# University of Alberta

Pathogenic and molecular characterization of single spore isolates of *Plasmodiophora brassicae* from Canada

by

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## Abstract

Clubroot, caused by *Plasmodiophora brassicae*, is an important new disease of canola (*Brassica napus*) in Alberta, Canada. A simple and efficient method to isolate single resting spores of the pathogen was developed, and the virulence of 25 isolates from Canada was characterized on two differential sets. The pathotype composition of *P. brassicae* appeared more diverse when isolates rather than populations of the pathogen were examined. In Alberta, pathotype 3 was predominant, but at least three and possibly four pathotypes were identified. Additionally, diversity was assessed by RAPD analysis, with isolates found to cluster according to population rather than pathotype. The utility of cleaved amplified polymorphic sequence analysis for molecular differentiation of pathotypes was also demonstrated. It appears that the *P. brassicae* population in Alberta is fairly diverse, and caution should be used in any breeding strategy, since rare pathotypes may quickly become predominant if susceptible host genotypes are continuously grown.

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# Chapter 1. Introduction

## 1.1. The clubroot pathosystem

### 1.1.1. Economic importance

Clubroot, caused by the soilborne, biotrophic pathogen *Plasmodiophora brassicae* Woronin, is one of the most important diseases of cruciferous crops worldwide (Voorrips, 1995). The infection of host roots by *P. brassicae* stimulates abnormal cell elongation and division, which results in club-like swelling (Ingram and Tommerup, 1972). The swelling of the roots blocks nutrient and water transport, stunts the growth of plants, and increases their susceptibility to wilting. Crête (1981) estimated that about 10% of *Brassica oleracea* L. crops in Japan were infected with clubroot. In Australia, clubroot damage to the *Brassica* vegetable industry reaches millions of dollars annually (Donald et al., 2001). In a soil testing survey carried out in a spring oilseed-growing area of Sweden, 78% of 190 fields sampled were found to be infested with clubroot (Wallenhammar, 1996). In Canada, the disease has traditionally been a problem in cole crop production in some areas of British Columbia, Quebec, Ontario and the Maritimes (Rimmer et al., 2003). However, in 2003, clubroot was found 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). Additional surveys in 2005 and 2006 revealed over 110 clubroot-infested fields in central Alberta (Strelkov et al., 2006a; Strelkov et al., 2007a). The disease could have a tremendous economic impact because canola is one of the major crops in Alberta and one-third the acreage is planted in acidic soils that could favor disease development (Tewari et al.,

2005). In 2003, when clubroot was first detected on canola in the province, a yield reduction of 30% was estimated in the most severely infested field (Strelkov et al., 2005; Tewari et al., 2005). However, disease losses seem to be intensifying. In one infested field in Sturgeon County just north of Edmonton, which had been cropped to canola for two consecutive years, the disease was so severe in 2006 that the crop was not harvested, and hence a 100% yield loss occurred (Strelkov et al., 2007a). In Quebec, yield losses of 80 to 91% were reported on canola genotypes grown in a field where *P. brassicae* had been prevalent for a number of years (Pageau et al., 2006).

### 1.1.2. Pathogenesis

The clubroot disease cycle consists of two stages (Fig. 1-1), the first occurring in the host root hairs and the second in the root cortex (Ingram and Tommerup, 1972). The first stage starts with the germination of haploid resting spores in the soil. The resultant zoospores subsequently penetrate the root hair wall and cause infection, which is followed by the production of plasmodia, i.e., multinucleate masses of cytoplasm formed by repeated mitotic nuclear divisions of the pathogen (Aist and Williams, 1971). These multinucleate plasmodia will develop into numerous uninucleate zoosporangia, from which haploid secondary zoospores are released to cause secondary infection of root hairs (Naiki et al., 1984).

Secondary infections are caused by the haploid secondary zoospores as well as dikaryotic zoospores that are formed as a result of the fusion of two haploid zoospores (Ingram and Tommerup, 1972; Buczacki, 1983). Like the primary zoospores, the

secondary zoospores infect the root hairs, but also spread to the root cortex. Although the mechanism of spread into the cortex is not known, the stimulated division of infected cells is presumed responsible (Dekhuijzen, 1981). Secondary infections are followed by the production of intracellular plasmodia, in which the haploid nuclei fuse in pairs at a later stage to form diploid nuclei (Tommerup and Ingram, 1971). The newly formed diploid nuclei develop undergo meiosis and develop into haploid resting spores, which are released into the soil as the host root decays (Tommerup and Ingram, 1971).

The study of the ultrastructure of resting spores, most of which are about 3 μm in diameter, has revealed a complex spore wall, which appears to consist of a proteinaceous matrix, lipoid granular material, chitin, and spore membrane (Buczacki, 1983). A study of intracellular contents revealed various cell structures including nuclei, mitochonchia, microbodies, and unidentified vesicles and granules (Tanaka et al., 2002). Germination of the resting spores is believed to be stimulated by the production of a germination stimulating factor (GSF) by the roots of the *Brassica* hosts (Suzuki et al., 1992). However, such a factor has not been isolated or characterized thus far. Nonhost plants, such as leek (*Allium porrum*) and winter rye (*Secale cereale*), have also been reported to stimulate the germination of *P. brassicae* resting spores (Friberg et al., 2006).

Research into the pathogenesis of *P. brassicae* has focused largely on the production of hormones in the host. Hypertrophy and hyperplasia, or the abnormal enlargement and division of host cells in the infected roots, is presumably associated with increased levels of auxin (Butcher et al., 1974; Ludwig-Müller et al., 1993) and cytokinin

(Siemens et al., 2006; Dekhuijzen, 1981). Increased levels of auxin have been detected in clubbed roots, although such increases may be transient (Butcher et al., 1974; Devos et al., 2005; Luwig-Müller et al., 1993; Ugajin et al., 2003). In contrast, in at least one study, a lower level of auxin was reported in infected *B. napus* roots versus non-inoculated control plants (Mousdale, 1981).

