely an effort to find a suitable

infection site instead of a broad advance of the pathogen in the field (Karling, 1968). Successive encystment and release into the rhizosphere of fortuitously oriented and choreographed zoospores could perhaps significantly advance movement of the pathogen, but even such efforts would be marginal compared to dispersal of resting spores in eroded soil.

Cyst formation on root hairs is initiated by contact of the zoospore with the root hair at the region of the zoospore distal to the flagella (Aist and Williams, 1971). Progressive flattening of the zoospore occurs while the now quiescent flagella wrap around the pseudopodic zoospore body (Aist and Williams, 1971). Retraction of the flagella completes the encystment of the zoospore, and creates the typical round morphology of the cysts (Aist and Williams, 1971). Production of vesicles with melanized inclusions and the concerted excretion of these vesicles into the wall of the cyst finalize the encystment process, creating the outer layer of the cyst wall (Aist and Williams, 1971). This fibrillar outer layer may be responsible for the adhesion of the cyst to the root hair (Aist and Williams, 1971).

Various structures are associated with the mature zoosporic cyst, most significantly the extracellular Rohr and Stachel (Aist and Williams, 1971). Aist and Williams (1971) describe the Rohr as a long tubular cavity while the Stachel is a sharp pointed rod contained within the Rohr. Penetration of the root hair involves the enlargement of the cyst vacuole and the formation of an adhesorium, presumably formed by the evagination of the Rohr. Further enlargement of the vacuole drives the stachel through the cell wall (Aist and Williams, 1971). Complete enlargement of the vacuole injects a small amoeba along the path of the

stachel into the host root hair (Aist and Williams, 1971). The host response to this process is limited to the formation of a papilla composed of callose (Aist and Williams, 1971; Buczacki, 1983). The papilla stage of Macfarlane (1970) and the papilla formation described by Aist and Williams, 1971) are distinct stages.

1.3.4. Primary plasmodia, secondary zoosporogenesis and zoospores

The early primary plasmodia are bound by a seven layer envelope (Buczacki, 1983), a feature similar to that of the secondary plasmodia as described by Williams and McNabola (1967). Development of primary plasmodia is loosely described by Aist and Williams (1971) and Buczacki (1983), while the details were studied by Ingram and Tommerup (1972). Colonization of the root hair from the injected plasmodium proceeds through synchronous mitotic nuclear divisions, which produces a multinucleate plasmodium (Buczacki, 1983). Zoosporogenesis progresses via the angular cleavage of the multinucleate plasmodium into bodies containing between one to six nuclei, which assume a rounded morphology and are bound by a membrane (Ingram and Tommerup, 1972). Further mitotic divisions in each developing zoosporangium lead to the formation of four to 16 small nuclei followed by the cleavage into uninucleate zoospores (four to 16) (Braselton, 2001; Dekhuijzen, 1979; Ingram and Tommerup, 1972). This mass of zoosporangia, referred completely as a sporangium (as the structure forms from one continuous plasmodium) or a sporangiosorus (as they may be considered a collection of individual sporangia) (Buczacki, 1983; Braselton, 2001; Dekhuijzen, 1979; Ingram and Tommerup, 1972;). Partial disintegration between adjacent sporangia and the formation of a discharge papilla (an aborted zoosporangium)

allow for a continuum between adjacent sporangia and the rhizosphere/root lumen (Buczacki, 1983; Buczacki and Clay, 1984; Braselton, 2001).

Chains of secondary zoosporangia form in the root hairs and release zoospores through exit chambers formed by the discharge papillae into the lumen of the root hair, or through the root hair wall into the rhizosphere (Buczacki and Clay, 1984; Braselton, 2001). Buczacki and Clay (1984) present very convincing images of these 'exit chambers' and the amoeboid movement of zoospores between adjacent zoosporangia. Plasmogamy between zoospores is very likely, based on the observations of binucleate zoospores (Tommerup and Ingram, 1971) and binucleate secondary plasmodia in the cortex. Karyogamy as described by Kole and Gielink (1961), however, has not been conclusively demonstrated (Buczacki, 1983; Ingram and Tommerup, 1972).

Encystment of these secondary zoosporangia has been predicted to occur in the trichoblast distal to the root hair, but has not been definitively shown (Aist and Williams, 1971; Ingram and Tommerup, 1972). With consideration for the consequent infection of the cortex and medullary rays, encystment in the trichoblast, as suggested by Aist and Williams (1971) isprobable (Mithin and McGrath, 1992; Ingram and Tommerup, 1972). It is also possible that direct cortical infection may occur after primary infection of epidermal cells and roots by primary zoospores (Ingram and Tommerup, 1972; Mithin and Magrath, 1992; Donald et al., 2008). It is evident that the transition between primary and secondary phases of *P. brassicae* is largely unresolved.

1.3.5. Secondary zoospores of *P. brassicae*

Secondary zoospores of *P. brassicae* are morphologically and functionally similar to primary zoospores (Dekhuijzen, 1979). Yet this similarity is often contentious, as conflicting reports exist regarding the importance or potential redundancy of these stages. Graveland et al. (1992) reported that primary zoospores were able to infect and produce secondary plasmodia without the observation of primary plasmodia or secondary zoosporangia in hairy root cultures, while Dobson and Gabrielson (1983) noted the necessity of both stages for cortical infection. Mithin and Magrath (1992) observed cortical infection coincident with secondary zoospore maturation, although their methods do not exclude the possibility of secondary zoospore infection from other unconsidered root hairs. As mentioned previously, zoospore fusion is enhanced in spatially constricted circumstances (in galls, in root hairs), and as binucleate amoebae are regularly encountered in early secondary plasmodial stages, plasmogamy of primary zoospores may allow for direct infection of epidermal and cortical cells (Ingram and Tommerup, 1972). Indeed, observed primary zoospore fusion *in situ* has been predicted as a mechanism for secondary spread of *P. brassicae* in galls (Ingram and Tommerup, 1972). It is evident that, while some accounts establish a redundancy between these two zoosporic phases, others substantiate an exclusive role for each respective stage.

The most conspicuous morphological similarity between the two zoospores is that both posses two atinsilate flagella in a “9 + 2” microfibril arrangement (Dekhuijzen, 1979). Morphological differences between the two

include the angle between two flagella (30-40 degrees for secondary zoospores; 45 degrees for primary zoospores) but are otherwise indistinguishable (Buczacki and Clay, 1984).

1.3.6. Plasmodia and gall formation

The term myxamoebae, while not formally correct considering the recent placement of *P. brassicae* in the Cercozoa (Archibald and Keeling, 2004; Cavalier-Smith and Chao, 2003), is applied to the early motile amoeboid phases observed in the cortex (Mithin and Magrath, 1992; Donald et al. 2008; Karling, 1968). Myxamoebae are typically characterized by a low concentration of lipid droplets, small size, the presence of one or two nuclei, pseudopodic movement, amyloplasts, and close association with host nuclei (Mithin and Magrath, 1992; Graveland et al., 1992). Additionally, they are frequently associated with cell wall breaks, suggesting active disruption and movement between the cells (Mithin and Magrath, 1992; Donald et al. 2008). The low concentration of lipid droplets and binucleate state are highly suggestive of a developmental relation to the injected amoebae from the secondary zoospore (Mithin and Magrath, 1992).

The presence of a myxameobal phase has been contested by numerous researchers (Buczacki, 1983). However, more recent observations in callus culture and *in planta* support its existence (Mithin and Magrath, 1992; Donald et al. 2008). Alternative modes of plasmodial distribution exist such as the synchronous division of host cells and plasmodia into infected daughter cells facilitated by the cytoplasmic streaming of plasmodia into the daughter cells (Asano and Kageyama, 2006; Tommerup and Ingram, 1971). Integration of both

modes of distribution may account for the observation of Krankheitsherde (regions of localized plasmodial distribution), where motile amoeboid phases transiently deposit sessile plasmodia that promote peri- and anticlinal divisions by passive means (Karling, 1968). It is likely that the formation of localized sites of infection may be attributable to the stochastic movement of *P. brassicae*, and have less to do with active plasmodial restriction by the host.

The fusion of adjacent plasmodia into a multinucleate, vegetative plasmodium has been correlated with an increased number of lipid droplets (Williams and McNabola, 1967). This phase develops asynchronically with other plasmodia in the gall, and fortuitously for researchers, all phases of plasmodial development can be observed in sectioned galls 23 days after infection (Ludwig-Müller et al., 2009). As of yet, karyogamy has not been clearly observed, although synaptonemal complexes have been reported in the anucleolate vegetative plasmodia, suggesting that meiotic processes are occurring (Garber and Aist, 1979). These processes occur concurrently with the stages of degradation of the plasmodial envelope into the three layered stage and the increase in cleavage vacuoles prior to resting sporogenesis (Garber and Aist, 1979; Williams and McNabola, 1967).

Fusion of vesicles containing host material, including organelles, around each haploid nucleus cleave the plasmodia into incipient resting spores surrounded by a host derived inter-sporangial matrix (Williams and McNabola, 1967; Garber and Aist, 1979). The deposition of this inter-sporangial matrix onto

the three layered membrane of the resting spore completes the formation of the typical five layered resting sporangia (Williams and McNabola, 1967).

1.4. Gall Formation

1.4.1. Host genotypes

Karling (1968) as adapted from Calhoun (1958) provides a comprehensive account of susceptible cruciferous hosts. In excess of 300 species in 61 genera of the Brassicaeae have been established to be susceptible to *P. brassicae* infection. A clear distinction in susceptibility exists between wild and cultivated species; a completely resistant commercial crucifer variety has been and continues to be an elusive goal (Karling, 1968). Even in wild cruciferous hosts, pathotype-specific susceptibility and resistance occur, suggesting some degree of selective pressure for resistance and responding pressure for virulence (Buczacki and Ockendon, 1979). The absence of clear resistance is further confounded by a multitude of coexisting pathotypes, considerable environmental variation, seasonal variation in field conditions and an incomplete understanding of the genetic basis of resistance.

One of the first commercially available and marketed clubroot resistant varieties was *Brassica oleracea* cv. Badger Shipper (Karling, 1968; Seaman et al., 1963; Table 1.1). Resistance in 'Badger Shipper' was short lived, as a highly virulent *P. brassicae* pathotype emerged (Seaman et al., 1963). A cursory search for clubroot research in the available reference databases revealed over 100

publications addressing resistance, accounting for over a sixth of the available clubroot research, with approximately half of this work being completed in the last 15 years. The search for clubroot resistant cultivars is, evidently, ongoing.

Table 1.1 provides an overview of resistant host genotypes currently available, the degree of resistance (and any pathotype or population specificity), the physiologic or genetic nature of resistance and the observation of spheroid galls (if any). Many of these accessions have been comprehensively reviewed by Karling (1968) and Xue (2008). The most promising sources of resistance come from *Brassica rapa* var. *rapifera* or fodder turnips (Toxopeus et al., 1986; Xue, 2008). Many of the resistant *B. rapa* species have been observed to routinely produce spheroidal galls, and are regularly used in Chinese cabbage breeding programs (Kuginuki et al., 1999; Xue, 2008).

1.4.2. Spheroid galls

Seaman et al. (1963), Williams (1966), Buczacki et al. (1975) and Osaki et al. (2008) all describe spheroidal galls as a distinct gall morphology typified by small size, spheroid shape, and strongly delimited margins. Spheroid galls have been regarded historically as resistance structures and researchers regularly ascribe a clubroot score of zero (meaning no symptoms) to any such observations (Williams, 1966). Typically, resistant host genotypes have been formally and anecdotally reported to form spheroid galls (Osaki et al., 2008, Table 1.1). Incidentally, spheroid galls have also been reported from opportunistic infection of the stem through wounds (Karling, 1968; Larson, 1934).

Actively motile plasmodial stages, associated with cell wall breaks, distribute plasmodia throughout the stele and cortex of primary tissues and upon infection of lateral meristems, cause an infection and proliferation of secondary tissues (Dekhuijzen, 1976; Donald et al., 2008; Gustaffson et al., 1986; Karling, 1968; Kobelt et al., 2000; Kunkel, 1918; Mithen and Magrath, 1992). The infection and proliferation of rays within the stele fragment the continuity of the vascular tissue, and the degree of tissue proliferation determines the size of the gall (Gustaffson et al., 1986; Kunkel, 1918). While this actively motile phase appears to be important for early spread of the pathogen in the roots, later stages involve the formation of multinucleate vegetative plasmodia, where the distribution of the pathogen progresses through the concurrent division and movement of pathogenic material into the mitotic derivatives (Dekhuijzen, 1976).

Spheroid galls can form on both the primary taproot and/or on lateral roots (Osaki et al., 2008). In heavily infected specimens, spheroid galls will manifest on all root tissues (Osaki et al., 2008). The manner in which spheroid galls form is largely unknown, but likely results from a deviation from spindle gall formation where isolated Krankheitsherde (localized clusters of anti- and periclinal cell division) are restricted from further plasmodial distribution through the gall (Karling, 1968). The involvement of lignification, or hypersensitive resistance mechanisms has not been observed in the formation of spheroid galls.

The observations of viable resting spore formation in spheroidal galls undermine the use of spheroidal gall enriching host genotypes in breeding programs (Osaki et al. 2008). Certainly, these cultivars are not resistant as

historically regarded in the literature, but using the terminology of Crute (1986), these galls could be regarded as partial resistance structures. The contribution (if any) of spheroidal galls to disease cycling in the environment is completely unknown.

1.4.3. Mechanisms for clubroot resistance

Comparative analysis of clubroot development indicates that root hair infection, secondary zoospore formation, and cortex infection occur in both susceptible and resistant varieties. Donald et al. (2008) found that a resistant *B. oleracea* host did not suffer secondary wall degradation in the xylem, whereas susceptible varieties did. Moreover, cell wall breaks were observed in both susceptible and resistant varieties, so, perhaps, the extent of plasmodial movement was altered, but not prevented, in the resistant variety (Donald et al., 2008). Inhibition of amoeboid movement was also observed by Dekhuijzen (1976; 1979) and Kobelt et al. (2000), where in all, a host hypersensitive response was observed preventing the further growth and movement of *P. brassicae.*

Resistance to clubroot of crucifers is not regularly observed in cultivated varieties, and a hypersensitive response is not typical. As extensive pathogen development, even in tolerant species, is often observed, and a complete lifecycle has been observed in non-host species, it is worth evaluating what truly confers resistance and susceptibility during the secondary (sporogenic) phase of the lifecycle (Donald et al., 2008; Ludwig-Müller et al., 1999).

Levels of glucosinolates are contentiously associated with resistant and susceptible species. High levels of indole glucosinolates have been associated

with susceptibility in some *Brassica* species and even some non-host species (Butcher et al., 1974; Ludwig- Müller et al., 1999), while increased production of aromatic glucosinolates has been observed in resistant varieties (Ludwig-Müller, 2009). This relationship may be underpinned by a potential dual role of glucosinolates in the clubroot pathosystem, as some glucosinolates function as defensive compounds, while others are precursors for auxin and auxin-like molecules (Ludwig-Müller, 2009). The up-regulation of myrosinase during *P. brassicae* infection would suggest a role for glucosinolates as auxin precursors for gall development in susceptible varieties, while the observed release of thiocyanates and isothiocyanates in tolerant varieties would indicate a resistance function (Grsic et al., 1999; Ludwig-Müller, 2009). Additionally, nitrilase expression (Grsic-Rausch et al., 2000) and activity (Grsic et al., 1999) were enhanced at various time points in Chinese cabbage (*B. rapa* subsp. *chinensis*) and *Arabidopsis thaliana,* respectively. Both myrosinase and nitralase are important in the biosynthesis of auxin, and are both enhanced and upregulated by the increase of jasmonic acid in clubbed roots (Grsic et al., 1999). The importance of jasmonic acid during clubroot development is worth investigating.

Trehalose, a common carbohydrate formed by microrganisms has been commonly associated with plant infection (Brodmann et al., 2002). Particularly, trehalose production by *P. brassicae* during infection has been found to interfere with carbon metabolism in hosts (Brodmann et al., 2002). The ATTRE1 (trehalase) gene has been associated with *P. brassicae* tolerance through the breakdown of trehalose (Brodman et al., 2002; Fig. 1.3). The intimate role of

trehalose is not completely understood, and is likely a critical aspect of *P. brassicae* pathogenesis.

The involvement of cytokinins in gall development has been reasonably suggested where cytokinin reporter proteins were heavily induced in the ARR5::GUS *A. thaliana* mutants (Devos et al., 2006). The hypothesized role in establishing a *de novo* meristematic area in the cortex has been suggested and is supported by preliminary experimentation (Devos et al., 2002; Devos et al., Interestingly, cytokinin oxidase/dehydrogenase overexpression has been associated with resistant varieties, and further substantiates the role of cytokinins in susceptible gall formation (Siemans et al., 2006; Fig. 1.3).

1.5. Detection of *Plasmodiophora brassicae* in fields and environmental samples

1.5.1. Quantitative PCR

1.5.1.1. General description

The polymerase chain reaction (PCR) effectively amplifies a region of deoxynucleic acid (DNA) by means of a heat stable DNA polymerase enzyme, nucleotides, and forward and reverse priming oligonucleotides (primers) in an optimized reaction buffer (Mullis and Faloona, 1987). The reaction is conducted in an automated thermocycler, whereby temperatures in the reaction tube are quickly heated to separate double stranded DNA, cooled to allow the respective annealing of forward and reverse primers to the 5'- end and 3' end of the

sequence, and slightly heated to allow the polymerization of nucleotides to the 3'-end of the priming oligonucleotides by DNA polymerase (Mullis and Faloona, 1987). This process repeats to a desired number of cycles producing 2^n (n = number of cycles) number of products, and will continue along this exponential trajectory as long as the PCR reaction remains 100% efficient. As reagents become limited during synthesis, the reaction no longer maintains it's maximal efficiency.

These PCR products are commonly separated by electrophoresis on agarose gels to analyze the resulting product size and reaction specificity (i.e., the presence of one band of expected size). This process allows for the coarse estimation of the amount of product present at the end of the reaction (commonly referred to as endpoint PCR) based on the intensity of the bands present in the agarose gel. Evaluating the product synthesized at the end of the PCR is not a particularly accurate or precise estimate of the starting template quantity as the synthesis plateau that precedes the endpoint is not 100% efficient and estimates will readily deviate from the true value.

Various methods exist to quantify the starting amount of DNA template in a reaction, and real time or quantitative polymerase chain reaction (qPCR, qRT-PCR, and variants) is a recent tool in assessing the quantity of double-stranded amplified product using either nucleic acid binding fluorescent dyes (SYBR green), or sequence specific fluorescently labeled nucleic acid probes. If proper controls are utilized in the SYBR green assay, these two fluorescence-based qPCR chemistries approach equivalency in terms of data analysis and

experimental design, where the only real considerations between the two are cost (probe-based assays are more expensive) and the specificity conferred by a third conserved region for probe annealing (Smith and Osborn, 2009). Both techniques estimate the copy number of a gene or sequence of interest by determining the cycle number in which florescence exceeds a baseline threshold of background fluorescence (C_T value), indicating that exponential amplification has begun. By utilizing reverse transcriptase, these techniques can be extended to estimate the amount of mRNA of an expressed gene at any particular time point. Other quantitative techniques, including competitive PCR and most probable number PCR, are cumbersome and expensive and have been largely replaced by fluorescence-based qPCR protocols (Smith and Osborn, 2009).

