Canola Agronomic Research Program

Final Report

Project Number: 2004-21

Evaluation of clubroot control with rotation, fungicides and soil amendments

(Characterization of the clubroot disease problem on canola)

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Evaluation of clubroot control with rotation, fungicides and soil amendments (Characterization of the clubroot disease problem on canola)

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CARP Project # 2004-21. Conducted at the University of Alberta 2004-2008

Key findings

1. *The clubroot pathogen (Plasmodiophora brassicae) is now established as a pest of canola in central Alberta, and may be spreading to other regions of the province*

2. *All currently available canola cultivars are susceptible to clubroot, but sources of resistance exist*

3. *The predominant strain of the pathogen is pathotype 3 or P_2, as classified on the systems of Williams or Somé et al., respectively*

4. *Pathogen populations are diverse, and resistance (when available) will have to be well-managed*

5. *A reliable method for the molecular detection of the clubroot pathogen has been developed; however, as with the bioassay, caution should be used when extrapolating the results obtained for a particular sample to an entire field*

Details

1. *The clubroot pathogen (Plasmodiophora brassicae) is now established as a pest of canola in central Alberta, and may be spreading to other regions of the province*

In 2003, clubroot was found on 12 fields of *Brassica napus* (Argentine) canola in Sturgeon County, Alberta, in the first report of this disease on canola in the Canadian prairies. Annual surveys for the occurrence of clubroot indicate that clubroot is spreading and more widespread than originally thought; as of 2007, at least 250 clubroot-infested fields had been identified in 10 counties in central Alberta, 1 county in southern Alberta, and a rural area of northeast Edmonton. The primary mechanism of spread between fields is the movement of infested soil on farm machinery. Yield losses ranging from 30% to 100% have been reported in severely infested canola fields. The occurrence of clubroot is not restricted to fields with acidic soils, but there is a significant negative correlation between disease severity and soil pH.

2. ***All currently available canola cultivars are susceptible to clubroot, but sources of resistance exist***

Greenhouse screening for clubroot resistance indicated that 48 canola (*B. napus* and *B. rapa*) cultivars included in the 2004 Prairie Canola Variety Trials were highly susceptible to a local population representing pathotype 3 (P_2) of *P. brassicae*. Similar results were found in an independent study from Quebec that examined the resistance of 31 canola varieties in the field. However, good sources of resistance were identified in European winter canola, rutabaga, and certain varieties of the *B. napus* parental species *B. rapa* and *B. oleracea*. In a separate study, the genetic resistance from these sources will be introgressed (transferred) into spring canola germplasm for the Canadian market.

3. ***The predominant strain of the pathogen is pathotype 3 or P_2, as classified on the systems of Williams or Somé et al., respectively***

Physiologic specialization (the existence of different strains or pathotypes of a pathogen) has long been known in *P. brassicae* and has important implications for breeding efforts. The choice of appropriate sources of resistance, and the durability of that resistance, will be influenced by the number and relative prevalence of pathotypes in a particular region. Testing of *P. brassicae* populations from central Alberta revealed that pathotype 3 or P_2, as classified on the systems of Williams or Somé et al., respectively, is predominant in the province.

4. ***Pathogen populations are diverse, and resistance (when available) will have to be well-managed***

As populations of *P. brassicae* often consist of a mixture of different pathotypes, a simple method to isolate single resting spores of the pathogen was developed and the virulence of 24 isolates, representing five populations from Alberta, Ontario and British Columbia, was characterized on the differentials of Williams and Somé et al. The pathotype composition of *P. brassicae* appeared more diverse when single spore isolates of the pathogen were examined. In Alberta, at least three and possibly four pathotypes were identified among the 14 isolates tested, whereas as a maximum of only two pathotypes were found when populations of the pathogen were studied. Testing of single spore isolates confirmed that pathotype 3 or P_2 is predominant in the province. However, the occurrence of other pathotypes at lower frequencies suggests that caution should be used in any breeding strategy, since rare pathotypes of *P. brassicae* may quickly become predominant if susceptible host cultivars are continuously grown.

5. ***A reliable method for the molecular detection of the clubroot pathogen has been developed; however, as with the bioassay, caution should be used when extrapolating the results obtained for a particular sample to an entire field***

A simple, one-step polymerase chain reaction (PCR) protocol was developed to detect *P. brassicae* in plant and soil samples. The primers TC1F and TC1R, based on a *P. brassicae* partial 18S ribosomal RNA gene sequence from GenBank, yielded a 548 bp

product in the optimized PCR. A second pair of primers, TC2F and TC2R, which amplified a fragment of the 18S and internal transcribed spacer (ITS) 1 regions of the rDNA repeat, was also tested and produced a 519 bp product. Neither set of primers amplified any DNA fragment from non-infected plant hosts, non-infested soil, or common soil fungi and bacteria tested in this study. Quantities of 100 fg or less of total *P. brassicae* DNA, or 1×10^3 resting spores per g of soil could be consistently detected using these primers and PCR protocol, corresponding to an index of disease of 11% or lower when the soil was bioassayed. The protocol also enabled detection of *P. brassicae* in symptomless root tissue 3 days after inoculation with the pathogen, and the PCR assay could provide a reliable diagnosis for routine detection of the pathogen in plant and soil materials in a specific and rapid manner. However, as with the bioassay, caution should be used when extrapolating the results obtained for a particular sample to an entire field, as clubroot often occurs in a patchy distribution.

Please refer to the complete report for full details.

Abstract

Clubroot, caused by *Plasmodiophora brassicae*, was initially found on *Brassica napus* canola in the Edmonton, Alberta region in 2003, in the first report of this disease on canola in the Canadian prairies. Surveys conducted from 2005 to 2007 indicate that clubroot is spreading and more widespread than originally thought, with 250 clubroot-infested fields identified in 10 counties in central Alberta, the centre of the outbreak. The disease has also been recently found in canola fields in a county in southern Alberta. The primary mechanism of spread between fields is the movement of infested soil on farm machinery. Yield losses ranging from 30% to 100% have been reported in severely infested canola fields. The occurrence of clubroot is not restricted to fields with acidic soils, but there is a significant negative correlation between disease severity and soil pH. Evaluation of the virulence of populations and single spore isolates of *P. brassicae* on differential hosts has revealed the presence of at least three, and possibly four, pathotypes in Alberta. Pathotype 3 or P_2, as classified on the differentials of Williams or Somé et al., respectively, is predominant and highly virulent on all canola cultivars currently available in Canada. However, good resistance was found in varieties of European winter canola, rutabaga, and the *B. napus* parental species *B. rapa* and *B. oleracea*, which will be introgressed into spring canola germplasm for the Canadian market. A simple, one-step polymerase chain reaction (PCR) protocol has been developed to identify *P. brassicae* in plant and soil samples, which could provide a reliable diagnosis for routine detection of the pathogen.

Acknowledgements

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General Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is one of the most economically important diseases of cultivated crucifers (Karling 1968), attacking virtually all members of this family. The pathogen induces galls on the roots of infected plants, reducing the capacity for water and nutrient uptake, resulting in stunting, wilting, lodging, and finally major yield and quality losses (Voorips 1995; Wallenhammar et al. 1999; Pageau et al. 2006). In Canada, clubroot has traditionally occurred on cruciferous vegetables, mainly in British Columbia and the eastern provinces, where it is a significant problem (Rimmer et al. 2003). However, the disease was recently identified in canola in Alberta (Tewari et al. 2005; Strelkov et al. 2005), and in the Lac Saint-Jean region of Quebec (Rimmer et al. 2004).

The identification of clubroot in Alberta is a matter of concern, as no management strategies have been developed for control of the disease on the canola crop in the Prairies. *Plasmodiophora brassicae* produces very long-lived resting spores (Karling 1968), and once a field becomes infested with clubroot, it is nearly impossible to eradicate the pathogen. Therefore, if the disease were to enter the main canola stream, it could have a significant negative impact on the agricultural industry in the province. The original proposal submitted to CARP in the fall of 2003 was focused exclusively on the evaluation of clubroot management strategies that would provide effective control of the disease on the prairies. However, as additional funding for this component of the research was not secured until 2007, the main objectives were revised (in consultation with CARP) to focus on the characterization of the clubroot disease problem in Alberta and on the development of an efficient technology to detect *P. brassicae* in soil and plant

samples. Clubroot research focused on the development of an integrated clubroot management strategy was initiated in 2007 with support from the Funding Consortium, and builds on the information presented here.

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Chapter 1: Clubroot on Canola in Alberta in 2003 and 2004

1.1 Methods

In September, 2003, a total of 70 commercial canola (*Brassica napus*) fields were surveyed in Parkland and Sturgeon Counties, Alberta, for the incidence of clubroot disease caused by *Plasmodiophora brassicae*. This survey was initiated as a result of the discovery of clubroot in a canola field near St. Albert, Alberta in late summer of 2003 (Buczacki 1977). The fields were surveyed after swathing, by inspecting the roots of 10 plants at 10 locations along the arms of a 'W' sampling pattern in each field. Using samples from the most severely infested field, clubroot was confirmed according to Koch's postulates (Buczacki 1977). In late August and early September, 2004, 41 fields were surveyed (15 in Parkland County and 26 in Sturgeon County), with particular emphasis on low lying sites and headland areas near field approaches (10-20 plants per site). Canola roots were not assessed for disease severity.

2.2. Results and Comments

In 2003, a total of 12 clubroot-infested fields were identified northwest, north and northeast of St. Albert, AB (Fig. 1). In one field, severe infection was observed, with 94% of plants infected. Estimated yield loss in this field was approximately 30%. Plants in water runs within the field showed the most severe infection. In another eight fields, infection was patchy and ranged from light to moderate; plants in small areas (less than 0.5 ha) had 30-50% infection, but in the majority of each field there was little or no infection. Two of these fields were adjacent to the severely infested field described

above, but diseased plants were found only on their bordering edges. Estimated yield losses in the light to moderately infested fields ranged from 0 to 15%. The remaining three fields exhibited only very light infestation, and infected plants were detected only near field approaches. Very little or no yield loss was expected in these fields. In 2004, no clubroot was detected in any of the 41 commercial fields surveyed. However, in both 2003 and 2004, clubroot was also identified in a field in northeast Edmonton (Fig. 1) at the Crop Diversification Centre North (CDCN), Alberta Agriculture, Food, and Rural Development. The disease was observed on canola plants growing in field plots at CDCN in 2003, and was also found on volunteer canola growing at the same location in August, 2004.

Among the 12 clubroot-infested fields identified in 2003, at least seven (including the most severely affected field) had been sown to canola every second year or more since 1997, with a general rotation of canola-cereal-canola-cereal. This may have contributed to inoculum build-up in these fields. Furthermore, the soil pH in the clubroot-infested fields ranged from 5.6 to 6.4, and acidic soils are known to favor clubroot disease development (Ayers 1957). It was surprising that clubroot was not detected in commercial fields in 2004, as the reappearance of the disease on volunteer canola at CDCN would indicate that environmental conditions were appropriate for its development. However, the survey conducted in 2004 was small, and a larger survey is planned for 2005. No other reports of clubroot occurred from anywhere else in the province.

1.3 References

Karling, J.S. 1968. The Plasmodiophorales. Hafner Publishing Co. New York.

Tewari, J. P., Strelkov, S. E., Orchard, D., Hartman, M., Lange, R. M., and Turkington, T.K. 2005. Identification of clubroot of crucifers on canola (*Brassica napus*) in Alberta. Can. J. Plant Pathol. 27: (in press).

1.4 Tables and Figures

Please refer to the following pages.

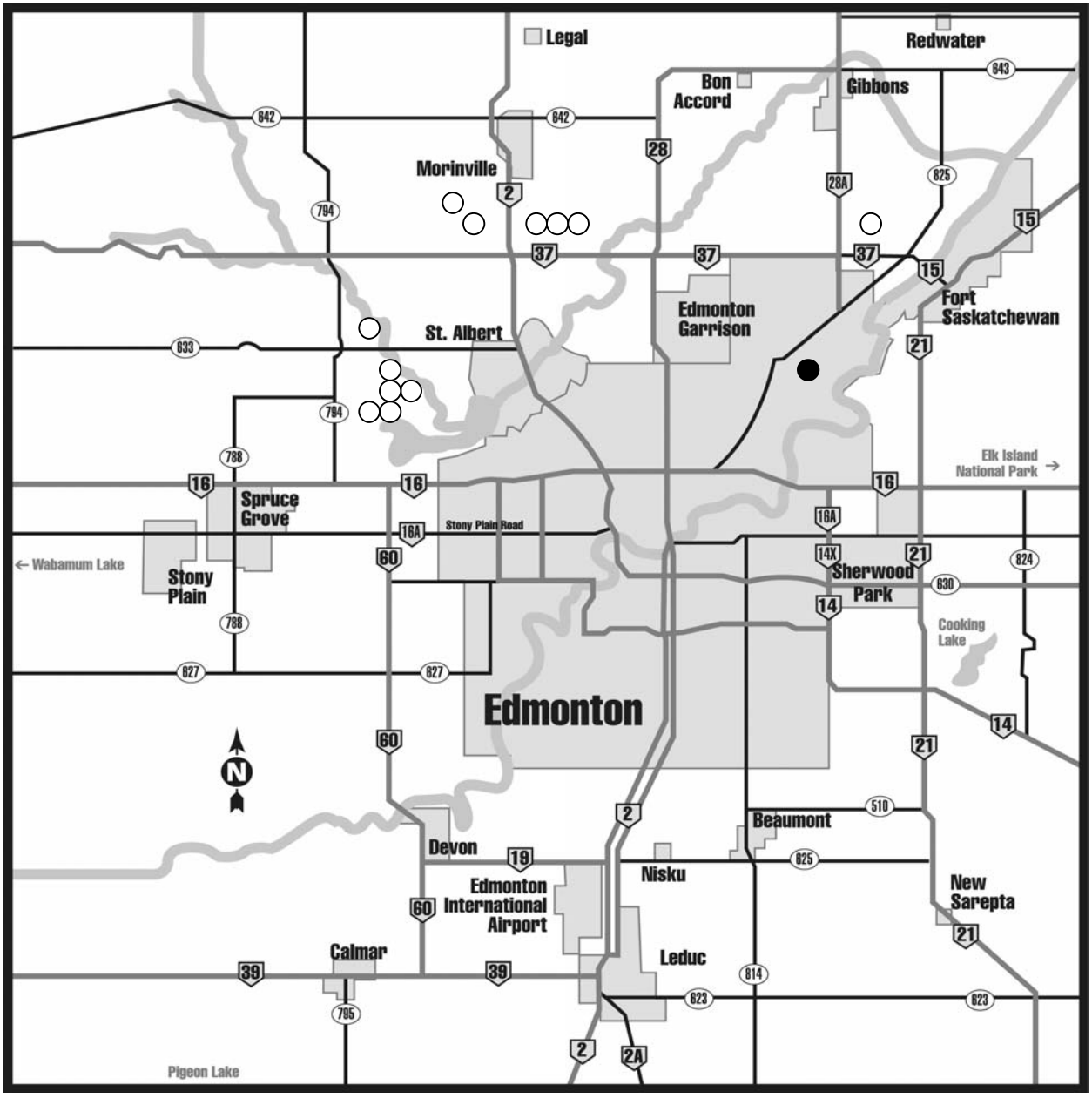

Figure 1-1. Incidence of clubroot on canola (*Brassica napus*) in the Edmonton, Alberta region. Each open circle represents the approximate location of a commercial canola field in which the disease was identified in 2003. The filled circle represents the Crop Diversification Centre North, Alberta Agriculture, Food and Rural Development, where clubroot was identified on canola in both 2003 and 2004.

Chapter 2: Incidence of Clubroot on Canola in Alberta in 2005

2.1 Methods

In September 2005, a total of 112 commercial canola (*Brassica napus* L.) fields were surveyed in Sturgeon County (77 fields), Parkland County (6 fields), Strathcona County (10 fields), the County of Leduc (1 field), the County of Wetaskiwin (1 field), and northeast Edmonton (17 fields), Alberta, for the incidence of clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin. The fields were surveyed after swathing by inspecting the roots of all plants within a 1 m^2 area at each of 10 locations along the arms of a 'W' sampling pattern. The presence of conspicuous galls on the roots was taken as an indication of clubroot infection. As infections tended to occur in patches, disease incidence in individual fields was calculated as the percentage of points (out of the 10 sampling points within each field) that were positive for clubroot. The severity of root infection was assessed on a 0 to 3 scale, adapted from Kuginuki et al. (1999), where 0 = no galling, 1 = a few small galls, 2 = moderate galling and 3 = severe galling. Representative soil and root samples were collected from each infested field for further analysis. The survey was conducted with a particular emphasis on areas where there had been reports of clubroot or clubroot-like symptoms, although 40 of the 112 fields were randomly selected. In addition, a smaller survey of 39 randomly selected canola fields was conducted in the acid soils of east-central Alberta, bringing to 151 the total number of fields visited in 2005.

2.2 Results and Comments

A total of 41 clubroot-infested canola fields were identified in 2005. The majority of infested fields were located in Sturgeon County (north of Edmonton) or in a rural area in the northeast corner of the city (Fig. 1-1). However, one clubroot-infested field was also identified east of Edmonton in Strathcona County (Fig. 1-1), and two infested fields were found south of the city, one in the County of Leduc (Fig. 1-1) and the other in the County of Wetaskiwin (not shown on map). No clubroot was identified in any of the 39 fields surveyed in east-central Alberta. The number of infested fields per region surveyed is given in Table 1-1. Within the 41 clubroot-infested fields, eight had a high incidence of disease (>70%), about half (21 fields) exhibited intermediate disease incidences, and 12 fields had a low incidence of disease (<30%) (Fig. 1-2).

Among fields showing a low level of disease, most clubroot-infected canola plants were identified in headland areas near field entrances, suggesting that the pathogen is introduced into fields on contaminated farming equipment. Furthermore, in all but the most heavily infested fields, the disease occurred in patches of varying size; these patches were sometimes associated with low-lying areas, perhaps reflecting the high moisture requirements of *P. brassicae* (Karling 1968). In contrast, the disease distribution was relatively uniform in fields with high incidences of clubroot. Most infested fields were in a canola-cereal-canola-cereal rotation, which may have contributed to the build-up of pathogen populations in affected areas. The occurrence of clubroot was not restricted to fields with acidic soils (the soil pH of infested fields ranged from 4.8 to 7.6, with an average value of 6.2), but there was a significant negative correlation between disease

severity and soil pH (results not shown). Acidic soils are known to favor development of clubroot (Karling 1968).

2.3 References

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Kuginuki, Y., Hiroaki, Y., and Hirai, M. 1999. Variation in virulence of *Plasmodiophora brassicae* in Japan tested with clubroot-resistant cultivars of Chinese cabbage (*Brassica rapa* L. spp. *pekinensis*). Eur. J. Plant Pathol. 105:327-332.

2.4 Tables and Figures

Please refer to the following pages.

Table 2-1. Distribution of clubroot-infested canola fields identified in Alberta in 2005.

Region	Total fields surveyed	Number of clubroot infested fields
Sturgeon County	77	27
Northeast Edmonton	17	11
Strathcona County	10	1
Parkland County	6	0
County of Leduc	1	1
County of Wetaskiwin	1	1
East-Central Alberta	39	0

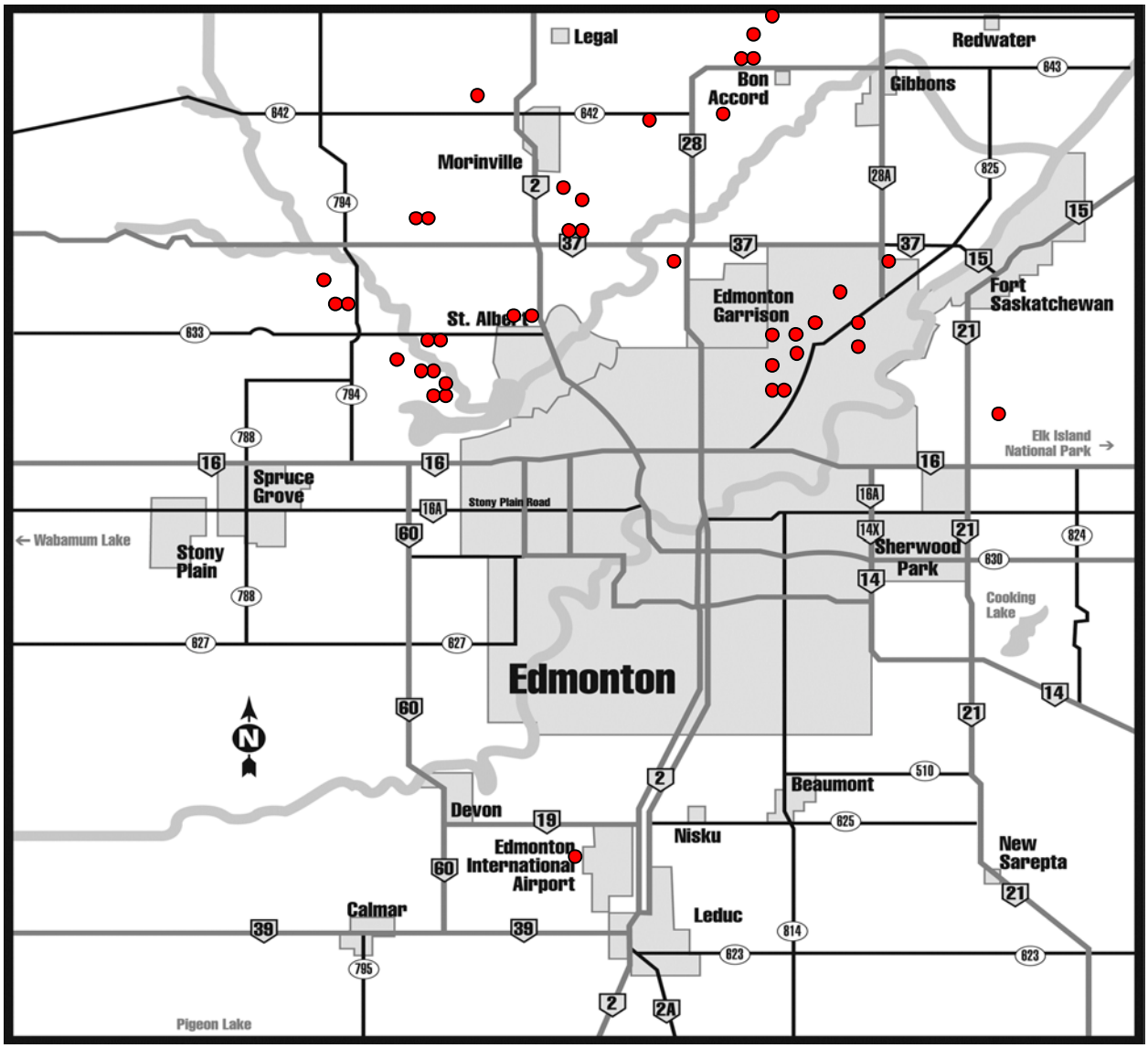

Figure 2-1. Incidence of clubroot on canola (*Brassica napus*) in the Edmonton, Alberta region. Each circle represents the approximate location of a commercial canola field in which the disease was identified in 2005. Forty fields are indicated on the map. Another infested field found near the City of Wetaskiwin (south of Leduc) is not shown.

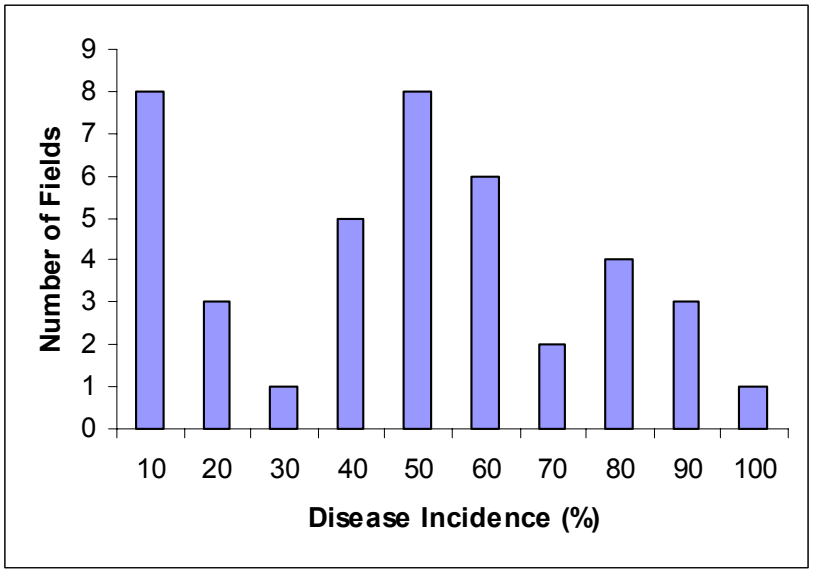

Figure 2-2. Incidence of clubroot in individual infested canola fields. Disease incidence was calculated as the percentage of points (out of a total of 10 sampling points within a field) that were positive for clubroot.