The detection, in the *P. brassicae* genome, of putative *ipt* genes encoding isopentenyltransferase, a key enzyme in cytokinin biosynthesis in microorganisms (Binns, 1994), has been a subject of great interest to a number of researchers (Ito et al. 1997). Dekhuijzen (1981) observed higher levels of cytokinins in clubbed versus healthy roots. These cytokinins are believed to be synthesized by plasmodia of the pathogen and released into the host cells, thereby triggering abnormal host cell division (Dekhuijzen, 1981; Müller and Hilgenberg, 1986). This theory was supported by Müller and Hilgenberg (1986), who deduced that *P. brassicae* produces *trans*-zeatin (a form of cytokinin), since isomers of zeatin and zeatin riboside were detected in clubbed root tissue. On the other hand, Ando et al. (2005) isolated cDNA fragments of five putative cytokinin synthase genes (*BrIPTs:* isopentenyltransferases) from *Brassica rapa*, and examined their expression using Northern blot analysis. Their study suggested that *P. brassicae* infection transiently stimulates the transcription of *BrIPTs* before club formation, and thereafter the expression is repressed in clubs. Therefore, they concluded that cytokinin biosynthesis by the plant is related to the primary development of clubroot disease, but is unlikely to contribute to club enlargement (Ando et al., 2005). The reduced expression of host isopentenyltransferase during clubroot development was

also confirmed by Siemens et al. (2006), who found that cytokinin synthases and cytokinin oxidases/dehydrogenases were strongly down-regulated, whereas the genes coding for enzymes involved in auxin homeostasis, such as nitrilases, were up-regulated.

Levels of the auxin indole-3-acetic acid (IAA) are thought to be increased by the synthesis and turnover of putative host auxin precursors, such as indole-3-acetaldoxime, indole-3-methylglucosinolate and indole-3-acetonitrile (IAN), in infected roots (Searle et al., 1982; Butcher et al., 1984). Although several pathways for the biosynthesis of IAA exist in plants (Normanly et al., 1995), myrosinase and nitrilase, which are involved in the IAA pathway in the Brassicaceae, have been the main subjects of study by researchers. Myrosinase converts indole glucosinolate (IG) to IAN, and nitrilase converts IAN to IAA (Butcher et al., 1974). The transcript level of the myrosinase gene was found to increase in clubbed roots of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) (Grsic et al., 1999). An increase in IAN was associated with an increase in nitrilase activity in *P. brassicae*-infected *B. napus* roots (Rausch et al., 1981). Using *Arabidopsis thaliana*, Grsic-Rausch et al. (2000) demonstrated that only during the exponential growth phase of the clubs was nitrilase prominently enhanced in infected roots compared with controls. In addition, nitrilase was mainly found in infected cells harboring sporulating plasmodia, and it was of plant rather than *P. brassicae* origin (Grsic-Rausch et al., 2000).

The conversion of tryptophan (Trp) to indole-3-acetaldoxime and IAN is believed to be the first step in indole glucosinolate biosynthesis in the Brassicaceae (Ludwig-Müller

and Hilgenberg, 1988). However, a pathway for the biosynthesis of IAA that does not involve Trp has been discovered in *A. thaliana* (Normanly et al., 1993). Indole-3-acetaldehyde (IAAld), derived from indole-3-acetaldoxime and indole-3-pyruvic acid, was also proposed as a precursor in IAA biosynthesis in plants (Bartel, 1997; Helmlinger et al., 1987). Aldehyde oxidase (OA) is considered to be the enzyme responsible for catalyzing IAAld to IAA (Koshiba et al., 1996). Two aldehyde oxidase genes (*BrAO1* and *BrAO2*) have been isolated from Chinese cabbage and their expression evaluated during clubroot development (Ando et al., 2006). The expression of *BrAO1* increased with clubroot development compared with uninoculated roots, whereas *BrAO2* expression was repressed (Ando et al., 2006).

### 1.1.3. Differential pathogenicity of *P. brassicae*

Differential pathogenicity or physiological specialization in *P. brassicae* was first demonstrated by Honig (1931). The physiologic specialization of field populations of the pathogen was confirmed in numerous later studies (Ayers, 1957; Seaman et al., 1963; Williams, 1966), which were followed by studies on the variation in single spore isolates derived from the same populations (Buczacki, 1977; Jones et al., 1982; Scott, 1985; Somé et al., 1996). Since no pathotypes of *P. brassicae* have been found to be virulent on a single species within a host genus, the taxonomic concept of *formae speciales* has not been applied to this pathogen (Crute et al., 1980). Buczacki et al. (1975) suggested the term "physiologic race" be applied to homogeneous populations of the pathogen, whereas others (Crute et al., 1980; Voorrips, 1995) proposed that

"pathotype" would be more appropriate, 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 pathogen (Parlevliet, 1985).

The variation in pathogenicity among populations of *P. brassicae* is presumably associated with the response of the pathogen to selection pressure (Voorrips, 1995). It was shown that five successive passages of a population through a partially resistant host line resulted in the population developing an increased degree of virulence on this genotype (Crute and Pink, 1989). The natural selection of pathotypes was also suspected to be responsible for the erosion of resistance in the cabbage (*Brassica oleracea* L. var. *capitata*) cv. Badger Shipper a few years after its release (Seaman et al., 1963).

A gene-for-gene model involving four gene pairs has been proposed to interpret interactions between *P. brassicae* pathotypes and cultivars of *B. napus* (Crute, 1986). From a study using some of the European Clubroot Differential (ECD) hosts, Gustafsson and Fält (1986) proposed virulence factors matching three clubroot resistance genes. On the cv. Nevin (ECD06), one resistance gene has been proposed (Gustafsson and Fält, 1986). In 'Badger Shipper', which is a selection from inbred progenies of a chance cross between kale (*B. oleracea* L. *acephala* DC) and cabbage, resistance is controlled by two or more recessive genes (Crute et al., 1980). In kale accessions, both dominant and recessive genes conferring resistance have been reported (Voorrips, 1995). A major gene with some minor genes is believed to be responsible for the control of resistance in some accessions of *B. rapa* (Yoshikawa, 1981). Similarly, Suwabe et al. (2003) reported that clubroot resistance in *B. rapa* is under oligogenic

control and at least two loci are involved. The inheritance of resistance in the broccoli line MSU 134 appears to be under the control of two genes: one is recessive, the other incompletely dominant. The resistance of the cabbages 'Bindsachsener' and 'Böhmerwaldkohl' is under recessive and polygenic control, with two genes likely in common (Crute et al., 1980).

Most recently, Hirai (2006) identified four independent loci conferring clubroot-resistance in Chinese cabbage. These genes are derived from the European turnips (*B. rapa* L. *rapifera*) 'Siloga' and 'Gelria R'. Using 202 double haploid lines of *B. napus* derived from the crosses Darmor-bzh × Yudal and Stellar × Darmor-bzh, three major specific genes have been identified as responsible for controlling resistance to three single spore isolates of the pathogen (Manzanares-Dauleux et al., 2006). Quantitative trait loci (QTLs) that control resistance to clubroot have been reported by a number of research groups in *B. oleracea* (Rocherieux et al., 2004; Nomura et al., 2005) and *B. napus* (Manzanares-Dauleux et al., 2000a).