Experiments are typically set up where either a standard curve is developed to express the C_T values compared to a standard dilution series of gene copy number (or other unit), or a relative expression experiment is designed which relates the copy number estimate normalized to a reference gene (typically a constitutively expressed gene such actin). Absolute unit measurements or gene copy numbers are determined from interpolating the observed C_T values to a prepared standard curve. Since the integrity of the standard curve largely determines the validity of estimates of gene copy number/unit measurement, the characteristics of the standard curve should be presented in any scientific publication (Smith and Osborn, 2009). Standard curve experiments are typical in the literature when assaying environmental samples. For determining *P. brassicae*

resting spore amounts in environmental samples, a standard curve protocol has been developed and will be discussed in depth in Chapter 3.

The effectiveness of any PCR protocol is contingent on the robustness and effectiveness of the DNA extraction protocol. PCR assays require high quality DNA template, however samples, especially soil and detritus samples, can contribute co-purified contaminants that inhibit PCR reactions (Ophel-Keller et al., 2008). Humic acids are the most notorious inhibitors of enzymatic activity, and have repeatedly demonstrated to inhibit PCR reactions (Tebbe and Vahjen, 1993; Cao et al., 2007). Therefore, careful validation of DNA extraction procedures and methods for discriminating false negatives due to amplification failure from genuine negative results are necessary.

Despite all efforts in designing an optimized PCR assay, the validity of any data collected is contingent on the experimental design and sampling protocol utilized. Inappropriate sampling designs can introduce bias into any data collected, however careful the analysis. This is especially true for soil sampling where the spatial patterns of organisms and environmental stratification may not be apparent.

1.5.1.2. Assay development

For successful assay development, the importance of clear experimental objectives and a hypothesis cannot be overstated; a thorough review of the pertinent literature may be just as important. Many optimized PCR protocols exist for agriculturally relevant plant pathogens, and a thorough analysis of preceding work can avoid redundant PCR optimization and validation. If a satisfactorily

developed PCR protocol does not exist, the development of a PCR assay progresses as depicted in Fig. 1.4.

The identification of a specific and exclusive region for specific PCR amplification is often difficult, especially in the case of *P. brassicae* where few published sequences exist. Primer design software is readily available and facilitates the design of specific and efficient primers for the region of interest. If the detection of a broad taxonomic level of organisms is desired (nematodes or Ascomycetes, for example), a fairly conserved diagnostic gene must be selected that is broadly present within the taxon of interest. If a narrow taxon is desired (a specific species or pathogenic race), a more variable diagnostic gene that is unique for the organism will have to be selected (Ophel-Keller et al., 2008).

PCR protocols require high quality DNA at an appropriate concentration and the potential effect of the sample matrix on the inhibition of PCR reactions has been extensively documented in the literature (Tebbe and Vahjen, 1993; Cao et al., 2007; Faggian and Strelkov, 2009). Different environmental and organism samples require different degrees of lysis, purification and concentration (Table 1.2). While moderately robust protocols and commercially available kits exist, consideration for sample matrix effects and organism biology are necessary (Saunders and Rossi, 2008). Unless previously reported, any DNA extraction protocol has to be validated to ensure that the technique does not introduce bias into the experiment, accurately and precisely extracts DNA from the pathogen of interest, extracts DNA in a timely manner (a special concern for ephemeral

organisms), and purifies DNA to an appropriate degree (Saunders and Rossi, 2008).

1.5.1.3. DNA extraction from plant and soil

High quality, purified DNA is a requirement for most molecular applications including PCR and PCR-based assays. DNA extraction procedures are based on a generic protocol involving preliminary sample preparation and homogenization, lysis of membranes, the stabilization of the DNA in the crude lysate, separation of the DNA from the sample matrix, removal of proteins and inhibitors, the concentration of the DNA and any ancillary purification steps (Saunders and Rossi, 2008). Each stage of this generalized protocol has to be optimized for the specific sample matrix, organism and downstream applications required (Saunders and Rossi, 2008).

Commercial kits for extracting DNA from soil are readily available and have been successfully employed to extract DNA from various soil samples (Cao et al., 2007; Dineen et al., 2010; and Etebu and Osborn, 2010). Likewise, lab-developed protocols exist that are sometimes superior to commercial kits in their ability to extract DNA cheaply and effectively (Huang et al., 2009; Ophel-Keller et al., 2008; Kageyama et al. 2003). In house produced kits do require considerable optimization and validation and often require the use of harsh chemicals in the extraction (Saunders and Rossi, 2008). Table 1.2 includes many of the soil extraction kits and protocols available and the generalized techniques involved. The suitability of a DNA extraction kit is determined by the requirements of the molecular application (Saunders and Rossi, 2008). The main

considerations are the purity of the DNA, the concentration of the DNA and the integrity of the purified DNA (Saunders and Rossi, 2008). Many of the protocols follow a generalized scheme where the soil containing the organism of interest is mechanically and/or chemically lysed, generating a coarse extraction mixture (Saunders and Rossi, 2008; Table 1.2). Various kits and protocols differ in the technique of lysis, and can impact the integrity of DNA (mechanical methods often shear DNA molecules). The nucleic acids are protected in the extraction buffer and are separated from the coarse lysate by centrifugation or chemical extraction and centrifugation, where the DNA is contained in the supernatant (Saunders and Rossi, 2008; Table 1.2). Purification of DNA can be achieved by various methods, which obviously impact the degree of purity in the resulting DNA sample (Saunders and Rossi, 2008; Table 1.2). These methods can employ affinity columns, chemical flocculation, and chelating agents (Saunders and Rossi, 2008). Concentration of DNA is typically achieved using either precipitation or commercial columns, although variants utilizing magnetic DNA affinity beads exist (Saunders and Rossi, 2008; Table 1.2).

DNA extraction from soils is not as straightforward as many routine extractions, where inhibitory substances to many downstream molecular applications can co-extract with DNA. These substances are regularly occurring in environmental substances such as soils, feces, and organic detritus. DNA extraction kits for soil typically employ protein denaturing salt solutions (Guanidine isothiocyanate), multiple wash steps and/or *post-hoc* precipitation or column extraction steps (Table 1.2). These procedures are time-consuming, yield-

reducing and essential to the purification of high quality DNA from soil (Saunders and Rossi, 2008). As environmental samples differ in their composition and, ultimately, their difficulty in DNA extraction, procedural modifications or careful kit selection and modification is often necessary. This can have impacts on experimental consistency and reproducibility, as a satisfactorily rigorous DNA extraction protocol has not yet been developed that can successfully handle all types of soil.

Routine spectrophotometric analysis of DNA extracts can highlight the purity of the samples, but often *ad hoc* PCR analysis is the definitive test for further successful PCR analysis and any additional sample treatment and purification.

1.5.2. Plant and Soil Sampling

1.5.2.1. Field Sampling for *P. brassicae*

A clear understanding of the patterns of pathogen dispersal in the environment is necessary for a thorough understanding of its epidemiology (Campbell and Noe, 1985). The spatial pattern, population size, aspect being sampled, sample area and spatial scale all impact the specific soil sampling strategy employed and as more is known about the spatial patterns, more effective and optimized sampling strategies can be employed (Campbell and Noe, 1985; Evans and Gleeson, 1980; Southwood, 1978). A clear objective and hypothesis must also be established when designing a sampling protocol, since different research objectives require different methods of spatial pattern analysis (for instance, mapping in two or three dimensions, goodness of fit to random probability distributions, computation of

indices of aggregation, spatial autocorrelation) and sampling requirements (Campbell and Noe, 1985; Campbell and Pennypacker, 1979; Southwood, 1978).

Cao et al. (2009) modified their sampling strategy to accommodate a quantitative assessment of clubroot, since most disease was observed at the entrances to agricultural fields, with disease incidence decreasing as samples were taken further into the field. By focusing sampling efforts at the entrance, Cao et al. (2009) improved the efficiency of sampling for clubroot in their experimental methods. This approach, however, was taken partly in an effort to minimize the sampling of fields where disease was most likely absent, and thereby enable surveyors to visit as many fields as possible. While this strategy was effective for the objectives of Cao et al. (2009), it may not be appropriate for assessing whole-field *P. brassicae* patterning, since the results will be extremely biased as all plants in the field do not have the same probability of being sampled, unlike other sampling protocols, namely the stratified random sampling design (SRSD).

When preliminary statistical information is available, either in the literature or from pilot studies, the effective sample size can be calculated to achieve a desired degree of precision while simultaneously minimizing effort and costs (Southwood, 1978). These calculations are again contingent on experimental design factors and are discussed in depth by Southwood (1978). Alternatively, the statistical power or effect size for a given sample size can be calculated using statistical power analysis (Champely, 2009; Cohen, 1988). Power analysis can be represented in graphical form and is highly amiable to analysis by the R statistical program (Champely, 2009; R Development Core Team, 2010).

Numerous hypotheses regarding the pattern of resting spores in the environment could be conceived, but very little experimental evidence exists to support or refute any of them. Commercial agricultural fields have historically utilized plow and tillage regimes that, to a degree, homogenize the soil environment (Campbell and Pennypacker, 1979). Plowing of clubroot galls has been shown to increase the soil inoculum levels, but the effects on resting spore patterning are not known, although the occasional observation of almost uniformly infected plants suggests that random patterning is possible (Murakami et al., 2004).

Campbell and Pennypacker (1979) observed a random patterning of *Rhizoctonia solani* infected plants in agricultural fields that were associated with cultivation and plowing. Additionally, Kim et al. (2000) found that *P. brassicae* resting spores were patterned evenly in the upper soil (zero to five cm.) with very little aggregation. Yet conflicting data exist as diseased plants are regularly observed aggregated at the field entrances where either favorable conditions exist for disease development, or reflect the site of initial introduction (Cao et al., 2009). As *P. brassicae* resting spores are sessile and disperse from decomposing galls, it is reasonable to expect that outside of instances of mechanical tillage, soil erosion, large scale vegetative propagation of clubroot infested materials, and/or dispersion by animals, an aggregated environmental pattern could be expected (Nicot et al., 1984; *sensu* Delp et al., 1986). Aggregated environmental patterning has been observed in *Verticillium dahliae,* a sclerotia producing soilborne fungus (Smith and Rowe, 1984). In recent decades, farmers in many regions of the world,

including western Canada, have adopted conservation tillage techniques in which plowing the fields is minimized or eliminated altogether.

The experimental design of a study to assess patterning of *P. brassicae* resting spores in the environment would have to include a record of the sample locations at an appropriate scale of resolution (Nicot et al., 1984). This would permit an assessment of patterning as spatial relationships, unlike a general frequency distribution that may obscure in-field patterns (Nicot et al., 1984; Delp et al., 1986). As some aggregation is expected in *P. brassicae* patterning, a SRSD will reduce any systematic error that is inherent in sampling an aggregated population with random methods (Southwood, 1978). A SRSD provides improvements in accuracy over data collected using transect or W-sampling designs, and can enable analysis of within field variance and patterning (this ability is lost when samples are bulked into composite samples) (Delp et al. 1986; Nicot et al., 1984; Goodell and Ferris, 1980). Once within-field variance is analyzed, repeated sampling of the field of interest can concentrate on these particular strata and develop fine resolution patterning maps (Goodell and Ferris, 1980).

In sampling nematodes, the deviation in estimating the mean is inversely proportional to sample size and sample number regardless of experimental design (Goodell and Ferris, 1980). In this case, the most efficient sampling program is the one that provides the most information at the least cost, and a SRSD was preferred as it is not especially expensive compared to other equally informative

sampling protocols, and provides estimates of within field variance (Goodell and Ferris, 1980; Delp et al., 1986).

Our current understanding of *P. brasicae* biology suggests that sampling protocols for aggregated environmental patterning, such as a SRSD as described by Delp et al. (1986) and Goodell and Ferris (1980), would be effective. The sampling intensity (sample size, quadrat number and size) would be contingent on the perceived inoculum density of the sampling area of interest, available resources, and information provided in the limited preliminary literature available (Cao et al., 2009; Delp et al., 1986; Goodell and Ferris, 1980; Kim et al., 2000). Information regarding the patterning and dispersion of *P. brassicae* would be extremely valuable in guiding pest management protocols, or in assessing details in the *P. brassicae* life history, since the environmental variables and factors that correlate with resting spore patterning could be identified. The precise and accurate application of lime or other chemical controls according to resting spore patterning in the field could overcome the high cost of chemical treatment of *P. brassicae* infestation.

1.5.2.2. Seed sampling

Seed borne infestation by *P. brassicae* has been sporadically addressed in the literature (Karling, 1968; Warne, 1943) and presents a potential mechanism for *P. brassicae* dispersal (Chapter 3). However, the degree to which seedborne infestation contributes to *P. brassicae* dispersal in the field is wholly unknown. Like soil sampling, the degree of homogeneity or heterogeneity expected in the seedlot influences the sampling protocol utilized (Remund et al., 2001).

For homogeneous, thoroughly mixed seedlots, a simple random sample can be obtained simply by obtaining samples anywhere in the lot (Remund et al., 2001). Non-random sampling effects (sample bias) are amplified in heterogeneous seedlots and careful sample selection methods must be used (Remund et al., 2001). Probe sampling schemes utilize a depth probe to obtain samples from various depths in seed containers within continuous grids subdividing the sample area (Remund et al., 2001). In probe sampling, all seeds have an equal chance of being sampled and a more random sample is obtained (Remund et al., 2001). SRSD regimes, akin to those used in soil sampling, can also be used if discernible strata are observed in the lot (Delp et al., 1986; Remund et al., 2001).

Batching of samples has proven to be an effective way to reduce costs in seed sampling, and has been utilized by various researchers to reduce the sample load, but eliminates any possibility of within-lot analysis (Evans and Gleeson, 1980; McNeil et al., 2004; Remund et al., 2001). Batching into composite lots is effective for studies concerned with bulk inoculum loads, but for fine resolution mapping of inoculum patterning in seedlots, location information would have to be included with any sample (Nicot et al., 1984; Remund et al., 2001).

1.6. Research objectives

1.6.1. The histopathology of spheroid galls

As the development of spheroid galls is often taken as an indication of host resistance to *P. brassicae*, this study will analyze anatomical and pathological aspects of spheroid gall formation. This work is timely, since anecdotal reports of spheroidal galls in recently released canola hybrids with clubroot resistance are already surfacing. The main objective of this research is to obtain an understanding of the anatomical basis of spheroidal gall formation, and to assess whether or not *P. brassicae* resting spores are produced within these galls.

1.6.2. Seedborne infestation

An understanding of *P. brassicae* patterning in the environment requires a complete knowledge of the modes of pathogen dissemination and epidemiology. With this information, effective direction of management plans that consider fine aspects of field patterning can be instated. To this end, the importance of seedborne infestation should be evaluated. Therefore, important objectives of this research include: (1) the development of an accurate and rapid qPCR-based protocol for the detection of *P. brassicae* on seeds and tubers of various field crops commonly grown in Alberta, and (2) an assessment of the amount of inoculum found on seed and tuber lots harvested from clubroot-infested fields, in order to better define the risk associated with seedborne dissemination of *P. brassicae*. This work will contribute to a more complete understanding of the

epidemiology of the clubroot pathogen, and result in the development of qPCR-based tool for routine quantification of *P. brassicae* inoculum on seed lots and other substrates.

1.7. Tables

Table 1.1. Contemporary and historical species and cultivars showing some degree of resistance to *Plasmodiophora brassicae*. The hypothesized basis for this resistance is presented along with the degree of spheroid gall formation.

Species	Common Name[1]	Degree of Resistance	Nature of Resistance	Spheroid gall formation[2]	References
Brassica rapa	ECD 01	High	Polygenic resistance	NA	Toxopeus et al., 1986; Piao et al., 2009
	ECD 02	High	,,	NA	Toxopeus et al., 1986; Piao et al., 2009
	ECD 03	High	,,	Regular	M. Plishka unpublished results; Toxopeus et al., 1986; Piao et al., 2009
	ECD 04	High	,,	NA	Toxopeus et al., 1986; Piao et al., 2009
	Siloga	Resistant to Date-01, Yuki-01, Rokunohe-01, and Ano-01	One major gene, two minor genes	NA	Kuginuki, 1999
	Gelria R	Resistant to Date-01, Yuki-01, Rokunohe-01, and Ano-01	,,	NA	Kuginuki, 1999
	CR Kanki 100	NA	Introgressed from Siloga and Gelria R	Frequent	Osaki et al. 2008
	CR Kanko	Resistant to Rokunohe-01 and	,,	Very Frequent	Osaki et al. 2008
	CR Kukai 65	Resistant to Rokunohe-01 and Ano-01	,,	Regular	Osaki et al. 2008
	Daifuku 234	NA	NA	Very Frequent	Osaki et al. 2008
	Daifuku 206	NA	NA	Frequent	Osaki et al. 2008
	CR Satokaze	NA	NA	Very Frequent	Osaki et al. 2008
	SW A 2044	Partial	NA	NA	Wallenhammer et al. 2000
Brassicae napus	Mendel	Partial resistance, highly race specific	One dominant gene, two recesive genes	NA	Diederichsen et al., 2006; Diederichsen et al., 2009
	Wilhelmsburger	Susceptible to 16/31/31, Williams 1, 4, 9, and Date-01	NA	Regular	M. Plishka unpublished results; Ayers, 1957; Crute et al., 1980; Kuginuki et al., 1999
	ECD 08	Susceptible to 16/31/31	NA	NA	Toxopeus et al., 1986
	ECD 09	Susceptible to 16/31/31	NA	NA	Toxopeus et al., 1986
	Swede NH 654-8	Resistant to six pathotypes	NA	NA	Ayers, 1957
	Swede NH 775-1	Partial	NA	NA	Ayers, 1957
	Swede NH 743-3	Partial	NA	NA	Ayers, 1957
Brassica oleracea	Bindsachsener	Partial	Quantitate trait loci, polygenic	Occasional	M. Plishka unpublished results; Crute et al., 1980; Luzny and Polach, 1961
	Böhmerwaldkohl	Partial	,,	NA	Crute et al., 1980; Nagaoka et al., 2009; Luzny and Polach, 1961; Karling, 1968
	MSU 134 broccoli	Partial	,,	NA	Crute et al., 1980; Nagaoka et al., 2009
	P.I. No. 165448	Partial		NA	Catovic-Catani and Rich, 1964; Karling, 1968
	Broccoli OSU CR-s to 8	Partial	,,	NA	Crute et al., 1980; Nagaoka et al., 2009
	Broccoli Oregon CR1	Partial	,,	NA	Toxopeus et al., 1986; Nagaoka et al., 2009
	Brocoli breeding lines OSU CR-2 - OSU CR-8	NA	,,	NA	Baggett and Kean, 1985; Nagaoka et al., 2009
	Cabbage Oregon 100, 123, 140 and 142	NA	,,	NA	Baggett, 1983; Nagaoka et al., 2009
	ECD 13	NA	,,	Frequent	M. Plishka unpublished results
	Cabbage Badger Shipper	NA	,,	Occasional, first reported in literature	M. Plishka unpublished results; Seaman et al., 1963; Williams, 1966
Brassica oleracea* var. *acephala plana	Markkohl Höner Blauer	High	NA	NA	Karling, 1968;
Arabidopsis thaliana	Ze-0	High resistance to isolate e	Single resistant gene, *RPB1* hypersensitive response	Necroticly delimited mini-galls	Kobelt et al., 2000
	Tsu-0	,,	,,	,,	Kobelt et al., 2000
	Ta-0	,,	,,	,,	Kobelt et al., 2000

[1]Represents the cultivar name or European Clubroot Differential (ECD) set designation

[2]NA (not applicable) given where spheroid galling is not observed or is not mentioned in the reviewed literature

Table 1.2. Common DNA soil extraction procedures and kits.