Chapter 3: Incidence of Clubroot on Canola in Alberta in 2006

3.1 Methods

In August and September 2006, a total of 250 commercial canola (*Brassica napus* L.) fields were surveyed in Sturgeon County (52 fields), Parkland County (63 fields), Strathcona County (24 fields), Flagstaff County (30 fields), Leduc County (46 fields), the County of Wetaskiwin (22 fields), and northeast Edmonton (13 fields), Alberta, for the incidence of clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin. Fields were surveyed after swathing. Since previous surveys indicated that the disease occurred most commonly in field entrances (Strelkov et al. 2005; Strelkov et al. 2006), the focus of this year's survey was on the entrance to each field. The roots of all plants within a 1 m^2 area at nine sampling points were inspected for disease development. The sampling points were at the field entrance and 150 and 300 m distant along each of four lines radiating from the entrance. The presence of conspicuous galls on the roots was taken as an indication of clubroot infection. The severity of root infection was assessed on a 0 to 3 scale, adapted from Kuginuki et al. (1999), where 0 = no galling, 1 = a few small galls, 2 = moderate galling and 3 = severe galling. The survey was conducted with a particular emphasis on areas where there had been reports of clubroot or clubroot-like symptoms, although many fields within those areas were randomly selected.

3.2 Results and Comments

A total of 71 clubroot-infested canola fields were identified in 2006. The majority of these fields were located in Sturgeon, Parkland and Leduc counties, although a number

of fields were also identified in a rural area of northeast Edmonton, as well as in Strathcona County (Fig. 3-1). No new cases of the disease were found in Flagstaff County or the County of Wetaskiwin in 2006. However, infected canola volunteers were found in a field in the County of Wetaskiwin, which had previously been identified as clubroot-positive (Strelkov et al. 2005). The number of infested fields per region surveyed is summarized in Table 3-1. In most fields, disease distribution was patchy and severity was light to moderate. However, at least 13 fields were heavily infested, including one in which clubroot was so severe that the canola crop was not harvested, and hence a 100% loss occurred.

Prior to 2006, nearly all clubroot-infested canola fields had been identified in Sturgeon County and northeast Edmonton, with only isolated cases reported in Leduc, Strathcona, Wetaskiwin and Flagstaff counties, and none in Parkland County. This year's survey indicates that the disease is more widely distributed, with many infested fields found in Leduc, Strathcona and Parkland counties, in addition to Sturgeon County and Edmonton. Nevertheless, clubroot does not appear to be widespread in Wetaskiwin and Flagstaff counties, and the outbreak remains centered mainly in the Edmonton region. A total of 113 fields are now known to be clubroot-infested, and the longevity of the pathogen resting spores (Karling 1968) suggests that they will remain so for the foreseeable future. Additional surveys are planned for 2007, in central Alberta and other canola growing areas of the province, in order to continue to evaluate pathogen distribution and spread.

3.3 References

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3.4 Tables and Figures

Please refer to the following pages.

Table 3-1. Distribution of clubroot-infested canola fields identified in Alberta as of September 2006.

Region	Number of Infested Fields (New Cases, 2006)	Total Number of Infested Fields (2005 + 2006)
Sturgeon County	28	55
Leduc County	21	22
Parkland County	15	15
Northeast Edmonton	4	15
Strathcona County	3	4
County of Wetaskiwin	0	1
Flagstaff County	0	1

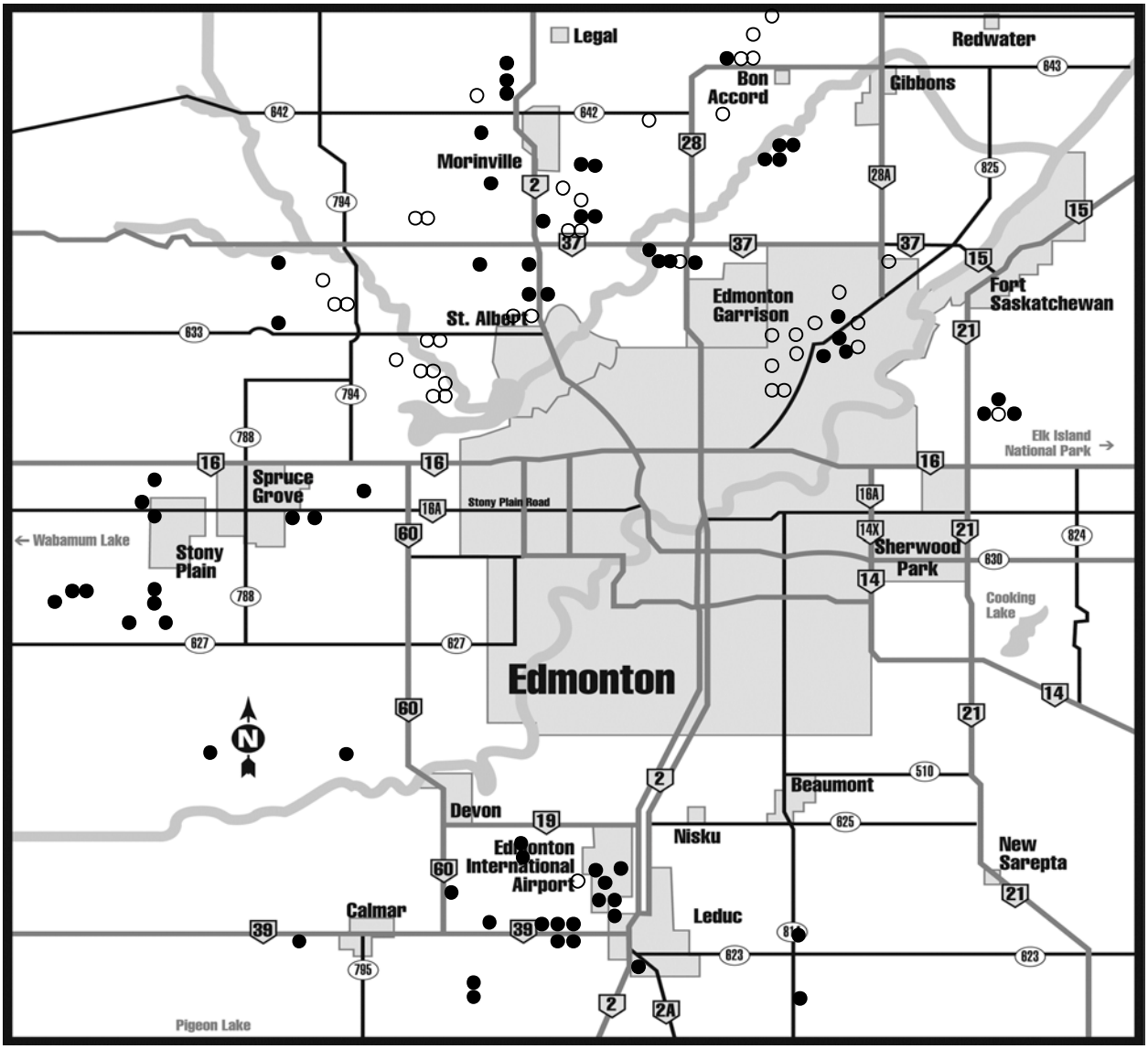

Figure 3-1. Incidence of clubroot on canola (*Brassica napus*) in the Edmonton, Alberta region. Each circle represents the approximate location of an infested canola field. Open circles represent fields identified in 2005, and solid circles represent fields identified in 2006. A total of 111 fields are indicated on the map. Two other infested fields found in Wetaskiwin and Flagstaff counties in 2005 are not shown.

Chapter 4: Incidence of Clubroot on Canola in Alberta in 2007

4.1 Methods

In August and September 2007, a total of 325 commercial canola (*Brassica napus* L.) fields were surveyed in 11 counties in central Alberta and one county in southern Alberta (Table 1) for the incidence of clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin. The fields were surveyed after swathing by inspecting the roots of all plants within a 1 m^2 area at each of 10 locations along the arms of a 'W' sampling pattern. The presence of conspicuous galls on the roots was taken as an indication of clubroot infection. As infections tended to occur in patches, disease incidence in individual fields was calculated as the percentage of points (out of the 10 sampling points within each field) that were positive for clubroot. The severity of root infection was assessed on a 0 to 3 scale, adapted from Kuginuki et al. (Ayers 1957), where 0 = no galling, 1 = a few small galls, 2 = moderate galling and 3 = severe galling. Representative soil and root samples were collected from each infested field for further analysis. The majority of surveyed fields were randomly selected, although some were visited because of reports of clubroot or clubroot-like symptoms.

4.2 Results and Comments

A total of 58 clubroot-infested canola fields were identified in 2007. The disease was found in 10 of 11 counties surveyed in central Alberta and in Newell County in southern Alberta (Table 1). The infestations in Newell, Barrhead, Lac St. Anne, Camrose and Westlock represent the first documented cases of clubroot in these counties.

However, no clubroot was found in 27 canola fields surveyed in Thorhild County. In Wetaskiwin and Flagstaff counties, multiple cases of the disease were found, whereas previously only one infested field had been reported in each county. A total of 171 infested fields have been identified in Alberta since the clubroot outbreak began in 2003. In addition, further surveying by agricultural fieldmen in Leduc, Sturgeon, Parkland and Barrhead counties revealed another 79 cases of the disease, for a grand total of 250 affected fields (C. Henkelmann, T. Prefontaine, E. Brock and M. Flock, personal communication). Within the 58 clubroot-infested fields identified as part of this year's main survey, five had a very high incidence of disease (>70%), 26 exhibited intermediate disease incidences (30-70%), and 27 fields had a low incidence of disease (<30%).

The percentage of infested fields in the 2007 survey (17.8%) was lower than in previous clubroot surveys conducted in Alberta (2, 3). This probably reflects the fact that this year's survey was not focused on areas where clubroot is known to be prevalent, but rather included a large number of randomly selected fields distributed throughout a much larger area. Indeed, in counties where clubroot was already known to occur, the proportion of infested fields tended to be quite high. For instance, in Sturgeon County, where the outbreak was first detected, 8 of 12 fields surveyed were clubroot positive.

4.2 References

Kuginuki, Y., Hiroaki, Y., and Hirai, M. 1999. Variation in virulence of *Plasmodiophora brassicae* in Japan tested with clubroot-resistant cultivars of Chinese cabbage (*Brassica rapa* L. spp. *pekinenses*). Eur. J. Plant Pathol. 105:327-332.

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Strelkov, S.E., Manolii, V.P., Cao, T., Hwang, S.F., and Orchard, D. 2007. Incidence of clubroot on canola in Alberta in 2006. Can. Plant Dis. Surv. 87:109-111.

4.4 Table

Please refer to the following page.

Table 4-1. Distribution of clubroot-infested canola fields identified in Alberta in 2007.

County	Total fields surveyed	Number of clubroot infested fields[a]
Barrhead	19	2 (2)
Camrose	22	3 (3)
Flagstaff	20	2 (3)
Lac Ste. Anne	15	1 (1)
Leduc	34	12 (34)
Newell	21	2 (2)
Parkland	40	14 (29)
Strathcona	26	4 (8)
Sturgeon	12	8 (63)
Thorhild	27	0 (0)
Westlock	39	3 (3)
Wetaskiwin	50	7 (8)

[a]The total number of infested fields identified from 2004 to 2007 is indicated in brackets for each county; an additional 15 infested fields were identified in a rural area of northeast Edmonton in 2005-2006, but this region was not surveyed in 2007.

Chapter 5: Characterization of *Plasmodiophora brassicae* Populations from Alberta, Canada

5.1 Abstract

Clubroot, caused by *Plasmodiophora brassicae* Woronin was identified in a number of canola (*Brassica napus* L.) fields in central Alberta in 2003. In order to characterize the virulence of the pathogen in the province, field populations from a number of locations in the Edmonton region were tested on the two most widely used sets of differential hosts, those of P.H. Williams and the European Clubroot Differential (ECD) series. Populations from British Columbia and Ontario were included for comparison. While the reaction of some hosts could be clearly defined as either resistant or susceptible, others showed intermediate disease index scores. If disease indices of 0 to 49% and 50 to 100% were regarded as resistant and susceptible, respectively, then populations from Alberta were classified as ECD 16/15/12 and 16/15/0 on the ECD set, or pathotypes 3 and 5, respectively, on the hosts of Williams. The population from British Columbia was classified as ECD 16/2/12 or pathotype 6, and the Ontario population as ECD 16/0/14 or pathotype 6. The Alberta populations were more virulent on the *B. napus* hosts than those from other provinces, perhaps a reflection of their canola origin. In addition, 48 canola cultivars included in the 2004 Prairie Canola Variety Trials were screened for resistance to a local population of the pathogen, and all appeared to be highly susceptible. If clubroot were to become more widely established in western Canada, it could have a major negative impact on yields.

5.2 Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is one of the most serious soilborne diseases of crucifers throughout the world. In Canada, the disease occurs mainly in eastern parts of the country and in British Columbia, where it can be an important problem in vegetable cole crop production (Rimmer et al. 2003). However, in 2003, clubroot was identified on canola (*Brassica napus* L.) plants in 12 commercial fields near St. Albert, Alberta, as well as in experimental field plots in northeast Edmonton (Strelkov et al. 2005; Tewari et al. 2005). Further surveys in 2005 revealed the presence of the disease in over 40 canola fields in the Edmonton region (Strelkov et al. 2006). Although clubroot had previously been identified in home and market gardens in the province, these were the first reported outbreaks of the disease on canola in Alberta. The identification of clubroot in canola fields is a matter of concern, given the significant yield losses associated with *P. brassicae* infection. In the Alberta outbreak, yield reduction because of clubroot was 30% in the most severely affected field (Strelkov et al. 2005; Tewari et al. 2005), but losses as high as 50% have been reported for *B. napus* (Wallenhammar et al. 1999).

Infection by *P. brassicae* causes the formation of galls on the roots of susceptible plants, which reduces their capacity for water and nutrient uptake, resulting in stunting, wilting and death when symptoms are severe. Acidic soils and high moisture favor development of the disease (Karling 1968). A number of strategies are recommended for managing clubroot, including liming of the soil, application of fungicides, use of resistant cultivars, and crop rotation. However, not all of these methods may be practical or affordable in canola cropping systems. For instance, large amounts of lime may be

necessary to increase soil pH sufficiently to reduce disease severity (Campbell et al. 1985; Myers and Campbell 1985; Webster and Dixon 1991), making this strategy impractical in field crops. Similarly, control of clubroot through the use of fungicides is not always consistent (Naiki and Dixon 1987), and may be prohibitively expensive. Crop rotation away from susceptible crops is an effective management strategy, but rotation breaks must be long, as resting spores of the pathogen can survive in the soil for extended periods of time (Wallenhammar 1996). The use of genetically resistant cultivars is one of the most economically and environmentally desirable strategies for clubroot control, but the resistance of most Canadian canola varieties is unknown.

Physiologic specialization has long been known in the pathogen (Honig 1931), and the differential hosts of Williams (1966) and the European Clubroot Differential (ECD) set (Buczacki et al. 1975) have been used extensively to analyze populations of *P. brassicae* (Williams 1966; Buczacki et al. 1975; Crute et al. 1980; Voorips 1995). However, little has been reported regarding the pathotype composition of *P. brassicae* populations from Canada. Williams (1966) tested two field populations from Quebec and one from British Columbia on his differential set; the pathotype ('race') designation of one of the Quebec populations was not clear, but the second Quebec population and the population from British Columbia were classified as pathotypes 2 and 6, respectively. Similarly, in a separate study, pathotype 6 was found to be the predominant pathotype from cole crops in Ontario (Reyes et al. 1974). Hildebrand and Delbridge (1995), also using the differentials of Williams (1966), characterized 10 populations of *P. brassicae* collected from various cruciferous crops in Nova Scotia, and identified eight as pathotype

3, and one each as pathotypes 1 and 2. To our knowledge, no ECD designations have been reported for any populations of the pathogen from Canada.

As clubroot was only recently identified on canola in Alberta, no information is available on the pathotype composition of *P. brassicae* populations in this province. Therefore, the objectives of the present study were to characterize populations of the pathogen from Alberta based on their virulence patterns on the differentials of Williams (1966) and the ECD set (Buczacki et al. 1975), and to test the resistance to clubroot of canola cultivars included in the 2004 Prairie Canola Variety Trials (PCVT), in order to assess the risk to the crop. Pathogen populations from British Columbia and Ontario were also characterized for comparison.

5.3 Materials and Methods

Terminology

A 'population' of *P. brassicae* refers to a collection of pathogen resting spores, obtained either from infested soil or from galls of a susceptible plant, and used to inoculate a set of differential hosts (Buczacki et al. 1975). As suggested by previous workers (Crute et al. 1980; Voorips 1995), the term 'pathotype' will be used instead of 'race,' since neither the populations of the pathogen nor the differential hosts possess the genetic uniformity necessary to apply the concept of races to the clubroot pathosystem (Parlevliet 1985). To avoid confusion, this terminology will be used regardless of the authors' original terminology (Crute et al. 1980).

Pathogen collections

Nine collections of *P. brassicae* were characterized in this study (Table 5-1). Five collections, termed SACAN03-1 to SACAN03-5, were obtained from diseased canola plants growing in a severely clubroot-infested commercial field, approximately 8 km northwest of St. Albert, AB. A sixth collection, CDCN04-1, was made from a volunteer canola plant exhibiting severe galling in an experimental field at the Crop Diversification Centre North (CDCN), Alberta Agriculture, Food and Rural Development (AAFRD), in northeast Edmonton, AB. The seventh collection tested, Leduc-1, was obtained from clubroot-infested soil from a market garden near Leduc, AB, and the eighth, AbotJE04-1, was made from infested soil collected near Abbottsford, BC, and kindly supplied by Dr. J. Elmhirst (Elmhirst Diagnostics, Abbotsford, BC). The ninth collection, ORCA04, was obtained from an infected cabbage root from Orton, ON, and was provided by Ms. K. Callow (Ontario Ministry of Agriculture and Food, Guelph, ON).

The roots and stems of the canola plants collected near St. Albert were allowed to air dry and stored at room temperature until processing. All other root gall material was kept frozen at -86^{o}C until needed. Soil samples were stored at 4^{o}C.

Plant material

The differentials of Williams (1966) and the ECD set (Buczacki et al. 1975) were used to characterize pathogen populations. All of the differentials are listed in Table 5-2. Three of Williams' differential hosts are also members of the ECD set (ECD 10, 11 and 13), while the fourth (*B. napus* var. *napobrassica* cv. Laurentian) is unique to the former. Seeds of the differential hosts were obtained from Horticulture Research International, Genetic Resources Unit, Wellesbourne, Warwick, UK. Additional seeds of 'Laurentian'

were purchased from the Crucifer Genetics Cooperative, Madison, Wisconsin. Forty-eight canola cultivars included in the 2004 PCVT were also tested for their resistance to *P. brassicae* (Table 5-3). Seeds of these cultivars were supplied by the canola breeding program at the University of Alberta.

Host inoculation

To extract *P. brassicae* resting spores from dried root tissue, approximately 1 g of dry root material was placed in a mortar and ground to a powder using a pestle. Twenty mL of sterile distilled water were added, and the homogenate was then mixed and filtered through six layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, N.C.). To extract spores from frozen tissue, a modification of the procedure described by Williams (1966) was employed, as previously described (Tewari et al. 2005). Collections of *P. brassicae* from soil samples were recovered by planting five-day-old Chinese cabbage (*B. rapa* var. *pekinensis*) cv. Granaat seedlings (the universal suscept) in the infested soil; plants were grown for 6 weeks, the galled roots harvested, and the spores extracted as described above. Resting spores were quantified with a haemocytometer (VWR, Mississauga, ON) and used immediately or stored at 4^{o}C for a maximum of 2 days.

Suspensions of *P. brassicae* resting spores, adjusted to a concentration of 1×10^7 spores/mL using sterile distilled water, were used to inoculate the host plants to be tested. Five-day-old seedlings, germinated on filter paper in Petri dishes, were inoculated by dipping the roots in the spore suspension. Controls were dipped in sterile distilled water. Seedlings were immediately planted in 4 × 4 cm plastic pots filled with Metro-Mix 290 soil (Scotts, Columbus, Ohio) at a density of one seedling per pot. The pots were

thoroughly watered and transferred to a growth cabinet kept at 21/18°C (day/night) with a 16 h photoperiod. The soil was kept saturated with water for the first week after inoculation, and then watered and fertilized as required.

Disease assessment

A minimum of 12 seedlings of each differential were used in inoculations of the differential sets, and tests were repeated three to five times with each *P. brassicae* population. In resistance screening of canola cultivars, a minimum of 24 plants per cultivar were inoculated, and tests were repeated two to three times, using the population SACAN03-1. Six weeks after inoculation, roots were dug from the soil, washed with water, and examined for symptom development. The severity of root infection was assessed on a 0 to 3 scale, adapted from Kuginuki et al. (1999), where 0 = no galling, 1 = a few small galls, 2 = moderate galling and 3 = severe galling. A disease index (ID) was calculated using the following formula, modified from Horiuchi and Hori (1980):

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

Where: Σ is the sum total; n is the number of plants in a class; N is the total number of plants; and 0, 1, 2 and 3 are the symptom severity classes. The results presented correspond to the mean ID of all of the tests performed for a particular host against a population.

5.4 Results

Virulence patterns of *P. brassicae* populations

The *P. brassicae* populations SACAN03-1 – SACAN03-5, obtained from a severely-infested canola field near St. Albert, AB, behaved similarly on all of the

differential hosts; the disease indices induced by one representative population (SACAN03-1) are illustrated in Figure 1. All five St. Albert populations produced no or low levels of disease on the *Brassica rapa* L. hosts ECD 01 – 04. On ECD 01, ID values ranged from 1.0% (SACAN03-3) to 13.1% (SACAN03-1), with standard deviations of 2.1 to 9.3%, respectively; on hosts ECD 02 and ECD 04, ID values induced by the populations were consistently 0%. Although levels of disease were also low on ECD 03, there was more variability observed, with the highest ID values (21.5 ± 18.8%) caused by SACAN03-2. Only one *B. rapa* host, ECD 05, was fully susceptible to the St. Albert populations, all of which produced IDs of 100 ± 0% on this host.

In contrast to *B. rapa*, most *B. napus* hosts were susceptible to the *P. brassicae* populations from St. Albert (Fig. 5-1). The only exception was ECD 10, a rutabaga cultivar on which disease levels were generally low, ranging from 17.0 ± 12.0% (SACAN03-4) to 22.7 ± 10.3% (SACAN03-2). The fodder rape (*B. napus* var. *napus*) hosts ECD 06 – 09 were all highly susceptible, with IDs near or equal to 100% 6 weeks after inoculation with any of the St. Albert populations. On rutabaga cv. Laurentian, the only host unique to Williams' (1966) differential set, ID values induced by the populations were also near or equal to 100%.

The virulence pattern of SACAN03-1 – SACAN03-5 on the *B. oleracea* L. differentials (ECD 11 – 15) was less clear, as IDs with intermediate values were often observed (Fig. 5-1). On ECD 11, ID values induced by the populations ranged from 18.8 ± 14.9% (SACAN03-3) to 38.6 ± 17.2% (SACAN03-1). Similar values were observed on ECD 12, with IDs ranging from as low as 12.4 ± 10.9% (SACAN03-4) to as high as 42.8 ± 7.5% (SACAN03-1). The hosts ECD 13 and ECD 14 appeared more susceptible,

with IDs ranging from 65.5 ± 7.5% (SACAN03-2) to 78.4 ± 7.2% (SACAN03-1) for ECD 13, and 86.5 ± 12.9% (SACAN03-5) to 100 ± 0% (SACAN03-1, SACAN03-3) for ECD 14. On ECD 15, IDs induced by the St. Albert populations ranged from 21.3 ± 14.9% (SACAN03-3) to 28.6 ± 10.1% (SACAN03-2).