### 1.1.4. Disease management

A number of strategies are recommended for the management of clubroot, including crop rotation, the application of chemicals to plants and soil, liming of the soil, improving drainage, and the use of genetically resistant cultivars. Crop rotation is probably the simplest and most effective approach for the control of clubroot, but may not be practical due to the longevity of the pathogen in infested fields (Karling, 1968). Liming of the soil increases the pH and calcium content (Myers and Campbell, 1985),

which serves to inhibit disease development. However, the effect of liming can be undesirable for other crops included in rotations, and in most cases, the strategy is not economically feasible (Hildebrand and McRae, 1998). The application of calcium cyanamide was found to reduce disease severity nearly three-fold (Klasse, 1996), but may be prohibitively expensive (Hildebrand and McRae, 1998). In addition, calcium cyanamide is considered to be a hazardous chemical (U.S. DHHS, 1993). In one study that evaluated the efficacy of various fungicides, only mercurous chloride showed consistent clubroot control (Naiki and Dixon, 1987). Fluazinam has been tested and registered in Australia as a soil drench to control clubroot of cole vegetables, but inconsistent results and fungicide phytotoxicity were also reported (Donald et al., 2001). The application of AquaGro 2000-L, a nonionic surfactant, increased the percentage of marketable broccoli heads from 4.7% to 86.7% in field experiments (Hildebrand and McRae, 1998). However, AquaGro 2000-L is only recommended for use in the production of ornamental plants in greenhouses and nurseries (http://www.plantprod.com). Genetic resistance to *P. brassicae* is considered a desirable approach to disease management (Kuginuki et al., 1999), but is not always available in desirable cultivars and may quickly break down if used in the absence of other management strategies (Seaman et al., 1963).

## 1.2. *Brassica* hosts of *Plasmodiophora brassicae*

### 1.2.1. Taxonomy of *Brassica* species

The *Brassica* genus includes a diverse set of species; Gómez-Campo (1999) estimated as many as 38 species and 41 subspecies within the genus, the majority of

established the relationships between these species, which is illustrated in what is now known as the triangle of U (U, 1935; Fig. 1-2). *Brassica oleracea* is the most diverse of the six cultivated species, consisting of at least 14 varieties. The subspecies designation is not recommended for these varieties, as different types of this species originated under domestication in a relatively short period of time (Gómez-Campo, 1999). The same consideration should also be applied to *B. napus*, although the subspecies classification has been traditionally used. For *B. rapa* (syn. *B. campestris*), only the most significant variants can be considered as subspecies, whereas some subspecies have been recognized in *B. juncea*.

### 1.2.2. Mechanisms of host resistance

The two stages in the life cycle of *P. brassicae* (the infection of root hairs by the primary zoospores and subsequent infection of the root cortex) have often been considered important for understanding the mechanisms of resistance in the host (Voorrips, 1995). However, since no correlation between resistance to root hair infection and resistance to clubroot development has been found, Voorrips (1995) proposed that only complete resistance to root hair infection would confer resistance to clubroot. This is because, as Voorrips (1995) explained, even a very low level of root hair infection would allow infection of the root cortex.

Indole glucosinolates, proposed precursors of IAA in the host (Butcher et al., 1974), have been studied for their possible role in the development of clubroot. Butcher et al. (1974) demonstrated a clear relationship between resistance and low glucosinolate

Indole glucosinolates, proposed precursors of IAA in the host (Butcher et al., 1974), have been studied for their possible role in the development of clubroot. Butcher et al. (1974) demonstrated a clear relationship between resistance and low glucosinolate content in cruciferous species. However, conflicting results were obtained by other researchers, who found no differences in indole glucosinolate content amongst resistant and susceptible rutabaga, turnip and Chinese cabbage hosts (Ludwig-Müller et al., 1993; Mullin et al., 1980). Voorrips (1995) attributed these discrepancies to the amount of auxin needed for clubroot development. Since only very low levels of auxin are required for gall formation (Rausch et al., 1983), it may be difficult to establish a general relationship between indole glucosinolate content and clubroot resistance (Voorrips, 1995). Nevertheless, as several pathways for the biosynthesis of IAA exist in plants (Normanly et al., 1995), the indole glucosinolate content may be related to the susceptibility of the host in specific cases.

Oxidases/dehydrogenases (CKO/CKX) irreversibly degrade cytokinins, including isopentenyladenine, zeatin, and their ribosides (Armstrong, 1994; Schmülling et al., 2003). A recent study revealed that lines over-expressing a cytokinin CKO/CKX were resistant to clubroot, suggesting that cytokinin is a key factor in clubroot disease development (Siemens et al., 2006). However, whether CKO/CKX themselves are important factors in resistance to the disease is not clear, since cytokinin reception and signaling are complex processes. For instance, in *A. thaliana*, a His kinase (HK) receptor and downstream His phosphotransfer proteins (HPs) and response regulators are reportedly involved in cytokinin reception and signaling pathways (Sheen, 2002; Heyl

A hypersensitive reaction has also been observed in response to host challenge by *P. brassicae*. Ecotypes Tsu-0 and Ze-0 of *A. thaliana* exhibited differential resistance to the pathogen, expressed as a hypersensitive reaction, which was controlled by the dominant allele of a single gene (*RPB*) (Fuchs and Sacristan, 1996). Similarly, along with genes involved in cell lignification, genes directing the hypersensitive response were also detected in clubroot-resistant *B. oleracea* and *B. napus* genotypes (Manzanares-Dauleux et al., 2006).

### 1.2.3. Clubroot resistance in *Brassica* species

Resistance sources summarized in this review are listed in Table 1-2. Crute et al. (1980) reported a number of resistant genotypes in *B. oleracea*, including the cabbage cvs. Badger Shipper, Bindsachsener, Böhmerwaldkohl, and broccoli lines MSU 134 and Oregon CR1. The resistance of broccoli breeding lines OSU CR-2, CR-3, CR-4 and CR-5 is derived from a cross involving the broccoli cv. Northwest Waltham (Baggett and Kean, 1985). However, based on a world-wide survey of host responses to *P. brassicae*, the fodder turnip (*B. rapa*. ssp. *rapifera*) lines of the European Clubroot Differential (ECD) set (Buczacki et al., 1975) were found to possess the strongest levels of resistance to the pathogen (Toxopeus et al., 1986). The fodder turnips 'Siloga' and 'Gelria R' are also highly resistant to four populations of *P. brassicae* from Japan (Kuginuki, 1999). Therefore, to develop resistant cultivars of Chinese cabbage for Japan, clubroot resistance genes from 'Siloga' and 'Gelria R' were introgressed into the

'Gelria R' are also highly resistant to four populations of *P. brassicae* from Japan (Kuginuki, 1999). Therefore, to develop resistant cultivars of Chinese cabbage for Japan, clubroot resistance genes from 'Siloga' and 'Gelria R' were introgressed into the Chinese cabbage genotypes. A number of resistant F1 hybrid varieties were obtained and later cultivated in that country (Yoshikawa, 1981; Kuginuki, 1997).