Reference	Commercial Name/Protocol	Manufacturer	Sample Preparation	Lysis Method	Concentration of DNA	Effective Sample Types	Notes
Cao et al. 2007; Dineen et al., 2010.	FastDNA Spin Kit for Soil	MP Biomedicals	Maximum 500mg sample size	Bead Mill, Proprietary lysis buffer	Silica DNA binding matrix packed into column, washed with ethanol salt solution	Loam,clay,sand, chernozem,	Cao et al., (2007) validated for *Plasmodiophora brassicae,* Authors report high yield, low quality, dilutions necessary
Lartey et al. 2010; Dineen et al., 2010	Powersoil DNA isolation kit	MoBio	Maximum 250mg sample size	Bead mill, proprietary lysis solution, optional heating step	Silica affinity column	Loam, sand, sugar beet field soil	None
Huang et al., 2009	Polyvinylpolyp yrrolidone (PVP)	NA[2]	Maximum sample size 500mg	Freeze-thaw with DNA extraction buffer,	Isopropanol precipitation, ethanol wash	Not specified	None
Kageyama et al., 2003	Glass bead-Skim milk-SDS[1]	NA	Maximum sample 200mg	Vortex and heating in DNA extraction buffer.	Isopropanol precipitation	Not specified	Validated for *Plasmodiophora brassicae*
Mingzhu et al., 2010	Magnetic DNA extraction[1]	Magextractor	Same as glass bead-skim milk-SDS	Same as glass bead	Magnetic DNA affinity beads	Forest soil, riverbed soil, and other plant and soil samples	None
Miller et al., 1999	Procedure 3	NA	100mg lyophilized and homogenized soil.	Bead mill, SDS-chloroform	Sephadex G-200 column	Collamer silt loam from forest, agricultural and wetland	Variations of this protocol are provided by the author

[1]SDS, sodium dodecyl sulphate
[2]NA (not applicable) given where the protocol is not commercially available

1.8. Figures

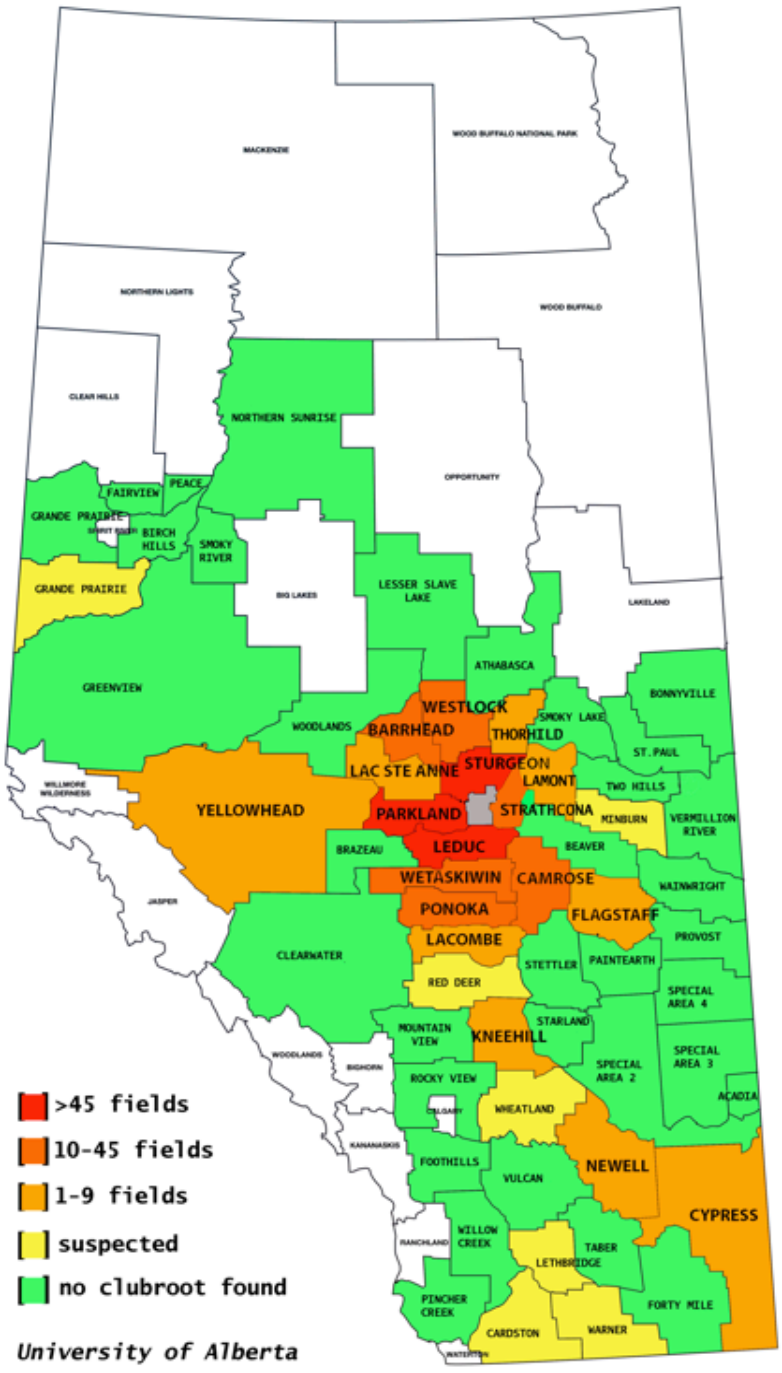

Figure 1.1. Occurrence of *Plasmodiophora brassicae* infested fields surveyed in Alberta as of October, 2010 (Strelkov et al., 2011). The shading reflects increased incidence of field infestation, totaling 566 confirmed fields.

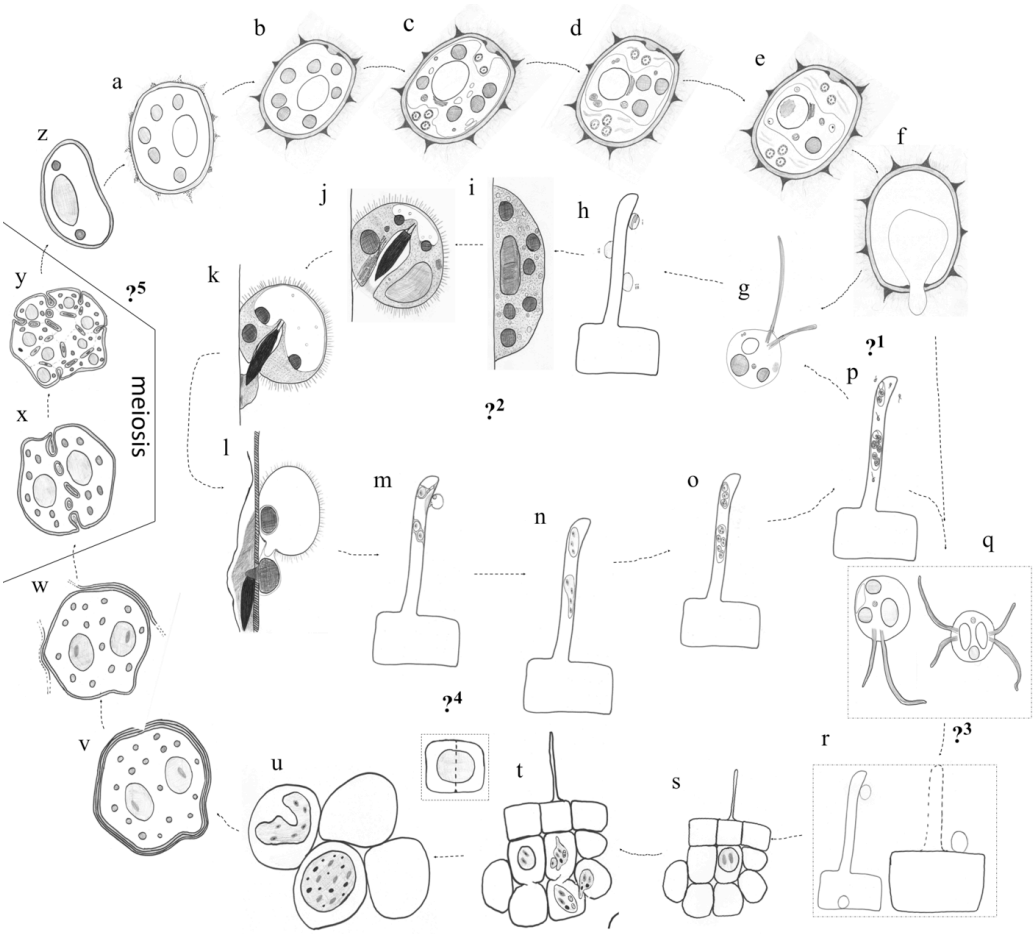

Figure 1.2. Life cycle of *Plasmodiophora brassicae* compiled from the research of Aist and Williams (1971), Donald et al. (2008), Mithin and Magrath (1992), Buczacki and Clay (1984), Buczacki (1983), Buczacki and Moxham (1983), Ingram and Tommerup (1971), Tommerup and Ingram (1972), Tanaka et al. (2001), Kageyama and Asano (2009), and Garber and Aist (1979). a, early resting spore; b, mature resting spore; c-e, zoospore differentiation; f, emergence of zoospore through germ pore; g, primary zoospore; h, encystment of zoospore on roothair; i, flattened early cyst; j, mature cyst; k, formation of apressorium; l, penetration of roothair by stachel; m, early primary plasmodium; n, synchronic mitotic divisions, multinucleate plasmodium; o, zoosporangium formation; p, secondary zoospore release into rhizosphere and roothair lumen; q, secondary zoospore and plasmogamy of zoospores; r, encystment of secondary zoospores on roothair, base of roothair lumen, or epidermal cell; s, bi-nucleate early plasmodia (amoeboid phase); t, motile amoeboid phase; u, coenocytic vegetative plasmodia; v-w, degeneration of outer plasmodial envelope; x-y, accumulation of vesicles concerted with meiosis; z, cleavage of immature resting spores. Question marks indicate stages of uncertainty involving: 1 and 2, direct infection of cortex by primary zoospores and possible re-infection of roothairs by secondary zoospores,

respectively; 3, cortical infection by uni-nucleate zoospores or binucleate zoospores and the specific area of this encystment; 4, the importance of passive dispersal by concerted division of the plasmodia with the plant cell; 5, the details of plasmodial fusion, karyogamy and meiosis (D. Rennie).

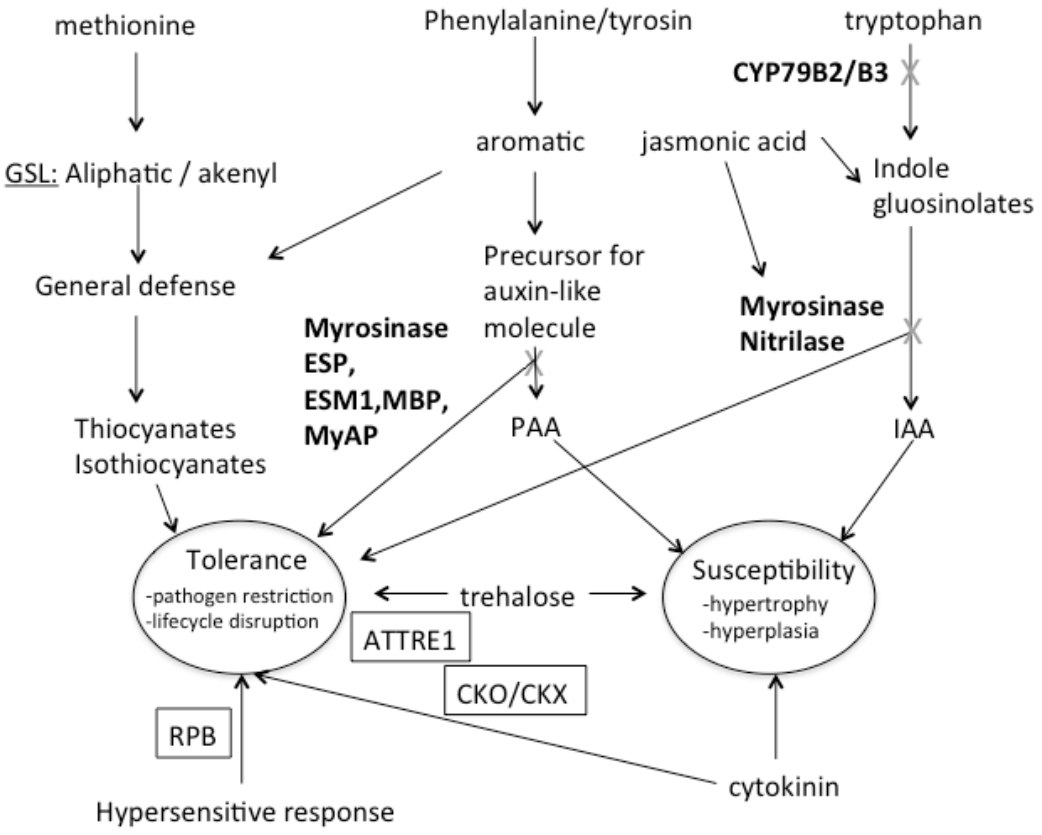

Figure 1.3. Metabolic pathways involved in tolerant and host reactions adapted from Ludwig-Müller (2009), Siemens et al. (2006), Kobelt et al. (2000), Brodman et al. (2002), Gresic et al. (1999), and Devos et al. (2002). Enzymes and genes in bold are metabolic enzymes responsible for the biosynthesis of metabolites involved in *Plasmodiophora brassicae* infection, the inhibition of which leads to tolerance (crosses). Bordered genes confer or contribute to tolerance in the respective host plants.
Abbreviations: GSL, glucosinolates; ESP, epithiospecifier protein; ESM1, *Epithiospecifier modifier 1* gene; MBP, myelin basic protein; MyAP, Myrosinase associated protein; PAA, phenylacetic acid; RPB, *Reaction to* P. brassicae 1 gene; ATTRE1, Arabidopsis thaliana *trehalose* 1 gene; CKO/CKX, Cytokinin oxidase/dehydrogenase ; CYP79B2/B3, cytochrome P450 enzyme; IAA, indole acetic acid

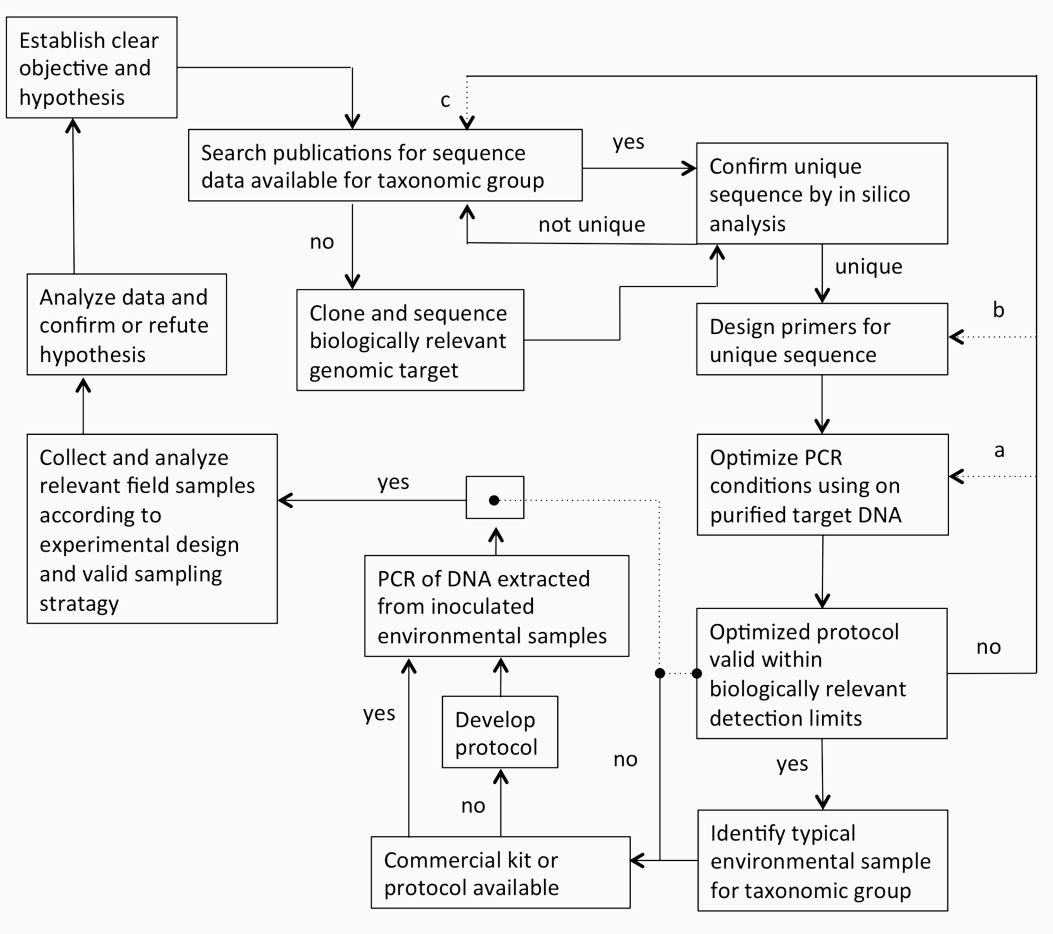

Figure 1.4. Decision tree for effective development of a generalized PCR assay for environmental samples. Stages a through c denotes sequential decisions to be made if a PCR assay does not detect within biologically relevant limits.

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Chapter 2: Histological analysis of spindle and spheroid root galls caused by *Plasmodiophora brassicae*

2.1. Introduction

Plasmodiophora brassicae is an obligate parasite and the soilborne causal agent of clubroot of crucifers, a disease affecting many species of the family Brassicaceae. Clubroot is typified by the formation of conspicuous root galls, which result from tissue proliferation in the infected roots. These galls are grossly distorted when compared to normal roots and hypocotyls, and usually have a club- or spindle-shaped morphology. The pathogen proliferates within the galled tissues, eventually producing very large numbers of resting spores that are released back into the soil, and which serve as inoculum for future infections (reviewed in Kageyama and Asano, 2009). Early clubroot researchers also recognized an alternative form of smaller galls, quite distinct from the typical spindle-shaped galls, which were termed 'spheroid galls' because of their spherical or nearly spherical form (Seaman et al., 1963; Williams, 1966). Spheroid galls have been reported to develop on numerous clubroot resistant Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) genotypes (Osaki et al., 2008; Seaman et al., 1963).