The *P. brassicae* population tested from Leduc, AB (Leduc-1) had a virulence pattern similar to that observed for the five populations from St. Albert (Fig. 5-1). As was the case with the St. Albert populations, all of the *B. rapa* hosts (with the exception of ECD 05) were resistant to Leduc-1, with the highest ID (27.8 ± 7.9%) observed on ECD 03. In contrast, ECD 05 was completely susceptible (ID = 100 ± 0%) to this population. As was also observed with the St. Albert populations, all of the *B. napus* hosts except for ECD 10 were susceptible to Leduc-1, with IDs near 100% for ECD 06 and ECD 08, and equal to 100 ± 0% for ECD 07 and ECD 09. Similarly, a relatively high ID value (84.7 ± 21.6%) was produced on 'Laurentian.' On the *B. oleracea* hosts, intermediate ID values were obtained after inoculation with the Leduc population, with ECD 11, ECD 12 and ECD 15 generally developing less severe symptoms than ECD 13 and ECD 14, a finding consistent with the results observed for the populations from St. Albert (Fig. 5-1).

The *P. brassicae* population characterized from the CDCN field in northeast Edmonton (CDCN04-1) possessed a somewhat different virulence pattern relative to the populations from St. Albert and Leduc. Like the other populations, CDCN04-1 was largely avirulent on hosts ECD 01 – ECD 04 and highly virulent on ECD 05 – 09 (Fig. 5-1). However, the amount of disease it caused on the *B. oleracea* hosts, particularly ECD 12 – 14, was significantly less. The CDCN population also produced fewer symptoms on

'Laurentian,' on which an ID of 33% was consistently observed. Furthermore, in contrast to the populations from St. Albert and Leduc, which induced IDs of 100% on a number of hosts, CDCN04-1 induced IDs greater than 90% on only two hosts (ECD 07 and ECD 08).

The two *P. brassicae* populations characterized from other provinces, AbotJE04-1 (BC) and ORCA04 (ON), produced unique virulence patterns. The British Columbia population was completely avirulent on ECD 06, ECD 08 – 09, and 'Laurentian' (ID values of 0%), hosts that were very susceptible to *P. brassicae* populations from Alberta (Fig. 5-1). On other hosts, however, AbotJE04-1 behaved similarly to the populations from St. Albert and Leduc, although the ID values it induced were generally lower, with a maximum ID of only 91.9 ± 7.1% on any one host (ECD 07). The population from Ontario was avirulent on most hosts, producing high levels of disease only on ECD 05 (ID = 77.8 ± 19.4%) and the *B. oleracea* hosts ECD 12 (ID = 62.5 ± 31.3%), ECD 13 (ID = 83.4 ± 23.5%) and ECD 14 (ID = 88.9 ± 15.7%) (Fig. 5-1). On ECD 15, an ID of 32.2% was observed, and on all other hosts, ID values ranged from 0% to less than 10%.

Resistance screening

In order to obtain a preliminary indication of clubroot resistance in current Canadian canola cultivars, 48 cultivars included in the 2004 PCVT were inoculated with *P. brassicae* population SACAN03-1. All varieties appeared highly susceptible to the disease (Table 5-3). The lowest level of symptom development was observed on 'SW 9803,' which had an ID of 85.0 ± 9.9%. All other cultivars had IDs greater than 90%, and nine had IDs of 100 ± 0%. In contrast, two clubroot-resistant winter oilseed rape

cultivars from Europe developed IDs of less than 40% after inoculation (results not shown).

5.5 Discussion

The virulence of seven *P. brassicae* populations from Alberta, as well as of one population from British Columbia and another from Ontario, was characterized on the differentials of Williams (1966) and the ECD set (Buczacki et al. 1975). However, while the reaction of some hosts to these populations could be clearly identified as resistant or susceptible, other differentials showed intermediate indices of disease (Fig. 5-1). This caused difficulty in classifying *P. brassicae* populations into pathotypes, as neither differential system refers to intermediate ID values (Williams 1966; Buczacki et al. 1975).

The inoculations in this study were conducted using field populations of *P. brassicae*. Therefore, it is possible that intermediate ID values were a result of the heterogeneity of the pathogen populations, as these may consist of a mixture of pathotypes (Tinggal and Webster 1981; Jones et al. 1982; Scott 1985). To preclude the possibility of mixed infections, Williams (1966) suggested that resting spores should be recovered from each differential host and used to re-inoculate the complete set of differentials. Similarly, Buczacki et al. (1975) stated that a population cannot be considered homogenous until at least two separate spore extractions and re-inoculations have not produced a change in host reaction. However, we repeated inoculations up to five times and still observed intermediate and fluctuating IDs for some hosts, particularly the *B. oleracea* subset (Fig. 5-1). In a previous study with Japanese populations of *P.*

brassicae, Kuginuki et al. (1999) also observed intermediate scores on several differentials, and suggested that indistinct reactions were due to genetic heterogeneity of the hosts rather than the pathogen. Indeed, resistance has not been conclusively shown to be differential in *B. oleracea*, and quantitative additive effects have been demonstrated in this species (Crute et al. 1980).

In an effort to accommodate intermediate ID values, Toxopeus et al. (1986) suggested that host reactions with an ID ≤ 20% should be regarded as resistant, and those with an ID ≥ 80% as susceptible. Although this criterion would allow classification of most of the *B. rapa* (ECD 01 – 05) and *B. napus* (ECD 06 – 10, 'Laurentian') hosts as either resistant or susceptible, the reactions of the majority of the *B. oleracea* hosts would still be indistinct (*sensu* Toxopeus et al. 1986), since these often developed IDs higher than 20% but lower than 80%. Therefore, in order to be able to assign pathotype designations to all populations tested, the range of values corresponding to a resistant and a susceptible reaction were expanded: hosts with an ID from 0 – 49% were regarded as resistant, and those with an ID from 50 -100 % as susceptible. Under this scheme, the populations from St. Albert and Leduc were classified as ECD 16/15/12 and the population from CDCN as ECD 16/15/0 on the differentials of Buczacki et al. (1975). On Williams' (1966) hosts, the St. Albert and Leduc populations were classified as pathotype 3 and the CDCN population as pathotype 5. Pathotype 3 was previously found to be predominant on cruciferous vegetables in Nova Scotia (Hildebrand and Delbridge 1995), while the population from CDCN represents the first reported occurrence of pathotype 5 in Canada. However, as noted above, little information is available on the pathotype composition of *P. brassicae* populations in Canada, and this pathotype may

occur elsewhere. The different populations and their pathotype classifications are summarized in Table 5-1.

The pathogen population from Abbotsford, BC, was classified as ECD 16/2/12 or pathotype 6 (Table 5-1). This pathotype 6 classification was consistent with a prior report (Williams 1966), which also identified a population from British Columbia as pathotype 6. The population from Orton, ON, was designated as ECD 16/0/14 or pathotype 6 (Table 5-1). This finding was also consistent with a previous report indicating that pathotype 6 is predominant in cole crops from Ontario (Reyes et al. 1974).

All *P. brassicae* populations examined were avirulent or of low virulence on the *B. rapa* hosts ECD 01 – 04 (Fig. 5-1). This observation was consistent with a survey of 299 ECD tests performed worldwide, in which these *B. rapa* hosts were found to be infected only rarely by the clubroot pathogen (Toxopeus et al. 1986). The remaining *B. rapa* host, ECD 05, is considered to be the universal suscept (Toxopeus et al. 1986) and was indeed susceptible to all populations characterized in this study (Fig. 5-1). Given the similar virulence patterns of all populations tested, the *B. rapa* subset did not appear useful for differentiating *P. brassicae* populations from Canada. In contrast, a complete range of reactions was observed on the *B. napus* hosts, indicating greater differentiating capacity in this subset. Interestingly, *P. brassicae* populations from Alberta were highly virulent on the fodder rape hosts ECD 06 – 09 (Fig. 5-1). In the case of the populations from St. Albert and CDCN, originally collected from canola, this may reflect a high degree of adaptation to a *B. napus* var. *napus* host. This relationship is not so clear for the Leduc population, which originated from infested soil from a market garden. Nevertheless, the populations from Ontario and British Columbia, obtained from regions

in which clubroot occurs mainly on cruciferous vegetables, were avirulent on all or all but one of the fodder rape varieties (Fig. 5-1). Seaman et al. (1963) reported that continuous cropping of a resistance source led to an erosion of its effectiveness, which was restored after a break of two years. This suggests that the pathotype composition of a population can shift quite rapidly, and is consistent with the increased virulence on *B. napus* observed in Alberta. It would be interesting to test whether *P. brassicae* populations collected from canola fields in Quebec, where clubroot has also been recently reported (Rimmer et al. 2004), also exhibit increased virulence on *B. napus*.

On the *B. oleracea* hosts, all pathogen populations produced at least some disease, a finding also consistent with previous reports (Toxopeus 1986; Voorips 1995). However, the CDCN population caused less disease on this subset than all other populations tested (Fig. 5-1). Given the fact that this population caused high amounts of disease on the *B. napus* hosts, it is unlikely that the low virulence observed on the *B. oleracea* varieties resulted from the experimental conditions or quality of inoculum used. Perhaps the CDCN population had parasitized canola for a longer period than other populations in Alberta, and therefore lost much of its virulence on the *B. oleracea* hosts; Crute et al. (1980) suggested that components of the pathogen population that are virulent on a particular host do not compete well with other components in the absence of selection. Indeed, no *B. oleracea* hosts had been grown on the CDCN site in recent years. It should be possible to test this hypothesis by re-isolating and re-inoculating the pathogen repeatedly on *B. oleracea* hosts, and observing whether symptom severity worsens over successive inoculations.

The reduced virulence of the CDCN population on the *B. oleracea* subset (and 'Laurentian') resulted in its unique pathotype designation, relative to the other populations from Alberta, under both classification systems used. As these differences in virulence were largely based on the reactions of the *B. oleracea* hosts, which produced intermediate reactions against all populations, these results should be treated with caution. Nevertheless, they seem to indicate the presence of genetic diversity in the pathogenicity of *P. brassicae* populations in Alberta. Moreover, if the intermediate ID values observed on some hosts were caused by pathotype mixtures, then presumably different pathotypes are present, even within single plants (Jones et al. 1982). In light of previous reports of clubroot in home and market gardens in Alberta, it was hypothesized that vegetable gardens may be acting as initial foci of infection, particularly through the import of infected transplants (Tewari et al. 2005). If this is the case, then a number of different initial populations may have been introduced, further contributing to pathogen heterogeneity. Characterization of single spore isolates from the Alberta populations may help to clarify this issue.

Unfortunately, screening of 48 canola cultivars included in the 2004 PCVT against a local population of *P. brassicae* indicated little if any resistance (Table 5-3). This is not surprising, considering that clubroot resistance has not been a focus of Canadian breeding efforts. Nevertheless, this high degree of susceptibility to the pathogen suggests that disease management may be particularly difficult if clubroot were to become more widely established in western Canada. As such, containment of the problem may be the most effective strategy for dealing with clubroot at this stage.

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5.7 Tables and Figures

Please refer to the following pages.

Table 5-1. Pathotype designations for field populations of *Plasmodiophora brassicae* collected in Alberta, British Columbia and Ontario, using a disease index of 50% as the cut-off between a resistant and susceptible reaction.

Population	Origin	Pathotype Designation[†]	
		ECD	Williams
SACAN03-1	St. Albert, AB (canola)	16/15/12	3
SACAN03-2	St. Albert, AB (canola)	16/15/12	3
SACAN03-3	St. Albert, AB (canola)	16/15/12	3
SACAN03-4	St. Albert, AB (canola)	16/15/12	3
SACAN03-5	St. Albert, AB (canola)	16/15/12	3
CDCN04-1	CDCN, Edmonton (canola)	16/15/0	5
Leduc-1	Leduc, AB (soil)	16/15/12	3
AbotJE04-1	Abbottsford, BC (soil)	16/2/12	6
ORCA04	Orton, ON (cabbage)	16/0/14	6

[†]As determined on the differential hosts of Williams (1966) or the European Clubroot Differential (ECD) set (Buczacki et al. 1975).

Table 5-2. Differential hosts used to characterize *Plasmodiophora brassicae* populations in the current study.

Common Name	Scientific Name	Cultivar or Line	ECD No. †
Polish Rape	*Brassica rapa* ssp. *rapifera*	line aaBBCC	01
Polish Rape	*Brassica rapa* ssp. *rapifera*	line AAbbCC	02
Polish Rape	*Brassica rapa* ssp. *rapifera*	line AABBcc	03
Polish Rape	*Brassica rapa* ssp. *rapifera*	line AABBCC	04
Chinese Cabbage	*Brassica rapa* var. *pekinensis*	'Granaat'	05
Fodder Rape	*Brassica napus* var. *napus*	'Nevin'	06
Fodder Rape	*Brassica napus* var. *napus*	'Giant Rape'	07
Fodder Rape	*Brassica napus* var. *napus*	Giant Rape Selection	08
Fodder Rape	*Brassica napus* var. *napus*	New Zealand Resistant Rape	09
Rutabaga	*Brassica napus* var. *rapifera*	'Wilhemsburger'	10
Cabbage	*Brassica oleracea* var. *capitata*	'Badger Shipper'	11
Cabbage	*Brassica oleracea* var. *capitata*	'Bindsachsener'	12
Cabbage	*Brassica oleracea* var. *capitata*	'Jersey Queen'	13
Cabbage	*Brassica oleracea* var. *capitata*	'Septa'	14
Kale	*Brassicae oleracea* var. *capitata* subvar. *laciniata*	'Verheul'	15
Rutabaga	*Brassica napus* var. *napobrassica*	'Laurentian'	n/a

†European Clubroot Differential (ECD) numbers denote the differential hosts of the ECD set (Buczacki et al. 1975); hosts ECD 10, 11, 13 and *B. napus* var. *napobrassica* represent the differentials of Williams (1966).

Table 5-3. Reaction of *Brassica napus* L. and *Brassica rapa* L. canola varieties, included in the 2004 Prairie Canola Variety Trials, to inoculation with *Plasmodiophora brassicae* population SACAN03-1, obtained from St. Albert, AB.

Variety	Index of Disease[†]	Variety	Index of Disease[†]
	Brassica napus		
3458_01	93.0 ± 5.7%	46H70	97.0 ± 4.2%
LBD 2393LL	100.0 ± 0%	v1032	100.0 ± 0%
46A65	90.0 ± 11.1%	46A76	100.0 ± 0%
NEX830CL	96.0 ± 5.7%	NEX 824CL	91.3 ± 7.6%
5030	97.0 ± 4.2%	9550	100.0 ± 0%
3140_01	92.5 ± 2.1%	163-12	100.0 ± 0%
46H23	95.3 ± 8.1%	5070	100.0 ± 0%
1841	98.5 ± 2.1%	GladiatoRR	100.0 ± 0%
34-55	97.0 ± 4.2%	IMC 209RR	98.5 ± 2.1%
AV 9505	97.0 ± 4.2%	46H02	98.5 ± 2.1%
V1031	96.0 ± 5.7%	LBD588RR	100.0 ± 0%
SP Craven	97.0 ± 4.2%	CNH1505R	98.5 ± 2.1%
SP Distinction CL	89.0 ± 9.8%	Cougar CL	94.0 ± 4.2 %
SP 442 CL	90.3 ± 4%	45H72	94.5 ± 7.8%
SP Deliver CL	92.7 ± 12.7%	289 CL	100 ± 0%
5020	96.0 ± 5.7%	33-95	93.3 ± 3.2%
Fortune RR	96.0 ± 5.7%	SW 6802	96.0 ± 5.7%
SW 9803	85.0 ± 9.9%	43A56	90.5 ± 2.1%
45H24	94.5 ± 7.8%	SP Desirable RR	98.5 ± 2.1%
1896	97.0 ± 4.2%	45H21	94.5 ± 7.8%
SP 451 RR	97.0 ± 4.2%	9451	94.3 ± 9.8%
SP Banner	97.0 ± 4.2%	Westar[‡]	99.3 ± 2.1%
	Brassica rapa		
AC Parkland	96.0 ± 5.7%	ACS-C7	91.0 ± 10.8%
SW Spirit River	91.0 ± 4.2%	AC Sunbeam	95.5 ± 6.4%

[†]A minimum of 24 plants per cultivar were rated for severity of root infection (six weeks after inoculation) on a 0 to 3 scale, where: 0 = no galling, 1 = a few small galls, 2 = moderate galling, and 3 = severe galling. Indices of disease were calculated using the formula:
$ID(\%) = \frac{\sum(n\times 0 + n\times 1 + n\times 2 + n\times 3)}{N\times 3}\times 100\%$, where: Σ is the sum total; n is the number of plants in a class; N is the total number of plants; and 0, 1, 2 and 3 are the symptom severity classes. Tests were repeated two to three times and the means are presented, followed by the standard deviations.
[‡]Canola cv. Westar was included as a susceptible check.

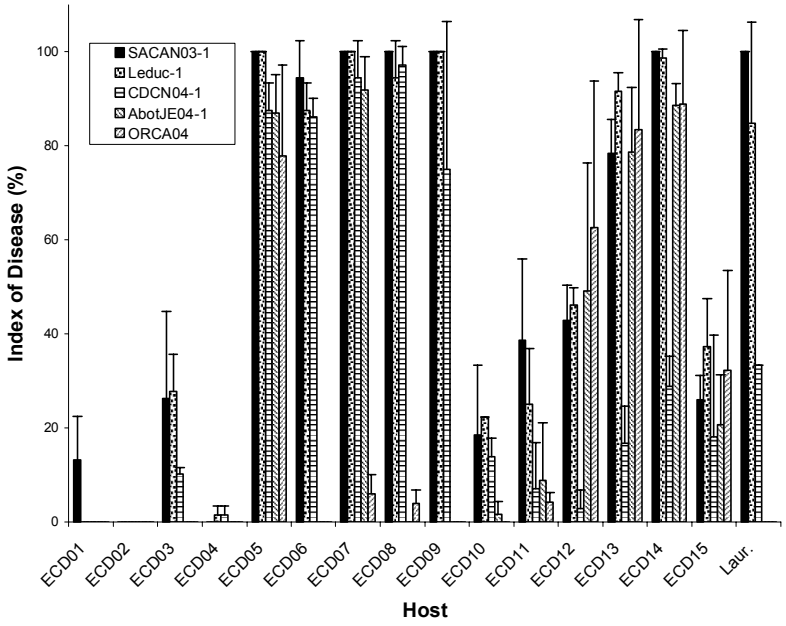

Figure 5-1. Virulence of *Plasmodiophora brassicae* populations on the differential hosts of Williams (1966) and the European Clubroot Differential (ECD) set. SACAN03-1 was collected near St. Albert, AB, Leduc-1 near Leduc, AB, CDCN04-1 in northeast Edmonton, AB, AbotJE04-1 near Abbotsford, BC, and ORCA04 near Orton, ON. Tests were repeated three to five times, and the mean indices of disease (ID) are shown as bars. Lines indicate standard deviations. ECD 01 = *Brassica rapa* ssp. *rapifera* line aaBBCC, ECD 02 = *B. rapa* spp. *rapifera* line AAbbCC, ECD 03 = *B. rapa* ssp. *rapifera* line AABBcc, ECD 04 = *B. rapa* ssp. *rapifera* line AABBCC, ECD 05 = *B. rapa* var. *pekinensis* cv. Granaat, ECD 06 = *Brassica napus* var. *napus* cv. Nevin, ECD 07 = *B. napus* var. *napus* cv. Giant Rape, ECD 08 = *B. napus* var. *napus* selection ex. "Giant Rape," ECD 09 = *B. napus* var. *napus* New Zealand clubroot resistant rape, ECD 10 = *B. napus* var. *napobrassica* cv. Wilhemsburger, ECD 11 = *Brassica oleracea* var. *capitata* cv. Badger Shipper, ECD 12 = *B. oleracea* var. *capitata* cv. Bindsachsener, ECD 13 = *B. oleracea* var. *capitata* cv. Jersey Queen, ECD 14 = *Brassica oleracea* var. *capitata* cv. Septa, ECD 15 = *B. oleracea* var. *acephala* subvar. *laciniata* cv. Verheul, Laur = *B. napus* var. *napobrassica* cv. Laurentian. Hosts ECD 10, 11 and 13 are also members of Williams' (1966) differential set, but 'Laurentian' is unique to this set.

Chapter 6: Pathotype Classification of *Plasmodiophora brassicae* and its Occurence in *Brassica napus* in Alberta, Canada

6.1 Abstract

A field survey for clubroot of crucifers, caused by *Plasmodiophora brassicae*, was conducted in the regions surrounding Edmonton, Alberta, Canada, in 2005. The presence of clubroot was confirmed in 41 of the 112 canola (*Brassica napus*) fields surveyed. These *P. brassicae*- infested fields were located in Sturgeon, Strathcona, Leduc, and Wetaskiwin counties, as well as in a rural area of northeast Edmonton. Infected roots were also received from an infested field in Flagstaff County, southeast of Edmonton. Although there was a significant negative correlation between index of disease (ID) and soil pH, the occurrence of clubroot was not restricted to fields with acidic soils. Populations of the pathogen were selected from 10 fields and used in pathotype classification on the differential hosts of Williams, Somé et al., and the European Clubroot Differential (ECD) set. Kruskal-Wallis analysis indicated no significant differences in the virulence of the 10 populations tested, suggesting that they are relatively homogenous. If a disease index of 50% was regarded as the cut-off between a resistant and a susceptible reaction, then all *P. brassicae* populations tested were classified as ECD -/15/12 on the hosts of the ECD set, or as pathotypes 3 or P_2 on the differentials of Williams or Somé et al., respectively. However, it may be difficult to detect rare or infrequent pathotypes when field populations of the pathogen are used for characterization.

6.2 Introduction

Clubroot of crucifers, caused by *Plasmodiophora brassicae* Woronin, is a devastating soil-borne disease of cruciferous crops including canola (oilseed rape), broccoli, brussels sprouts, cabbage, kale, kohlrabi, radish, rutabaga, cauliflower, turnip, and black mustard. The pathogen induces root galls on infected plants, reducing the capacity for water and nutrient uptake, resulting in stunting, wilting, lodging, and finally major yield losses of 50% or more (Voorrips 1995; Wallenhammar et al. 1999; Strelkov et al. 2005). Growing susceptible crops on the same land exacerbates the disease because *P. brassicae* resting spores can build up as an inoculum in the soil after the root galls decay. Moist and acidic soils are most favorable for disease development (Karling 1968).

Clubroot has long been a problem in cruciferous crops (Woronin 1878). The disease is a limiting factor in oilseed production in Australia, Japan, Scotland, England, Wales, France, and Germany (Voorrips 1995; Wallenhammar et al. 2000). A survey of a spring oilseed rape growing area of central Sweden indicated that 78% of 190 fields visited were infested with *P. brassicae* (Wallenhammar 1996). In Canada, clubroot has traditionally been a problem in vegetable cole crop production in eastern regions of the country and in British Columbia (Rimmer et al. 2003). However, the disease has been recently identified on canola in the provinces of Alberta (Tewari et al. 2005; Strelkov et al. 2005) and Quebec (Pageau et al. 2006), and field surveys indicate that *P. brassicae* has become established as a pest of canola in central Alberta (Tewari et al. 2005; Strelkov et al. 2005, 2006a). Clubroot could pose a serious threat to the oilseed and cole crop industries, because of the long persistence of the disease in the soil (Karling 1968) and

the possibly heterogeneous composition of *P. brassicae* pathotypes in the same gall (Tinggal 1980; Jones et al. 1982a, 1982b).

Physiological characterization of *P. brassicae* was initiated many decades ago (Honig 1931), and numerous sets of differential hosts have been proposed for the assessment of virulence in the pathogen (Ayers 1957; Williams 1966; Buczacki et al. 1975; Somé et al. 1996). In studies of pathogen populations from Canada, the differential set of Williams (1966), which consists of two cabbage and two rutabaga cultivars, has been most widely used. In his original paper, Williams (1966) identified pathotypes ('races') 2 and 6 in populations of *P. brassicae* obtained from Quebec and British Columbia, respectively. More recently, pathotype 6 was also reported from another population from British Columbia (Strelkov et al. 2006b). Similarly, pathotype 6 was determined to be the predominant pathotype in cole crops from Ontario (Reyes et al. 1974), a finding that was confirmed in a more recent study (Strelkov et al. 2006b). In populations of *P. brassicae* from Nova Scotia, pathotype 3 was found to be predominant (Hildebrand and Delbridge 1995). In Alberta, a total of seven populations have been characterized: five populations obtained from a commercial canola field near St. Albert were identified as pathotype 3, as was a population from a market garden near Leduc, while another population from experimental field plots located in northeast Edmonton was given a pathotype 5 designation (Strelkov et al. 2006b). The virulence of the Alberta populations was also evaluated on the hosts of the European Clubroot Differential (ECD) set (Buczacki et al. 1975), which consists of 15 genotypes representing three subsets (a *Brassica rapa* L., a *Brassica napus* L. and a *Brassica olearacea* L. subset). On the hosts of the ECD set, the populations from St. Albert and Leduc were designated as ECD

16/15/12, while the population from northeast Edmonton was designated as ECD 16/15/0 (Strelkov et al. 2006b).