In *B. napus*, Ayers (1957) found that the Swede NH 654-8 was completely resistant to six pathotypes of *P. brassicae* from North America, and the Swedes NH 775-1 and NH 743-3 were highly resistant. The rutabaga 'Wilhelmsburger' showed good resistance to Williams' (1966) pathotypes 2, 3, 5, 6, 7 and 8, but was susceptible to pathotypes 1, 4 and 9 (Williams, 1966). The fodder turnip line AABBCC (*B. rapa*. ssp. *rapifera*), the kale 'Verheul' (*B. oleracea* var. *acephala*), and the cabbage 'Böhmerwaldkohl' were selected to initiate a *B. napus* resistance-breeding program in Germany (Diederichsen and Sacristán, 1996). The resynthesized *B. napus* lines obtained were resistant to populations of the pathogen that were virulent on the parental lines and all of the *B. napus* differential hosts of the ECD set. A genetic study indicated that three dominant pathotype ("race")-specific resistance genes were present in the early breeding lines, whereas *B. napus* cv. Mendel, which was derived from those breeding lines, possessed only one of the three genes (Diederichsen et al., 2006).

## 1.3. Characterization of *Plasmodiophora brassicae*

1.3.1. Necessity for and method of single spore isolation

Since field populations of *P. brassicae* may consist of a mixture of pathotypes (Tinggal and Webster, 1981; Jones et al., 1982; Somé et al., 1996), the isolation of single spores of the pathogen is necessary to obtain homogeneous populations (Kageyama et al., 1995). Homogenous populations are desirable because inoculation of hosts using a mixture of pathogen genotypes can result in indistinct reactions, making it difficult to distinguish between a resistant and a susceptible response. Such complications can make assessment of virulence patterns in *P. brassicae* difficult (Williams, 1966; Toxopeus et al., 1986). It was also suggested that one pathotype might alter the response of a differential host to a second pathotype present in the same inoculum (Jones et al., 1982). In addition, in screening for resistance to *P. brassicae*, pathotypes present at low frequencies may not be detected when heterogenous populations of the pathogen are used. These rare pathotypes may be pathogenic to host genotypes scored as resistant based on their reaction to the predominant pathotypes (Jones et al., 1982). Thus, the use of single spore-derived isolates with defined pathogenicity is recommended for screening for resistance (Jones et al., 1982; Somé et al., 1996).

Unfortunately, given the very small size of the resting spores and the fact that *P. brassicae* cannot be grown in culture, the isolation of single spores of the pathogen can be a challenging task. A number of different approaches have been used, with varying success, to improve the efficiency of spore isolation. The agar (or agarose)-based

method, in which spores are placed on a layer of agar and the agar is sectioned into pieces containing a single spore, has been commonly used for isolating single spores of the pathogen (Tinggal and Webster, 1981; Jones et al., 1982; Scott, 1985; Somé et al., 1996). Alternatively, Buczacki (1977) located single spores in a diluted spore suspension ($1 \times 10^5$ spores $mL^{-1}$) using a microscope, but experienced difficulties in confirming the number of spores in droplets of the suspension (Jones et al., 1982). Using an approach similar to the method proposed by Buczacki (1977), Kageyama (1995) placed 0.5 µl droplets containing a single resting spore on the glass coverslip of a microscope slide. A seedling was then placed on the coverslip, and the seedling and coverslip were transferred together into a polycarbonate bottle filled with autoclaved soil.

The germination rates of resting spores are quite low when examined *in vitro* (Suzuki et al., 1992), and there is common agreement that stimulants in host plants induce germination (Macfarlane, 1970). To improve the efficiency of single spore isolation (including successful infection of the host by a single spore), root leachates were employed to increase germination rates (Jones et al., 1982). The use of decomposing galls as the starting material in the isolation process may also yield increased germination rates, as a result of resting spore maturation and changes in the permeability of the spore walls (Macfarlane, 1970). High germination rates were observed in resting spores obtained from rotted galls, while long term freezing significantly reduced germination (Suzuki et al., 1992).

1.3.2. Characterization of *P. brassicae* with differential hosts

Pathogenic variation in *P. brassicae* populations from North America was first demonstrated by Ayers (1957) using 23 cruciferous varieties, among which six hosts were effective for differentiating between six pathotypes (Table 1-2). A new pathotype was recognized by Seaman et al. (1963), who added the clubroot-resistant cabbage cv. Badger Shipper to the differential set of Ayers (Table 1-2). The pathotype classification system in North America was further modified by Williams (1966), who retained three differentials ('Wilhelmsburger', 'Laurentian' and 'Badger Shipper') used by Seaman et al. (1963) and added the cabbage cv. Jersey Queen to his differential set. However, Williams (1966) excluded the wild crucifer species used by Ayers (1957) and Seaman et al. (1963) because of the difficulties in maintaining genetic uniformity in those wild species. Since it uses four differentials, a maximum of 16 possible pathotypes ($4^2$) can be detected with Williams' system, although he found only 9 in a survey of 125 populations from throughout the world (Williams, 1966). Five of the pathotypes identified by Williams (1966) (pathotypes 1, 2, 3, 6 and 7) corresponded to pathotypes described by Seaman et al. (1963), while the other four (pathotypes 4, 5, 8 and 9) represented new designations.

In Europe, the European Clubroot Differential (ECD) series was proposed for the study of physiologic specialization in *P. brassicae* (Buczacki et al., 1975). The ECD set consists of 15 genotypes, including five from each of three *Brassica* species: *B. rapa*, *B. napus* and *B. oleracea* (Table 1-3). The 15 genotypes are ranked in a fixed order and the

five genotypes in each subset are assigned a denary number (1, 2, 4, 8 or 16) that corresponds to a binary number ($2^0$, $2^1$, $2^2$, $2^3$ or $2^4$) (Table 1-4). A pathotype can be defined by three population numbers, which are the sums of the denary numbers from the corresponding susceptible genotypes within the same species or subset. Since any combination of the five genotypes within a species gives a unique population number, the pathogenicity of the pathotype on these differentials can be deduced from the number designation. For example, if a pathotype is designated as ECD18/31/5, the pathotype is pathogenic on ECD 02 and 05 (denary numbers: 2 and 16) of the *B. rapa* differentials, on ECD 06, 07, 08, 09 and 10 (denary numbers summing up to 31) of the *B. napus* differentials, and on ECD 11 and 13 of the *B. oleracea* differentials (Buczacki et al., 1975). The ECD set is limitated in its ability to differentiate pathotypes because ECD 05 and 07 are almost universally susceptible (Toxopeus et al., 1986). Furthermore, poor differentiating capacity was demonstrated on ECD 13 and ECD 14, and indistinct reactions are often associated with ECD 12 and 15. The *B. rapa* hosts ECD 01 to 04 might be closely related and could be represented by 03 alone. The greatest differentiating capacity has been found on the *B. napus* hosts (ECD 06, 08, 09 and 10) (Toxopeus et al., 1986).