Seaman et al. (1963) and Williams (1966) both describe spheroid galls, ostensibly, as resistance structures. The basis for this determination was the assumption that the smaller gall size and spherical form of these galls results from

a restriction of the pathogen within the galled tissues. While this is a reasonable hypothesis, there is little information available to support or refute this theory. Nonetheless, the assessment of spheroid galls as resistance structures appears to be supported by the more contemporary work of Osaki et al. (2008), in which spheroid galls (also referred to as small spheroid galls (SSG)) were regularly observed on clubroot resistant Chinese cabbage genotypes. Host plants that develop spheroid galls are commonly regarded as being completely resistant to clubroot when these are screened for symptom severity, suggesting the absence of infected material (Osaki et al, 2008; Williams et al., 1966). This view of spheroid galls as host resistance structures may be considered problematic, since infective resting spores are still produced within these galls under greenhouse conditions (Osaki et al., 2008). If spheroid gall-forming host genotypes are incorporated into breeding programs under the erroneous assumption that they possess complete resistance to clubroot, a situation could arise wherein purportedly resistant varieties are contributing to inoculum buildup in the field, despite their supposed resistance.

The lifecycle of *P. brassicae* is composed of two phases, firstly, the infection of root hairs and formation of secondary (2°) zoospores (sporangial phase), and secondly, the formation of resting spores (sporogenic phase) (Ingram and Tommerup, 1972). The 2° zoospores are thought to be responsible for initiating the sporogenic phase of the lifecycle in the host cortex (Ingram and Tommerup, 1972). The 2° zoospores, through a currently unknown mechanism, infect the cortex and develop into an actively motile, myxameoboid phase

(Donald et al., 2008; Mithen and Magrath, 1992). The active movement of this myxameoboid phase is likely responsible for the distribution of the pathogen within the host during the early stages of disease development (Donald et al., 2008; Mithen and Magrath, 1992). Eventually, the myxameoboid masses coalesce into coenocytic vegetative plasmodia, whereby further distribution is achieved through synchronous divisions with the host (Asano and Kageyama, 2006). The movement and development of the plasmodial phases in the host vascular rays, vascular cambium and cortex leads to the proliferation of tissues and the consequent expansion of the root (Asano and Kageyama, 2006; Gustafsson et al., 1986; Kageyama and Asano, 2009).

Synthesizing what is known regarding spheroidal galls, it seems plausible that their formation results from the restriction of the actively motile myxameoboid phase during the sporogenic phase of the lifecycle. The development of plasmodia would continue in these galls, but the pathogen would be restricted in its spread to the region of initial infection, unlike in spindle galls, in which *P. brassicae* spreads more extensively throughout the root tissues. As such, we hypothesize that spheroidal galls do not represent wholly resistant structures, but rather a limitation of myxamoebal movement in the host. Histological analysis of spindle and spheroid galls was undertaken to examine this possibility and establish the anatomical basis for spheroid gall formation.

2.2. Materials and Methods

2.2.1. Plant material

Seeds of *Brassica rapa* var. *rapifera* (European Clubroot Differential (ECD) 03), *B. napus* var. *napobrassica* cv. Wilhemsburger, (ECD 10), *B. oleracea* var. *capitata* cv. Badger Shipper, (ECD 11), *B. oleracea* var. *capitata* cv. Bindsachsener, (ECD 12), *B. oleracea* var. *capitata* cv. Jersey Queen, (ECD 13), and *B. oleracea* var. *acephala* subvar. *laciniata* cv. Verheul (ECD 15) were obtained from Horticulture Research International, Genetic Resources Unit (Wellesbourne, Warwick, UK).

2.2.2. Pathogen material and inoculum production

The *P. brassicae* populations SACAN03-1, SACAN03-4, AbotJE04-1 and CDCN04-1 were originally collected from western Canada (Strelkov et al., 2006) and were maintained as frozen (-86 °C) root galls. A population of the pathogen collected from China (China01) was also included in some of the histological analyses as described below. SACAN03-1 and SACAN03-4 are classified as pathotype 3 on the differentials of Williams (1966), whilst CDCN-04-1 and AbotJE04-1 are classified as pathotypes 5 and 6, respectively (Strelkov et al. 2006). The pathotype of China01 is undetermined at the current time (Cao and Strelkov, University of Alberta, *personal communication*).

Resting spore suspensions for host inoculation purposes were prepared by homogenizing the frozen galls in an electric blender (Warring, Torrington, CT) and filtering the homogenate through a 450µm sieve (W.S. Tyler, St. Catherines,

ON), as described in Rennie et al. (2011). The resting spore concentration in the extract was measured with a haemocytometer (VWR, Mississauga, ON), and the final spore concentration adjusted to approximately 1×10^7 resting spores/mL with sterile distilled water. The spore suspensions were stored at 4°C until needed and utilized within a week of preparation.

2.2.3. Virulence assays

The *Brassica* hosts ECD 03, ECD 10, ECD 11, ECD 12, ECD 13 and ECD 15 were inoculated separately with the pathogen populations SACAN03-1, SACAN03-4, AbotJE04-1 and CDCN04-1, in order to assess the relative incidence of spheroid (versus spindle) gall formation (Table 2.1). Five day-old seedlings, which were pre-germinated on moistened filter paper in Petri dishes, were inoculated by briefly dipping the roots in a spore suspension (Tewari et al., 2005) prepared as above, and planted in plastic pots (5.0 cm × 5.7 cm × 5.7 cm) filled with Sunshine Mix LA7 potting mixture (Sunshine Growers, Vancouver, BC) at a density of one seedling per pot. The seedlings were maintained in a growth chamber (21°C/18°C with a 16 h photoperiod at a light intensity of 180 $\mu mol/m^2/s$) or a greenhouse (20 ± 2°C with a 16 h photoperiod under natural light supplemented by artificial light) for 45 days. The potting mixture was kept saturated for the first two weeks after inoculation by placing the pots on trays filled with water, following which they were removed from the trays and watered daily from above. Fertilizer (20N:20P:20K) was sparingly applied when needed to ensure abundant plant growth and disease development. Control plants were grown in the same manner as the inoculated plants, but were not inoculated.

The plants were harvested at 45 days after inoculation by gently digging out the roots from the potting mixture, washing with water, and carefully examining the roots for symptom development. Between 3 to12 seedlings were inoculated per treatment, and data from three separate experiments were compared. The general proclivity of a particular host-pathogen combination to form spheroid galls was classified as follows: 2+ = spheroid galls on ≥25% of plants examined; + = spheroid galls on <25% of plants examined; sd = only spindle galls observed; and - = no galling.

2.2.4. Histological analysis

Based on the degree of spheroid gall formation observed in the virulence assays, additional inoculations were conducted with specific host-pathogen combinations in order to produce infected material for histological analysis. The *P. brassicae* populations SACAN03-1 and AbotJE04-1 were used to inoculate the cabbage 'Jersey Queen' (ECD 13), while the population China01 was used to inoculate the rutabaga 'Wilhemsburger' (ECD 10). Inoculations were conducted as described above, except that instead of dipping the roots in a spore suspension, a 100 µL aliquot of *P. brassicae* inoculum (1×10^7 resting spores/mL) was applied to the potting mixture at the base of the 'Jersey Queen' seedlings. Seedlings were grown at a density of one plant per 5.0 cm × 5.7 cm × 5.7 cm pot with a total of 24 plants per treatment, and maintained in a growth cabinet under a 16 h photoperiod at 21°C/18°C (day/night) with a light intensity of 180 µmol/m^2/s.

Twenty four seedlings were sampled at 5, 12, 19, 21, 26, 33, 40 and 45 days after inoculation and examined for clubroot symptom development as above.

Galls were identified by macroscopic observation, and if very small, were confirmed under low magnification with a stereomicroscope. (Wild Heerbrugg, Heerbrugg, Switzerland). The galls were dissected from the host plant, photographed and promptly immersed in 10% neutral buffered formilin solution for at least one week (A. Oatway, University of Alberta, *personal communication*). Large galls were coarsely sectioned with a sharp scalpel to expedite fixation and facilitate sectioning. In cases where multiple galls were observed on the same host, the individual galls were fixed and sectioned separately.

2.2.5. Tissue sectioning and staining

Fixed specimens were dehydrated through an ethanol series (70%-90%-100%-100%), transferred to an ethanol:toluene solution (1:1), passed through two successive toluene washes and impregnated with paraffin wax. The processed tissue was placed into sectioning cassettes and embedded in paraffin wax. All tissue processing stages were performed in a Leica Tissue Processor 1020 (Leica, Richmond Hill, ON). Embedded samples were sectioned longitudinally at 5-7µm, ideally through the median of the specimen using a rotary microtome (The American Optical Company, Southbridge, MA). Radial sections were made of similarly embedded specimens and sectioned at 5-7µm. Serial sections were taken when additional detail was required. Sectioned paraffin ribbons were adhered to slides, de-paraffinized with toluene, rehydrated through an ethanol series and stained with either a standard Harris' hematoxylin eosin staining protocol (HE) or with a standard safranin-fast green staining protocol (A. Oatway, University of

Alberta, *personal communication*). All slides were mounted under a glass coverslip with DPX mounting medium (Sigma-Aldrich, Oakville, ON).

2.2.6. Microscopic observation

Tissue sections were examined with an Axio Imager M1 brightfield/fluorescence microscope (Zeiss, Toronto, ON) or a Primo Star light microscope (Zeiss). Host autofluorescence was visualized using a blue fluorescence filter set at an excitation of 365 μm and an emission of 445/450μm. Pseudo-coloured composite images and bright field images were recorded with Axiovision 4.6 software (Zeiss) or MetaImage (Molecular Devices, Sunnyvale, CA). Tagged image file format images were stored and, to render a complete image, were aligned and composited together using the GNU image manipulation program (The GIMP Team, 2011). The GNU image manipulation program (The Gimp Team, 2011) was also utilized to artificially colour relevant anatomical features to aid in analytical clarity.

Morphological characteristics considered during microscopic observation of the tissue samples included: degree of infection in the stele, degree of infection outside of the stele, degree of pathogen maturation, presence of mature resting spores, presence of lignification and host autofluorescence, aberrant vascular strand initiation, degree of secondary tissue development/infection, and lateral root development/infection.

2.3. Results

2.3.1. Virulence assays

The capacity of certain host genotypes to produce spheroid galls and/or spindle galls when inoculated with particular populations of *P. brassicae* is summarized in Table 2.1. Exclusive spindle galling was only observed in a few cases, and in the majority of the interactions some spheroid galling was observed. Spheroid galls were observed on all hosts analyzed, although not in response to every population of the pathogen (Table 2.1).

Limited spheroid gall production was found when ECD 03 was inoculated with the pathogen populations SACAN03-4 or CDCN04-1. Spindle galls were observed when ECD 03 was inoculated with SACAN03-1, while insufficient data were available to discern the typical response after inoculation with AbotJE04-1. In contrast, consistent and extensive spheroid galling was observed when ECD 10 was inoculated with SACAN03-1. Inconsistent spheroid gall production was found when ECD 10 was inoculated with SACAN03-4, while there was inconsistent spindle gall development after inoculation with CDCN04-1. No galling was observed after inoculation of ECD 10 with AbotJE04-1.

Spheroid galls were regularly observed when ECD 11 was inoculated with CDCN04-1, while spheroid galling was also found after inoculation with AbotJE04-1. The reaction of ECD 11 to inoculation with SACAN03-4 was inconsistent, with this host producing either spindle and spheroid galls, or no galls at all. No galling was observed when ECD 11 was inoculated with SACAN 03-1.

ECD 12 inconsistently formed spheroid galls in response to inoculation with SACAN03-1, SACAN03-4, and consistently with CDCN04-1. However, this host produced only spindle galls after inoculation with AbotJE04-1. The most consistent development of spheroid galls was observed in the cabbage ECD 13 after inoculation with SACAN03-1, SACAN03-4 or AbotJE04-1, with the greatest degree of spheroid galling found in response to SACAN03-1. Inoculation of ECD 13 with CDCN04-1 resulted in the inconsistent formation of spindle galls. Finally, the kale ECD 15 consistently developed spheroid galls when inoculated with the population CDCN04-1, and to a lesser degree, developed these structures after inoculation with AbotJE04-1. Spheroid gall formation by ECD 15 in response to SACAN03-1 or SACAN03-4 was rare and nonexistent, respectively (Table 2.1).

2.3.2. Anatomy of healthy root tissue

In lateral sections of mature plant roots that had not been inoculated with *P. brassicae,* periderm surrounded the expanded secondary cortex that enveloped a central stele of secondary vascular tissues (Fig. 2.2). The stele is defined in this work as the central region of complex tissue including (but not limited to) the xylem, rays and phloem, that is delimited from the secondary cortex by the secondary phloem (Fig. 2.2).

In cross sections of roots of 'Jersey Queen' (ECD13) that had not undergone secondary thickening, two prominent strands of phloem alternated with the diarchly arranged xylem in a manner comparable to the root anatomy of *Arabidopsis thaliana* (Dolan et al., 1993; Larson, 1934; Fig. 2.3a). In samples that

had undergone secondary thickening, the secondary cortex was observed outside of the secondary vascular elements (as in the lateral sections), where a row of thickened fiber cells could be observed in cross-section (Fig. 2.3b).

During the development of secondary tissue, the primary cortex (characterized by large diameter, isodiametric and undifferentiated parenchyma) was sloughed along with the epidermis (Fig. 2.3b). Remnants of the senescent primary cortex and epidermis were seen outside of the developing periderm (Fig. 2.3b). The endodermis could not be clearly identified, likely because it would have been sloughed off during secondary tissue development.

2.3.3. General observations of infected root tissue

Nine of the fixed spheroid galls and 11 of the spindle galls were of a sufficient quality for detailed microscopic analysis. In gall sections stained with HE and examined by light microscopy, the host cell walls and nuclei were a uniform purple, while pathogen structures varied from light purple to light red in color (Fig. 2.4). During resting spore formation, the cleaving plasmodia and immature resting spores stained a dark purple, while mature resting spores stained a light red (Fig. 2.5). In fast green/safranin stained sections, lignified or suberized cells stained red, while other plant cells stained a light blue. Pathogen material stained a similar light blue colour (Fig. 2.3). The nuclei, whether observed in pathogen or host cells, stained red (data not shown).

Various stages in the development of vegetative plasmodia and resting spores could be observed in the specimens examined. As the harvesting and fixation process damaged the root hairs, no primary plasmodia (which are

typically observed in the root hairs) could be seen. Anatomical details were readily observed in longitudinal sections through the median of the roots, where the stele was delimited from the secondary cortex by the phloem (Fig. 2.4; Fig. 2.5). The endodermis could not be conclusively determined and would not be present in samples that had undergone secondary thickening. The phloem could be identified by the characteristic elongate cells, and the vascular cambium could be inferred from the position of the secondary xylem and secondary phloem (Fig. 2.2). These lignified and suberized tissues readily fluoresced and helped to confirm the identity of such tissues (data not shown).

2.3.4. Spindle gall morphology and anatomy

Spindle galls could be regularly observed on roots at 26 days after inoculation. These galls typically had a distorted stele as a result of the expansion of rays and other secondary tissues (Fig. 2.3d; Fig. 2.5). Phloem and associated tissues were often distended by the expansion of secondary tissues in the stele (Fig. 2.3d; Fig. 2.5). The diameter of the vascular cylinder was small and not fragmented in uninfected portions of the gall, but begun to enlarge deeper into the infected tissues as a consequence of expansion and fragmentation of the stele (Fig. 2.3d; Fig. 2.5). In radial sections, the vascular cylinder was contiguous (Fig. 2.3d).

Infection and proliferation of tissues outside of the stele was regularly observed, along with infection and proliferation of the stelar tissues (Fig. 2.5; Table 2.2). Mature resting spores were also commonly observed, and were typically found in the tissues external to the stele (Fig. 2.5c; Table 2.2). Resting spore maturation was concentrated in localized areas of pathogen proliferation

(Krankheitsherde) and was principally observed in the external proliferating tissues, and occasionally in the stele (Fig. 2.1c and 2.1d; Fig. 2.5). The area of the spindle gall longitudinal sections examined microscopically could not be accurately measured, as they comprised many fields of view and had an irregular shape.

2.3.5 Spheroid gall morphology and anatomy

Spheroid galls were not observed on infected roots until 45 days after inoculation. These galls appeared as strongly delimited, round structures that were considerably and without exception smaller then typical spindle galls, with the average area of nine longitudinal section galls being 1.2 mm^2 . Spheroid galls were finely textured and uniformly buff coloured (data not shown).

Spheroid galls were typically observed on lateral roots, and when observed on tap roots, were not associated with extensive spindle gall development (Fig. 2.1b; Fig. 2.4). Despite the capacity of all host genotypes tested to form both spindle and spheroid galls, extensive spindle galling was not coincident with spheroid galling on any individual host examined (data not shown).

In longitudinal section, the spheroid galls typically had a region of proliferating tissue that corresponded to the secondary cortex and periderm of the healthy plants (Fig. 2.4). The outer proliferating tissue of the spheroid gall was less infected than the inner portions of the gall (Fig. 2.4). The underlying host tissue had limited secondary tissue development, was largely uninfected, and, where infection occurred, a continuous stele was maintained (Fig. 2.4). Proliferation of the stelar tissues and the pathogen within these tissues was limited

in all sections examined (Fig. 2.4). This observation was confirmed in radial section, where proliferating tissue was observed outside of a largely intact stele (Fig. 2.3c). Mature resting spores were observed in one longitudinal section of a spheroid gall (Fig. 2.6; Table 2.2)

2.4. Discussion

The present study, along with the earlier work of Kunkel (1918), Larson (1934), Gustaffson et al. (1986) and Graveland et al. (1992), assessed the anatomical details of spindle galling in the roots of *Brassica* hosts. In all cases, spindle gall development in the infected roots was coincident with the expansion of the stele and infection of secondary tissues (Kunkel, 1918; Larson, 1934; et al., 1992; Fig. 2.3d; Fig. 2.5). Graveland et al. (1992) reported infection and hyperplasia of the stele in galls developing in hairy root culture, with the cortex found to senesce. A mature clubroot gall evidently results largely from the infection of the vascular cambium, where the size of the gall is a function of the degree of spread in the vascular cambium (Kunkel, 1918). The division of the stele into fragments by the enlargement of rays has been shown to be an essential mechanism for gall development by the work of Kunkel (1918) and Gustaffson et al. (1986). This is evidenced further by the current observation that where a great deal of secondary tissue infection and expansion occurs in the stele, the development of a spindle gall is likely (Fig. 2.3d; Fig. 2.5;). This finding confirms the work of Gustaffson et al. (1986) and Larson (1934), who reported

extensive secondary tissue infection and proliferation were observed in spindle galls. It is, however, difficult to conclusively attribute this proliferation to vascular cambium infection, as the vascular cambium could not be conclusively observed in all samples. Logically, however, the infection of this meristem is likely for such a degree of coincident secondary tissue proliferation and infection. It is possible (although improbable) that plasmodia actively infected all of the observed secondary tissues by virtue of coincidental infection.

Spheroid galling was observed to occur in both clubroot resistant and susceptible hosts (Table 2.1), contrary to the earlier report of Osaki et al. (2008) who reported spheroid galling only on resistant cultivars. In the virulence assays, the cabbage 'Jersey Queen' was found to enrich for spheroid and spindle galls when inoculated with SACAN03-1 and AbotJE04-1, respectively (Table 2.1). There were no spheroid galls observed with AbotJE04-1 inoculation during our histological analysis (not included in Table 2.1). As spheroid galls form on resistant (*B. oleracea* var. *capitata* cv. Badger Shipper) and non-resistant varieties alike (Table 2.1), it seems that spheroid gall formation is not exclusively associated with clubroot-resistant host genotypes. It may be worth testing if spheroid gall formation is more frequent in clubroot resistant varieties other than 'Badger Shipper', as has been demonstrated by Osaki et al., (2008).