Although *P. brassicae* populations in Alberta appeared to be fairly homogenous (Strelkov et al. 2006b), most of the populations tested came from the same field. While this was a justified approach for evaluating pathogen diversity when only 12 tightly clustered fields were known to be infested with clubroot (Strelkov et al. 2005), the disease has since been found to be much more widespread in central Alberta (Strelkov et al. 2006a), and the diversity of *P. brassicae* over this larger region needs to be assessed. Such information is important in the development of canola cultivars with resistance to the predominant pathotypes of *P. brassicae*, as well as for the appropriate management of this resistance once it is obtained. In this study, we present the results of a larger survey of canola fields in central Alberta, and characterize the virulence of 10 *P. brassicae* populations selected from geographically separated locations, in order to gain a better understanding of the diversity in the pathogen. Furthermore, the disease severity of all fields visited is correlated with soil pH, to evaluate the risk posed by clubroot to the total canola acreage in Alberta and the Canadian Prairies. A preliminary report on this work has been published (Strelkov et al. 2006a).

6.3 Materials and Methods

Terminology

The terminology used will be as defined in earlier research (Strelkov et al. 2006b), with the term 'population' referring to a collection of *P. brassicae* resting spores made from galls of a susceptible plant or from infested soil, and used to inoculate a set of

differential hosts (Buczacki et al. 1975). The term 'pathotype' will be used in place of 'race' (Crute et al. 1980; Voorips 1995), regardless of the authors' original terminology, since neither the pathogen populations nor the differential hosts possess the genetic uniformity required to apply the concept of races to the clubroot pathosystem (Parlevliet 1985).

Clubroot survey and collection of pathogen field populations

In September 2005, a total of 112 commercial canola fields were surveyed for clubroot in central Alberta, including 77 fields in Sturgeon County, 10 in Strathcona County, 6 in Parkland County, 17 in northeast Edmonton, and 1 field each in Leduc and Wetaskiwin counties. The fields were visited after swathing, and the roots of all plants within a 1 m^2 area were inspected at each of 10 locations along the arms of a 'W' sampling pattern. The presence of galls was taken as an indication of *P. brassicae* infection, and the severity of root infection was assessed on a 0 to 3 scale and used to calculate a disease index, as described below.

Galled roots and soil samples were collected from each infested field, allowed to air dry, and stored in brown paper bags at room temperature until further processing. In addition to the roots collected in the survey, galled roots collected by Dr. Godfrey Chongo (Bayer Crop Science) from an infested field in Flagstaff County, AB, were also included in the analysis. A total of 10 *P. brassicae* populations were selected for pathotype testing; these were chosen because they represented relatively isolated clubroot-infested fields or clusters of fields (Fig. 6-1). Each population was derived from a collection of *P. brassicae* resting spores made from a single plant from each field. Only one field population was chosen for characterization within each group of infested fields.

Plant materials

Three sets of differential hosts, including those of Williams (1966), the ECD series (with the omission of the first four genotypes of the first subset) (Buczacki et al. 1975), and the differentials of Somé et al. (1996), were used for pathotype differentiation in this study (Table 6-1). Three of Williams' (1966) differentials are also members of the ECD series (ECD 10, 11, and 13), as are two of the differentials used by Somé et al. (1996) (ECD 06 and ECD 10). In contrast, the hosts *B. napus* var. *napobrassica* cv. Laurentian and *B. napus* var. *napus* cv. Brutor are unique to the differential sets of Williams (1966) and Somé et al. (1996), respectively. Seeds of all of the differential hosts, except for 'Laurentian' and 'Brutor,' were obtained from Horticulture Research International, Genetic Resources Unit (Wellesbourne, Warwick, UK). Seeds of 'Laurentian' and 'Brutor' were purchased from the Crucifer Genetics Cooperative (Madison, Wisconsin) and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Genebank (Gatersleben, Germany), respectively.

Host inoculation

Resting spores of *P. brassicae* were extracted as previously described (Tewari et al. 2005), with some minor modifications. Briefly, 2.5 to 3.0 g of dried root material were ground in a mortar with a pestle, 50 ml of sterile deionized water (sdH_2O) were added, and the homogenate was mixed and filtered through eight layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, NC). The resting spores were quantified with a hemocytometer (VWR, Mississauga, ON) and suspensions of 2×10^7 to 3×10^8 resting spores/ml used for inoculation of host roots. One-week old seedlings of the various differential hosts, which were pre-germinated on a piece of moistened filter paper

in Petri dishes, were inoculated by dipping the entire root system in the resting spore suspension for 10 s. The inoculated seedlings were then immediately planted in 6 × 6 × 6 cm plastic pots (Kord Products Inc., Brampton, ON) filled with Metro-Mix 290 soil (Scotts, Columbus, OH) at a rate of one seedling per pot. The pots were thoroughly watered and transferred to either a growth cabinet kept at 21/18^{o}C (day/night) with a 16 h photoperiod, or a greenhouse at 20 ± 2 ^{o}C with a 16 h photoperiod. The soil in the pots was kept saturated with water for the first week after inoculation, and then watered and fertilized as required. Twenty four seedlings of each host line were used in inoculation of the differentials with each *P. brassicae* population, and inoculations were repeated a total of four times (two repetitions each in the greenhouse and growth chamber).

Disease assessment and statistical analysis

Six weeks after inoculation, the roots of each differential host line were dug out, washed with tap water, and examined for gall formation. The severity of root galls was evaluated on a 0 to 3 scale as described previously (Kuginuki et al. 1999), where 0 = no galling, 1 = a few small galls, 2 = moderate galling, and 3 = severe galling. A disease score was calculated for each differential line using the formula:

$Score = \sum (n \times 0 + n \times 1 + n \times 2 + n \times 3)$, where n is the number of plants in each class and 0, 1, 2 and 3 are the symptom severity classes. The average disease scores obtained from the four repetions of the experiment were analyzed using the Kruskal-Wallis Test, a non-parametric significance test between two populations. All statistical analyses were conducted with SAS 8.2 software (SAS Institute Inc. 1999).

An index of disease (ID) was also calculated for each host differential line, using the method of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006b):

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

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

Determination of soil pH in water

During the field survey, approximately 1 kg of soil was sampled from the top soil layer (0 to 15 cm) of a clubroot-positive spot in each field. Only one soil sample was collected from each clubroot-positive field. Soil pH in water was determined according to Peech (1965) with some modifications. Briefly, 50 g of air-dried soil (soil particles <1 mm) were suspended in 50 ml sdH_2O with occasional agitation for one hour. The soil particles were allowed to settle and the pH of the supernatant determined using an Accumet AB15/15+ pH meter (Fisher Scientific, Ottawa, ON).

6.4 Results

Clubroot survey

A total of 41 clubroot-infested fields were identified in 2005. The majority of infested fields (Strelkov et al. 2006) were located in Sturgeon County (north of the City of Edmonton) or in a rural area in the northeast corner of the city (11 fields). In addition, two clubroot-infested canola fields were identified south of the city, in Wetaskiwin and Leduc counties, and one field was found east of Edmonton, in Strathcona County. Within clubroot-infested fields, disease severities ranged from low (IDs ≤ 10%) to moderate (IDs 10 to 40%) to high (IDs ≥ 40%); in most infested fields, however, severity was low to

moderate. The index of disease of the infested fields was found to be negatively correlated with the soil pH as determined in water (Fig. 6-2).

Virulence of *P. brassicae* field populations

Pairwise comparisons using the Kruskal-Wallis Test indicated no significant differences in the virulence of the 10 *P. brassicae* field populations characterized in this study (results not shown). There were, however, signficantly different responses to inoculation among the host genotypes. The host reactions, which ranged from highly susceptible to highly resistant, are summarized as IDs in Table 6-2 in order to facilitate comparisons with most of the existing literature. As expected, the universal suscept, ECD 05, was highly susceptible to all of the populations tested. Similarly, all of the pathogen populations produced very high levels of disease on the fodder rape (*B. napus* var. *napus*) hosts ECD 06-ECD 09, with IDs ranging from 80% ± 6% to 100% ± 0% (Table 6-2). Most of the other *B. napus* hosts were also highly susceptible; the rutabaga "Laurentian" and the spring oilseed rape "Brutor" developed IDs ranging from 89% ± 4% to 100% ± 0% in response to inoculation. In contrast, the reaction of the remaining *B. napus* host, ECD 10, ranged from highly to moderately resistant, and IDs of 9% ± 2% to 33% ± 13% were obtained.

The reactions of the *B. oleracea* differentials were generally less distinct than hose of the *B. napus* genotypes (Table 6-2). Inoculation of ECD 11 and ECD 12 resulted in development of IDs from as low as 8% ± 3% to as high as 45% ± 2%. On ECD 15, IDs ranging from 21% ± 7% to 43% ± 2% were observed, indicating that this genotype was also moderately resistant to infection. Although intermediate ID values were also observed on ECD 13, these tended to be higher than on ECD 11, 12 or 15, and ranged

from a low of 62% ± 4% to a high of 86% ± 8%. Thus, this host appeared to be moderately to highly susceptible to the *P. brassicae* populations tested. However, the highest ID values of any of the *B. oleracea* genotypes were observed on ECD 14. On this highly susceptible cabbage, IDs ranged from 86% ± 9% to 99 ± 1%.

6.5 Discussion

The detection of over 40 clubroot-infested fields in 2005 suggests that the disease may be more prevalent on canola in central Alberta than previously thought. It is not clear whether these cases represent new infestations, or simply increased awareness of and surveying for the disease. Most clubroot-infested canola plants were identified near field entrances, suggesting that the pathogen is introduced into fields on contaminated farming equipment. In all but the most heavily infested fields, disease distribution was patchy, which is common for soilborne pathogens. In severely infested fields, clubroot distribution was relatively uniform. Although acidic soils are known to favor clubroot development (Karling 1968), the occurrence of the disease was not restricted to fields with acidic soils, and the pH of infested fields ranged from 4.8 to 7.6, with an average value of 6.2. Nevertheless, there was a significant negative correlation between disease index and soil pH ($R^2 = 0.1467$, $P < 0.0134$) (Fig. 6-2), which indicates that acidic soils may be most at risk.

In our present analysis of the virulence pattern of *P. brassicae* populations from Alberta, most members of the *B. rapa* subset of the ECD set (Buczacki et al. 1975) were excluded, as we had previously found that this subset was not useful for differentiating pathogen populations from Canada (Strelkov et al. 2006b). However, *Brassica rapa* var.

pekinenses cv. Granaat (ECD 05) was retained as a susceptible check. We also examined the reactions of the *B. napus* and *B. olreacea* subsets of the ECD, *B. napus* var. *napobrassica* cv. Laurentian (Williams, 1966), and *B. napus* cv. Brutor, a differential used by Somé et al. (1996). The latter was not included in a previous characterization of *P. brassicae* from Canada (Strelkov et al. 2006b), but allowed us to obtain pathotype classifications as per Somé et al. (1996). The virulence of the pathogen populations on these differential genotypes was not significantly different (as determined by non-parametric analysis), and was consistent with what was reported previously for a smaller number of populations tested from Alberta (Strelkov et al. 2006b). Generally, the fodder rape hosts ECD 06-09 and 'Brutor' were highly susceptible, which may reflect a high degree of adaptation by local populations to a *B. napus* host, likely because these populations were collected from canola fields (Strelkov et al. 2006b). Similarly, *B. napus* var. *napobrassica* cv. Laurentian was also highly susceptible.

However, as has previously been observed (Toxopeus et al.1986; Strelkov et al. 2006b), the hosts of the *B. oleracea* subset of the ECD tended to develop intermediate ID values (Table 6-2). Intermediate and fluctuating IDs have been attributed to the heterogeneity of pathogen populations (Tinggal and Webster 1981; Jones et al. 1982a; Scott 1985) and/or to the heterogeneity of the differential hosts themselves (Kuginuki et al. 1999). Repeated spore extractions and reinoculations failed to yield clearly distinguishable host reactions (Strelkov et al. 2006b), as did inoculation of the differential hosts with single spore-derived isolates of *P. brassicae* (Chapter 8). Therefore, we believe that host heterogeneity may be a major factor in the intermediate ID values observed on some differentials. Furthermore, resistance to *P. brassicae* has not been

conclusively shown to be differential in *B. oleracea*, and quantitative additive effects have been reported in this species (Crute et al. 1980; Crute et al. 1983). This makes classification of the pathogen into individual pathotypes problematic, since a qualitative designation (i.e. resistant or susceptible) has to be applied to what in many cases appears to be a quantitative reaction.

A number of approaches have been proposed for dealing with this problem. Toxopeus et al. (1986) suggested that hosts developing an ID of 20% or less should be classified as resistant, while those with an ID of 80% or more should be classified as susceptible. However, this rating scheme does not accommodate the reactions of most of the *B. oleracea* differentials, which generally developed IDs between 20 and 80% (Table 6-2). Kuginuki et al. (1999) used the Wilcoxson method to test the differences between the ID of each host genotype and IDs of the genotypes with minimum or maximum IDs in an experiment. Hosts with an ID that was not significantly different from the genotype with the minimum ID were regarded as resistant, while those with an ID not significantly different from the genotype with the maximum ID were regarded as susceptible; those in between were designated as partially resistant (Kuginuki et al. 1999). However, it is difficult to assign a broad designation to hosts exhibiting intermediate disease reactions, since these can range from moderately susceptible to moderately resistant. In our present study, the 13 differential hosts could be split into six groups (at a significance level of 5% using the Kruskal-Wallis Test) in response to inoculation with the homogenous *P. brassicae* populations from Alberta (results not shown). This again suggests a continuum of host reactions, an observation that must be confirmed using single spore isolates rather than populations of the pathogen. If this continuum exists, a rating scale for evaluating

the response of host genotypes, with designations ranging from highly susceptible to highly resistant, may be more appropriate for the clubroot pathosystem.

Nevertheless, if pathotype designations are to be assigned to the *P. brassicae* populations tested in the current study, a cut-off point between a resistant and susceptible reaction must be selected. Previously, Strelkov et al. (2006b) used an ID of 50% as this cut-off when analyzing populations from Alberta. In contrast, using *P. brassicae* populations and single-spore isolates from France, Somé et al. (1996) regarded an ID of 25% as the cut-off between a resistant and susceptible reaction. However, we found that while there was a fairly continuous range of host IDs from 0 to about 45%, and from about 65 to 100%, very few genotypes developed IDs in the 45 to 65% range. Therefore, a cut-off of 50% seems to represent a more natural or less arbitrary separation point between a susceptible or resistant reaction, at least with *P. brassicae* populations from Canada (Table 6-2; Strelkov et al. 2006b). Using this criterion, all *P. brassicae* populations tested in the present study would be classifed as ECD -/15/12 on the differentials of the ECD set (Table 6-3) (Buczacki et al. 1975), which would correspond to pathotype 3 according to the system of Williams (1966), or P_2 on the hosts of Somé et al. (1996). Nevertheless, if a cut-off of 25% is used, then the populations from fields 1, 30, 64, 90, 112, and 113 would be classifed as P_2, whereas fields 44, 52, 92 and 111 would be classified as P_1. The different classification would be based solely on the reaction of ECD 10 (*B. napus* var. *napobrassica* cv. Wilhemsburger), which developed IDs ranging from 10% ± 1% to 33% ± 13% in response to inoculation with the populations tested.

It appears that *P. brassicae* field populations from Alberta are fairly homogenous. However, field populations may consist of a mixture of pathotypes, and rare pathotypes within that mixture may not be detected, particularly if they occur at very low frequencies (Jones et al. 1982b). Therefore, the diversity of the pathogen is not necessarily fully assessed when tests are conducted using *P. brassicae* populations. Indeed, in a previous study, at least one population from Alberta gave a pathotype 5 or ECD 16/15/12 designation on the hosts of Williams (1966) and the ECD set (Buczacki et al. 1975), respectively (Strelkov et al. 2006b). Since it appears that the pathotype composition of a population can shift quite rapidly, particularly when a resistance source is continuously cropped (Seaman et al. 1963), it will be important to compare the virulence patterns of single spore isolates of *P. brassicae* with the field populations from which they were derived. This will help ensure that clubroot-resistance is properly managed in any canola cultivars that are introduced. Such work is currently the focus of ongoing research.

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6.7 Tables and Figures

Please refer to the following pages.

Table 6-1. Differential hosts used to identify *Plasmodiophora brassicae* pathotypes in Alberta, Canada.

ECD No.*	Differential host
ECD 05	*Brassica rapa* var. *pekinensis* cv. Granaat
ECD 06	*Brassica napus* var. *napus* cv. Nevin
ECD 07	*Brassica napus* var. *napus* cv. Giant Rape
ECD 08	*Brassica napus* var. *napus* selection ex. 'Giant Rape'
ECD 09	*Brassica napus* var. *napus* New Zealand clubroot resistant rape
ECD 10	*Brassica napus* var. *napobrassica* cv. Wilhemsburger
ECD 11	*Brassica oleracea* var. *capitata* cv. Badger Shipper
ECD 12	*Brassica oleracea* var. *capitata* cv. Bindsachsener
ECD 13	*Brassica oleracea* var. *capitata* cv. Jersey Queen
ECD 14	*Brassica oleracea* var. *capitata* cv. Septa
ECD 15	*Brassica oleracea* var. *acephala* subvar. *laciniata* cv. Verheul
na	*Brassica napus* var. *napobrassica* cv. Laurentian
na	*Brassica napus* cv. Brutor (spring oilseed rape)

*European Clubroot Differential (ECD) numbers denote the differential hosts of the ECD set (Buczacki et al. 1975); hosts ECD 10, 11, 13 and "Laurentian" represent the differentials of Williams' (1966), whereas hosts ECD 06, ECD 10 and "Brutor" represent the differentials of Somé et al. (1996).

Table 6-2. Virulence of *Plasmodiophora brassicae* populations from Alberta, Canada, on the differential hosts of Williams (1966), Somé et al. (1996) and the European Clubroot Differential (ECD) set.

Differential host	Index of disease (%) induced by populations of *Plasmodiophora brassicae**									
	Field 1	Field 30	Field 44	Field 52	Field 64	Field 90	Field 92	Field 111	Field 112	Field 113
ECD05	100 ± 0	100 ± 0	100 ± 0	100 ± 0	99 ± 1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
ECD06	95 ± 2	94 ± 3	95 ± 2	88 ± 4	92 ± 3	94 ± 5	86 ± 6	95 ± 4	80 ± 6	94 ± 4
ECD07	100 ± 0	100 ± 0	100 ± 0	99 ± 1	99 ± 1	99 ± 1	100 ± 0	100 ± 0	98 ± 2	99 ± 1
ECD08	99 ± 1	100 ± 0	100 ± 0	97 ± 3	95 ± 2	98 ± 2	99 ± 1	100 ± 0	99 ± 1	100 ± 0
ECD09	99 ± 1	96 ± 4	98 ± 2	94 ± 4	90 ± 4	99 ± 1	99 ± 1	97 ± 2	93 ± 6	97 ± 2
ECD10	10 ± 1	18 ± 3	33 ± 13	28 ± 5	16 ± 4	20 ± 8	29 ± 5	27 ± 4	22 ± 2	9 ± 2
ECD11	31± 6	40 ± 14	39 ± 3	45 ± 2	8 ± 3	41 ± 2	23 ± 5	21 ± 3	20 ± 4	44 ± 5
ECD12	27± 2	24 ± 3	36 ± 12	31 ± 4	42 ± 13	33 ± 2	22 ± 6	18 ± 4	16 ± 5	42 ± 7
ECD13	71± 8	72 ± 8	84 ± 4	86 ± 8	65 ± 5	83 ± 7	63 ± 11	62 ± 4	77 ± 6	82 ± 5
ECD14	94 ± 0	95 ± 2	99 ± 1	99 ± 1	86 ± 9	93 ± 3	89 ± 4	94 ± 4	90 ± 2	96 ± 2
ECD15	40 ± 4	34 ± 7	34 ± 3	30 ± 3	34 ± 6	39 ± 14	26 ± 6	23 ± 1	21 ± 7	43 ± 2
'Laurentian'	94 ± 3	93 ± 4	100 ± 0	99 ± 1	89 ± 4	92 ± 3	94 ± 3	91 ± 3	93 ± 2	99 ± 1
'Brutor'	100 ± 0	100 ± 0	100 ± 0	97 ± 2	94 ± 3	98 ± 2	100 ± 0	100 ± 0	99 ± 1	99 ± 1

* Means of four replicates ± 1 standard error. Indices of disease were calculated using the formula:

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

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

Table 6-3. Pathotype designations for field populations of *Plasmodiophora brassicae* collected from commercial canola fields in Alberta, Canada, in 2005.

Population	Pathotype designation*		
	ECD	Williams	Somé et al.
Field 1	-/15/12	3	P_2
Field 30	-/15/12	3	P_2
Field 44	-/15/12	3	P_2
Field 52	-/15/12	3	P_2
Field 64	-/15/12	3	P_2
Field 90	-/15/12	3	P_2
Field 92	-/15/12	3	P_2
Field 111	-/15/12	3	P_2
Field 112	-/15/12	3	P_2
Field 113	-/15/12	3	P_2

*As determined on the differential hosts of the European Clubroot Differential (ECD) set (Buczacki et al. 1975), Williams (1966), or Somé et al. (1996). For classification into pathotypes, an index of disease of 50% was used as the cut-off between a resistant and a susceptible reaction.

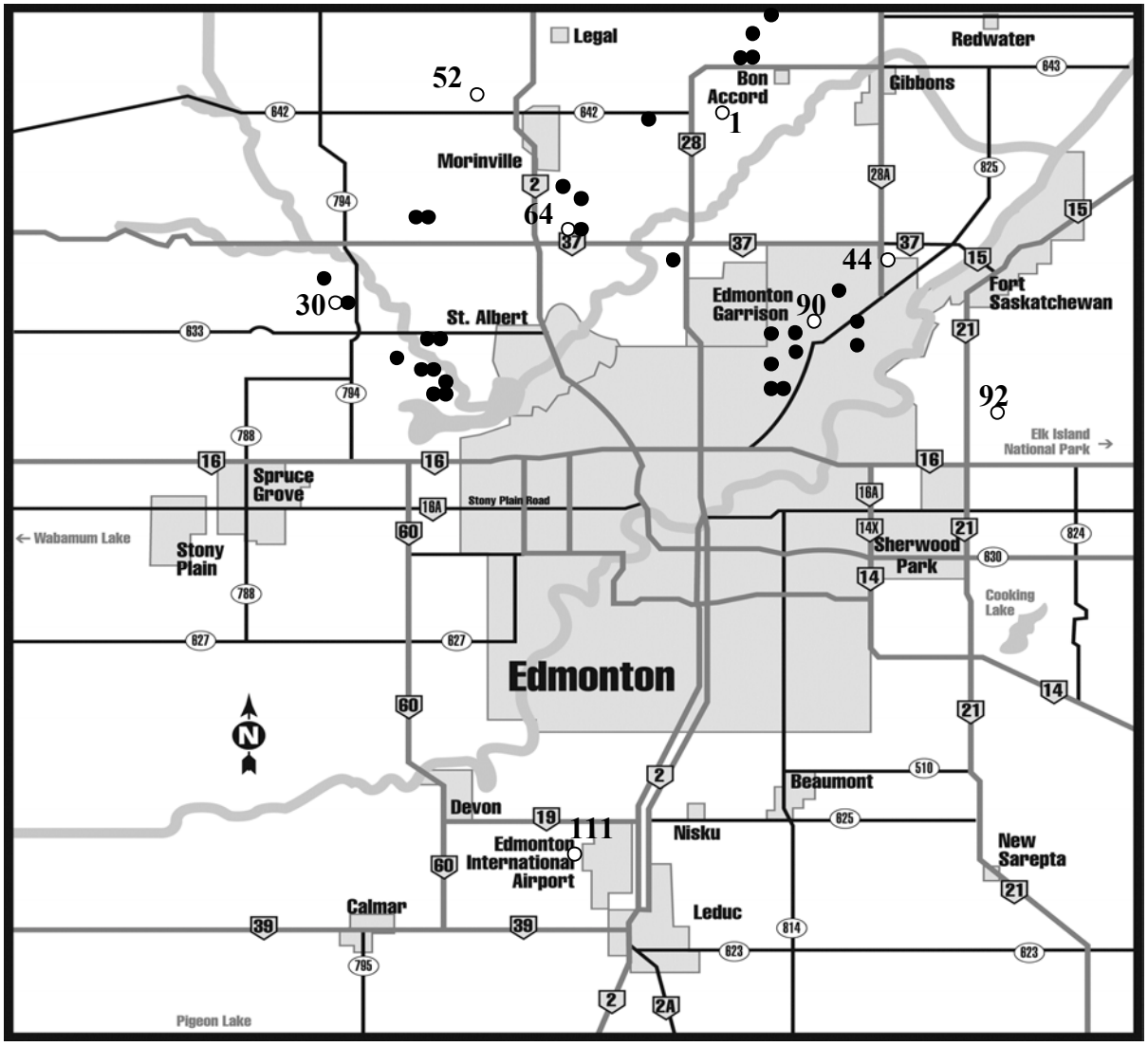

Figure 6-1. Incidence of clubroot on canola (*Brassica napus*) in the Edmonton, Alberta region. Each circle represents the approximate location of a commercial canola field in which the disease was identified. Two fields identified in Wetaskiwin and Flagstaff counties, southeast of Edmonton, are not shown. White circles represent fields from which populations of *Plasmodiophora brassicae* were selected for characterization in the current study. Two of the populations (Fields 112 and 113) were selected from the fields in Wetaskiwin and Flagstaff counties and are therefore not shown.