In addition to those described above, numerous other classification systems have been proposed for isolates and populations of *P. brassicae*. Unlike *B. oleracea* and *B. rapa*, *B. napus* is a predominantly inbreeding species. Therefore, it is easier to maintain genetically uniform stocks of these differentials (Johnston, 1968; Somé et al., 1996). Partly for this reason, Somé et al. (1996) proposed a system involving three differential

Similarly, there has been substantial variation in the methods used to classify the reaction of hosts inoculated with *P. brassicae.* Although neither Williams (1966) nor Buczacki et al. (1975) referred to intermediate disease severities (indices of disease), these are often observed (Toxopeus et al., 1986; Kuginuki et al., 1999; Strelkov et al., 2006b). To accommodate intermediate host reactions, Toxopeus et al. (1986) regarded an index of disease (ID) of less than 20% as a resistant reaction, an ID of greater than 80% as a susceptible reaction, and reactions resulting in IDs between 20 and 80% as indistinct (Toxopeus et al., 1986). Somé et al. (1996) regarded an ID of 25% as the cut-off between a resistant and susceptible reaction, while Ayers (1957) used least significant differences to group host reactions. In contrast, Kuginuki et al. (1999) used a non-parametric significance test (the Wilcoxon method) to determine whether the reaction of the host genotypes was significantly different in response to inoculation; a host with an ID not significantly different from the host with the minimum ID in a test was classified as resistant, while a host with an ID not significantly different from the host with the maximum ID in a test was classified as susceptible. Genotypes with IDs significantly different from hosts with minimum and maximum IDs were treated as partially resistant. More recently, in order to be able to assign pathotype designations to all pathogen populations tested from Alberta, Strelkov et al. (2006b) regarded an ID of less than 49% as a resistant reaction, and an ID of greater than 50% as a susceptible reaction.

1.3.3. Analysis of *P. brassicae* isolates with molecular markers

host with the maximum ID in a test was classified as susceptible. Genotypes with IDs significantly different from hosts with minimum and maximum IDs were treated as partially resistant. More recently, in order to be able to assign pathotype designations to all pathogen populations tested from Alberta, Strelkov et al. (2006b) regarded an ID of less than 49% as a resistant reaction, and an ID of greater than 50% as a susceptible reaction.

1.3.3. Analysis of *P. brassicae* isolates with molecular markers

A number of molecular techniques have been used to analyze diversity in isolates of *P. brassicae* and to differentiate between pathotypes of the pathogen. Molecular approaches are desirable since studies on the variation of pathogenicity using differential hosts can be time-consuming, labor-intensive, and variable as result of environmental conditions and phenotype/genotype plasticity (Manzanares-Dauleux et al., 2000b). Moreover, isolation and characterization of genetically uniform single spore isolates may not be feasible as a routine procedure for large collections of the pathogen because of the time required and the variable success rate (Voorrips, 1996; Manzanares-Dauleux et al., 2000b). Thus, the use of molecular markers specific to isolates or pathotypes may serve as a valuable tool for the identification and monitoring of *P. brassicae* populations (Manzanares-Dauleux et al., 2000b).

Polymerase chain reaction (PCR)-based methods have the advantage of allowing the use of very small quantities of DNA and can be suitable for distinguishing distantly related isolates (Buhariwalla et al., 1995; Faggian et al., 1999; Yano et al., 1997). Pathotypes of *P. brassicae* were characterized by patterns of PCR-amplified DNA

fragments using arbitrary primers (Möller and Harling, 1996; Yano et al., 1997). However, molecular and pathogenicity-based classifications were not unequivocally correlated, although some markers were specific to a pathotype or to particular isolates (Buhariwalla et al., 1995; Manzanares-Dauleux et al., 2001; Möller and Harling, 1996). The lack of correlation between pathogenicity and RAPD patterns presumably resulted from the high degree of polymorphism among isolates within the same pathotype (Manzanares-Dauleux et al., 2001) and from the fact that pathotype evolution is independent of the evolution of neutral DNA markers (Manzanares-Dauleux et al., 2001). Nevertheless, amplification of *P. brassicae* with arbitrary primers can be useful for analyzing genetic diversity (Manzanares-Dauleux et al., 2001, Möller and Harling, 1996), and one RAPD marker has been converted to a pathotype-specific sequence characterized amplified region (SCAR) (Manzanares-Dauleux et al., 2000b).

Buhariwalla et al. (1995) established comparable partial genomic libraries that were subjected to Southern analysis in order to select clones containing repetitive elements, which could be used to establish sequence-specific primers. The resultant sequence-generated primers produced a small number of DNA fragments that were easy to distinguish and were reproducible (Buhariwalla et al., 1995). Using the *P. brassicae*-specific primers developed by Buhariwalla et al. (1995), Klewer et al. (2001) amplified putatively isolate-specific bands using PCR. One primer pair was designed based on the sequence of one of those bands, yielding one isolate-specific fragment 1700 bp in size.

However, the use of PCR markers may be a less appropriate tool for analysis of closely related isolates, since few reproducible differential PCR-markers have been

found (Klewer et al., 2001). Restriction fragment length polymorphism (RFLP) markers have also been developed to characterize single spore isolates of *P. brassicae*. When using pathogen-specific clones as hybridization probes, RFLP markers can be used with a mixture of host and pathogen DNA as the template (Klewer et al., 2001). Fingerprint-like patterns were also obtained using Southern analysis, which were unique among four different isolates (Klewer et al., 2001).

### 1.3.4. Chromosome polymorphisms in *P. brassicae*

The comparison of chromosome polymorphisms or karyotypes (chromosome size and number) has been used as another approach to characterize isolates of *P. brassicae* (Graf et al., 2004; Ito et al., 1994). This technique utilizes contour-clamped homogeneous electric field gel electrophoresis (CHEF) to separate individual chromosomes of the pathogen. In combination with Southern analysis, the hybridization pattern between *P. brassicae*-specific DNA fragments and chromosome bands may be used as a tool for genetic mapping, as well as for the characterization of different isolates (Graf et al., 2004). The hybridization probes used so far include ribosomal RNA gene fragments and pathogen-specific DNA fragments.