Larson (1934) observed spheroid gall formation as a result of wound-facilitated *P. brassicae* infection of stems of a Fusarium Yellows resistant *B. oleracea* genotype, and upon sectioning these galls, found tissue proliferation outside of the stele but little expansion of the stelar tissues. It is unclear why the

tissues outside of the stele were regularly infected, while infection of the stele was limited (Table 2.2). As spheroid gall production was consistently observed in certain host/pathogen combinations (Table 2.1), and as no mechanical facilitation was employed in the current study, it seems unlikely that wounding is absolutely necessary for spheroidal gall formation in the root proper (Larson, 1934). Nonetheless, it is uncertain if wounding, which may occur as the lateral root emerges from the overlaying tissues, can contribute to *P. brassicae* infection (Malamy and Benfey, 1997). If wounding does occur during lateral root formation, this may facilitate the development of spheroid galls in a manner akin to that observed on stems (Larson, 1934; Malamy and Benfey, 1997). However, even if similar modes of development exist, it is difficult to compare gall formation on the stem with that on the root, as the anatomical differences between the two are significant, despite the statement of Kunkel (1918) that similar modes of disease development occur on both the stem and root.

Infection by *P. brassicae* leads to considerable anatomical disruption, and consequently the exact identity or origin of the proliferating tissue in spheroid galls is difficult to determine. It is uncertain whether these tissues are genuine cells of the secondary cortex or periderm (large, polyhedral, thin walled and undifferentiated), or are products of hypertrophy and hyperplasia of the vascular cambium (Dolan et al, 1993; Larson, 1934; Webster and Radin, 1972; Fig. 2.3a and 2.3b). Secondary cortex proliferation is an aspect of storage root formation in radish (*Raphanus sativus*), and the similarities between clubroot formation and storage root formation have been described previously by Gustafsson et al.

(1986).

Despite the confounding nature of *P. brassica* gall anatomy, it seems likely that the mechanism for spheroid gall development may be characterized by a lack of secondary tissue development, infection and proliferation in the subtending host tissues during disease progression. This has been evidenced in the work of Larson (1934) and in the current study. Stele distention, secondary tissue development, and ray infection and expansion are all means for generating large spindle-shaped galls, and all of these aspects are clearly absent from spheroid galls observed in this study (Kunkel, 1918; Fig. 2.4; Fig. 2.2). It seems that the distribution of *P. brassicae* within the vascular cambium and the proliferation of vascular cambium derivatives is either absent from spheroid galls, or is inhibited as infection and movement within this meristem would lead to infected secondary tissue proliferation in a manner described by Kunkel (1918). It is uncertain if the spheroid galls described by Larson (1934), Seaman et al. (1963), Buczacki et al. (1975) and Osaki et al. (2008) are comparable to the small, necrotically delimited 'minigalls' of Kobelt et al. (2000). These minigalls were demarcated by necrotic tissue, with the pathogen developing to the mature resting spore stage while encapsulated by the host hypersensitive reaction. The minigalls were observed between the stele and cortex, where the stele was largely intact (Kobelt et al., 2000).

With consideration for the treatment of spheroid galls as resistance structures, it appears that a cellular or developmental mechanism is minimizing either the movement of the pathogen in the host or the impact of the pathogen on

gall development. Whether an inhibition of the pathogen results from a restriction of myxameoboid movement was not conclusively demonstrated in the current study, but is likely due to the lack of pathogen spread in the observed tissues. An active host defensive reaction, in the form of cell lignification or the hypersensitive response, was not observed in this study, but was reported in *A. thaliana* ecotypes containing the RPB1 gene (Kobelt et al., 2000).

The observation, both in this study (Fig. 2.6) and by Osaki et al. (2008), that resting spores of *P. brassicae* are produced in spheroid galls, underscores the need to re-evaluate the view that these structures are indicative of complete resistance to clubroot and further suggests that spheroid galls can contribute to soil inoculum loads in the field. Spheroid galls appear to result from the expansion of extra-stelar tissues, since infection and expansion of the stele characteristic of spindle galls is not observed. The results from this study are consistent with our hypothesis that spheroid gall formation represents a limitation of myxamoebal movement in the host.

2.6. Tables

Table 2.1. Interaction of various *Brassica* species with populations of *Plasmodiophora brassicae* and the degree of spheroid gall formation in three distinct bioassays.

		Plasmodiophora brassicae population			
Host	ECD code	SACAN03-1	SACAN03-4	CDCN04-1	AbotJE04-1
Brassica rapa var. *rapifera*	3	na[a] / -[b] / sd[c]	+[d] / na / +	+ / na / +	na / na / -
B. napus var. *napobrassica* cv. Wilhemsburger	10	+ / 2+[e] / +	sd / - / +	na / - / sd	na / - / -
B. oleracea var. *capitata* cv. Badger Shipper	11	na / - / -	na / - / +	+ / 2+ / +	- / 2+ / +
B. oleracea var. *capitata* cv. Bindsachsener	12	na / - / +	+ / - / +	+ / 2+ / +	na / sd / sd
B. oleracea var. *capitata* cv. Jersey Queen	13	2+ / 2+ / 2+	na / 2+ / 2+	na / - / sd	+ / 2+ / 2+
B. oleracea var. *acephala* subvar. *laciniata* cv. Verheul	15	na / - / +	na / - / -	+ / 2+ / 2+	+ / na / +

[a] na indicates insufficient data available
[b] - indicates no galls were observed
[c] sd indicates that only spindle galls were observed
[d] + indicates that <25 percent of the galled plants exhibited spheroidal galls
[e] 2+ indicates that ≥25 percent of the plants exhibited spheroidal galls.

Table 2.2. Summary of the typical anatomy of spindle galls, spheroid galls and normal roots.

	Region of tissue proliferation				Resting spores	
	Stele	n	Extra-steler	n	Mature	n
Spheroid gall	Yes	1	Yes	9	Yes	1
	Limited	8	Limited	0	Limited	0
	No	0	No	0	No	8
Spindle gall	Yes	7	Yes	8	Yes	8
	Limited	1	Limited	0	Limited	1
	No	0	No	0	No	0

Sample sizes are given as the total number of samples (n) exhibiting this trait.

2.7. Figures

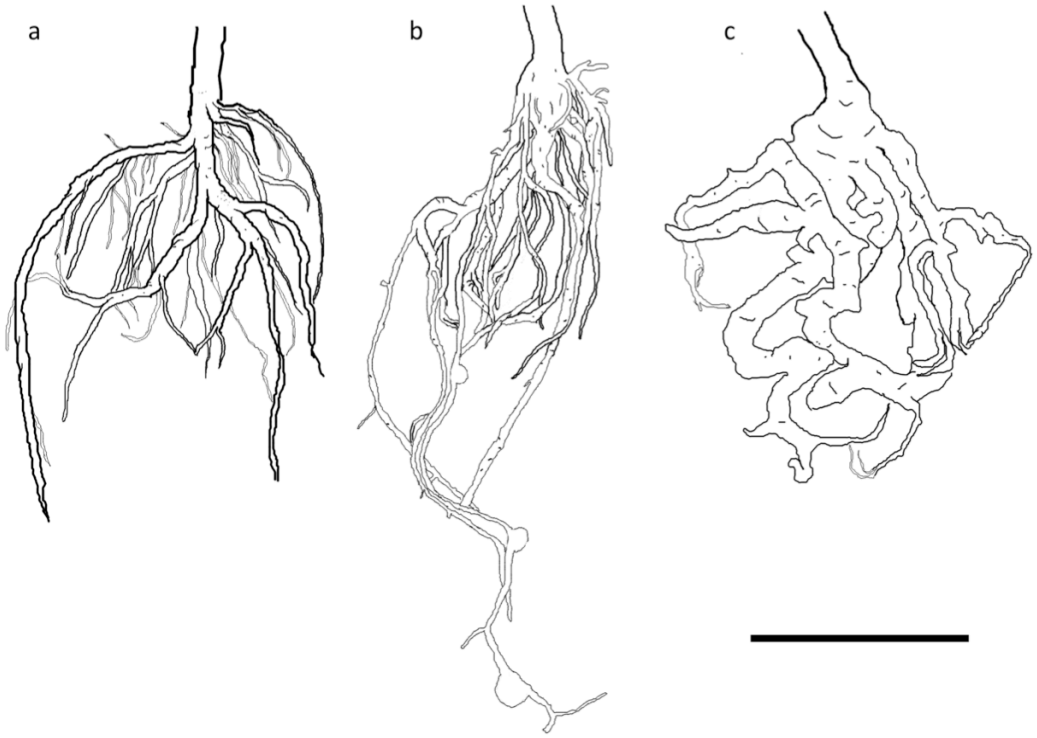

Figure 2.1. Line drawings depicting a healthy *Brassicae oleracea* root (a), and roots with spheroidal galls (b) and spindle galls (c) caused by *Plasmodiophora brassicae* infection. The scale bar denotes 2.5 cm (D. Rennie).

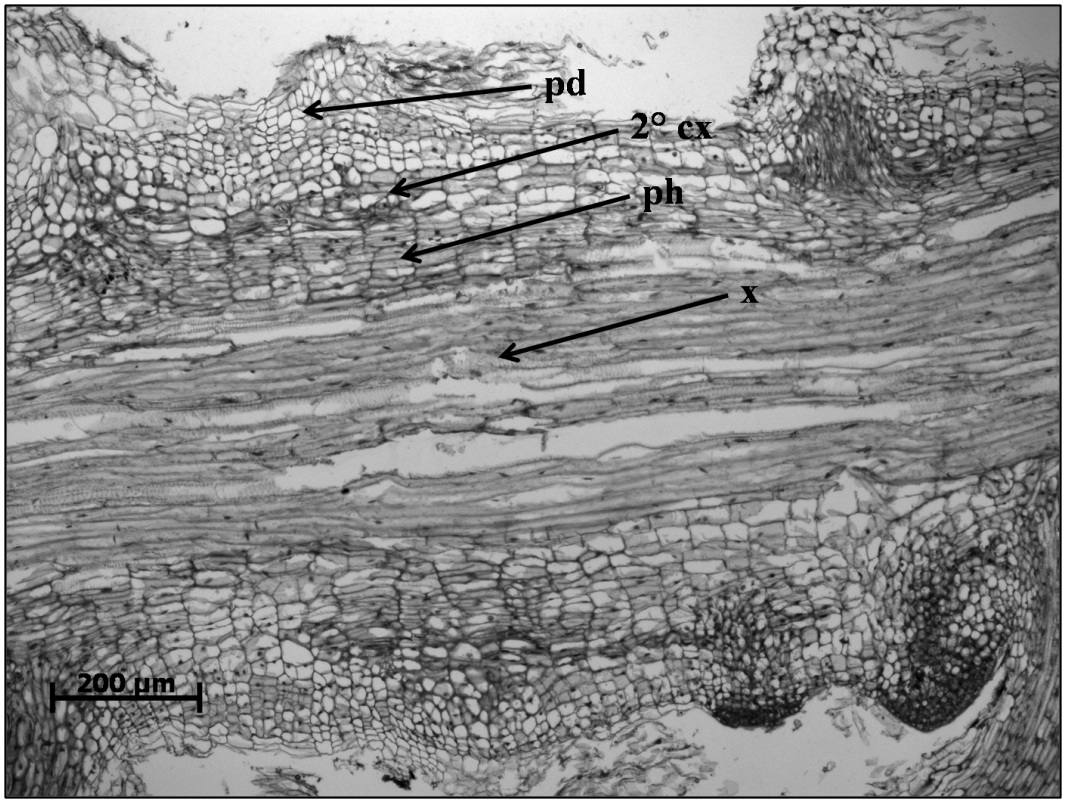

Figure 2.2. Median longitudinal section through a healthy *Brassica oleracea* var. *capitata* cv. Jersey Queen root. Image was taken with a brightfield microscope at 10× magnification. Annotations are as follows: x, xylem (primary and secondary); ph, phloem (primary and secondary); 2° cx, secondary cortex; pd, periderm.

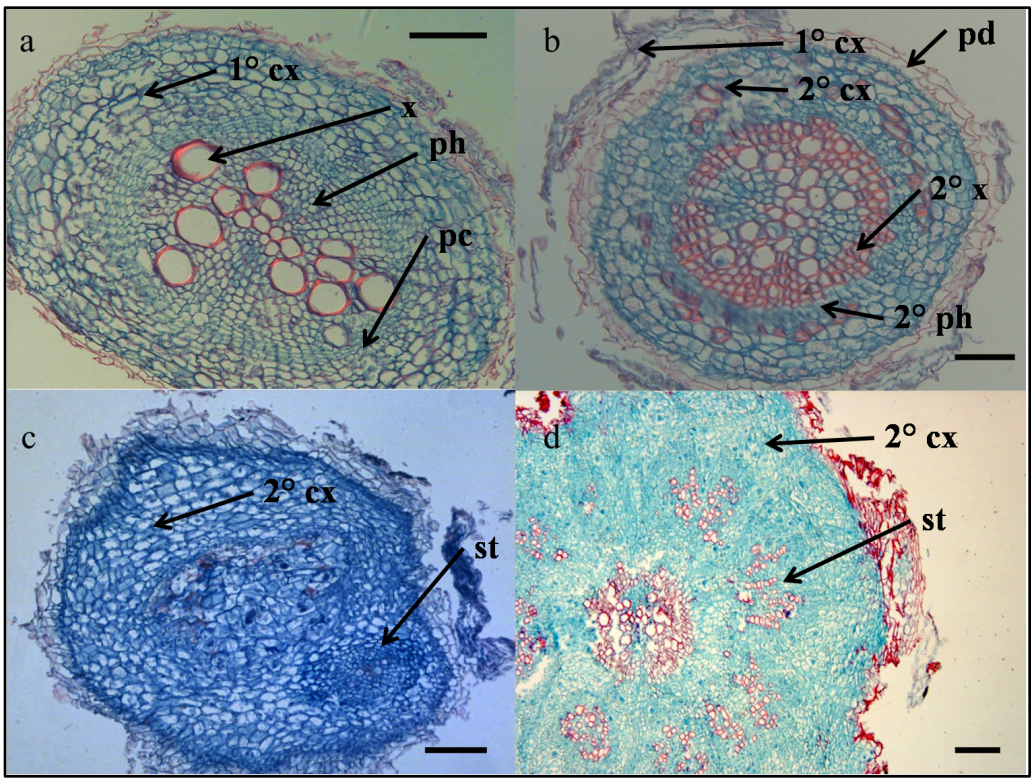

Figure 2.3. Safranin fast green -stained radial sections through healthy and *Plasmodiophora brassicae* infected plants. a. 20x view of a healthy root of *Brassica oleracea* var. *capitata* cv. Badger Shipper, b. 10× view of a healthy root of *B. oleracea* var. *acephala* cv. Verheul, c. 10× view of a spheroid gall on *Brassica napus* var. *rapifera* cv. Wilhemsburger after infection with the *P. brassicae* population SACAN03-1, d. 10× view of a spindle gall harvested from *B. oleracea* var. *capitata* cv. Bindsachsener 45 days after inoculation with SACAN03-1.
Annotations are as follows: 1° x, primary xylem; 2° x, secondary xylem; 1° ph, primary phloem; 2° ph, secondary phloem; pc, pericycle; 1° cx, primary cortex; 2° cx, secondary cortex; pd, periderm; st, stele (central region of vasculature surrounded by cortex and delimited by phloem).
Scale bars are all 100µm.

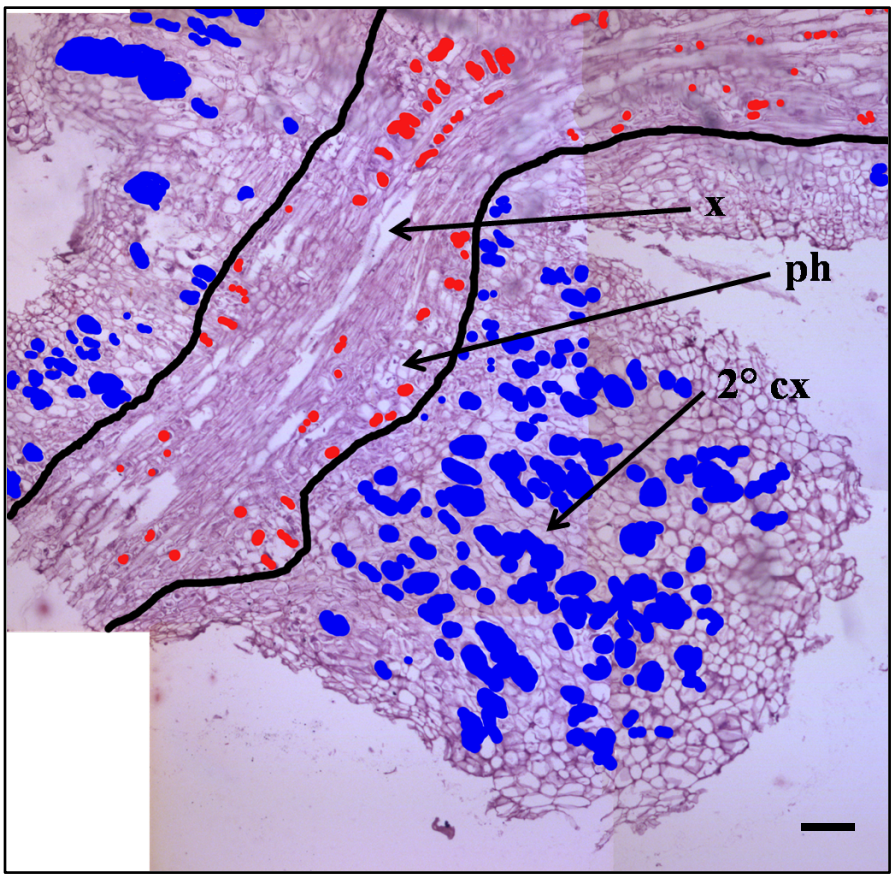

Figure 2.4. Median longitudinal section through a spheroid gall harvested from *Brassica oleracea* var. *capitata* cv. Jersey Queen 45 days after inoculation with the population SACAN03-1 of *Plasmodiophora brassicae*. Complete view of the gall was made by aligning three images at 10× magnification. The image is overlaid with an anatomical map, where plasmodia developing within (red) and external (blue) to the stele (black lines) are highlighted. Annotations are as follows: x, xylem (primary and secondary); ph, phloem (primary and secondary); 2° cx, secondary cortex. Scale bar denotes 100µm.