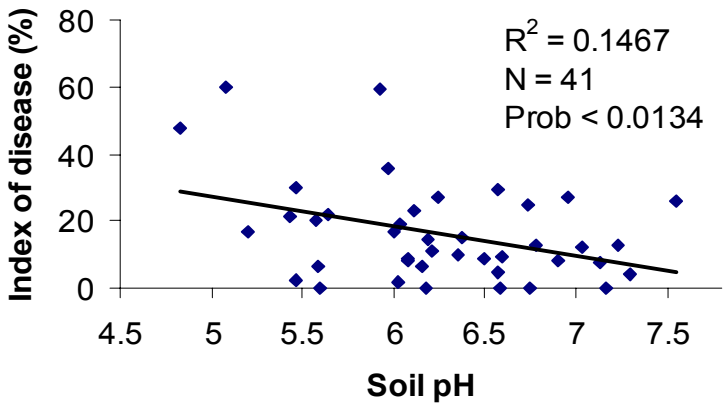

Figure 6-2. Correlation between index of disease (ID) and soil pH among the 41 *Plasmodiophora brassicae*-infested fields identified in Alberta in 2005. The average ID of 10 sampling points within each field was used for correlation analysis. Soil pH was determined in 50 ml sdH_2O using 50 g air-dried soil collected from one clubroot positive sampling point in each field.

Chapter 7: Molecular Detection of *Plasmodiophora brassicae*, Causal Agent of Clubroot of Crucifers, in Plant and Soil

7.1 Abstract

Clubroot of crucifers, caused by *Plasmodiophora brassicae* Woronin, has been recently identified in canola (*Brassica napus* L.) fields in Alberta, Canada. An effective strategy for managing the disease is to avoid planting cruciferous crops in *P. brassicae*-infested soil, because the pathogen produces resting spores that can remain infectious for many years. A simple, one-step polymerase chain reaction (PCR) protocol was developed to detect the pathogen in plant and soil samples. The primers TC1F and TC1R, based on a *P. brassicae* partial 18S ribosomal RNA gene sequence from GenBank, yielded a 548 bp product in the optimized PCR. A second pair of primers, TC2F and TC2R, which amplified a fragment of the 18S and internal transcribed spacer (ITS) 1 regions of the rDNA repeat, was also tested and produced a 519 bp product. Neither set of primers amplified any DNA fragment from non-infected plant hosts, non-infested soil, or common soil fungi and bacteria tested in this study. Quantities of 100 fg or less of total *P. brassicae* DNA, or 1×10^3 resting spores per g of soil could be consistently detected using these primers and PCR protocol, corresponding to an index of disease of 11% or lower when the soil was bioassayed. The protocol also enabled detection of *P. brassicae* in symptomless root tissue 3 days after inoculation with the pathogen. Therefore, the PCR assay described in this study could provide a reliable diagnosis for routine detection of *P. brassicae* in plant and soil materials in a specific and rapid manner.

7.2 Introduction

Plasmodiophora brassicae Woronin is an obligate parasite that causes clubroot, an important disease of crucifers worldwide. Clubroot was recently identified in a number of canola (oilseed rape) fields in central Alberta (Strelkov et al. 2005; Tewari et al. 2005), and there is increasing evidence that the disease has become established on canola in the region (Strelkov et al. 2006a). This is a cause for concern, given the high yield losses associated with *P. brassicae* infection. The pathogen typically causes the formation of club-shaped tumor-like growths on both tap and lateral roots, and occasionally on the base of the stem, in affected plants. The clubs stunt the growth of the infected plants by interfering with nutrient and water transport, and increase the susceptibility to wilting (Karling 1968; Voorips 1995). Consequently, yield losses are inevitable if symptoms are severe. The pathogen produces resting spores that can remain viable in the soil for at least 7 years (Karling 1968). Resting spores released from decayed galls provide a source for inoculum buildup in the soil in areas where crucifers are repeatedly cropped. Acid soils with high moisture and cool temperatures are most favorable for disease development (Karling 1968). Control measures, such as soil fumigation, application of fungicides (Donald et al. 2001; Shimotori et al. 1996) and surfactants (Hildebrand and McRae 1998), and supplemental calcium and liming to increase soil pH (Murakami et al. 2002) may reduce disease pressure, but are not always sufficient to keep the crops healthy. Biological control measures, such as using the fungal endophyte *Heteroconium chaetospira* (Narisawa et al. 2005) and bait crops like leafy daikon (Murakami et al. 2000) to suppress resting spore population levels in the soil, have not proved practical. Brassica cultivars resistant to all *P. brassicae* races or pathotypes are currently

unavailable. Thus, the best way to avoid clubroot is to sow susceptible crops in pathogen-free soils.

A number of diagnostic assays for detection of *P. brassicae* in soil samples have been developed in the past. Reliable bioassays were established by growing cabbage (Colhoun 1957) or rape (Melville and Hawken 1967) seedlings in *P. brassicae*-infested soil in a greenhouse and evaluating gall formation after five to six weeks. Estimates of spore loads in naturally infested soils could be achieved by comparing disease indices obtained from the infested soils with those from soils inoculated with known spore loads (Colhoun 1957). While soil bioassays are generally reliable, they are also labor intensive, time consuming, and costly, and can sometimes require large amounts of greenhouse space. Other diagnostic methods, such as checking for root-hair infection under a microscope (MacFarlane 1952), or observing resting spores stained with fluorochromes under a fluorescence microscope (Takahashi and Yamaguchi 1988; Takahashi and Yamaguchi 1989), are also time consuming, costly, or involve highly specific skills. Serological detection (Lange et al. 1989; Wakeham and White 1996) requires the availability of pathogen-specific antibodies and may also be associated with problems in terms of sensitivity or affinity, because of the possible coexistence of multiple *P. brassicae* races or pathotypes in the same gall (Jones and Ingram 1982). Therefore, these diagnostic assays may not meet the requirements for a routine test that is rapid, reliable, sensitive, and specific.

Polymerase chain reaction (PCR)-based techniques have been extensively applied to the detection of fungal pathogens in plant and soil samples, because they can provide rapid, sensitive, and reliable results. Ito and coworkers (1999) developed a single-tube

nested PCR assay for specific detection of *P. brassicae* in soils, using three primers based on the DNA sequence of the pentyltransferase gene (Ito et al. 1997). However, to improve the sensitivity of the assay and its ability to detect the pathogen in naturally-infested soils, the PCR products obtained from the nested reaction had to be subjected to a second PCR amplification (Ito et al. 1999). Faggian and coworkers (1999) reported another nested PCR technique for specific detection of *P. brassicae* in soil and water, utilizing two sets of primers designed from the ribosomal repeat and internal transcribed spacer (ITS) regions of rDNA. Although the sensitivity of this technique was greatly improved because of the high copy number of rDNA sequences in the pathogen genome (Faggian et al. 1999), it still required a two-step PCR. More recently, Wallenhammar and Arwidsson (2001) reported another two-step, nested PCR technique for detection of *P. brassicae,* using primers developed by Ito et al. (1999) and Faggian et al. (1999), or generated based on a DNA sequence published by Ito and coworkers (1997). However, a simple, diagnostic one-step PCR assay for specific detection of *P. brassicae* in soil and plant debris has not yet been developed. A one-step procedure would be faster than a two-step protocol, and would reduce the risk of contamination during sample manipulation. Thus, in this study we present a single-tube, non-nested PCR protocol for specific detection of *P. brassicae* in plant and soil material, which may be used as a routine test by non-specialist personnel.

7.3 Materials and Methods

P. brassicae populations

Four field populations of *P. brassicae* were used in this study: (i) SACAN03-1 was collected from a diseased canola (*Brassica napus* L.) plant growing near St. Albert, Alberta, in 2003, (ii) CDCN04-1 was obtained from a volunteer canola plant growing at the Crop Diversification Centre North (CDCN), Alberta Agriculture, Food and Rural Development, Edmonton, Alberta, in 2004, (iii) Leduc-1 was collected from *P. brassicae*-infested soil near Leduc, Alberta, in 2004, and (iv) AbotJE04-1 was obtained from infested soil collected near Abbottsford, British Columbia, in 2004 (Strelkov et al. 2006b).

Isolation of resting spores

Plasmodiophora brassicae resting spores were isolated according to the procedure of Castlebury et al. (1994). Approximately 2 g of fresh galled roots of canola cv. Westar or Chinese cabbage (*Brassica rapa* var. *pekinensis*) cv. Granaat were homogenized in a blender (Waring, Torrington, CT) in 50 ml sterile distilled water (sdH_2O) for 2 min at high speed, and the resultant homogenate was filtered through eight layers of cheesecloth (American Fiber & Finishing Co., Albemarle, NC). The filtrate was centrifuged at 4°C in 50 ml centrifuge tubes at 4000 × g for 10 min in a swinging-bucket rotor. The supernatant was discarded, the pellet re-suspended in 5 ml of 50 % sucrose, and centrifuged again at 2000 × g for 10 min. The resulting supernatant was transferred to a new 50 ml tube and diluted with 30 ml sdH_2O before centrifuging at 4000 × g for 10 min. The pellet obtained was re-suspended in 5 ml sdH_2O and centrifuged as above to remove any remaining sucrose. The supernatant was removed and the resting spore pellet re-suspended in 5 ml

sdH_2O. The resting spores were then purified using a continuous gradient of LUDOX (HS-40 colloidal silica, 40 wt. % suspension in water, Sigma-Aldrich Canada Ltd., Oakville, ON), as described by Castlebury et al. (1994). The purified *P. brassicae* resting spore suspension was normally stored at 4^{o}C for less than 48 hours before DNA extraction. Spore concentrations were quantified using a haemocytometer (VWR, Mississauga, ON).

Naturally infested soils

Two black chernozemic soil (Clayton et al. 1977) samples, previously collected in Sturgeon County, Alberta, and confirmed to be naturally infested with *P. brassicae*, were included in the study. The two samples exhibited different degrees of infestation, with bait plants grown in the soils developing disease indices of 100% and 78%, when bioassayed as per Strelkov et al. (2006b).

Common soil fungi and bacteria

Twenty-seven fungal, four bacterial, and two plant species (Table 7-1) were used in this study to test the specificity of the *P. brassicae*-specific primers employed in the PCR reaction. *Alternaria brassicae*, *A. brassicicola*, *A. raphani*, and *Leptosphaeria maculans* were grown on V8 juice medium supplemented with Rose Bengal (0.05 g/liter) and $CaCO_3$ (3.0 g/liter) (Bansal et al. 2002), *Allomyces javanicus* was grown on cornmeal dextrose peptone agar (Carolina Biological Supply, Burlington, NC), and *Rhizophlyctis rosea* was grown on 683 Koch's K-1 agar medium containing 0.6 g/liter peptone, 0.4 g/liter yeast extract, 1.8 g/liter glucose, and 15 g/liter agarose (ATCC, Manassas, VA). All other fungal and bacterial species were grown on potato dextrose agar (Difco

Laboratories, Detroit, MI). Cultures were maintained at room temperature and harvested at 7 to 10 days for genomic DNA extraction.

DNA extraction

Genomic DNA was extracted from roots of canola cv. Westar, Chinese cabbage cv. Granaat, fungal mycelia and bacteria according to the protocols of Rogers and Bendich (1985; 1994). Total DNA from soil samples was extracted from 0.5 g of soil using the FastDNA® Spin® Kit (Qbiogene Inc., Irvine, CA) as per manufacturer's instructions. DNA of *P. brassicae* was extracted from LUDOX purified resting spores using either the FastDNA® Spin® Kit or the procedures of Rogers and Bendich (1985; 1994). When the quantity of galled root material was limiting, DNA from infected roots was also extracted using the FastDNA® Spin® Kit. DNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm, and working solutions were adjusted to 2 ng/µl with either sdH_2O or 1 × Tris-ethylenediaminetetraacetic acid (TE) buffer (pH 8.0). DNA samples were placed at -20°C for long term storage or at 4°C for immediate processing.

Primer design

Two sets of 30-base primers were designed using Primer3 software (Rozen and Skaletsky 2000). Primers TC1F (5'-GTGGTCGAACTTCATTAAATTTGGGCTCTT-3') and TC1R (5'-TTCACCTACGGAACGTATATGTGCATGTGA-3') were based on a partial 18S ribosomal RNA gene sequence from *P. brassicae* available in GenBank (accession no. AF231027). Primers TC2F (5'-AAACAACGAGTCAGCTTGAATGCTAGTGTG-3') and TC2R (5'-CTTTAGTTGTGTTTCGGCTAGGATGGTTCG-3'), which amplified a fragment in the

18S and ITS1 regions of the rDNA repeat, contained the entire sequences of primers PbITS6 and PbITS7, respectively, developed previously by Faggian et al. (1999). A third set of primers, attTC1F (5'-AAAAAGCAGGCTGTGGTCGAACTTCATTAAATTTGGGCTCTT-3') and attTC1R (5'-AGAAAGCTGGGTTTCACCTACGGAACGTATATGTGCATGTGA-3'), was used to clone the amplicon obtained with primers TC1F and TC1R into the pDONR221 sequencing vector (Invitrogen Life Technologies, Carlsbad, CA), as per manufacturer's instructions.

PCR conditions and gel electrophoresis

All PCR amplifications were conducted using a GeneAmp® PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA) in a 25-µl volume containing 1 U of Platinum® *Taq* DNA polymerase, 2 mM $MgCl_2$, 1 × PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl) (Invitrogen Life Technologies, Carlsbad, CA), 0.4 µM of each forward and backward primer, 0.2 mM of each dNTP, and either 10 ng of *P. brassicae* DNA or 10 ng of total DNA extracted from soil, plant, bacterial or fungal material. A positive control containing all of the reaction components and 10 ng *P. brassicae* DNA, and a negative control consisting of all the components except a DNA template, were included in each PCR assay to ensure proper reaction conditions and the absence of DNA contaminants in the reaction mixtures. The amplification cycle consisted of an initial heat denaturation step at 94°C for 2 min followed by 45 cycles of 94°C for 30 s, 65°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products (10 µl reaction mix per loading well) were resolved on ethidium bromide-stained 1% agarose gels in 1 × TAE

buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3.), and were visualized under UV light using a Syngene BioImaging System (Synoptics Inc., Frederick, MD).

DNA sequencing

pDONR221 plasmid DNA, containing the DNA fragment amplified with primers TC1F and TC1R, was purified using the QIAprep® Spin Miniprep Kit (QIAGEN Inc., Mississauga, ON) according to the manufacturer's instructions, and submitted to the Molecular Biology Service Unit of the University of Alberta, Edmonton, for sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Primer specificity and sensitivity

The specificity of primer pairs TC1F/TC1R and TC2F/TC2R was tested by attempting to amplify, using the PCR protocol described above, DNA isolated from a variety of common soil microorganisms and plant root tissue (Table 7-1). The sensitivity of the assay was assessed by using purified *P. brassicae* genomic DNA, serially diluted from 10 ng to 1 fg with 1 × TE buffer, as a template for PCR. In addition, total DNA was extracted from 0.5 g samples of an orthic black chernozemic soil (Clayton et al. 1977), inoculated with 100 µl suspensions of *P. brassicae* (population SACAN03-1) resting spores at concentrations of 5×10^5 to 0 spores/ml (corresponding to infestation levels of 1×10^5 to 0 spores/g soil, respectively), and subjected to PCR as above. The soil was collected from the Edmonton Research Station, Edmonton, Alberta, from a site cropped mainly to cereals and with no history of clubroot. The detection limit of the PCR protocol was also directly compared with that of a bioassay (see below).

Comparison with bioassay

In an attempt to correlate the PCR detection limit to clubroot development in *P. brassicae*-infested soil, a bioassay was conducted by growing canola cv. Westar plants in a non-sterilized orthic black chernozemic soil inoculated with pathogen resting spores at rates ranging from 1×10^5 to 0 spores/g soil. For inoculation purposes, *P. brassicae* (population SACAN03-1) spore suspensions were adjusted to a concentration of 36×10^5 spores/ml, and diluted tenfold from 36×10^5 to 36×10^1 spores/ml with sdH_2O. Inoculation of soil with the pathogen was made by adding 1 ml of the various dilutions of the spore suspension to plastic pots (4 × 4 × 5 cm, Kord Products Inc., Brampton, ON), each filled with 36 g of air-dried soil (soil particle < 1 mm), in order to produce infestation levels corresponding to 1×10^5, 1×10^4, 1×10^3, 1×10^2, or 1×10^1 resting spores/g soil. Controls were inoculated with sdH_2O only (i.e. 0 spores/g soil). Immediately after inoculation, 15 ml sdH_2O were added to each pot to disperse the resting spores evenly in the soil. Control pots received an equivalent amount of sdH_2O. Approximately four hours after inoculating the soil with *P. brassicae* spores, germinating canola seeds were sown into the soil at a rate of one seed per pot. To favor *P. brassicae* infection, the pots were soaked in water on a plastic tray for one week immediately after sowing, and were then bottom watered and fertilized as required. Plants were kept in a greenhouse with a 16 h photoperiod (natural light supplemented with artificial lighting) at 20 ± 2^oC for six weeks, at which point they were pulled from the soil, and the roots washed and examined for symptom development. A disease index was calculated for each treatment as described in Strelkov et al. (2006b). Fifty-four seeds were sown per treatment (i.e. soil infestation level), and experiments were repeated five times. The

average disease index of the five experiments was calculated for each treatment and used for comparison with the PCR results. In one repetition of the bioassay, 1 g soil samples were collected from each plastic pot 4 h after inoculation, pooled according to treatment, air-dried at room temperature and used as templates for total DNA extraction using the FastDNA® Spin® Kit.

Pathogen detection at different stages of disease development

To assess our ability to detect *P. brassicae* infection prior to the development of visible symptoms, 7 day old canola cv. Westar seedlings were germinated on filter paper in Petri dishes and inoculated by dipping the roots in a resting spore suspension (1×10^7 spores/mL) as previously described (Strelkov et al. 2006b). Control plants were dipped in sdH_2O. Seedlings were then planted in 4 × 4 cm plastic pots filled with Metro-Mix soil (Scotts, Columbus, OH) at a rate of one seedling per pot and placed in a greenhouse at 22 ± 2°C with a 16 h photoperiod (natural light supplemented with artificial lighting). To ensure infection, the soil was kept saturated with water for the first week after inoculation, and then fertilized and watered as required. Seedlings were harvested at 3, 10, 24 and 36 days after inoculation and the roots washed in H_2O and examined for symptom development. Total DNA was then extracted from the tissue according to the protocols of Rogers and Bendich (1985; 1994) and used as a template in the PCR protocol described above.

Dilution of infested soil with non-infested soil

Plasmodiophora brassicae-infested soil was mixed with increasing quantities of non-infested soil in order to evaluate how dilution of the infested sample would affect PCR detection of the pathogen. An air-dried, *P. brassicae*-infested black chernozemic

soil [producing an ID of 40.7% when bioassayed as previously described (Strelkov et al. 2006b)] was mixed 1:1, 3:7, 1:4, and 1:9 (w/w), respectively, with non-infested air-dried soil of the same type, to a total mass of 20 g for each composite sample. Total DNA was extracted from 0.5 g sub-samples of each dilution and subjected to PCR as above, using the primers TC1F and TC1R.

7.4 Results

PCR amplification and sensitivity

Amplification of purified *P. brassicae* DNA using the primers TC1F and TC1R resulted in a PCR product of the expected 548 bp size (Fig. 7-1A). Similarly, amplification using the primers TC2F and TC2R also produced a fragment of the predicted 519 bp size (Fig. 7-1B). The product obtained with the primers TC1F/TC1R was cloned and sequenced, and a GenBank search using the BLASTN program (Altschul et al. 1990) revealed that the fragment (accession no. DQ 533682) was 100% homologous to a sequence from a *P. brassicae* isolate from Harpenden, United Kingdom (accession no. PBY12831), and 99% homologous to another sequence from an Australian isolate of the pathogen (accession no. AF231027).

PCR amplification of varying quantities of *P. brassicae* genomic DNA with either set of primers resulted in the production of a strong band when using as little as 100 fg of template (Fig. 7-1). Below that level, results were more variable. When using primers TC1F/TC1R, amplifiable products could be obtained from as little as 1 fg of template, but the intensity of the resulting band was very weak (Fig. 7-1A). With the primers TC2F/TC2R, a weak band was observed at 10 fg, but no product was visible at 1 fg (Fig.

7-1B). In soils, both pairs of primers could consistently amplify a visible PCR product at infestation levels as low as 1×10^3 spores/g soil (Fig. 7-2), although occasionally spore loads as low as 1×10^1 spores/g soil could be detected (results not shown).

Comparison with bioassay

In order to compare the detection limit of the bioassay with the PCR test, canola plants were grown on a black chernozemic soil inoculated with varying quantities of *P. brassicae* resting spores. As the soil used was not sterilized, a small percentage of the test plants succumbed to damping-off and were therefore excluded from the analysis. After six weeks of growth, plants grown at infestation levels corresponding to 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, and 0 spores/g soil exhibited average disease indices (± 1 standard error) of 41.8 ± 15.9 %, 24.4 ± 10.9 %, 11.0 ± 6.5 %, 1.4 ± 0.8 %, 0.8 ± 0.5 % and 0%, respectively, over the five repetitions of the experiment. In one of the repetitions, total DNA was extracted from soil samples collected from each contamination level, as described in the Materials and Methods, and subjected to PCR using primer sets TC1F/TC1R or TC2F/TC2R. This direct test of biossayed soil with the PCR protocol confirmed that a strong 548 bp band was produced with primers TC1F/TC1R when DNA from soil infested with as few as 1×10^3 *P. brassicae* spores/g soil was used as a template (results not shown). A much weaker band could also be detected at an infestation level of 1×10^1 spores/g soil, but not at 1×10^2 spores/g soil (results not shown). Similarly, a strong 519 bp band was obtained with primers TC2F/TC2R at 1×10^3 spores/g soil (results not shown).

Primer specificity

Amplification of DNA from 27 common soil fungal and four bacterial species (Table 7-1), using the primer pairs TC1F/TC1R or TC2F/TC2R, yielded no visible PCR products (Fig. 7-3). Furthermore, no amplified products were observed when total DNA was extracted from non-infested soil or from healthy canola and Chinese cabbage roots (Fig. 7-4). In contrast, strong single bands were obtained with both sets of primers when PCR was conducted on total DNA extracted from clubbed canola roots (infected with the *P. brassicae* populations AbotJE04-1, CDCN04-1 or Leduc-1), or from a black chernozemic soil naturally-infested at different levels with the pathogen (Fig. 7-4).

Detection of the pathogen at different stages of disease development

When using either set of primers, a visible product could be amplified from DNA extracted from canola roots as few as 3 days after inoculation with *P. brassicae* (Fig. 7-5). Strong bands were also observed with both sets of primers at 10, 24 and 36 days after inoculation (Fig. 7-5). Clubroot disease symptoms were not visible to the naked eye until 24 days after inoculation, when a slight swelling could be observed upon careful examination of the roots. No macroscopic disease symptoms were evident at 3 or 10 days after inoculation with the pathogen.