Using spheroplasts as the starting material, Ito et al. (1994) were able to distinguish 13 chromosomal bands, ranging from 1.9 Mb to 750 kb in size, in *P. brassicae*. In another study, 16 chromosomal bands from 2.2 Mb to 680 kb were identified (Graf et al., 2001). As these numbers of chromosomal bands are much less than the 20

synaptonemal complexes estimated by electron microscopy (Braselton, 1982), additional chromosomal bands may not have been revealed (Graf et al., 2004).

## 1.4. Research objectives

Previously, the virulence patterns of nine field populations of *P. brassicae* from a number of locations in the Edmonton, Alberta region were evaluated on the differentials of Williams and the ECD set (Strelkov et al., 2006b). The populations were classified as pathotypes 3 and 5 or ECD 16/12/15 and ECD 16/12/0, respectively, using the systems of Williams (1966) and the ECD set (Buczacki et al., 1975). Two other collections, from Abbottsford, BC, and from Orton, ON, were also characterized and classified as pathotype 6 (Strelkov et al., 2006b). The objectives of the present study included development of an efficient technique for the isolation of single resting spores of *P. brassicae* from these populations, and characterization of the single spore-derived isolates on the differentials of Williams (1966) and Somé et al. (1996). Multiple isolates were characterized from each population in order to evaluate variation in virulence within each population, since different pathotypes may be present in the same gall (Jones et al., 1982; Scott, 1985; Tinggal, 1980). An additional objective of this study was the molecular characterization of the single spore-derived isolates, using several techniques modified from the literature. The significance of the results is discussed with respect to the clubroot outbreak in Alberta, as well as within the larger context of the *P. brassicae*/crucifer pathosystem.

## 1.5. References


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Table 1-1. Taxonomy of cultivated *Brassica* species and their allied subspecies or varieties.

| Species | Classification within species | |
|---|---|---|
| | *Subspecies or variety | Common name |
| *B. rapa* (syn. *B. campestris*) (AA, n=10) | [GW]*chinensis* (L.) Hanelt | Pak choi |
| | [PG]*dichotoma* (Roxb.) Hanelt | Toria |
| | [GW]*narinosa* (Bailey) Hanelt | Pak choi |
| | [GW]*nipposinica* (Bailey) Hanelt | Pak choi |
| | [PW]*oleifera* | Turnip rape |
| | [W]*parachinesis* | Choy |
| | [PGW]*pekinensis* (Lour.) Hanelt | Chinese cabbage |
| | [W]*perviridis* | Mustard spinach |
| | [PW]*rapifera* | Turnip |
| | [PGW]*trilocularis* (Roxb.) Hanelt | Sarson |
| | [W]*utilis* | Broccoli raab |
| *B. nigra* (L.) Koch (BB, n = 8) | [P]*occidentalis* Sinsk. | Western black mustard |
| | [P]*orientalis* Sinsk. | Asia black mustard |
| *B. oleracea* L. (CC, n = 9) | [W]*aboglabra* | Chinese kale |
| | [PW]*acephala* DC. | Kales |
| | | Cauliflower, heading broccoli |
| | [PW]*capitata* | Cabbage |
| | [PW]*costata* | Portuguese cabbage |
| | [PW]*gemmifera* DC | Brussels sprouts |
| | [PW]*gongylodes* L. | Kohl-rabi |
| | [PW]*italica* | Broccoli calabrese |
| | [PW]*medullosa* | Narrow stem kale |
| | [W]*palmifolia* DC | Tree cabbage |
| | [PW]*sabauda* L. | Savoy cabbage |
| | [PW]*sabellica* | Collards |
| | [PW]*selensia* | Bore cole |
| | [P]*ramosa* | Thousand head kale |
| *B. carinata* A. Br. (BBCC, n = 17) | | Ethiopian mustard (Abyssinian mustard) |
| *B. juncea* L. (AABB, n = 18) | [W]*capitata* | Head mustard |
| | [W]*crispifolia* | Cut leaf mustard |
| | [W]*faciliflira* | Broccoli mustard |
| | [C]*foliosa* | |
| | [C]*intigrifolia* | |

| | | |
|---|---|---|
| | [C]*juncea* | |
| | [W]*lapitala* | Large petiole mustard |
| | [W]*multiceps* | Multishoot mustard |
| | *napiformis* | |
| | [W]*oleifera* | Indian mustard |
| | [W]*rapifera* | Root mustard |
| | [W]*spicea* | Mustard |
| | [W]*tsa-tsa* | Big stem mustard |
| *B. napus* *(AACC, n = 19)* | *napobrassica* | |
| | *pabularia* | |
| | [W]*oleifera* | Oil rape |
| | [W]*rapifera* | Swede rutabaga |

*Letters in front of the variety or subspecies names indicate the corresponding reference: P = Prakash and Hinata (1980), G = Gómez-Campo (1999), W= Williams and Hill (1986), and C = Chopra and Prakash (1996).

Table 1-2. Potential resistance sources to *Plasmodiophora brassicae* in crucifer genotypes.

| Resistance source | Nature of resistance | Reference |
|---|---|---|
| ***Brassica rapa*** | | |
| ECD 01 (Fodder turnip) | Rarely infected | Toxopeus et al., 1986 |
| ECD 02 (Fodder turnip) | Rarely infected | Toxopeus et al., 1986 |
| ECD 03 (Fodder turnip) | Rarely infected | Toxopeus et al., 1986 |
| ECD 04 (Fodder turnip) | Rarely infected | Toxopeus et al., 1986 |
| Fodder turnip Siloga | Resistant to 4 populations from Japan | Kuginuki, 1999 |
| Fodder turnip Gelria R | As in Siloga | Kuginuki, 1999 |
| ***Brassica napus*** | | |
| Mendel | One dominant, two recessive gene: | Diederichsen et al., 2006 |
| Wilhelmsburger[a] | Susceptible to '16/31/31', Williams' 1, 4 & 9 | Ayers, 1957; Crute et al., 1980 |
| ECD 08 | Susceptible to '16/31/31' | Toxopeus et al., 1986 |
| ECD 09 | As in ECD 08 | Toxopeus et al., 1986 |
| ECD 10 | As in ECD 08 | Toxopeus et al., 1986 |
| Swede NH 654-8 | Complete to 6 pathotypes | Ayers, 1957 |
| Swede NH 775-1 | Partial, to 6 pathotypes | Ayers, 1957 |
| Swede NH 743-3 | Partial, to 6 pathotypes | Ayers, 1957 |
| ***Brassica oleracea*** | | |
| Bindsachsener[b] | Partial resistance | Crute et al., 1980 |
| Böhmerwaldkohl[b] | Partial resistance | Crute et al., 1980 |
| MSU 134 broccoli | Partial resistance | Crute et al., 1980 |
| Broccoli OSU CR-s to 8 | Partial resistance | Toxopeus et al., 1986 |
| Broccoli Oregon CR1 | Partial resistance | Crute et al., 1980 |
| Broccoli breeding lines OSU CR-2 - OSU CR-8 | Using 'Northwest Waltham' as R source | Baggett and Kean, 1985 |
| Cabbage Oregon 100, 123, 140 and 142 | Danish Ballhead × Accession 2311 | Baggett, 1983 |

| | | |
|---|---|---|
| Cabbage Badger Shipper | Partial resistance | Ayers, 1957; Crute et al., 1980 |