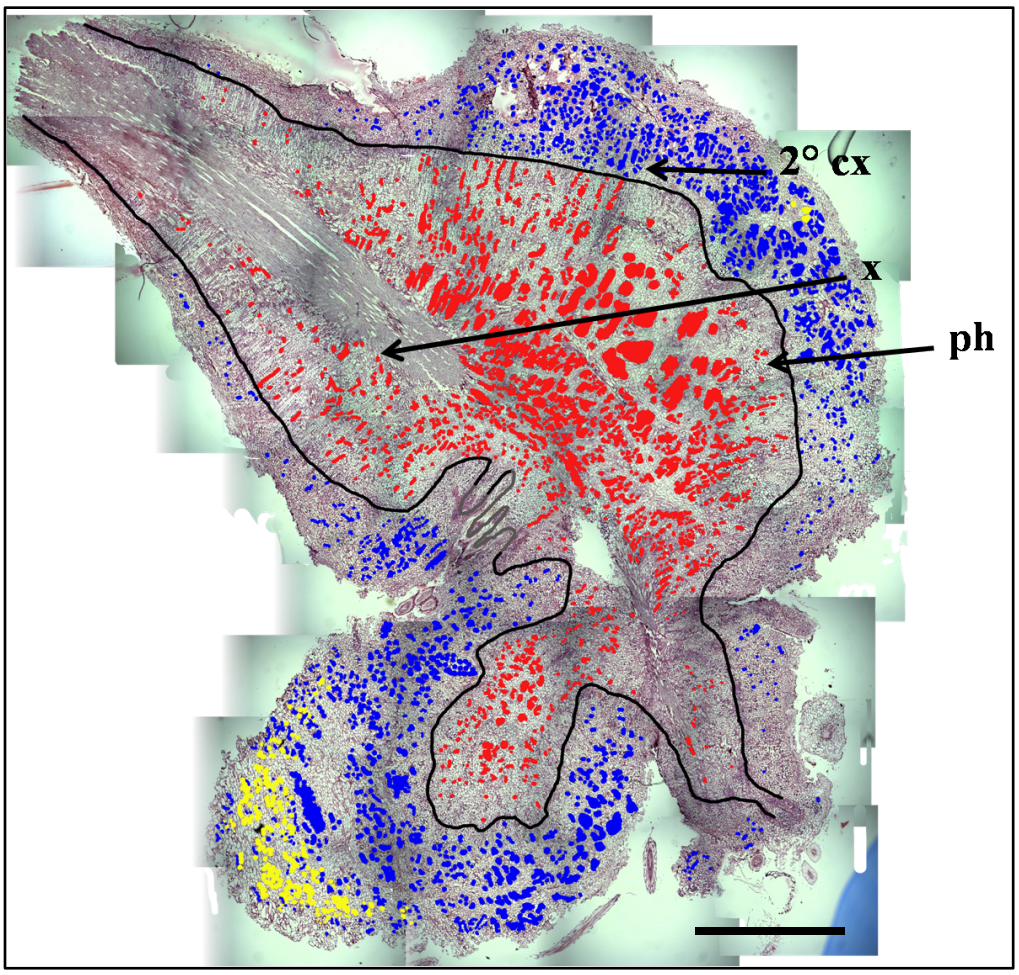

Figure 2.5. Median longitudinal section through a spindle gall harvested from *Brassica oleracea* var. *capitata* cv. Jersey Queen 45 days after inoculation with population SACAN03-1 of *Plasmodiophora brassicae*. Complete view of the gall was made by aligning multiple images at 4× magnification. The image is overlaid with an anatomical map, where plasmodia developing within (red) and external (blue) to the stele (black lines) are highlighted. Resting spore development and maturation is indicated (yellow). Annotations are as follows: x, xylem (primary and secondary); ph, phloem (primary and secondary); 2° cx, secondary cortex. Scale bar denotes 1cm.

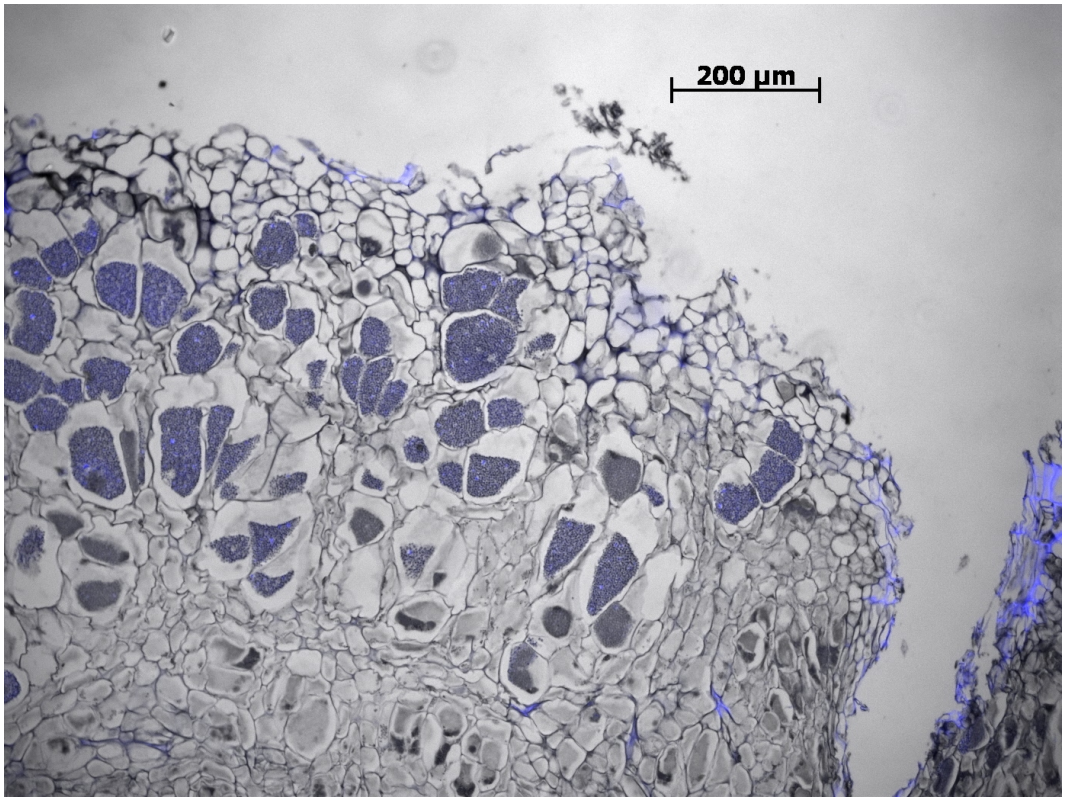

Figure 2.6. Median longitudinal section through a spheroid gall harvested from *Brassica oleracea* var. *capitata* cv. Jersey Queen 45 days after inoculation with the population SACAN03-1 of *Plasmodiophora brassicae.* The image is rendered from the superimposition of a fluorescent (blue fluorescence) and a brightfield image. Infected host cells filled with mature resting spores are self evident and readily fluoresce blue.

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Chapter 3: Direct evidence of surface infestation of seeds and tubers by *Plasmodiophora brassicae* and quantification of spore loads[1]

3.1. Introduction

The obligate parasite *Plasmodiophora brassicae* is the causal agent of clubroot, an important disease of both cultivated and wild species in the Brassicaceae family. Infection by this pathogen can result in considerable yield and quality losses in susceptible crops, accounting for a 10-15% reduction in yields on a global scale (Dixon, 2009). Clubroot has recently emerged as a major constraint to canola (*Brassica napus*) production in the province of Alberta, Canada (Howard *et al.*, 2010). While only 12 clubroot-infested fields were found when the disease was first identified on canola in central Alberta in 2003 (Tewari *et al.*, 2005), more than 450 infested fields were reported by 2009 (Strelkov *et al.*, 2010), with the outbreak spreading to southern and eastern regions of the province.

The life history of *P. brassicae* consists of primary and secondary phases occurring in the root hairs and periderm of the host, respectively (Buczaki, 1983).

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Clubroot development is initiated by the germination of pathogen resting spores and encystment of primary zoospores in the root hairs, followed by the formation of primary plasmodia and release of secondary zoospores that infect the root cortex. Gall formation is a consequence of this cortical infection and the resulting hyperplasia and hypertrophy of the affected root tissues. Resting spores are formed within the galled roots and are dispersed by various means after root decomposition (Buczaki, 1983). The resting spores can remain viable in the soil for many years, with the potential to infect any nearby host (Wallenhammar, 1996).

While clubroot-resistant cultivars of *B. napus, Brassica oleracea* and *Brassica rapa* are available, their extensive use is often limited by the short durability and race or pathotype-specificity of the resistance (Diederichsen et al., 2009; Voorrips, 1995). An effective clubroot management plan, therefore, requires the use and integration of various tools for disease control (Diederichsen *et al.*, 2009; Donald and Porter, 2009). Sanitary practices, crop rotation, chemical control and other means are promoted to effectively manage clubroot disease (Donald and Porter, 2009). In Alberta and the neighboring provinces of western Canada, efforts to contain the clubroot outbreak have focused largely on the use of best management practices, including cleaning and disinfesting of field equipment by physical and chemical means, since *P. brassicae* resting spores can be disseminated with infested soil carried on farm machinery, vehicles, tools,

footwear and other types of equipment operated in fields with a history of clubroot (Cao et al., 2009; Howard et al., 2010).

While soilborne transmission of clubroot on tools and equipment represents one of the principal modes of pathogen dispersal (Cao et al., 2009; Donald and Porter, 2009), the dissemination of *P. brassicae* resting spores as external seed contaminants has also been suggested. Eriksson (1930) described an account of clubroot on turnips in Sweden that reportedly was caused by seedborne inoculum, while Gibbs (1931) also documented several cases of clubroot that were anecdotally attributed to seedborne infestations. More recently, two cases of clubroot in New South Wales, Australia, were confirmed in white mustard (*Sinapis alba*) plants grown from a Canadian seed stock, but it is unknown if the seeds were the source of the inoculum (Hind-Lanoiselet and Parker, 2005). To our knowledge, however, only one early study (Warne, 1943) has explicitly examined the question of seedborne transmission of *P. brassicae*. This research was limited to the sowing of untreated and surface-sterilized seeds of two swede (*B. napus*) cultivars in pots, with subsequent assessment of the roots for clubroot symptom development. While restricted in its scope, this study did provide indirect evidence for the presence of *P. brassicae* inoculum on some of the seeds (Warne, 1943).

Given the concern associated with the potential for clubroot to infect Canadian canola crops on a wide geographical scale, it is important to properly

understand the extent of the risk associated with seedborne transmission of this disease. Hypothetically, infestation of seeds and tubers by *P. brassicae* may represent a secondary mode of inoculum dispersal, and it is possible that the level of inoculum found on seeds harvested from clubroot-infested fields could be sufficient to cause disease symptoms. In this context, the objectives of the present study were to: (1) develop a reliable, quantitative PCR (qPCR)-based assay to measure the numbers of *P. brassicae* resting spores present in soil and dust associated with seeds and potato tubers, (2) estimate the levels of inoculum found on seeds of common field crops and potato tubers grown on clubroot-infested fields in Alberta, (3) assess the viability of this inoculum through staining and microscopic observation, and (4) compare resting spore levels found on seeds and tubers to those required to cause disease under greenhouse conditions. Knowledge of whether or not seedborne dissemination of *P. brassicae* represents a viable mechanism for clubroot spread is critical to properly understand the epidemiology of this pathogen in agricultural systems, as well as for the development of effective strategies to limit the spread of clubroot in Canada and elsewhere. Seedborne dissemination could undermine the effectiveness of the rigorous sanitation and exclusion practices currently employed by many in the agricultural industry.

3.2. Materials and Methods

3.2.1. Inoculum of *Plasmodiophora brassicae*

Galled roots of *Camelina sativa* (false flax), which had been inoculated with population SACAN03-1 of *P. brassicae* (Séguin-Swartz et al., 2009), were used as the source of inoculum for all components of this study. SACAN03-1 is a highly virulent population of the pathogen originally collected from infected canola plants in central Alberta (Strelkov et al., 2006). This population has been classified as pathotype 3, 16/15/12, or P_2 on the differentials of Williams (1966), the European Clubroot Differential series (Buczacki et al., 1975), and Somé et al. (1996), respectively (Strelkov et al., 2006). Clubroot galls were stored at -20°C until needed. The resting spores were isolated by homogenization of infected root tissue with a blender, filtration of the homogenate through a 450 µm sieve, and centrifugation in a 50% sucrose gradient as per the protocols of Castlebury et al. (1994) and Cao et al. (2007). The resulting spore pellets were re-suspended in 5% (v/v) glycerol, and the resting spore concentrations were quantified with a hœmocytometer and adjusted as needed with 5% (v/v) glycerol. Glycerol facilitates the dispersal of *P. brassicae* spores, which often tend to clump in aqueous suspensions.

3.2.2. Artificial infestation of canola seeds

Seeds of the clubroot-susceptible canola cv. 34-65 RR (Monsanto Canada) were surface-sterilized by soaking in 1% (v/v) sodium hypochlorite (bleach) for 5 minutes and rinsing with sterile distilled water (sd-H_2O), after which they were air

dried. Aliquots (10.5 g) of the surface-sterilized seeds were placed in labeled, sterile 50 mL tubes (Corning, New York, NY). The approximate number of seeds per g was estimated by weighing three replicates of 100 seeds each, and a 2 mL volume of a resting spore suspension (1.3×10^9 resting spores per mL) was added to one of the aliquots to infest the seeds at a concentration of 1×10^6 resting spores per canola seed. Following addition of the spore suspension, the seeds were vortexed and shaken vigorously to ensure uniform distribution of the inoculum. The remaining resting spore suspension was serially diluted with 5% (v/v) glycerol and used to infest additional 10.5 g seed aliquots at concentrations of 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, and 1×10^0 resting spores per canola seed, as described above. Untreated, surface-sterilized seeds were used as a control. The artificially infested seed samples were placed in a glass seed desiccator over silica desiccant and stored at 4°C until dry.

3.2.3. Seeds and potato tubers assessed for clubroot infestation

A total of 45 different seedlots and one lot of potato tubers were assessed for the occurrence and quantity of *P. brassicae* inoculum via qPCR analysis and resting spore viability staining. These included two canola, two barley (*Hordeum vulgare*) and three wheat (*Triticum aestivum*) samples from crops grown on clubroot-infested fields in central Alberta; these samples, which ranged in mass from 50 g to 2000 g, were kindly donated by area farmers shortly after harvest and had not been commercially cleaned. In addition, 100 g aliquots of commercially cleaned seedlots of rye (*Secale cereale*; one sample), oat (*Avena sativa*; two samples), barley (six samples), wheat (six samples) and pea (*Pisum*

sativum var. *arvense*; 15 samples) were collected from a seed cleaning plant in Sturgeon County, Alberta, located in the centre of the clubroot outbreak, and provided for the study by Mr. M. Hartman (Alberta Agriculture and Rural Development, Lacombe). The selection of seeds from the seed cleaning plant was random and no information was available as to which seedlots, if any, actually came from clubroot-infested fields. One lot of potatoes (*Solanum tuberosum*) (consisting of six small tubers) and four (100 g) samples of peas grown on a clubroot-infested field in Newell County, southern Alberta, were also included in the analysis, as were an additional four (100 g) pea seed samples grown on an infested field in Leduc County, central Alberta (R.J. Howard, *unpublished data*).

3.2.4. Seed/tuber washes and DNA extraction

The washing protocol used to collect resting spores from seed or tuber surfaces was based on MacNeil *et al.* (2004) with some modifications. A 10 g aliquot of seeds was placed in a 250 mL Erlenmeyer flask and 25 mL sd-H_2O was added, or in the case of the potatoes, two tubers were placed in a generic 1 L Pyrex dish and 100 mL sd-H_2O was added. The flask or dish was then agitated on an orbital mixer at 150 rpm for 1 h. Each seed wash was decanted into a 50 mL conical tube (Corning), while each tuber wash was transferred into a 250 mL polypropylene bottle (Nalgene, Rochester, NY). An additional 25 or 100 mL of sd-H_2O was added to each flask or dish containing the seeds or tubers, respectively, and these were agitated again for a brief period. The supernatants were decanted and pooled with the previously collected washes. Seed and tuber wash suspensions were centrifuged at 4894 × *g* for 20 min in a swinging bucket rotor. The supernatants

were discarded, and the pellets re-suspended in the remaining supernatant and transferred to 2 mL Lysing E Matrix tubes (MP Biomedicals). The tubes were centrifuged at 14000 rpm for 1 min and the supernatants discarded. Total DNA was then extracted from the resting spore pellets with a FastDNA® Spin Kit for Soil (MP Biomedicals) as per the manufacturer's instructions.

3.2.5. PCR analysis

Quantitative PCR analysis was performed on undiluted DNA extracted from the seed or tuber washes, or after dilution (1/2, 1/10, or 1/100 v/v) with sd-H_2O if the presence of PCR inhibitors was suspected. The primers DC1F (5'-CCTAGCGCTGCATCCCATAT-3') and DC1R (5'-CGGCTAGGATGGTTCGAAAA-3') were designed using Primer Express Software v. 3.0 (Rozen and Skaletsky, 2000) based on a partial 18S rRNA gene sequence from *P. brassicae* available in GenBank (accession no. AF231027). All qPCR amplifications were conducted using a StepOnePlus Real Time PCR System (Applied Biosystems, Carlsbad, CA) in a 10 µL volume containing 2.5 µL of the template DNA solution, 2.5 µL of a 3.2 mM DR1F and DR1R primer mix, and 5 µL Dynamite qPCR Mastermix (Molecular Biology Service Unit, University of Alberta, Edmonton, Canada), which contains SYBR Green (Molecular Probes) as the detection dye. Reaction conditions consisted of an initial heat denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 15 s and 60°C for 60 s. A melting point analysis was conducted at the end of each reaction, and the presence of a single amplification product confirmed (Scherr et a*l.* 2001; Etebu and Osborn 2010).

A preliminary qPCR screen was performed on DNA extracted from a single subsample of each of the 46 seed and tuber samples. Each sample that tested positive for the presence of *P. brassicae* in this initial screening was evaluated further by the extraction of DNA from three additional subsamples, which were subsequently analyzed by qPCR in three parallel reactions per subsample (for a total of nine qPCR amplifications per original seed or tuber lot). Infestation by the clubroot pathogen was further confirmed by conventional PCR as described previously (Cao et al., 2007), using the *P. brassicae*-specific primers TC1F (5′-GTGGTCGAACTTCATTAAATTTGGGCTCTT-3′) and TC1R (5′-TTCACCTACGGAACGTATATGTGCATGTGA-3′). The amplification products were resolved on 1% agarose gels stained with SYBR® Safe (Invitrogen, Carlsbad, CA) and visualized under UV light in a Syngene BioImaging System (Synoptics Inc., Frederick, MD).

Standard curves for the quantification of *P. brassicae* resting spores were generated with DNA isolated from known quantities of spores, which had been purified on a sucrose gradient as noted above. Briefly, total DNA was extracted from 1×10^9 resting spores and serially diluted with sd-H_2O at 10-fold intervals down to a dilution of 1×10^{-2}; DNA from each dilution in the series was then used as a template in qPCR under the aforementioned conditions. The average threshold cycle (C_T), linear regression coefficient (R^2), line equation and PCR efficiency (E) were calculated from four independent sets of serial DNA dilutions. In order to validate the seed wash, DNA extraction and qPCR protocols, as well as the standard curves, total DNA was extracted from washes of canola seeds that

had been artificially infested at known concentrations ranging from 1×10^{6} to 1×10^{-2} resting spores per seed; these DNA samples were subjected to qPCR as above. The C_T value and 95% confidence interval for each infestation level were calculated based on analysis of four independent sets of artificially infested seeds.