Effect of soil dilution on pathogen detection

Using the primers TC1F/TC1R, amplification of DNA extracted from *P. brassicae*-infested soil (ID = 40.7 %) mixed with increasing quantities of pathogen-free soil yielded a strong band down to a dilution factor of five (Fig. 7-6). However, a very faint band could also be observed after a 10-fold dilution of the infested soil with non-infested soil (Fig. 7-6).

7.5 Discussion

The single-step PCR assay developed in the current study enabled reliable detection of *P. brassicae* in plant and soil samples. The primer pairs TC1F/TC1R and TC2F/TC2R could be used to consistently detect 100 fg or less of total *P. brassicae* DNA (Fig. 7-1) in a single-tube, non-nested procedure. The ability to detect very low amounts of *P. brassicae* DNA was likely a consequence of the high copy number of the rDNA sequences (Russel et al. 1984) on which these primers were based. Moreover, while they reliably amplified a visible product from *P. brassicae*-positive materials, the primers did not yield a product from non-infected host species, common soil fungal and bacterial species (Fig. 7-3), or non-infested soils (Fig. 7-4), demonstrating their high specificity for the pathogen. In infected root tissue, *P. brassicae* could be detected 3 days after inoculation (Fig. 7-5), whereas clubroot symptoms were not visible until 24 days.

Australian researchers (Faggian et al. 1999) reported a nested PCR protocol that could detect as little as 0.1 fg of *P. brassicae* DNA, and they also found that the sensitivity of their nested PCR (1×10^3 to 1×10^4 resting spores/g potting mix) matched the bait plant detection threshold (1×10^3 resting spores/g soil). However, the artificial potting mix that they utilized may differ from natural soil in that the former contains fewer microflora than the latter, in addition to its ameliorated porosity. Thus, DNA samples extracted from artificial potting mix may contain less DNA species and PCR inhibitors than DNA extracted from natural soils. Using a single-tube nested PCR method, Japanese researchers (Ito et al. 1999) could detect one resting spore of *P. brassicae* per g of soil. However, Ito and coworkers employed a total of 75 cycles for their entire nested PCR method, and in order to improve the sensitivity of their assay in

naturally-infested soils, they had to subject samples to a second round of PCR (double PCR). Furthermore, the Australian and Japanese researchers did not directly correlate the detection limits of their respective assays to disease indices induced by corresponding levels of *P. brassicae* resting spores in natural or artificial soils.

In contrast, Wallenhammar and Arwidsson (2001) did compare the detection limit of their two-step, nested PCR assay with disease development in bait plants, and were able to detect the pathogen in soils with inoculum levels corresponding to a disease severity index of 21% or greater (more than 35% infected plants in a bioassay). Nevertheless, the PCR protocol described in the present study could be used to consistently detect *P. brassicae* in a black chernozemic soil inoculated with spore loads of 1×10^3 resting spores/g soil, corresponding to an index of disease of only 11% (14% infected plants, Fig. 7-2). Occasionally, the detection limit was even better, allowing detection of as little as 1×10^1 spores/g soil or an index of disease of 0.8% (results not shown). This variation in the detection limit at the lower levels of infestation likely reflected the non-homogenous distribution of the pathogen resting spores in the soil. Furthermore, as the quantity of the DNA template decreases, the quality of the reagents and primers used could also have a greater effect on the ability to detect the pathogen, likely contributing to inconsistent results when testing soils with only trace levels of infestation. Therefore, for practical application of this or any of the other protocols reported, it would be advisable to regard the lowest amount of DNA or spores that can be consistently detected as the actual detection limit, thereby precluding the possibility that a clubroot-positive sample could be scored as negative. In this context, the simple one-step PCR test described in this study provided detection limits equivalent or superior to those

achieved using the previous nested PCR procedures. Inoculum levels of approximately 1 $\times 10^5$ resting spores/g soil may be required to produce significant clubroot symptoms under field conditions (G.R. Dixon, *personal communication*).

For conducting efficient PCR reactions, extraction of high quality genomic DNA from representative soil samples is of primary importance. In our attempts to disrupt the physically and chemically strong walls of *P. brassicae* (Buczacki 1983), we tried several DNA extraction procedures, including freezing/thawing, grinding, and boiling (results not shown). However, none of these methods proved satisfactory. The best and most consistent results were achieved by extracting DNA using a commercial kit, the FastDNA® Spin® Kit (Qbiogene Inc.). Furthermore, dilution of the soil-extracted DNA to 2 ng/µl was usually necessary prior to conducting the PCR, because of the presence of reaction inhibitors such as humic acids and other organic compounds in the soil extracts (Yeates et al. 1998).

An additional challenge associated with testing a particular soil or field for the occurrence of *P. brassicae* is the patchy distribution in which it often occurs (Strelkov et al. 2005; Strelkov et al. 2006a). Thus, the absence of the pathogen from a particular soil sample may not necessarily indicate its absence from the field in which it was collected. To increase the chances of detection, it may be necessary to sample soil from numerous points in a field. These samples could be processed more simply and efficiently with a PCR test as opposed to a bioassay. However, to simplify testing even further, it would be ideal if soil samples taken from different points in a field could be pooled, and the PCR test performed on only one composite sample. In order to evaluate the feasibility of this approach, we mixed *P. brassicae*-infested soil (producing an ID of 40.7%) with

equivalent quantities of non-infested soil of the same type (black chernozemic). A strong band could be obtained when the infested soil was diluted up to five times with "clean" soil (Fig. 7-6), despite the moderate infestation level of the starting sample. Therefore, such an approach seems possible, although further refinement and validation are necessary.

The PCR assay yielded the same or nearly the same sized amplicon for all four *P. brassicae* populations tested in this study (Fig. 7-4), suggesting that the fragment amplified in the rRNA repeat is conserved among these populations (SACAN03-1, CDCN04-1, Leduc-1, and AbotJE04-1). In an earlier study (Strelkov et al. 2006b), SACAN03-1 and Leduc-1 were classified as pathotype 3, CDCN04-1 as pathotype 5, and AbotJE04-1 as pathotype 6 on the differential set of Williams (1966). Therefore, the PCR products obtained with the primers TC1F/TC1R or TC2F/TC2R may not serve as appropriate probes for pathotype differentiation among these four populations. The observation that the 548 bp DNA fragment (Genbank accession no. 533682) obtained with primers TC1F/TC1R is 100% homologous to a sequence from a *P. brassicae* isolate from Harpenden, United Kingdom (Ward and Adams 1998), and 99% homologous to a sequence from an isolate collected in Manjimup, Australia (Faggian et al. 1999), suggests that the *P. brassicae* population (SACAN03-1) from Alberta may have the same evolutionary origin as those from both the United Kingdom and Australia.

7.6 References

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7.7 Tables and Figures

Please refer to the following pages.

Table 7-1. Fungal, bacterial and plant species used to test the specificity of *Plasmodiophora brassicae*-specific primers.

Species and Isolate Number (Where Available)	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*
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

*Deposited at the U of A Microfungus Collection and Herbarium (UAMCH), Devon, AB

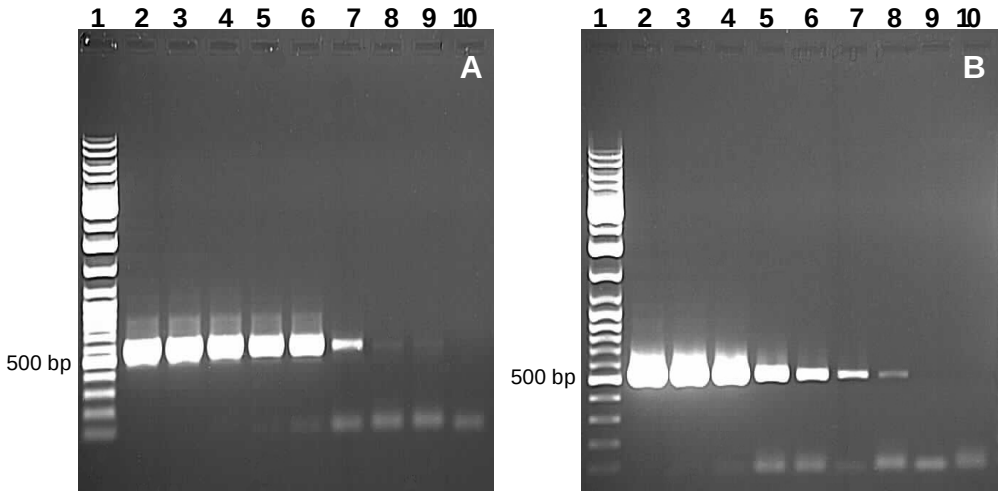

Fig. 7-1. Ethidium bromide-stained 1% agarose gels of PCR products amplified with primers TC1F/TC1R (A) or TC2F/TC2R (B) from a 10-fold serial dilution of purified *Plasmodiophora brassicae* DNA. DNA was serially diluted from 10 ng to 1 fg with 1 × TE buffer and used as a template for PCR. Lane 1: DNA ladder (O'GeneRuler DNA ladder mix, Fermentas Canada Inc.), lane 2: 10 ng *P. brassicae* template DNA, lane 3: 1 ng template DNA, lane 4: 100 pg template DNA, lane 5: 10 pg template DNA, lane 6: 1 pg template DNA, lane 7: 100 fg template DNA, lane 8: 10 fg template DNA, lane 9: 1 fg template DNA, and lane 10: sdH_2O negative control.

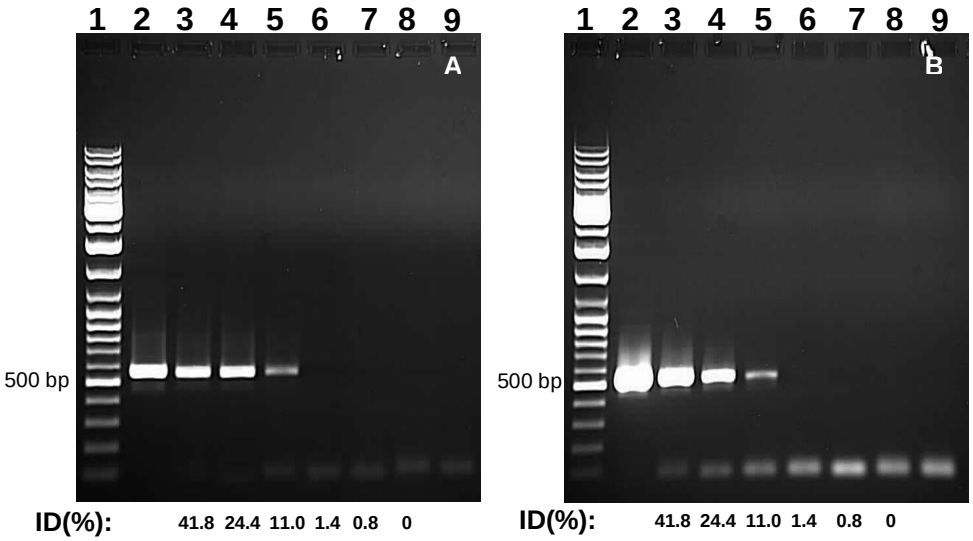

Fig. 7-2. Ethidium bromide-stained 1% agarose gels of PCR products amplified with primers TC1F/TC1R (A) or TC2F/TC2R (B) from DNA extracted from *Plasmodiophora brassicae*-infested black chernozemic soil. Total DNA was extracted using a FastDNA® Spin® Kit (Qbiogene Inc.) from 0.5 g soil samples inoculated with 100 µl spore suspensions at concentrations of 5×10^5, 5×10^4, 5×10^3, 5×10^2, 5×10^1, or 0 spores/ml. DNA concentration was adjusted to 2 ng/µl, and 10 ng template DNA was used in each PCR reaction. Lane 1: DNA ladder (O'GeneRuler DNA ladder mix, Fermentas Canada Inc.), lane 2: *P. brassicae* DNA (population SACAN03-1), lane 3: 1×10^5 *P. brassicae* spores/g soil, lane 4: 1×10^4 spores/g soil, lane 5: 1×10^3 spores/g soil, lane 6: 1×10^2 spores/g soil, lane 7: 1×10^1 spores/g soil, lane 8: 0 spores/g soil, and lane 9: sdH_2O. Indices of disease (ID) were determined by growing *Brassica napus* L. plants (cv. Westar) for 6 weeks in a greenhouse in black chernozemic soil inoculated with 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, or 0 *P. brassicae resting* spores/g soil, respectively.

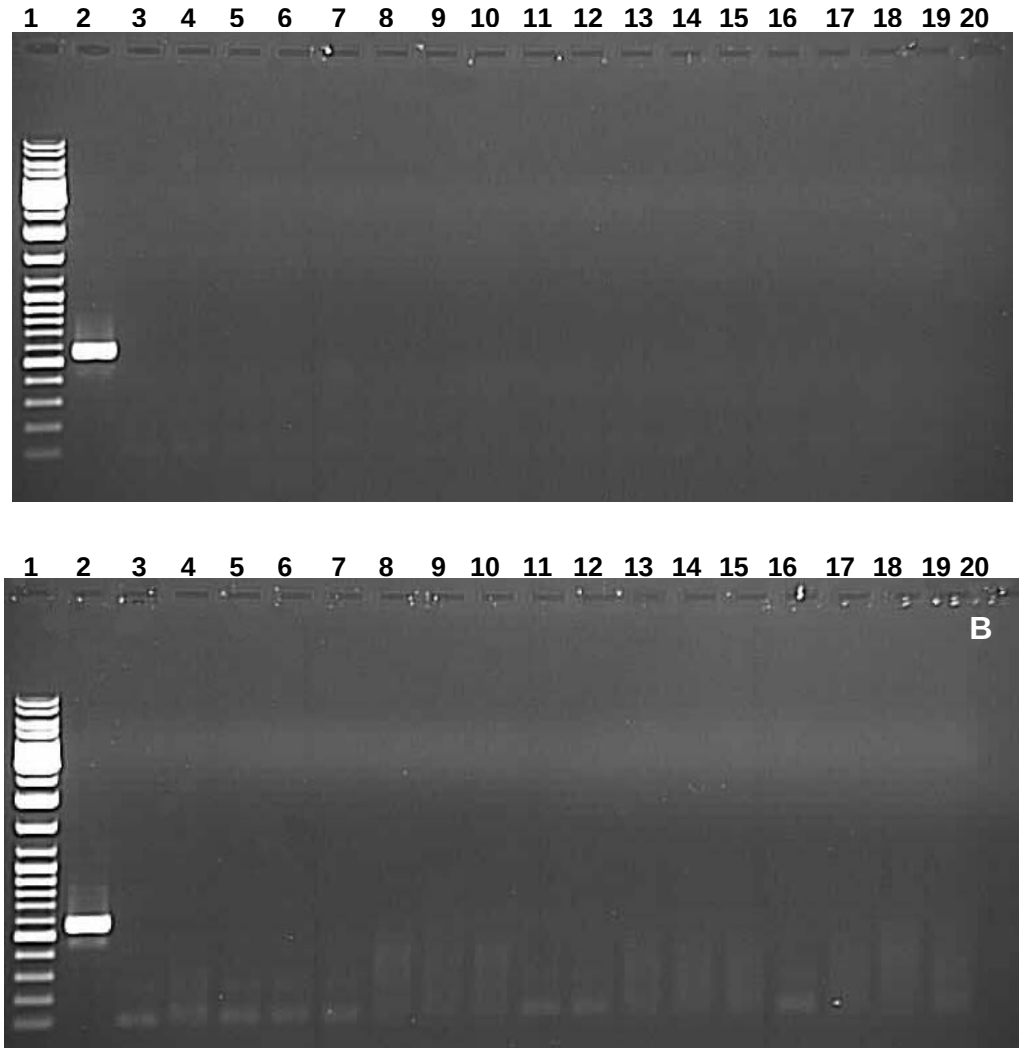

Fig. 7-3. Ethidium bromide-stained 1% agarose gels of PCR products amplified with primers TC1F/TC1R from DNA extracted from plant, fungal, and bacterial species. Identical results were obtained with the primers TC2F/TC2R and are not shown. DNA concentration was adjusted to 2 ng/µl, and 10 ng template DNA was used in each PCR reaction.

A. Lane 1: DNA ladder (O'GeneRuler DNA ladder mix, Fermentas Canada Inc.), lane 2: *Plasmodiophora brassicae*, lane 3: *Brassica napus* L., lane 4: *Brassica rapa* var. *pekinensis*, lane 5: *Allomyces javanicus*, lane 6: *Alternaria alternata*, lane 7: *Alternaria brassicae*, lane 8: *Alternaria brassicicola*, lane 9: *Alternaria raphani*, lane 10: *Armilaria mellea*, lane 11: *Aspergillus niger*, lane 12: *Bipolaris* sp., lane 13: *Botrytis* sp., lane 14: *Cladosporium* sp., lane 15: *Colletotrichum damentium*, lane 16: *Cyathus olla*, lane 17: *Fusarium avenaceum*, lane 18: *Fusarium graminearum*, lane 19: *Fusarium oxysporium*, and lane 20: sdH_2O.

B. Lane 1: DNA ladder (O'GeneRuler DNA ladder mix, Fermentas Canada Inc.), lane 2: *P. brassicae* DNA, lane 3: *Gliocladium roseum*, lane 4: *Leptosphaeria maculans*, lane 5: *Myrothecium verrucaria*, lane 6: *Penicillium* sp., lane 7: *Periconia* sp., lane 8:

Phanerochaete chrysosporium, lane 9: *Pythium pythioides*, lane 10: *Rhizoctonia solani*, lane 11: *Rhizopus* sp., lane 12: *Rhizophlyctis rosea*, lane 13: *Trichoderma harzianum*, lane 14: *Verticillium albo-atrum*, lane 15: *Bacillus subtilis*, lane 16: *Pseudomonas atrofaciens*, lane 17: *Pseudomonas fluorescens*, lane 18: *Streptomyces* sp., lane 19: sdH_2O, and lane 20: empty.

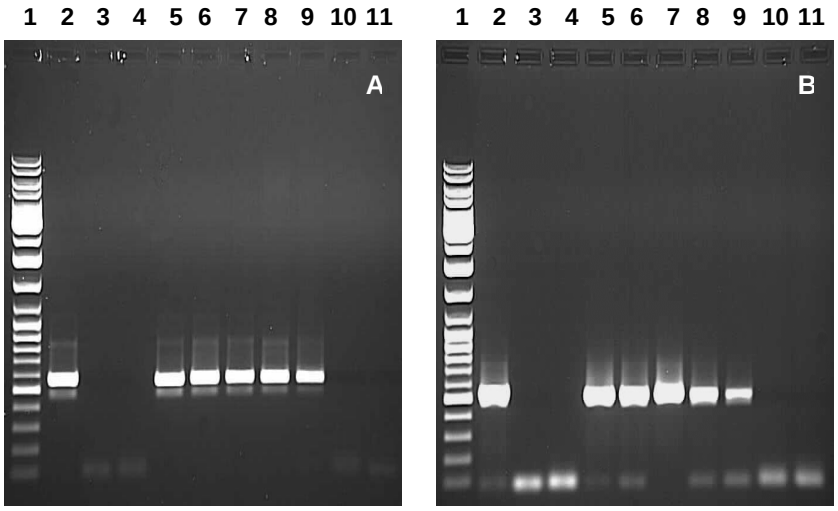

Fig. 7-4. Ethidium bromide-stained 1% agarose gels of PCR products amplified using primers TC1F/TC1R (A) or TC2F/TC2R (B) from DNA extracted from healthy plant roots, galled roots, and soil samples. DNA concentration was adjusted to 2 ng/µl, and 10 ng template DNA was used in each PCR reaction. Lane 1: DNA ladder (O'GeneRuler DNA ladder mix, Fermentas Canada Inc.), lane 2: *Plasmodiophora brassicae* population SACAN03-1, lane 3: healthy *Brassica napus* L. root, lane 4: healthy *Brassica rapa* var. *pekinensis* root, lane 5: clubbed *B. napus* root (infected by population AbotJE04-1), lane 6: clubbed *B. napus* root (infected by CDCN04-1), lane 7: clubbed *B. napus* root (infected by Leduc-1), lane 8: *P. brassicae*-infested black chernozemic soil (index of disease [ID] = 100%), lane 9: *P. brassicae*-infested black chernozemic soil (ID = 78%), lane 10: non-infested black chernozemic soil, and lane 11: sdH_2O.

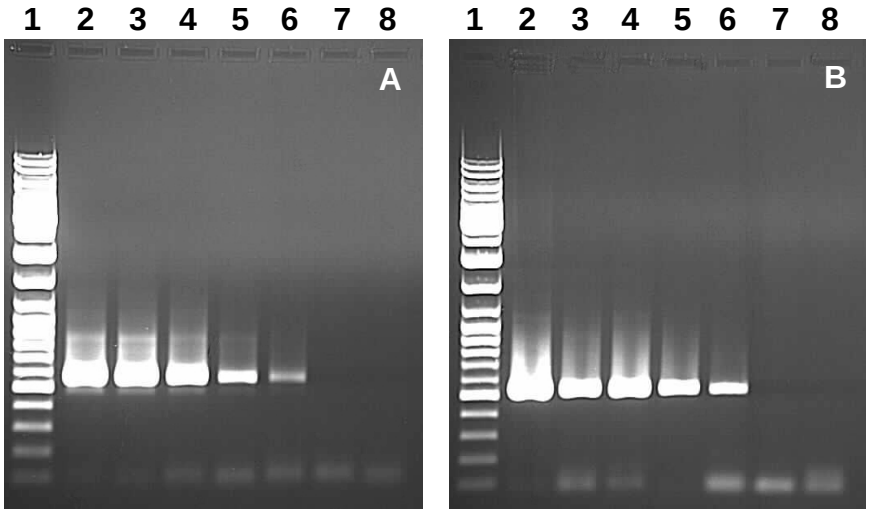

Fig. 7-5. Ethidium bromide-stained 1% agarose gels of PCR products amplified using primers TC1F/TC1R (A) or TC2F/TC2R (B) from DNA extracted from healthy and infected *Brassica napus* L. (cv. Westar) roots at different times after inoculation with *Plasmodiophora brassicae*. DNA concentration was adjusted to 2 ng/µl, and 10 ng template DNA was used in each PCR reaction. Lane 1: DNA ladder (O'GeneRuler DNA ladder mix, Fermentas Canada Inc.), lane 2: *P. brassicae* population SACAN03-1, lane 3: root 36 days after inoculation with *P. brassicae*, lane 4: root 24 days after inoculation, lane 5: root 10 days after inoculation, lane 6: root 3 days after inoculation, lane 7: non-infected root, and lane 8: sdH_2O.

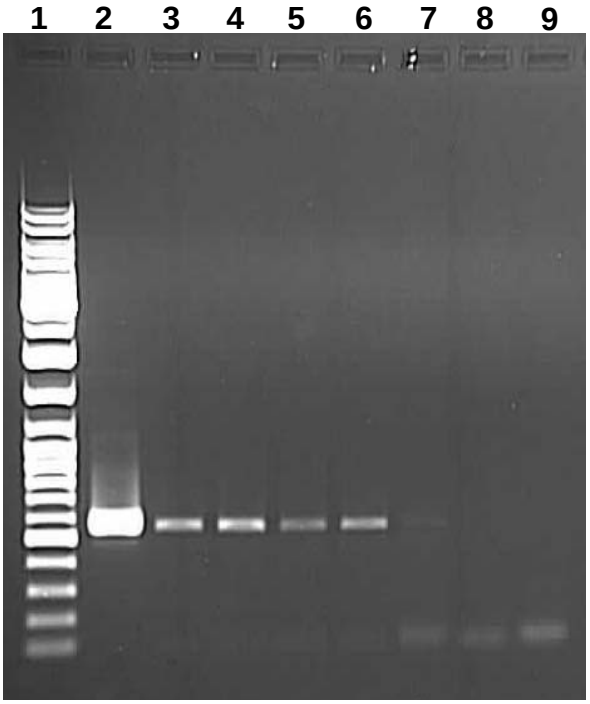

Fig. 7-6. Ethidium bromide-stained 1% agarose gel of PCR products amplified with primers TC1F and TC1R from DNA extracted from *Plasmodiophora brassicae*-infested black chernozemic soil mixed with increasing quantities of non-infested soil. Total DNA was extracted using a FastDNA® Spin® Kit (Qbiogene Inc.) from 0.5 g soil samples. DNA concentration was adjusted to 2 ng/µl, and 10 ng template DNA was used in each PCR reaction. Lane 1: DNA ladder (O'GeneRuler DNA ladder mix, Fermentas Canada Inc.), lane 2: *P. brassicae* population SACAN03-1, lane 3: *P. brassicae*-infested soil (ID = 40.7 %), lane 4: 1 part infested soil mixed with 1 part non-infested soil, lane 5: 3 parts infested soil mixed with 7 parts non-infested soil, lane 6: 1 part infested soil mixed with 4 parts non-infested soil, lane 7: 1 part infested soil mixed with 9 parts non-infested soil, lane 8: non-infested soil, and lane 9: sd H_2O.