[a]Resistant cultivars derived from 'Wilhelmsburger' include 'Richelain' and cabbage line 8-41 (Voorrips, 1995).

[b]Resistant lines and cultivars developed from these two genotypes include 'Respla' and 'Resista' (Voorrips, 1995).

Table 1-3. Differential hosts used by Ayers (1957) and Seaman et al. (1963) to classify populations of *Plasmodiophora brassicae* into pathotypes ('races').

| | Pathotype[a] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ayers (1957) | | | | | | Seaman et al. (1963) | | | | | | |
| Differential host | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| *B. oleracea* L. var. *capitata* L. | | | | | | | | | | | | | |
| Badger Shipper | | | | | | | − | + | − | + | − | − | + |
| Cabbage Danish Ballhead | + | + | + | + | + | + | | | | | | | |
| *B. napus* (Rutabaga) | | | | | | | | | | | | | |
| Wilhelmsburger | +[b] | − | − | − | − | − | + | − | − | − | − | − | − |
| Laurentian | + | + | + | + | − | − | + | + | + | + | − | − | − |
| Wild crucifers | | | | | | | | | | | | | |
| *Sisymbrium altissimum* | + | − | + | − | − | − | + | − | + | − | − | − | − |
| *B. campestris* | + | − | + | + | | | + | − | + | + | + | − | |
| *B. nigra* | − | − | − | − | + | − | − | − | − | − | + | − | − |

[a]The term 'race' was originally used by all of the authors.

[b]The difference between a susceptible reaction (+) and a resistant reaction (-) was determined by using the LSD ($p$ = 0.01) by Ayers (1957), while Seaman et al. (1963) did not specify the criteria.

Table 1-4. Hosts of the European Clubroot Differential (ECD) set and their assigned denary and binary values (Buczacki et al., 1975).

| ECD no. | Differential hosts | Denary number | Binary number |
|---|---|---|---|
| | *Brassica rapa* L. (2n = 20) | | |
| 01 | Line aaBBCC | 1 | $2^0$ |
| 02 | Line AAbbCC | 2 | $2^1$ |
| 03 | Line AABBccc | 4 | $2^2$ |
| 04 | Line AABBCC | 8 | $2^3$ |
| 05 | cv. Granaat | 16 | $2^4$ |
| 06 | *Brassica napus* L. (2n = 38) | | |
| | Dc101 (Nevin) | 1 | $2^0$ |
| 07 | Line Dc119 (Giant Rape) | 2 | $2^1$ |
| 08 | Line Dc128 (Selection ex Giant Rape) | 4 | $2^2$ |
| 09 | Line Dc129 (NZ Clubroot Resistant Rape) | 8 | $2^3$ |
| 10 | Line Dc130 (Wilhelmsburger) | 16 | $2^4$ |
| 11 | *Brassica oleracea* L. (2n = 18) | | |
| | cv. Badger Shipper | 1 | $2^0$ |
| 12 | cv. Bindsachsener | 2 | $2^1$ |
| 13 | cv. Jersey Queen | 4 | $2^2$ |
| 14 | cv. Septa | 8 | $2^3$ |
| 15 | cv. Verheul | 16 | $2^4$ |

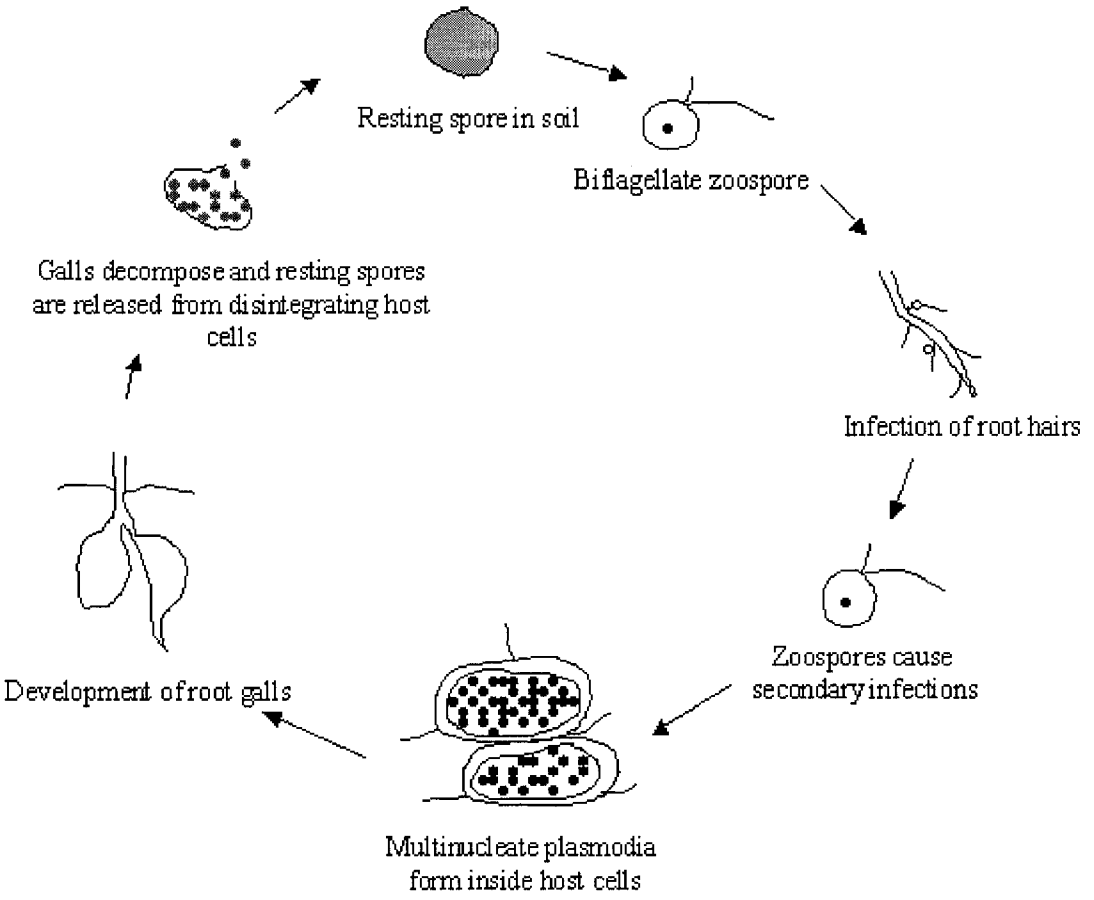


Fig. 1-1. Diagrammatic representation of the life cycle of *Plasmodiophora brassicae*, causal agent of clubroot of crucifers (S.E. Strelkov).