The specificity of primers DC1F and DC1R was assessed by subjecting 5 ng and 50 ng aliquots of DNA from each of two plant, five bacterial and 26 fungal species (Table 3.1) to qPCR analysis as above. Genomic DNA from each of these species was obtained as described in Cao et al. (2007). The C_T value, average product melting temperature (T_M) and 95 % confidence intervals for both C_T and T_m were calculated for a minimum of two parallel qPCR reactions conducted with aliquots of DNA from each plant, fungal or bacterial species. Primer specificity was further confirmed by conventional PCR analysis (*data not shown*).

3.2.6. DNA sequencing

The amplicon generated with primers DC1F and DC1R from DNA of *P. brassicae* population SACAN03-1 was subjected to sequence analysis to confirm that the expected product was obtained. The amplification products from two independent qPCR assays were visualized on agarose gels and purified with a Qiaquick Gel Extraction Kit (Qiagen, Toronto, ON) as per the manufacturer's instructions. The purified amplicons were then sequenced in the forward and reverse directions using primers DR1F and DR1R, respectively, with sequencing performed on a 3740 DNA Analyzer (Applied Biosystems) at the Molecular Biology Services Unit, University of Alberta, using a BigDye® Terminator Sequencing Kit (Applied Biosystems).

3.2.7. Viability staining

The viability of *P. brassicae* resting spores on seed and tuber samples was assessed using an Evan's blue vital staining protocol developed by Tanaka et al. (1999). Briefly, Evan's blue stain (20 mg/mL) (Sigma-Aldrich, St. Louis, MO) was mixed in equal proportions with 500 µL of the concentrated seed and tuber washes described above. After a 15 to 30 min incubation period, the seed wash/Evan's blue solution was diluted 1:4 in 5% (v/v) glycerol and a 30 µL aliquot transferred to a glass microscope slide. Thirty resting spores from each sample were examined for cytoplasmic staining (an indication of non-viability) using bright-field microscopy on a Primo Star light microscope (Carl Zeiss) and the numbers of dead and viable spores were recorded.

3.2.8. Bioassays with infested seeds

In order to determine the infestation level at which visible clubroot symptoms could be obtained, two separate plant bioassays were conducted with artificially infested canola seeds. Limited quantities of naturally infested seeds precluded their inclusion in these bioassays. The artificially infested canola seeds were sown either in 39 × 26 × 15 cm plastic trays (bioassay 1) or 7.5 cm diameter plastic pots (bioassay 2) filled with, respectively, 2000 or 500 g of Sunshine #4 Mix/LA7 soil medium (SunGro Horticulture). Seeds were sown at a density of 120 seeds/m^2 in both bioassays, as this corresponds to the recommended seeding rate for canola in Alberta (Anonymous, 2007). In the trays, 12 seeds were sown as two discrete rows, whilst in the pots three seeds were spaced out equally over the entire surface area. The trays in bioassay 1 were kept in a greenhouse at an average temperature

of 20 ± 2°C, under natural light supplemented by artificial light and a 16 h photoperiod. The pots in bioassay 2 were maintained in a growth cabinet with a 16 h photoperiod at 21°C/18°C (day/night). Trays and pots were kept saturated with water for the first two weeks after sowing. Thereafter, the plants were watered and fertilized as required. Treatments in both bioassays were arranged in a randomized complete block design, with four replicates (each consisting of one tray or four pots) per treatment.

Six weeks after sowing, the plants were gently uprooted, washed with water and scored for clubroot symptom development on a 0 to 3 scale as per Kuginuki et al. (1999), where: 0 = no galling; 1 = a few small galls; 2 = moderate galling; and 3 = severe galling. These scores were used to calculate an index of disease (ID) for each treatment according to the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006). Any galls that were not obviously symptoms of clubroot were subjected to conventional PCR analysis (Cao et al., 2007) to confirm *P. brassicae* infection. The 95% confidence interval for each treatment was calculated and all data were plotted using R software (R Development Core Team, 2008).

3.3. Results

3.3.1. Validation of qPCR

Sequence analysis of the amplicon obtained with primers DC1F/DC1R in two separate qPCR assays revealed it to be 90 bp in size with 100% sequence

similarity to 13 *P. brassicae* accessions deposited in GenBank, confirming that the correct product was amplified. The experimentally determined average (± 95% confidence intervals) T_M of this amplicon was 85.18 ± 0.12°C. Primer specificity was assessed using genomic DNA from a collection of 33 plant, fungal and bacterial species as (Table 3.1) the template in qPCR, and further corroborated by conventional PCR (*data not shown*). Non-specific amplification of DNA from these species was on average not observed until a C_T of greater than 29.76 ± 0.56 when using 5 ng of template, or 28.28 ± 1.04 with 50 ng of template. A two-tailed t-test indicated a significant difference ($p = 0.006$) between these two C_T values and, therefore, 28.28 ±1.04 was taken as the lower limit of detection. Similarly, reactions in which the C_T was less than 11 were not regularly reproducible, and were also excluded from the analysis. All non-*P. brassicae* templates also could be clearly distinguished from true positives (i.e., *P. brassicae* DNA) through the T_M analysis. Thus, only those samples with a C_T value between 11 and 28.28, and which had a melting point consisting of a single peak that was comparable to the experimentally verified T_M of 85.18 °C, were considered accurate for inoculum quantification purposes.

Reproducible linear standard curves could be generated from DNA extracted from between 1×10^7 to 1×10^3 resting spores of *P. brassicae* (Fig. 3.1). A good PCR amplification efficiency ($E = 0.98$) was confirmed from the slope of these standard curves, with a strong correlation ($R^2 = 0.99$) observed between the quantity of *P. brassicae* DNA and the corresponding C_T value (Fig. 3.1). The estimated numbers of resting spores calculated based on the qPCR analysis were

on average 56% lower than the theoretical levels of infestation, as defined on the artificially infested canola seed samples over the established detection range (Fig. 3.1). The average confidence interval for C_T value in the artificially infested seedlots was 1.01, indicating that the extraction procedures were consistent over the established detection range (Fig. 3.1).

3.3.2. Quantification of *P. brassicae* inoculum on seed and tuber lots

Of the 46 seed and tuber lots analyzed in this study, resting spores of *P. brassicae* could be reliably and unequivocally quantified on seven (15.2%), all of which also tested positive by conventional PCR (Table 3.2). The highest level of infestation was detected on wheat seeds harvested from a clubroot-infested field in central Alberta (sample 1), which yielded an average C_T value of 19.94 ± 0.45 (equivalent to approximately 3.43×10^4 resting spores per 10 g of seeds). Potato tubers harvested from an infested field in southern Alberta (sample 4) gave a C_T value of 24.63 ± 0.27 after a 10-fold dilution of the extracted DNA, which corresponded to approximately 1.40×10^4 resting spores per tuber when corrected for this dilution. An average C_T value of 23.08 ± 0.91 (equivalent to 4.04×10^3 resting spores per 10 g of seeds) was obtained for a lot of canola seeds harvested from another infested field in central Alberta (sample 2). Resting spores of *P. brassicae* were also found on four separate lots of pea seeds, one originating from a seed cleaning plant in the center of the clubroot outbreak (sample 3), and three more originating from clubroot-infested field plots in southern Alberta (samples 5, 6 and 7). The levels of infestation on these pea samples were fairly low, with C_T values ranging from 25.94 ± 9.54 to 28.00 ± 0.60 (or less than 1.00×10^3 resting

spores per 10 g of seeds). Sample 3 was the only commercially cleaned sample, of 30 tested, on which quantifiable levels of inoculum were found.

3.3.3. Spore viability staining

Resting spores of *P. brassicae* could be observed microscopically after Evan's blue staining of all seed and tuber washes that had tested positive by qPCR, serving to corroborate that analysis (Table 3.2). In those spores presumed to be non-viable (Tanaka *et al.*, 1999), the cytoplasm became prominently stained with the Evan's blue dye. Based on the cytoplasmic staining reaction, the vast majority of resting spores examined appeared to be viable. Indeed, 100% of resting spores from pea sample 7 and 98% of spores from pea sample 3 were viable. Resting spores examined from pea samples 5 and 6 appeared to be 97% viable, while spore viability was 90% in the canola seed (sample 2) and potato tuber washes (sample 4). The lowest viability was observed for resting spores from the wheat seed washes (sample 1), at 80% unstained spores (Table 3.1).

3.3.4. Bioassays with infested seeds

Canola plants were examined for the presence and severity of clubroot symptoms six weeks after sowing of the artificially infested seeds. At that time, mature inflorescences had developed on most plants and root development was extensive. The results from the bioassays are summarized in Fig. 3.2. In bioassay 1, in which the plants were grown in 39 × 26 × 15 cm plastic trays, no symptoms of disease were detected in the non-infested control (0 spores per seed) or at infestation levels of 1×10^1 or 1×10^2 resting spores per seed. A single small gall, however, was observed at 1×10^0 resting spore per seed, resulting in an ID of

1.85 ± 3.63% for this treatment. Given its small size and isolated occurrence, this gall was tested by conventional PCR with *P. brassicae*-specific primers (Cao et al., 2007) and confirmed to be positive for clubroot. In contrast, a moderate amount of galling was found at infestation levels of 1×10^3 resting spores per seed (ID = 13.47 ± 2.64%) and 1×10^4 resting spores per seed (ID = 12.86 ± 12.13%). The most severe symptoms of clubroot were detected at infestation levels of 1×10^5 resting spores per seed (ID = 50.38 ± 15.59%) and 1×10^6 resting spores per seed (ID = 34.97 ± 8.66%) (Fig. 3.2).

Similar results were obtained in bioassay 2, in which the seeds were sown in 7.5 cm diameter plastic pots. No symptoms of clubroot were observed at 0, 1×10^0, 1×10^1 or 1×10^2 resting spores per seed, while consistent galling was found at the higher infestation levels. At 1×10^3 resting spores per seed, an ID of 3.33 ± 6.53% was obtained. The disease severity increased considerably at an infestation level of 1×10^4 resting spores per seed, with an ID of 36.23 ± 0.25%. At an infestation level of 1×10^5 resting spores per seed, the ID was 37.73 ± 15.15%, while at 1×10^6 resting spores per seed, it was 42.99 ± 10.76% (Fig. 3.2.).

3.4. Discussion

Given the persistence of *P. brassicae* resting spores in the soil, eradication of this pathogen is difficult if not impossible once it becomes established in a field. Therefore, much of the clubroot management effort in the Canadian outbreak on canola has been focused on exclusion of *P. brassicae* from disease-free fields

(Howard et al., 2010). For such an approach to be successful, however, all mechanisms of pathogen dispersal must be considered. The present assessment of seeds and tubers from clubroot-infested fields in Alberta supports the hypothesis that infestation of propagative materials by *P. brassicae* may represent a secondary mode of inoculum dispersal, although the level of inoculum found on most samples was lower than that required to cause consistent symptoms of disease.

While the infestation of seeds by *P. brassicae* has been suggested previously (Warne, 1943), it has not been demonstrated directly, since the pathogen is an obligate parasite that cannot be cultured on axenic medium. In recent years, the detection of *P. brassicae* has been facilitated by the development of molecular techniques to identify the pathogen (Faggian and Strelkov, 2009). However, most of these approaches have been qualitative in nature, and if the risk posed by seedborne clubroot is to be properly evaluated, an assessment of the amount of inoculum present is also required. The qPCR protocol developed in the current study represents an important tool for conducting such a quantitative assessment. Analysis of the artificially infested seedlots revealed that the resting spore numbers calculated using the qPCR assessment were about 56% lower than the theoretical values, which likely reflected spore and/or DNA loss during the extraction procedures. Nevertheless, a strong linear correlation ($R^2 = 0.99$) was observed between the amount of *P. brassicae* DNA and the corresponding C_T value. Moreover, while the numbers of spores on the artificially infested seeds may have been somewhat underestimated, the small confidence intervals indicate

that the data were consistent (Fig. 3.1). Therefore, the qPCR-based assay appears to be a reliable method to estimate the numbers of *P. brassicae* resting spores on soil and dust associated with seeds and tubers, and this method was used to assess the levels of infestation on propagative materials from clubroot-infested regions in Alberta.

Quantifiable numbers of *P. brassicae* resting spores were found on 7 of 46 field-grown seed and tuber lots analyzed by qPCR (Table 3.2). The level of infestation found on four of these samples (nos. 3, 5, 6 and 7, representing peas) was very low and likely would not have been of concern in a field situation. The number of resting spores found on the remaining three samples (representing wheat, potatoes and canola) was higher, but still generally lower than that required to cause consistent development of clubroot symptoms in greenhouse bioassays. The infestation level of 4.04×10^3 resting spores per 10 g of seed found on one of the canola seedlots (sample 2) corresponded to about two resting spores per seed, at which an ID of 1.85 ± 3.63% was obtained in bioassay 1 (Fig. 3.2). The highest levels of inoculum, however, were found on wheat seeds (sample 1) and potato tubers (sample 4) harvested from clubroot-infested fields (Table 3.2). While these crops do not serve as hosts for *P. brassicae*, they may serve as vectors for the dispersal of this pathogen, particularly if they are more commonly contaminated with soil or dust than are seeds of canola. Of course, any resting spores introduced into a field along with a non-host crop would have to survive in the soil until canola or some other susceptible species was planted. Since resting spores of *P. brassicae* have been estimated to have a half-life of 3.6

years (Wallenhammar, 1996), the longer the period prior to cropping of a susceptible host, the smaller the chance that a clubroot infestation could become established.

Whether any of these *P. brassicae* resting spores could successfully cause infection would also depend on the prevailing environmental conditions, as well as on the final distribution and concentration of the spores in the field. The distribution of resting spores in a field as a result of seed infestation would likely be a function of the distribution of the resting spores within a particular seedlot. While the inoculation protocol employed in the current study likely resulted in a fairly homogenous distribution of resting spores on the artificially infested seeds, such uniformity cannot be assumed in naturally infested samples. Since the confidence intervals in the qPCR analysis of the artificially infested seedlots were small and the correlation coefficient was high, variation observed in the C_T values of naturally infested seedlots likely reflected, at least in part, a heterogenous distribution of the inoculum (Fig. 3.1, Table 3.2). Thus, the mean infestation level does not necessarily capture the extremes that may occur within a particular seedlot, and some seeds could be infested at much lower or higher rates than the average. Such a situation could arise, for example, if there were small pieces of soil matter or plant debris mixed with the seeds, or if the seeds were coated with muddy soil. This scenario serves to highlight the importance of appropriate commercial cleaning of seeds and other propagative materials in mitigating any risk posed by seedborne dissemination of *P. brassicae*. In the current study, quantifiable levels of infestation were found in only 1 of 30 commercially cleaned

seed samples, as opposed to 6 of the 16 seed and tuber samples that had not been cleaned.

While the presence of *P. brassicae* can be confirmed and quantified by PCR-based protocols, these methods assess only the amount of pathogen DNA, and not necessarily the viability of the inoculum. The Evan's blue staining assay employed in this study was developed to assess the impact of temperature damage on resting spore viability (Tanaka et al., 1999), and was further validated prior to use in the current study (*data not shown*). The results obtained with this assay suggest that the vast majority of *P. brassicae* resting spores recovered from the seed and tuber samples were viable (Table 3.1), and that any exposure to heat or other physical stresses during handling did not have a large impact on this inoculum. As such, fungicidal seed treatments may represent another tool, in addition to seed cleaning, for further reducing the risk of seedborne dissemination of the clubroot pathogen.

Although the current results provide direct evidence for the occurrence of quantifiable numbers of *P. brassicae* resting spores on seeds and tubers of various crops harvested from clubroot-infested fields, seedborne transmission likely plays a very minor role in clubroot spread relative to the movement of infested soil on farm equipment and other machinery (Cao et al., 2009). The amount of soil (and therefore number of resting spores) carried on equipment far exceeds that found on even the most heavily infested of seedlots. Nevertheless, transmission of resting spores on seeds and tubers could, under the right conditions, lead to the dissemination of *P. brassicae* over longer distances than might typically be

associated with the movement of infested soil on farm machinery, and could also result in the introduction of novel races or pathotypes to particular regions. Therefore, the possibility of seedborne transmission should not be ignored in the development of clubroot-containment strategies, and appropriate measures to mitigate this risk, such as commercial cleaning of seeds and other propagative material, should be employed by farmers.

3.5. Tables

Table 3.1. List of fungal, bacterial and plant species used to test the specificity of *Plasmodiophora brassicae*-specific primers.

Species and Isolate Number (Where Available)[a]	Origin
Allomyces javanicus P7-15-5916 (17)	Carolina Biol. Supply Co. Burlington, NC
Alternaria alternata	J.P. Tewari, University of Alberta (U of A), Edmonton, AB
Alternaria brassicae CA2	J.P. Tewari, U of A[b]
Alternaria brassicicola French 719	J.P. Tewari, U of A
Alternaria raphani French 725	J.P. Tewari, U of A
Armilaria mellea HKG 86-217	J.P. Tewari, U of A
Aspergillus niger	J.P. Tewari, U of A
Bipolaris sp.	J.P. Tewari, U of A
Botrytis sp.	J.P. Tewari, U of A
Cladosporium sp. Ken 2	J.P. Tewari, U of A
Colletotrichum dematium ATCC 18013	American Type Culture Collection
Cyathus olla DAOM 197563	Canadian Collection of Fungal Cultures
Fusarium avenaceum Lacombe	J.P. Tewari U of A
Fusarium graminearum	Randall Clear, Grain Research Laboratory, Winnipeg, MB
Fusarium oxysporum (E)	J.P. Tewari, U of A
Gliocladium roseum #17	J.P. Tewari, U of A
Leptosphaeria maculans V77	J.P. Tewari, U of A
Myrothecium verrucaria	J.P. Tewari, U of A
Penicillium sp.	J.P. Tewari, U of A
Periconia sp. #1 K1103	J.P. Tewari, U of A
Phanerochaete chrysosporium 3642	UAMCH
Pythium pythioides 88-1-8	J.P. Tewari U of A
Rhizoctonia solani C51-25	J.P. Tewari, U of A
Rhizopus sp.	J.P. Tewari, U of A
Rhizophlyctis rosea ATCC (R) 24054	American Type Culture Collection
Trichoderma harzianum AC 85-46-5A	J.P. Tewari, U of A
Verticillium albo-atrum	J.P. Tewari, U of A
Bacillus subtilis	J.P. Tewari, U of A
Pseudomonas atrofaciens #3894	Andy Tekauz, Agriculture and Agri-Food Canada, Winnipeg Research Centre, Winnipeg, MB
Pseudomonas fluorescens	Dept. of Biological Sciences, U of A
Streptomyces sp. 147-54	J.P. Tewari, U of A
Brassica napus L.	U of A Canola Breeding Program
Brassica rapa var. *pekinensis*	Horticulture Research International, Genetic Resources Unit, Wellesbourne, Warwick, UK

[a] Genomic DNA from each of these species was obtained as described in Cao et al. (2007)
[b] Deposited at the U of A Microfungus Collection and Herbarium (UAMCH), Devon, AB

Table 3.2. Levels of seed and tuber infestation by resting spores of *Plasmodiophora brassicae*, as determined via qPCR with the *P. brassicae*-specific primers DR1F and DR1R. Quantifiable numbers of resting spores were found on 7 of 46 samples analyzed.