Chapter 8: Isolation and Variation in Virulence of Single Spore Isolates of *Plasmodiophora brassicae* from Canada

8.1 Abstract

Clubroot of crucifers, caused by *Plasmodiophora brassicae*, is emerging as an important disease of canola (*Brassica napus*) in Alberta, Canada. Populations of the pathogen often consist of a mixture of different pathotypes. Therefore, a simple and efficient method to isolate single resting spores of *P. brassicae* was developed, based on serial dilution of spore suspensions. The virulence of 24 single spore isolates, representing five populations of the pathogen from Alberta, Ontario and British Columbia, was characterized on the differentials of Williams and Somé et al. Symptoms were rated six weeks after inoculation and Fisher's least significant difference (LSD) ($p<0.05$) was used to differentiate resistant from susceptible host reactions. The pathotype composition of *P. brassicae* in Canada appeared more diverse when single spore isolates were examined rather than populations of the pathogen. In Alberta, at least three and possibly four pathotypes were identified among the 14 isolates tested, whereas a maximum of only two pathotypes had been previously reported when populations of the pathogen were examined. Pathotype 3 or P_2, as classified on the differentials of Williams and Somé et al., respectively, was found to be predominant in the province. The occurrence of other pathotypes at lower frequencies suggests that caution should be used in any breeding strategy, since rare pathotypes of *P. brassicae* may quickly become predominant if susceptible host genotypes are continuously grown.

8.2 Introduction

As a soil-borne biotrophic pathogen, *Plasmodiophora brassicae* Woronin causes clubroot, a serious disease affecting cruciferous crops worldwide. In Canada, clubroot has traditionally been a major problem in cole crop production in certain areas of British Columbia, Quebec, Ontario, and the Maritime provinces (Rimmer et al. 2003). However, clubroot was also recently identified on canola (*Brassica napus* L.) in Alberta (Strelkov et al. 2006a; Tewari et al. 2005) and Quebec (Pageau et al. 2006). In Alberta, the disease was initially found in 2003, in 12 commercial fields near St. Albert and in an experimental field in northeast Edmonton. However, additional surveys in 2005 and 2006 revealed more than 110 infested canola fields, which are distributed over a fairly wide geographic area in central Alberta (Strelkov et al. 2007a; Strelkov et al. 2005). The rapid increase in the number of clubroot-infested fields has raised concerns, since canola is one of the major crops in the province, one-third of which is traditionally planted in acidic soils that favor disease development (Tewari et al. 2005). In a study from Quebec (Pageau et al. 2006), it was found that losses were as high as 91% when canola was planted in *P. brassicae*-infested soil, with a 4.7 to 6.1% reduction in oil content in infected plants. In Alberta, a yield loss of 100% occurred in one infested field in 2006, which had been consecutively cropped to canola for two years (Strelkov et al. 2005).

Crop rotation is the simplest and most effective approach for the management of clubroot, but may not be practical due to the longevity of the pathogen in infested soil (Karling 1968). Application of lime suppresses disease development, but is not economically feasible since large quantities of lime and repeated applications are required (Hildebrand and McRae 1998). The effect of fungicide treatment is not always consistent

(Naiki and Dixon 1987), and may be prohibitively expensive. Although the application of calcium cyanamide is considered an effective management option (Klasse 1996), it is also expensive. Furthermore, its efficacy has not been confirmed in a canola cropping system under prairie conditions. To date, no clubroot-resistant canola cultivars are available in Canada (Pageau et al. 2006; Strelkov et al. 2007b), although the development of such varieties is a desirable alternative approach for controlling this disease (Kuginuki et al. 1999).

A good breeding strategy and appropriate deployment of plant resistance require an understanding of the diversity in the virulence of *P. brassicae* (Manzanares-Dauleux et al. 2001). The pathogenic diversity in field populations of *P. brassicae* from North America has been evaluated by a number of researchers (Ayers 1957; Hildebrand and Delbridge 1995; Seaman et al. 1963; Strelkov et al. 2007b; Strelkov et al. 2006b; Williams 1966). Among the various systems proposed for pathotype designations, the differentials of Williams (Williams 1966) have been commonly used to characterize pathogen populations from Canada (Hildebrand and Delbridge 1995; Reyes et al. 1974; Strelkov et al. 2007b;Williams 1966). In Europe, the European Clubroot Differential (ECD) set, which consists of 15 genotypes from three *Brassica* species, has been frequently used (Buczacki et al. 1975; Crute et al. 1980; Voorips 1995). Somé et al. (1996) proposed a differential set consisting of three *B. napus* genotypes, which may be useful for characterizing the pathogen in Alberta, where it occurs predominantly on *B. napus* canola.

Previously, nine *P. brassicae* field populations from various regions of Canada were tested using two sets of differential hosts (Strelkov et al. 2007b). Seven populations

from Alberta were classified as pathotypes 3 and 5 based on their reactions on the differential hosts of Williams (1966), or as ECD16/15/12 and ECD16/15/0, respectively, on the ECD set (Buczacki et al. 1975). Two other populations, originating from Abbottsford, BC, and Orton, ON, were classified as pathotype 6. Concerns, however, were raised over the intermediate and fluctuating results of certain differential hosts following inoculation with the populations tested (Strelkov et al. 2007b). This phenomenon, which was also reported by others (Kuginuki et al. 1999; Toxopeus et al. 1986; Williams 1966), may result from heterogeneity in the pathogen populations (Toxopeus et al. 1986; Williams 1966) or from genetic heterogeneity in the differential hosts themselves (Kuginuki et al. 1999). The use of single spore-derived isolates of *P. brassicae* has been suggested as a way to more accurately assess virulence in the pathogen, and a number of agarose-based methods for the isolation of single resting spores have been developed (Jones et al. 1982; Tinggal and Webster 1981; Somé et al. 1996). However, these agarose-based techniques are generally time-consuming (Kageyama et al. 1995), and inoculation efficiencies can be very low. Therefore, the objectives of the present study were: (i) to develop a simple and efficient technique for isolating single spores of *P. brassicae*, and (ii) to assess variation in virulence among single spore isolates derived from pathogen populations from Canada.

8.3 Materials and Methods

Terminology

A 'population' of *P. brassicae* refers to a collection of resting spores recovered from infested soil or clubs of an infected plant, and used to inoculate a set of differential

hosts (Buczacki et al. 1975), whereas a 'single spore isolate' refers to a population derived from a club inoculated with a single resting spore and maintained in isolation (Voorips 1995). To conform with the suggestions of Voorips (1995) and Crute et al. (1980), the term 'pathotype' will be used instead of 'race,' regardless of the authors' original terminology.

Pathogen populations

Five populations of *P. brassicae* were used for the isolation of single resting spores of the pathogen: (i) SACAN03-1, originally obtained from clubbed canola roots collected near St. Albert, AB, (ii) CDCN04-1, collected from a diseased volunteer canola plant growing near the Crop Diversification Centre North, Alberta Agriculture and Food, Edmonton, AB, (iii) Leduc-1, recovered from infested soil from a market garden near Leduc, AB, (iv) ORCA04, collected from an infected cabbage root (*Brassica oleracea* L. var. *capitata*) from Orton, ON (provided by Ms. K. Callow, Ontario Ministry of Agriculture and Food, Guelph, ON), and (v) AbotJE04-1, recovered from infested soil collected near Abbottsford, BC (supplied by Dr. J. Elmhirst, Elmhirst Diagnostics, Abbotsford, BC). These populations, which were previously characterized by Strelkov et al. (Strelkov et al. 2007b), were reproduced and maintained on the universally susceptible Chinese cabbage 'Granaat' (*Brassica rapa* L. ssp. *pekinensis*).

Plant materials

The virulence of 25 single spore isolates of *P. brassicae* was investigated following inoculation of six differential hosts. The differentials of Williams (1966), purchased from the Crucifer Genetics Cooperative (Madison, WI), include the rutabaga (*Brassica napus* var. *napobrassica* Mill.) cultivars 'Wilhelmsburger' and 'Laurentian',

and the cabbage cultivars 'Jersey Queen' and 'Badger Shipper'. The differentials of Somé et al. (1996) include 'Wilhelmsburger' and the oilseed rape cultivars 'Nevin' and 'Brutor' (*B. napus* L. var. *napus*). 'Nevin' was obtained from the Czech Genebank, Research Institute of Crop Production, Prague-Ruzyne, Czech Republic, and 'Brutor' from the Leibniz Institute of Plant Genetics and Crop Research (IPK) Genebank, Gatersleben, Germany. Chinese cabbage 'Granaat' was purchased from B & T World Seeds (Paguignan, France), and used as a susceptible control.

Isolation of single spores and host inoculation

Single spores were isolated from 8-week-old galls of 'Granaat' inoculated with the individual populations of *P. brassicae*. Approximately 3 g of clubbed roots, either freshly harvested, frozen (-80^{o}C), or partially decayed (kept for about two months at 4^{o}C in a sealed container), were homogenized in 50 ml of sterile distilled water (sdH_2O) in a commercial blender, followed by filtration through eight layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, N.C.). A pellet of resting spores was recovered from the filtrate by centrifugation (1000×g, 4^{o}C, 10 min) and washed five times with sdH_2O, with the pellet re-suspended and centrifuged each time. The final pellet was stored at 4^{o}C for no more than 2 days before use.

Immediately prior to use, resting spores were re-suspended in either sodium phosphate buffer (pH 8.0, 10 mM) or in 5% (v/v) glycerol in sdH_2O. The spore concentration was quantified using a haemocytometer (VWR, Mississauga, ON) and adjusted to approximately 2×10^3 spores/ml with phosphate buffer or 5% (v/v) glycerol. A 0.5-µl drop of spore suspension was placed on the glass coverslip of a microscope slide (Fisher Scientific, Edmonton, AB) and examined at 100 fold magnification. Upon

confirmation of the presence of a single spore, the droplet was soaked up by gently moving the roots of a 1- or 2-week-old 'Granaat' seedling (pre-germinated on moistened sterile filter paper in a Petri dish) horizontally through the drop. The seedling was then placed in a Petri dish on a piece of filter paper moistened with sdH_2O (pH 6.5 to 7.0) or tap water (pH 6.0), or in a Petri dish with the roots soaked in 100 µl of sdH_2O (pH 6.5 to 7.0) or tap water (pH 6.0). The pH of the tap water was adjusted using HCl. Following a 2-day incubation in darkness at 21°C (the incubation solution was replenished on the second day), the seedlings were transplanted into 7.5 cm diameter plastic pots filled with Metro-Mix 290 soil (Scotts, Columbus, OH), at a density of one plant per pot, and maintained in a growth cabinet at 24°C (light) /18°C (dark) with a 16 h photoperiod and a light intensity of 180 µmol/m^2/s. The soil was kept saturated for 19 days after transplanting by soaking the pots in a layer of low pH (6.0) tap water. The plants were watered thereafter as required and fertilized (15N-30P-15K) once a week.

Inoculation of differential hosts

Resting spores were collected (as described above) from the galled roots of 8-week-old 'Granaat' plants inoculated with single spore isolates of *P. brassicae*, and the spore concentration was diluted to approximately 1×10^7 spores/ml with sdH_2O. One-week-old seedlings, which were pre-germinated on moistened sterile filter paper, were inoculated by dipping the roots in the spore suspension for 10 s (Strelkov et al. 2007b). The inoculated seedlings were then immediately planted in 4 by 4 cm plastic pots filled with Metro-Mix 290 soil, placed in flat containers without drainage holes at the bottom, and transferred to a greenhouse maintained at 21°C (light) / 18°C (dark) with a 16 h photoperiod (natural light supplemented by high pressure sodium light). The soil was

kept saturated using tap water (pH 6.0) for the first week after inoculation, and then watered with regular tap water as required. Fertilizer (15N-30P-15K) was applied once a week.

Disease assessment

'Granaat' plants were assessed for symptom development 8-weeks after inoculation with single spores, while the differential hosts were examined 6-weeks after inoculation for pathotype designation. The plants were pulled from the soil, the roots washed free of soil mix and visually evaluated for disease development using a 0 to 3 scale (Kuginuki et al. 1999), where 0 = no galling, 1 = a few small galls (small galls on less than one-third of the roots), 2 = moderate galling (small to medium-sized galls on one-third to two-thirds of the roots), and 3 = severe galling (medium to large-sized galls on more than two-thirds of the roots). An index of disease (ID) was calculated using the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2007b):

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

Where: n is the number of plants in a class; N is the total number of plants in an experimental unit; and 0, 1, 2 and 3 are the symptom severity classes. The mass of the clubbed roots (total root mass minus mass of non-affected roots) was also recorded for each infected plant.

Experimental design and data analysis

For each combination of inoculation method and population, 20 to 118 'Granaat' seedlings were inoculated with a suspension containing a single resting spore of *P.*

brassicae. The number of seedlings inoculated and the number of galled plants obtained were recorded upon completion of the 8-week incubation period. The proportion of infected plants was first analyzed for the various populations and inoculation methods (Table 8-1) using the PROC RELIABILITY in SAS 9.1 Software (SAS Institute Inc., Cary, NC), to test the hypothesis of equality of the proportions (i.e. that the proportion of infected plants was not significantly different). For any given rejection of the hypothesis, the PROC MULTTEST was then used to contrast the infection rates if there were more than two proportions.

For characterization of the virulence of the single spore-derived isolates on the differential hosts, five separate tests were conducted, each involving five different single spore isolates used separately to inoculate the six differential hosts. For each combination of host and single spore isolate, experimental units consisted of 12 plants and were replicated three times in a split-plot design with isolates as main plots and differential hosts as subplots. Since normal score plotting indicated that 90% of the data points (ID values) were normally distributed, analysis of variance (ANOVA) was conducted, and Fisher's least significant difference (LSD) at a significance of $p<0.05$ was calculated to differentiate resistant from susceptible reactions. Genotypes developing IDs that were not significantly different from the genotype with the lowest ID in the same treatment were regarded as resistant, while those with IDs that were significantly different from the genotype with the lowest ID were regarded as susceptible. In addition, nonparametric analysis was conducted by transforming the mean disease ratings of each experimental unit into midranks (Shah and Madden 2004), followed by analysis using the PROC MIXED in SAS 9.1 software. Finally, the mass of clubbed roots (6 weeks after

inoculation with the single spore isolates) was also analyzed parametrically for each host genotype, using the PROC MIXED.

8.4 Results

Single spore isolation

The infection rates obtained with the different populations and inoculation methods (Table 8-1) were treated as proportions and the PROC RELIABILITY was used to test the hypothesis of equality of the proportions. The proportion of infected plants was found to be significantly different across the five populations ($\chi^2 = 11.598$, $p<0.0206$), and further analysis using the PROC MULTTEST revealed that the only significant contrast ($p<0.0216$) for overall infection rate was between SACAN03-1 (8.4%) and Leduc-1 (1.9%). The hypothesis of equality of proportions was also rejected within the populations SACAN03-1 ($\chi^2 = 10.5685$, $p<0.0051$), AbotJE04-1 ($\chi^2 = 4.9708$, $p<0.0258$), and ORCA04 ($\chi^2 = 18.67$Strelkov et al. 2006a, $p<0.0001$), indicating that the infection rates obtained within these populations were significantly different. In contrast, testing of the hypothesis of equality revealed no significance (and therefore acceptance of the hypothesis) within the populations Leduc-1 ($\chi^2 = 3.2126$, $p>0.2006$) and CDCN04-1 ($\chi^2 = 6.3911$, $p>0.1718$).

When the data were pooled across populations according to the type of water used for incubation of the seedlings, a significant difference ($\chi^2 = 13.4568$, $p<0.0002$) was found between low pH and distilled water. Infection rates of 9.0% and 2.8% were obtained with the distilled and low pH water, respectively. However, within the population SACAN03-1, the type of water did not have a significant effect ($\chi^2 = 0.6836$,

$p>0.4083$). When the infection rates were pooled across populations according to buffer ($\chi^2 = 2.1314$, $p>0.1443$), seedling age ($\chi^2 = 0.9029$, $p>0.3420$), or the condition of the galls used for single spore isolation ($\chi^2 = 2.6793$, $p>0.2619$), no significant differences were found. Nevertheless, differences with respect to these parameters were observed within some populations. Within population SACAN03-1, the infection rate was significantly higher ($\chi^2 = 6.9446$, $p<0.0084$) when using 2-week-old seedlings (13.25%) instead of 1-week-old seedlings (0%). A significant difference ($\chi^2 = 18.67$Strelkov et al. 2006a, $p<0.0001$) was also observed within ORCA04 when decomposing rather than fresh or frozen galls were used for isolation of resting spores. In the case of inoculations conducted with single spores derived from AbotJE04-1, the use of fresh rather than frozen galls significantly ($\chi^2 = 4.9708$, $p<0.0258$) increased the infection rate (Table 1).

Characterization of single spore isolates

The virulence of five single spore isolates derived from each of the five *P. brassicae* populations was compared on the differential hosts of Williams (1966) and Somé et al. (1996). ANOVA revealed no significant interactions between the differential hosts and the single spore isolates derived from SACAN03-1 (SACAN-ss1 to SACAN-ss5) ($p>0.8458$), nor any significant interactions between the differential hosts and the single spore isolates from AbotJE04-1 (AbotJE-ss1 to AbotJE-ss5) ($p>0.9999$). Therefore, the five single spore isolates derived from each of the two populations appeared to be fairly homogeneous (Table 8-2). The reactions of the hosts were classified as resistant or susceptible through LSD grouping ($p<0.05$), with any genotype in the same group as the genotype with the lowest ID considered resistant. Using this criterion, two hosts, 'Wilhelmsburger' and 'Badger Shipper,' were classified as resistant to isolates

SACAN-ss1, SACAN-ss2, SACAN-ss4, and SACAN-ss5, corresponding to a pathotype 3 or P_2 designation on the differentials of Williams' (1966) or Somé et al. (1996), respectively (Table 8-3). However, only 'Wilhelmsburger' was classified as resistant to SACAN-ss3, corresponding to a pathotype 2 designation on the hosts of Williams' (1966), but which would still correlate to P_2 on the system of Somé et al. (1996). Thus, although ANOVA indicated a non-significant interaction between differential hosts and isolates derived from SACAN03-1, the LSD groupings suggested some diversity. In response to inoculation with single spore isolates derived from the population AbotJE04-1 (AbotJE-ss1 to AbotJE-ss5), only 'Brutor' and 'Jersey Queen' appeared susceptible. Therefore, isolates AbotJE-ss1 to AbotJE-ss5 were classified as pathotype 6 according to Williams (1966) or as P_3 on the differentials of Somé et al. (1996).

Significant interactions were found between differential hosts and single spore isolates derived from the populations CDCN04-1 ($p<0.0005$), Leduc-1 ($p<0.0001$) and ORCA04 ($p<0.0001$). Three of the five single spore isolates from CDCN04-1 (CDCN-ss2, CDCN-ss4 and CDCN-ss5) possessed similar virulence patterns, with only 'Wilhemsburger' and 'Badger Shipper' showing resistance. Thus, these isolates were classified as pathotype 3 on the differentials of Williams (1966), or as P_2 on the hosts of Somé et al. (1996) (Table 8-3). In contrast, one of the isolates, CDCN-ss3, exhibited reduced virulence on 'Jersey Queen,' in addition to its avirulence on 'Wilhemsburger' and 'Badger Shipper' (Table 8-2); this resulted in a pathotype 8 classification on the differentials of Williams (1966), but still corresponded to a P_2 designation on the system of Somé et al. (1996) (Table 8-3). Similarly, isolate CDCN-ss1 also exhibited reduced virulence on 'Jersey Queen,' resulting in a pathotype 8 designation according to Williams

(1966). However, the host differential 'Brutor' was also resistant to CDCN-ss1, producing a virulence pattern that was not described by Somé et al. (1996) in their original report. Nonetheless, Manzanares-Dauleux et al. (2001) [using the differentials of Somé et al. (1996)] identified isolates with this virulence pattern and classified them as a novel pathotype, P_6, a designation that we have retained (Table 8-3).

The Leduc-1 population also appeared to be heterogeneous in its composition (Table 8-3). The single spore isolates Leduc-ss1, Leduc-ss4, and Leduc-ss5 were classified as pathotype 3 or P_2 according to Williams (1966) or Somé et al. (1996), respectively, based on the resistant reactions of 'Wilhemsburger' and 'Badger Shipper' (Tables 8-2 and 8-3). In contrast, only 'Jersey Queen' and 'Brutor' were susceptible to Leduc-ss2, corresponding to a pathotype 6 designation on the system of Williams (1966), or a P_3 designation according to Somé et al. (1996). Inoculation of the differential hosts with Leduc-ss3 revealed that the viability of this isolate was poor, since even the universal suscept 'Granaat' developed an ID of only 44% (Table 8-2). Hence, this isolate was excluded from further analysis. The hosts 'Wilhemsburger' and 'Badger Shipper' were resistant to the single spore isolates ORCA-ss1 and ORCA-ss5, derived from the population ORCA04. All other hosts were susceptible to these isolates, corresponding to a pathotype 3 designation according to Williams (1966), or a P_2 designation according to Somé et al. (1996). Isolate ORCA-ss2, derived from the same population, was avirulent on 'Jersey Queen' as well as on 'Wilhemsburger' and 'Badger Shipper,' which resulted in a pathotype 8 classification on the system of Williams (1966). However, as 'Jersey Queen' was not used as a differential by Somé et al. (1996), ORCA-ss2 was still classified as P_2 according to their system. The remaining single spore isolates, ORCA-ss3

and ORCA-ss4, were avirulent on all hosts except for 'Brutor,' corresponding to a pathotype 5 or P_3 designation on the differentials of Williams (1966) and Somé et al. (1996), respectively.

Parametric analysis of the average mass of clubbed roots in each experimental unit using the PROC MIXED of SAS yielded very similar results to those from the analysis of IDs, when pair-wise comparisons of differential host within isolates were conducted using the most resistant host as a control. In contrast, the results obtained using nonparametric analysis of disease indices showed greater disparity with those from the analysis of clubbed root masses (data not shown). The highly significant positive correlation between IDs and masses of clubbed roots ($R^2 = 0.6746$, $p < 0.0001$, N = 5152) also confirmed that the IDs accurately reflected the degree of disease development.

6.5 Discussion

Single spore isolation

Two technical problems, the minute size of *P. brassicae* resting spores and their tendency to aggregate, can hamper the isolation of single spores of the pathogen. These problems have often been dealt with by the use of agarose-based methods to isolate single spores of *P. brassicae*, which rely on the even distribution of resting spores on a thin film of agarose placed on a microscope slide (Some et al. 1996); pieces of agarose containing a single resting spore are excised and used to inoculate host plants. To facilitate this process, special equipment, such as a dummy microscope objective lens on which a punch is mounted to cut and lift out a disc of agar containing a single spore, has occasionally been employed (Jones et al. 1982; Tinggal and Webster 1981). In the current

study, we experienced great difficulties in lifting up tiny pieces of agarose without the aid of special apparatus. Moreover, the agarose attached to a spore might prevent it from making direct contact with the root hairs of the host.

An alternative to agarose-based methods is the use of highly diluted *P. brassicae* spore suspensions, in which the presence of a single resting spore can be confirmed per unit volume. For instance, Buczacki (1977) located single spores of the pathogen in droplets of a diluted spore suspension placed in wells of a glass cavity slide, and withdrew and expelled the droplets onto the roots of seedlings when the presence of a single spore was confirmed. Using this procedure, however, a considerable proportion of the spore suspension was left in the pipette tips and in the wells of the slide, resulting in reduced infection rates. Kageyama et al. (1995) deposited a 0.5-µl droplet of spore suspension on the glass cover slip of a microscope slide. The presence of a single resting spore in the droplet was confirmed by microscopic examination, and the cover slip carrying the single spore was placed on the surface of autoclaved soil in a container. A 1-day-old Chinese cabbage seedling was laid over the spore droplet and covered with a small amount of additional soil. Although we evaluated this method, we obtained very low infection rates (0 to 4.2%), perhaps as a result of the attachment of resting spores to the glass cover slip.

Therefore, one of the objectives of the current study was to develop a relatively simple and efficient method to isolate single resting spores of *P. brassicae* that allows for the inoculation and infection of host roots with increased success. The procedure we developed has three advantages: (i) the tendency for resting spores to aggregate was reduced by using 5% (v/v) glycerol in sdH_2O for the preparation of the spore suspension,

thereby facilitating the identification and isolation of single spores, (ii) the protocol allowed direct contact of the resting spore with the root hairs of the host, resulting in higher infection rates, and (iii) less technical difficulties were encountered, since pieces of agarose did not have to be cut and removed. Using this procedure with a properly diluted spore suspension, 2 to 4 seedlings could be inoculated per hour, and infection rates ranging from 4 to 17% were obtained with the various pathogen populations (Table 8-1). The relative simplicity and efficiency of this technique should facilitate research that requires the use of single spore-derived isolates of *P. brassicae*, including breeding for clubroot resistance and analysis of the genetic diversity of field populations using molecular markers.

Since a germination stimulating factor from *Brassica* root exudates has been proposed to stimulate germination of pathogen resting spores (Suzuki et al. 1992), we hypothesized that infection rates would be higher in 2-week-old versus 1-week-old seedlings, because they would presumably release more root exudates. However, while the data obtained with SACAN03-1 appeared to support such a hypothesis, the data obtained with CDCN04-1 did not (Table 8-1). Hence, additional experiments are required to clarify this issue. The condition of the galls used for the isolation of resting spores also had an inconsistent effect in the different populations (Table 8-1). In the case of ORCA04, spores obtained from decomposing galls produced significantly higher infection rates than spores from fresh or frozen galls. Suzuki et al. (1992) reported similar results and hypothesized that resting spores from decomposing galls possessed higher rates of germination, perhaps because of increased maturity. However, in Leduc-1, inoculum from decomposing galls did not yield significantly higher infection rates, and

when the data were pooled across populations, no significant difference between gall types was observed.

Characterization of single spore-derived isolates

Various systems have been proposed for the classification of *P. brassicae* into pathotypes or races. A recent report used the hosts of Williams (1966) and the ECD set (Buczacki et al. 1975) to analyze the virulence of pathogen populations from Canada (Strelkov et al. 2007b). In the current study, we retained the differentials of Williams (1966) to enable comparisons between the virulence of single spore isolates (examined here) and the populations from which they were obtained (Strelkov et al. 2007b). However, since most isolates examined were originally obtained from canola (*B. napus*), we also included the *B. napus* differential 'Brutor,' which allowed us to obtain pathotype designations according to Somé et al. (1996). The differentials of the ECD set (Buczacki et al. 1975), with the exception of Chinese cabbage 'Granaat' (ECD 05), were excluded from the current study because of space limitations; 'Granaat' was retained as a susceptible control.

While physiologic specialization has long been known in the pathogen (Honig 1931), many of the differential hosts that have been proposed to characterize *P. brassicae* develop intermediate ID values in response to inoculation (Kuginuki et al. 1999; Strelkov et al. 2007b; Toxopeus et al. 1986). Intermediate and fluctuating IDs have been attributed not only to the heterogeneity of *P. brassicae* populations (Jones et al. 1982; Scott 1985; Tinggal and Webster 1981), but also to the heterogeneity of the differential hosts themselves (Kuginuki et al. 1999). The results from the current study do suggest that genetic heterogeneity in some hosts contributes to the development of indistinct reactions

(Toxopeus et al. 1986), since intermediate IDs were often obtained after inoculation with single spore isolates that should represent a single genotype of the pathogen (Table 8-2). This was particularly evident with the cabbage 'Jersey Queen', which commonly developed IDs ranging from 30% to 60%. However, in the case of this and other cabbage cultivars, indistinct reactions may result not only from genetic heterogeneity in the host genotypes, but also from possible quantitative additive effects that have been reported for clubroot resistance in this species (Crute et al. 1980).

The occurrence of indistinct host reactions makes it difficult to classify the pathogen into races or pathotypes, since a qualitative designation (resistant or susceptible) has to be applied to what in many instances appears to be a quantitative reaction (Strelkov et al. 2007b). A number of strategies have been used to accommodate indistinct host responses. Most recently, Strelkov and coworkers (2006b; 2007b) regarded an ID of 50% as the cut-off between a resistant and a susceptible reaction. However, this represents a somewhat arbitrary criterion, and other workers have used different cut-offs [for example, Somé et al. (1996) used 25%]. In the current study, ANOVA of the IDs was conducted and LSD ($p<0.05$) used to differentiate resistant from susceptible reactions (Table 8-2). For comparison, we also performed parametric analysis using the average mass of clubbed roots and nonparametric analysis using the mean disease ratings. While parametric analysis of the mass of clubbed roots yielded similar results to those from the analysis of IDs, the results were very different from those of the nonparametric analysis. Given that the mass of clubs provides a biological measure of susceptibility, this suggests that parametric analysis is more appropriate than nonparametric analysis for study of the *Brassica-P. brassicae* interaction.

Using LSD ($p<0.05$) to distinguish resistant from susceptible reactions, we found that the pathotype designation of most isolates obtained from the populations SACAN03-1, Leduc-1 and AbotJE04-1 were similar to the populations from which they were derived. In the case of AbotJE04-1, all single spore isolates possessed the same virulence pattern as this population, and were classified as pathotype 6 according to the system of Williams (1966) [or as P_3 on the differentials of Somé et al. (1996)], suggesting that this population is fairly homogenous (Table 8-3). Similarly, ANOVA revealed no significant interaction between differential hosts and isolates derived from SACAN03-1, which was previously classified as pathotype 3 (Strelkov et al. 2007b) on the differentials of Williams (1966). However, while four of five single spore isolates from this population shared a pathotype 3 designation based on LSD groupings, isolate SACAN-ss3 was classified as pathotype 2 because of a susceptible reaction by 'Badger Shipper' (Table 8-3). The actual ID of 'Badger Shipper' in response to SACAN-ss3 was nevertheless only 6% to 14% higher than in response to the other isolates, and further testing may be required to confirm whether SACAN-ss3 does in fact represent a different pathotype. On the differentials of Somé et al. (1996), which do not include 'Badger Shipper', all isolates derived from SACAN03-1 were classified as P_2 (Table 8-3). In the case of Leduc-1, a population previously classified as pathotype 3 (Strelkov et al. 2007b), three of four single spore isolates also shared the same classification [equivalent to P_2 on the differentials of Somé et al. (1996)]. However, isolate Leduc-ss2 was designated as pathotype 6 or P_3 on the hosts of Williams (1966) and Somé et al. (1996), respectively. The different virulence patterns in isolates derived from Leduc-1 were confirmed by

ANOVA, which indicated a significant interaction between differential hosts and single spore isolates, strongly suggesting that heterogeneity exists in this population.

The isolates obtained from the other two *P. brassicae* populations, CDCN04-1 and ORCA04, possessed very different virulence patterns relative to the original populations and also appeared to be a mixture of pathotypes. While CDCN04-1 was previously classified as pathotype 5 (Strelkov et al. 2007b) on the differentials of Williams (1966), three of the five single spore isolates tested were classified as pathotype 3 in the present study, with the remaining isolates classified as pathotype 8 (Table 8-3). However, Strelkov et al. (2007b) cautioned that the pathotype 5 classification obtained in their study was based largely on the reactions of the *B. oleracea* hosts, which produced intermediate and fluctuating reactions against all populations; hence, it is possible that this classification may have simply reflected the inconsistent reactions of these hosts. Nonetheless, 'Laurentian,' a *B. napus* host that generally produces distinct reactions, was more susceptible to the single spore isolates than to the original population, and the discrepancy in the pathotype designations between CDCN04-1 and its derived single spore isolates may have been due to the interactions of different pathotypes in the original population (Jones et al. 1982; Toxopeus et al. 1986). One pathotype can alter the response of a differential host to a second pathotype present in the same inoculum (Jones et al. 1982), and indistinct reactions may result from the presence of multiple pathotypes in the same population (Toxopeus et al. 1986; Williams 1966). On the hosts of Somé et al. (1996), four of the isolates from CDCN04-1 were classified as P_2, while the fifth (CDCN-ss1) was classified as P_6 (Table 8-3).

Similarly, while ORCA04-1 had been classified as pathotype 6 (Strelkov et al. 2007b) on the hosts of Williams (1966), none of the single spore isolates from this population shared that designation (Table 8-3). Two of the five isolates were classified as pathotype 3, two were classified as pathotype 5, and one was classified as pathotype 8. On the differentials of Somé et al. (1996), three isolates were designated as P_2, and two were classified as P_3. The differential classification of three of the isolates (ORCA-ss1, ORCA-ss2 and ORCA-ss5) resulted in part from the fact that they exhibited greatly increased virulence on 'Nevin' and 'Laurentian' (Table 8-2) relative to the original population (Strelkov et al. 2007b). The lower virulence of the latter may have been due to its heterogeneous composition, since reduced virulence has been previously reported in pathotype mixtures, and may reflect competition between pathogenic and nonpathogenic isolates (Voorips 1995). Nonpathogenic isolates may also induce resistance mechanisms in the host, thereby reducing infection by pathogenic isolates. A reduction in virulence resulting from a pathotype mixture cannot explain the results obtained with ORCA-ss3 and ORCA-ss4, however, as these isolates (and ORCA-ss2) exhibited greatly reduced virulence on 'Jersey Queen' relative to the original population (Table 8-2). It is possible that nonpathogenic isolates can also rely on pathogenic isolates present in the same population to proliferate within otherwise resistant hosts. Clearly, complex interactions between pathotypes and host genotypes appear to be at play.

The pathotype composition of *P. brassicae* in Canada appeared more diverse when single spore isolates rather than populations of the pathogen were examined. In Alberta, at least three and possibly four pathotypes were identified among 14 isolates characterized on two differential sets; these isolates were derived from a small number of

populations, and additional pathotypes may be detected with further testing. In contrast, only two pathotypes were found when populations of *P. brassicae* were tested (Strelkov et al. 2007b), and another study found only one (Strelkov et al. 2006b). Nevertheless, the predominant pathotype among isolates from Alberta remains pathotype 3 or P_2, as classified on the differentials of Williams (1966) and Somé et al. (1996), respectively. This is consistent with studies of field populations, in which pathotype 3 or P_2 was also predominant (Strelkov et al. 2007b; Strelkov et al. 2006b). Pathotype 3 was shown to be highly virulent on all canola cultivars tested from Canada (Strelkov et al. 2007b), and is also highly virulent on the spring oilseed rape differential host 'Brutor' (Table 8-2). Therefore, this pathotype appears to be a reasonable choice to use in screening for clubroot resistance in *B. napus* canola in Alberta. However, caution should be used in any breeding strategy, since rare pathotypes of *P. brassicae* may quickly become predominant if susceptible host genotypes are continuously grown (Seaman et al. 1963). Genetic resistance will have to be utilized in conjunction with other management strategies, including crop rotation and proper sanitation, to ensure its durability.

8.6 References

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8.7 Tables

Please refer to the following pages.

Table 8-1. Infection rates obtained for 1 and 2 week-old seedlings of Chinese cabbage cv. Granaat inoculated with single spores of *Plasmodiophora brassicae* under different conditions.

Population[a]	Seedling age	Clubs used for isolation[b]	Spore suspension[c]	Water for incubation[d]	Percent infection rate (no. infected/total inoculated)[e]
SACAN03-1	1 week	Frozen	phosphate buffer	Low pH	0.00 (0/48) a
	2 week	Frozen	phosphate buffer	Distilled	4.17 (1/24) ab
	2 week	Frozen	phosphate buffer	Low pH	16.95 (10/59) b
AbotJE04-1	2 week	Frozen	phosphate buffer	Low pH	0.00 (0/77) a
	2 week	Fresh	phosphate buffer	Low pH	6.25 (5/80) b
Leduc-1	2 week	Frozen	phosphate buffer	Low pH	0.00 (0/75) a
	2 week	Fresh	phosphate buffer	Low pH	1.25 (1/80) a
	2 week	Decomposing	phosphate buffer	Low pH	3.45 (4/116) a
ORCA04	2 week	Frozen	phosphate buffer	Low pH	0.00 (0/118) a
	2 week	Fresh	phosphate buffer	Low pH	0.00 (0/63) a
	2 week	Decomposing	phosphate buffer	Distilled	10.00 (8/80) b
CDCN04-1	2 week	Decomposing	phosphate buffer	Low pH	0.92 (1/109) a
	2 week	Fresh	phosphate buffer	Low pH	4.00 (2/50) a
	1 week	Decomposing	5% glycerol	Low pH	5.00 (1/20) a
	2 week	Decomposing	5% glycerol	Low pH	4.08 (2/49) a
	2 week	Decomposing	5% glycerol	Distilled	10.34 (3/29) a

[a]Populations were maintained on Chinese cabbage cv. Granaat.
[b]Frozen clubs were stored at −80°C; fresh clubs were used 1 or 2 days after harvest (stored at 4°C prior to use); decomposing clubs were stored 1 to 2 months at 4°C in parafilm-sealed beakers prior to use.
[c]Refers to solutions used to resuspend the resting spores prior to microscopic examination and inoculation of seedlings; phosphate buffer = sodium phosphate buffer (pH 8, 10 mM); 5% glycerol = 5% (v/v) glycerol in sdH_2O; both solutions were autoclaved prior to use.
[d]Refers to water used to incubate seedlings in Petri dishes for 2 days after inoculation; low pH = tap water with pH adjusted to 6.0; distilled = distilled water (pH = 6.5 to 7.0); both low pH and distilled water were autoclaved prior to use.
[e]Percentages followed by the same letter are not significantly different at $p<0.05$ within each population, as determined using the PROC RELIABILITY or PROC MULTTEST in SAS 9.1 Software (SAS Institute Inc., Cary, NC).

Table 8-2. Disease indices on six *Brassica* differential hosts following inoculation with single spore-derived isolates of *Plasmodiophora brassicae* from Canada.

Single spore isolate	Control ('Granaat')	*Brassica napus*				*Brassica oleracea*		$LSD_{0.05}$
		'Brutor'	'Laurentian'	'Nevin'	'Wilhelmsburger'	'Badger Shipper'	'Jersey Queen'	
SACAN-ss1	100	99.0	81.9	65.2	4.7R	17.5R	41.8	12.9
SACAN-ss2	90.9	96.8	86.0	62.0	12.0R	23.9R	48.3	22.0
SACAN-ss3[a]	97.2	96.7	67.0	62.5	8.0R	31.6	44.0	20.9
SACAN-ss4	100	96.0	80.7	69.3	9.3R	25.8R	46.4	17.1
SACAN-ss5	100	97.7	82.0	67.7	17.8R	24.3R	39.3	16.6
AbotJE-ss1	84.8	57.0	0.0R	3.8R	0.0R	4.8R	77.0	15.0
AbotJE-ss2	83.3	65.3	0.0R	0.0R	6.0R	8.9R	82.3	18.3
AbotJE-ss3	94.4	64.1	0.0R	0.0R	1.0R	3.9R	84.3	17.7
AbotJE-ss4	88.9	63.7	0.0R	0.9R	0.0R	8.3R	84.7	20.7
AbotJE-ss5	88.9	62.8	0.0R	1.9R	0.0R	5.5R	85.7	12.0
CDCN-ss1	72.2	17.7R	37.8	68.5	8.3R	10.2R	21.4R	29.2
CDCN-ss2	100	99.0	77.0	83.7	13.9R	25.7R	50.1	14.4
CDCN-ss3	100	100	61.7	67.5	13.4R	15.8R	49.5R	40.0
CDCN-ss4	100	99.1	95.5	90.1	28.0R	17.0R	75.2	15.6
CDCN-ss5	100	97.8	95.1	85.3	19.5R	6.1R	48.6	24.0
Leduc-ss1	100	99.0	68.0	82.7	7.4R	17.3R	56.0	29.4
Leduc-ss2	91.6	16.6	0.0R	0.0R	0.0R	2.8R	80.7	14.0
Leduc-ss3[b]	44.4	4.6	0.9	0.0	0.0	0.0	3.0	7.4
Leduc-ss4	97.2	93.7	24.0	74.7	0.9R	4.6R	30.8	7.4
Leduc-ss5	100	99.1	83.1	79.5	16.7R	2.8R	55.2	31.6
ORCA-ss1	100	100	61.7	73.3	5.3R	25.7R	48.0	21.7
ORCA-ss2	96.7	94.0	48.5	79.0	5.5R	3.7R	26.8R	23.3
ORCA-ss3	72.7	24.7	3.7R	21.3R	0.0R	0.0R	2.8R	21.8

ORCA-ss4	66.7	33.4	1.9R	14.2R	0.9R	0.9R	9.4R	22.3
ORCA-ss5	100	98.1	100	93.3	21.9R	13.5R	71.5	16.7

[R]Indicates a resistant reaction; LSD (p<0.05) was used to differentiate resistant from susceptible reactions [genotypes developing disease indices (IDs) that were not significantly different from the genotype with the lowest ID in the same treatment were regarded as resistant; all others were regarded as susceptible].
[a]'Badger Shipper' was only tentatively classified as susceptible to SACAN-ss3, as ANOVA indicated no significant interactions between the differential hosts and SACAN-ss1 to SACAN-ss5.
[b]The reactions of the differential hosts to Leduc-ss3 were not classified, as the ID on the susceptible control (Chinese cabbage cv. Granaat) was only 44%.

Table 8-3. Reactions of six *Brassica* differential hosts in response to inoculation with single spore-derived isolates of *Plasmodiophora brassicae* from Canada[a].

Single spore isolate	*Brassica napus*				*Brassica oleracea*		Pathotype[b]
	'Brutor'	'Laurentian'	'Nevin'	'Wilhelmsburger'	'Badger Shipper'	'Jersey Queen'	
SACAN-ss1	+	+	+	-	-	+	$3/P_2$
SACAN-ss2	+	+	+	-	-	+	$3/P_2$
SACAN-ss3	+	+	+	-	+	+	$2/P_2$[c]
SACAN-ss4	+	+	+	-	-	+	$3/P_2$
SACAN-ss5	+	+	+	-	-	+	$3/P_2$
AbotJE-ss1	+	-	-	-	-	+	$6/P_3$
AbotJE-ss2	+	-	-	-	-	+	$6/P_3$
AbotJE-ss3	+	-	-	-	-	+	$6/P_3$
AbotJE-ss4	+	-	-	-	-	+	$6/P_3$
AbotJE-ss5	+	-	-	-	-	+	$6/P_3$
CDCN-ss1	-	+	+	-	-	-	$8/P_6$
CDCN-ss2	+	+	+	-	-	+	$3/P_2$
CDCN-ss3	+	+	+	-	-	-	$8/P_2$
CDCN-ss4	+	+	+	-	-	+	$3/P_2$
CDCN-ss5	+	+	+	-	-	+	$3/P_2$
Leduc-ss1	+	+	+	-	-	+	$3/P_2$
Leduc-ss2	+	-	-	-	-	+	$6/P_3$
Leduc-ss4	+	+	+	-	-	+	$3/P_2$
Leduc-ss5	+	+	+	-	-	+	$3/P_2$
ORCA-ss1	+	+	+	-	-	+	$3/P_2$
ORCA-ss2	+	+	+	-	-	-	$8/P_2$
ORCA-ss3	+	-	-	-	-	-	$5/P_3$
ORCA-ss4	+	-	-	-	-	-	$5/P_3$
ORCA-ss5	+	+	+	-	-	+	$3/P_2$

[a] + indicates a susceptible (compatible) host reaction; − indicates a resistant (incompatible) host reaction; LSD ($p<0.05$) was used to differentiate resistant from susceptible reactions [genotypes developing disease indices (IDs) that were not significantly different from the genotype with the lowest ID in the same treatment were regarded as resistant; all others were regarded as susceptible].

[b]As determined on the differential hosts of Williams (Williams 1966) and Somé et al. (Some et al. 1996).
[c]SACAN-ss3 was classified as pathotype 2 on the differentials of Williams based on LSD groupings, although ANOVA indicated no significant interactions between the differential hosts and SACAN-ss1 to SACAN-ss5.

Appendix: Publications Arising from this Project

A. Refereed Papers Published in Scientific Journals

Xue, S., Cao, T., Howard, R.J., Hwang, S.F., and Strelkov, S.E. 2008. Isolation and variation in virulence of single-spore isolates of *Plasmodiophora brassicae* from Canada. Plant Disease, 92:456-462.

Strelkov, S.E., Manolii, V.P., Cao, T., Xue, S., and Hwang, S.F. 2007. Pathotype differentiation of *Plasmodiophora brassicae* and its occurrence in *Brassica napus* in Alberta. J. Phytopathology, 155:706-712.

Cao, T., Tewari, J.P, and Strelkov, S.E. 2007. Molecular detection of *Plasmodiophora brassicae*, causal agent of clubroot of crucifers, in plant and soil. Plant Disease, 91:80-87.

Strelkov, S.E., Tewari, J.P., and Smith-Degenhardt, E. 2006. Characterization of *Plasmodiophora brassicae* populations from Alberta, Canada. Can. J. Plant Pathol. 28:467-474.

B. Contributions to the Canadian Plant Disease Survey

Strelkov, S.E., Manolii, V.P., Hwang, S.F., Howard, R.J., Manolli, A., Zhou, Q., Holtz, M., and Yang, Y. Incidence of clubroot on canola in Alberta in 2007. Can. Plant Dis. Survey, 88: In Press.

Strelkov, S.E., Manolii, V.P., Cao, T., Hwang, S.F., and Orchard, D. 2007. Incidence of clubroot on canola in Alberta in 2006. Can. Plant Dis. Survey, 87:109-111.

Strelkov, S.E., Cao, T., Manolii, V.P., Lange, R.M., Smith-Degenhardt, E., Orchard, D., and Tewari, J.P. 2006. Incidence of clubroot on canola in Alberta in 2005. Can. Plant Dis. Survey, 86:91-93.

Strelkov, S.E., Tewari, J.P., Hartman, M., and Orchard, D. 2005. Clubroot on canola in Alberta in 2003 and 2004. Can. Plant Dis. Survey, 85:72-73.

C. Abstracts Published in Refereed Journals

Strelkov, S.E., Manolii, V.P., Cao, T., and Hwang, S.F. 2007. Occurrence of clubroot [*Plasmodiophora brassicae*] on canola in Alberta in 2006. Can. J. Plant Pathol. 29:212.

Xue, S., Howard, R., Rahman, M.H., Hwang, S.F., and Strelkov, S.E. 2007. Variation in single spore-derived isolates of *Plasmodiophora brassicae* from Canada. Can. J. Plant Pathol. 29:212.

Xue, S., and Strelkov, S.E. 2006. Evaluation of spore isolation techniques for *Plasmodiophora brassicae*, causal agent of clubroot of crucifers. Can. J. Plant Pathol. 28: 321.

Manolii, V.P., Strelkov, S.E., Bansal, V.K., and Howard, R.J. 2005. Liming and calcium-fertilizer application for clubroot control in canola (*Brassica napus*). Can. J. Plant Pathol. 27:472.

Strelkov, S.E., Tewari, J.P., and Smith-Degenhardt, E. 2005. Characterization of *Plasmodiophora brassicae* populations from Alberta, Canada. Can. J. Plant Pathol. 27:478.

Xue, S., and Strelkov, S.E. 2005. Comparison of single spore isolation techniques for *Plasmodiophora brassicae*. Can. J. Plant Pathol. 27: 481.

D. Conference Proceedings

S.E. Strelkov, M.H. Rahman, S.F. Hwang, and R.J. Howard. 2008. Clubroot of crucifers: A new challenge to canola production in Alberta. *In* Agronomy Update 2008: Conference Proceedings. Jan. 15-16, 2008, Red Deer, AB. *Edited by* N. Whatley and M. Tunney. Alberta Agriculture and Food. pp. 67-71.

S.E. Strelkov, S.F. Hwang, R.J. Howard, V.P. Manolii, S. Xue, G.D. Turnbull and T. Cao. .2008. Clubroot: an emerging threat to the canola industry. *In* FarmTech 2008 Proceedings. Jan. 30 – Feb. 1, 2008, Edmonton, AB. pp. 100-101.

Dosdall, L., Turkington, T.K., Kutcher, R., Strelkov, S., Klein-Gebbinck, H., Lange, R., Clayton, G., Hartman, M., and Brandt, S. 2006. Managing insects and diseases in canola. *In* FarmTech 2006 Proceedings. Jan. 25-27, 2006, Edmonton, AB. pp. 52-62.

Kutcher, H.R., Turkington, T.K., Rashid, K., Strelkov, S., Lange, R., and Brandt, S. 2006. Management of oilseed diseases. *In* Proceedings of the 18th Annual Meeting, Conference and Trade Show of the Saskatchewan Soil Conservation Association. Feb. 15-19, 2006, Regina, SK. pp. 118-125.