Sample[a]	Crop	Description	C_T Value (± 95% Confidence Intervals)	qPCR Estimated Resting Spore Number[b]	T_M (°C) (± 95% Confidence Intervals)	Conventional PCR Result[c]	Evan's Blue Viability Assay[d] (% viable; n=30)
1	Wheat	Farmer harvested, not commercially cleaned	19.94±0.45	3.43×10^4	85.24±0.03	Positive	80
2	Canola	Farmer harvested, not commercially cleaned	23.08±0.91	4.04×10^3	85.20±0.03	Positive	90
3	Pea	Farmer harvested, commercially cleaned	25.94±9.54	$<1.00\times10^3$	84.41±0.06	Positive	98
4[e]	Potato	Harvested from field plots, not commercially cleaned	24.63±0.27	1.40×10^4	85.29±0.05	Positive	90
5	Pea	Harvested from field plots, not commercially cleaned	27.56±0.47	$<1.00\times10^3$	84.95±0.16	Positive	97
6	Pea	Harvested from field plots, not commercially cleaned	26.70±2.17	$<1.00\times10^3$	85.03±0.21	Positive	97
7	Pea	Harvested from field plots, not commercially cleaned	28.00±0.60	$<1.00\times10^3$	85.02±0.17	Positive	100
NA	Various	DNA from each of 28 plant, fungal or bacterial species as template	28.28 ± 1.04[e]	NA	83.77±0.15[f]	Negative	NA

[a] Samples 1, 2 and 3 were collected from Sturgeon County, Alberta, Canada, while samples 4, 5, 6 and 7 were collected from the County of Newell, Alberta, Canada.

[b] Estimates of resting spore number were based on 10 g of seeds or 2 potato tubers for each repetition of the qPCR analsysis.

[c] Conventional PCR was performed with primers TC1F and TC1R as per Cao et al. (2007).

[d] The Evan's blue viability assay was performed as per Tanaka et al. (1999).

[e] Sample 4 was diluted 10-fold prior to quantification and the relative spore load was adjusted accordingly.
[f] This value represents the average from two independent qPCR amplifications using DNA extracted from each of 33 plant, fungal or bacterial species as a template.

3.6. Figures

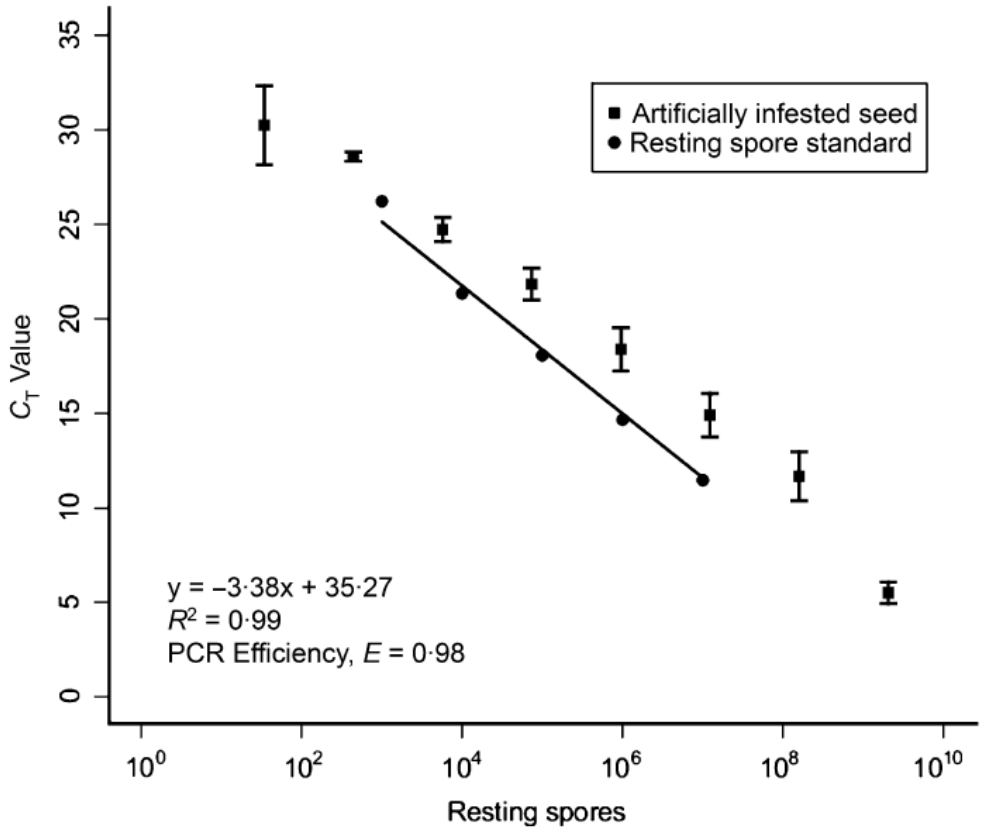

Figure 3.1. Cycle threshold (C_T) values obtained using DNA extracted from different numbers of *Plasmodiophora brassicae* resting spores as a template in quantitative PCR. Standards were prepared from a serial dilution of 1×10^7 to 1×10^3 resting spores, and the averages from four replications of the experiment are shown (circles). Quantitative PCR was also performed using DNA extracted from artificially infested canola seeds as a template, with the averages from four replications also shown (squares). Bars indicate the 95% confidence intervals for each data point, and the line represents the linear regression of the resting spore standard over the reliable range of detection. The R^2 was calculated from the regression line as was the line formula. The PCR efficiency (E) was determined according to the formula: $E = 10^{-1/slope} - 1$.

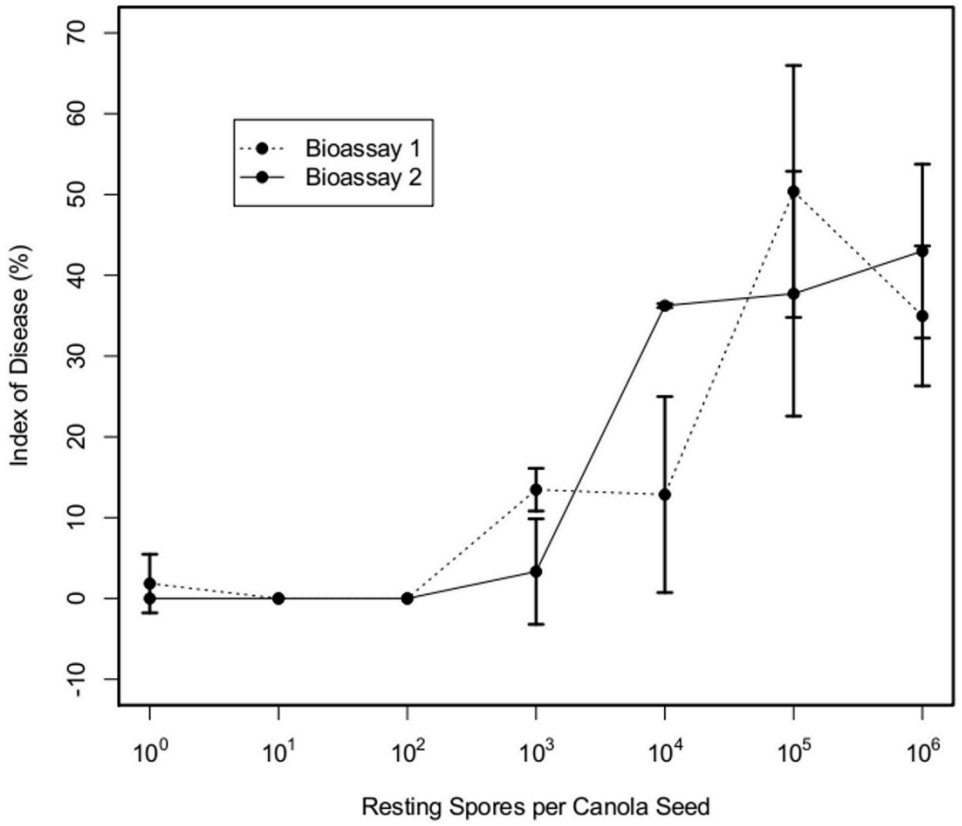

Figure 3.2. Average index of disease on canola seedlings grown from seeds artificially infested with resting spores of *Plasmodiophora brassicae* at different rates. Infested seeds were sown at a density of 120 seeds m^{-2} in 39 × 26 × 15 cm plastic trays (bioassay 1) or 7.5 cm diameter plastic pots (bioassay 2). The trays or pots were filled with Sunshine #4 Mix/LA7 soil medium (SunGro Horticulture) and maintained in a greenhouse or growth cabinet, respectively. Treatments were arranged in a randomized complete block design. Plants were rated for symptom development six weeks after seeding on a four-point scale, where: 0 = no galling, 1 = mild galling, 2 = moderate galling, and 3 = extensive galling. The individual scores were used to calculate indices of disease for the various treatments as per Strelkov et al. (2006). Bars indicate the 95% confidence intervals for each data point. Control plants inoculated at a rate of 0 resting spores per seed did not show any symptoms of *P. brassicae* infection in either bioassay (not shown).

3.7. References

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Chapter 4: Conclusions

4.1. Synthesis and implications

The current work has addressed two simple but important questions: (1) what is the anatomical morphology of spheroid galls compared to spindle galls? and (2) is seedborne infestation a prevalent and important aspect of clubroot epidemiology? From the answers to these questions, we can ascertain the implications of spheroid galls and seedborne infestation on the biology and epidemiology of *P. brassicae.*

While it is prudent to consider the role of spheroid galls in the epidemiology of *P. brassicae*, aside from the report of Osaki et al. (2008), this issue has been largely overlooked. The detection of mature resting spores in spheroid galls, both in the current study and by Osaki et al. (2008), is sufficient cause for a reconsideration of these galls as host resistance structures. In Chapter 2, all *Brassica* host genotypes analysed, including some clubroot resistant varieties were found to produce at least some spheroid galls (Table. 2.1), and the absence of any defined resistance structures or processes (such as lignification and/or the hypersensitive response) suggests that active resistance mechanisms are not involved in spheroid gall formation.

It is evident that spheroid galls result from the infection and proliferation of tissues outside of the stele. This suggests that plasmodial development in the root is restricted. Whether this is comparable to the necrotically defined minigalls described by Kobelt et al. (2000) is unlikely, as a host hypersensitive response was not observed in the present study (Chapter 2). Kunkel (1918) reported the

occurrence of knob-like extensions that resulted from the infection of lateral root primordia, and it is possible that these structures were similar if not identical to spheroid galls. However, since Kunkel (1918) had no manner by which to non-destructively sample these knoblike galls, it is difficult to confirm that they were indeed lateral root primordia prior to infection. Indeed, these early observations may have been confounded by the development of a meristematic area in the cortex, which has been reported to be associated with early clubroot development (Devos et al., 2006).

Alternatively, a hormonal imbalance caused by the production of cytokinins by *P. brassicae* could be responsible for the extent of secondary cortex proliferation in the absence of secondary stele expansion (Peterson, 1972; Ting and Wren, 1980; Webster and Radin, 1972). Cytokinins are predicted to be responsible for the development of the meristematic area, as described by Devos et al. (2006), and it is likely that hormonal regulation would underpin early clubroot development as well.

Similarities exist between spheroid gall development on stems and spheroid gall development on roots (Kunkel, 1918). The importance of wounding in the development of stem spheroid galls is clear, but the role of wounding in spheroid gall development in the roots is less clear. If wounding is absolutely necessary for spheroid gall development in both organs, the formation of spheroid galls may require root injury in the rhizosphere, perhaps during lateral root development. However, wounding as an exclusive prerequisite for spheroid gall formation is unlikely, since root wounding is relatively common, while spheroid

galling certainly is not.

Further to the epidemiology of *P. brassicae,* it seems from the current work that seeds and tubers of various crops can be infested by resting spores of the pathogen, potentially contributing to transmission of clubroot over longer distances (Chapter 3). The extent or degree of this transmission under field conditions was not addressed, since this was not the aim of Chapter 3. The current work builds on the early report of Warne (1943), which suggested the presence of *P. brassicae* inoculum on seeds, and highlights some of the risks associated with planting common, untreated seed harvested from clubroot-infested fields. While the risk posed by seedborne transmission of *P. brassicae* appears to be small relative to that posed by the movement of the pathogen in soil carried on field equipment, it should be considered as part of as a proactive clubroot-management strategy.

4.2. Questions and future work

The aspects of *P. brassicae* biology and ecology that have been addressed in this thesis include an analysis of the histopathology of spheroid galls, the potential for spheroid galls to contribute to the disease cycle, and the occurrence of viable pathogen inoculum as an external contaminant on seed and tuber lots. Further work to establish the importance of seedborne infestation under field conditions and to assess the utility of spheroid galling in the development of genetically resistant *Brassica* germplasm would represent a natural progression from the

present work.

To move the issue of seedborne *P. brassicae* infestation forward, we must conduct field experimentation to determine the threshold resting spore level required for clubroot development under 'natural' conditions. Similarly, to assess the role of spheroid galls in an environmental context, the prevalence of these structures on different host genotypes under field conditions must be examined.

The design of a large-scale clubroot bioassay for a field experiment, comparable to that described in Chapter 3 for the greenhouse, would be relatively straightforward and would establish the threshold of infestation necessary for disease development under field conditions. The importance of *P. brassicae*-infested non-host seeds and tubers on the dispersal of the pathogen would be more difficult to ascertain, but could be determined by sowing the infested seeds of the non-host crop one season, followed by the cropping of a highly susceptible bait crop in the following year.

Sampling to assess the prevalence of spheroid galls under field conditions would allow us to evaluate the relative role of these structures, if any, in field resistance to *P. brassicae* in the field. As widespread field surveys for clubroot are already in progress, and spheroid galling appears to be relatively common amongst our tested genotypes (Chapter 2), the simple task of recording the occurrence of spheroid galls would provide an estimate of the prevalence of these structures in the field. Information regarding the cultivar and pathogen population present would support our observations of spheroid gall enriching cultivars, and provide insight into the importance of spheroid galls in the epidemiology of *P.*

brassicae. This would be especially true if spheroid galls were observed and enriched on recently developed canola genotypes with clubroot resistance. The impact of spheroid galling on agronomic parameters (e.g., yield, plant mortality, and/or oil quality) could be assessed from fields exhibiting enhanced spheroid galling.

Other aspects of *P. brassicae* biology, ecology, and epidemiology worth evaluating include: the environmental patterning/dispersion of resting spores in the environment, the interaction of roots and zoospores in the rhizosphere, the interactions of invertebrates and *P. brassicae,* and the transitory stages between sporangial and sporogenic phases of the lifecycle. All of these topics are virtually unexplored, and need to be investigated.

The patterning of resting spores in the environment is affected by many factors. A comparison of inoculum loads found in dust traps and water runs would, for example, enable an assessment of the relative importance of soil erosion as a dispersal mechanism. Research along these lines has recently been initiationed, and the results may contribute to an improved understanding of *P. brassicae* epidemiology (SE Strelkov, University of Alberta, *personal communication*) Furthermore, comparisons of the amount of inoculum originating from fields cultivated in different ways (i.e., under conventional tillage vs. minimum or zero tillage) would allow the relative importance of these dispersal mechanisms to be linked back to agronomic practices. Indeed, even a thorough study focusing on resting spore patterning exclusively in the soil would be a step forward in understanding clubroot epidemiology.

Recent research regarding the foraging behavior of plant roots in the rhizosphere has particular implications for the interactions of zoospores and roots in the environment (McNickle and Cahill, 2009). Decomposing galls are certainly a localized source of nutrients, and plant roots have been found to optimally forage around localized sources of nutrients in the rhizosphere, conforming to the marginal value theorem (MVT) (McNickle and Cahill, 2009). The MVT (amongst other postulates) states that as the quality of a nutrient patch increases, the time an organism stays in that patch also increases (McNickle and Cahill, 2009). This has particular ramifications for clubroot disease, as the nutritional contribution of decomposing clubroot galls to soils is likely substantial, and the proximity and duration of root exposure to resting spores is likely important in disease development.

There have also been long-standing questions regarding the cues required for zoospore germination and the role of chemotaxis in zoospore movement towards plant roots. A recent report by Feng et al. (2010), who cloned and characterized the serine protease Pro1 from *P. brassicae*, advanced our understanding of the process of resting spore germination. These workers found that the treatment of root exudates with Pro1 enhanced their stimulatory effect on the germination of resting spores, and hypothesized that this protease may play a role in the pathogenesis of *P. brassicae* by helping to degrade host exudates, resulting in products that in turn stimulate spore germination. This is certainly a reasonable hypothesis, although since the outer resting spore is largely proteinaceous in nature, Pro1 could have also contributed directly to the

degradation of this structure. Such a Pro1-mediated degradation, in the presence of other enzymes or factors necessary to degrade chitinous components of the wall, would have permitted the release of the developed zoospore, thereby enhancing resting spore germination. This possibility of a direct impact of Pro1 on spore germination was not excluded by the methods of Feng et al. (2010), where resting spores were exposed to Pro1 with and without root exudates. Nevertheless, the requirement of a protease in the germination of resting spores was clearly highlighted by the workers.

Commercial fields are not isolated from ecosystem processes and interactions, and these interactions are wholly unknown. The anecdotal observation of root maggot proliferation in clubroot galls is a very interesting phenomenon (S.E. Strelkov, University of Alberta, *personal communication*). Is there a role for root maggots or other soil organisms in the lifecycle of *P. brassicae*? Do these organisms serve as vectors for *P. brassicae*? Do they passively consume plasmodia and resting spores during their herbivorous lifecycle? Further, does clubroot affect the soil invertebrate community structure? Simple invertebrate trapping between clubroot infested fields and non-infested fields would highlight some of these possible changes.

Developmental and life history studies are regularly conducted with light and electron microscopes that necessitate the use of fixed specimens. Unfortunately, it can be difficult to attribute dynamic developmental processes to static images. Advances in microscopy now permit the visualization and optical sectioning of live material, and would be particularly well suited to the study of

root hair infection. Experiments with *Arabidopsis thaliana,* on account of its size, susceptibility to clubroot, and the availability of anatomical aids (tissue/cell-specific green fluorescent protein or glucuronidase reporters) will enable additional histological analyses of clubroot disease (especially gall development) and better understanding of host and pathogen structures. Visualization of the roots and root hairs of infected hosts by confocal microscopy will reveal key aspects of the transition between the sporangial and sporogenic phases of the *P. brassicae* lifecycle, and help to elucidate the dynamic processes involved in clubroot development.

A thorough synthesis of the mass of clubroot research, along with the development of consistent terminology, will be essential to furthering the science of *P. brassicae.* This process may have to wait until the phylogenetic relationships of this pathogen are conclusively established, since different taxonomic groups have their associated terminology. It is evident, however, that progress in clubroot research is accelerating. Indeed, our understanding of clubroot disease has been advanced by the work presented in this thesis. Principally, the general risks associated with seedborne *P. brassicae* infestation have been assessed. The quantitative polymerase chain reaction methods utilized in Chapter 3 are novel and provide much-needed tools for quantifying resting spore abundance in a variety of environmental samples. Secondly, the analysis of clubroot galls presented in Chapter 2 is, to our knowledge, the first histological description of spheroid galls, and one of few histological assessments of spindle gall anatomy to date.

4.3. References

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