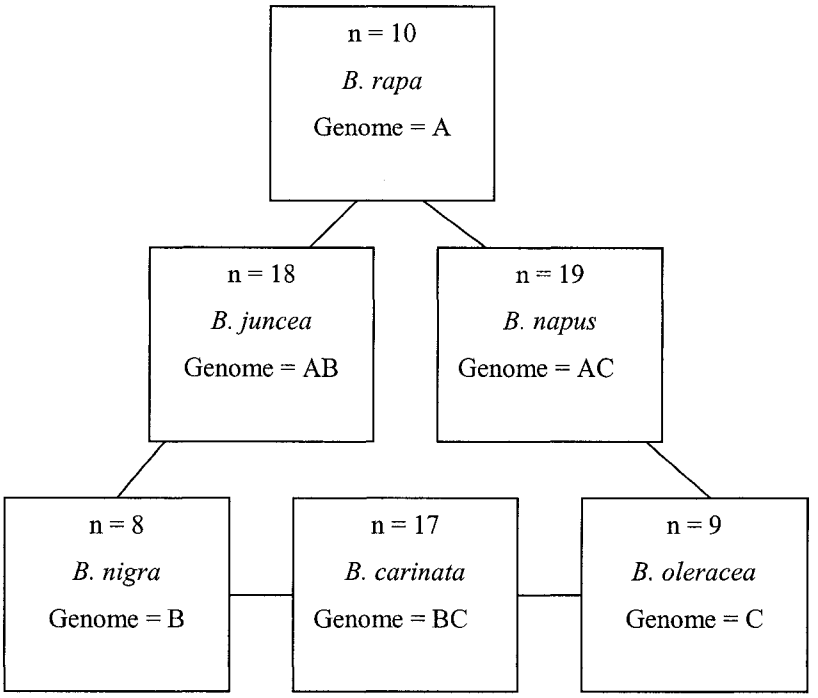


Fig. 1-2. The triangle of U (U, 1935). This chart illustrates the relationship between the different species within the *Brassica* genus. *Brassica rapa* was formerly known as *B. campestris*.

# Chapter 2. Isolation and variation in virulence of single spore isolates of *Plasmodiophora brassicae* from Canada

## 2.1. Introduction

The 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). Recently, however, clubroot was also identified on canola (*Brassica napus* L.) in Alberta (Strelkov et al., 2005; 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., 2006a; Strelkov et al., 2007a). 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 Quebec (Pageau et al., 2006), 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., 2007a).

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 alleviates disease development, but is not always economically feasible since large quantities of lime and repeated applications may be required (Hildebrand and McRae, 1998). The effect of fungicide treatment is not always consistent (Naiki and Dixon, 1987), and fungicides may be prohibitively expensive. Although the application of calcium cyanamide is considered an effective management option (Klasse, 1996), its efficacy has not been confirmed in a canola cropping system under prairie conditions. To date, no clubroot-resistant *B. napus* canola cultivars are available in Canada (Pageau et al., 2006; Strelkov et al., 2006b), although the development of such varieties is considered 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., 2006b; Williams, 1966). Among the various systems proposed for pathotype designations, the differentials of Williams (1966) have been commonly used to characterize pathogen populations from Canada (Hildebrand and Delbridge, 1995; Reyes et al., 1974; Strelkov et al., 2006b; 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, Voorrips, 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., 2006b). 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 observed in certain differential hosts following inoculation with the populations tested (Strelkov et al., 2006b). This phenomenon, which was also reported previously 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., 1982b, Tinggal and Webster, 1981, Somé et al., 1996). However, these agarose-based techniques are generally laborious (Kageyama et al., 1995). Therefore, the objectives of the present study were: (1) to develop a simple and efficient technique for isolating single spores of *P. brassicae*, and

(2) to assess variation in virulence among single spore isolates derived from pathogen populations from Canada.

## 2.2. 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 (Voorrips, 1995). To conform with the suggestions of Voorrips (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: (1) SACAN03-1, originally obtained from clubbed canola roots collected near St. Albert, AB, (2) CDCN04-1, collected from a diseased volunteer canola plant growing near the Crop Diversification Centre North (CDC-N), Alberta Agriculture and Food, Edmonton, AB, (3) Leduc-1, recovered from infested soil from a market garden near Leduc, AB, (4) 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 (5) 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. (2006b), were reproduced and maintained on the universally susceptible Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) cv. Granaat.

**Plant materials**

The virulence of 25 single spore isolates of *P. brassicae* (see below) 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 (RICP), Prague-Ruzyne, Czech Republic, and 'Brutor' from the Leibniz Institute of Plant Genetics and Crop Research (IPK) Genebank, Gatersleben, Germany. Chinese cabbage cv. Granaat (*B. rapa* ssp. *pekinensis*) was purchased from B & T World Seeds (Paguignan, France), and used as a susceptible control.

**Isolation of single spores**

Single spores were isolated from 8 week-old galls of 'Granaat' inoculated with the individual populations of *P. brassicae*. Approximately three grams of clubbed roots, either freshly harvested, frozen (-80°C ), or partially decayed (kept for about two months at 4°C in a sealed container), were homogenized in 50 ml of distilled water 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°C, 10 min) and washed five times with sterile distilled water, with the pellet re-suspended and centrifuged each time. The final pellet was stored at 4°C for 1 to 2 d before use.

Immediately prior to use, resting spores were re-suspended in either phosphate buffer (pH 8.0, 10 mM) or sterilized 5 % (v/v) glycerol in distilled water. The spore concentration was quantified using a haemocytometer (VWR, Mississauga, ON) and adjusted to approximately $2 \times 10^3$ spores/ml with buffer or 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 × 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 (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 sterilized distilled water (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 sterilized distilled water (pH 6.5 to 7.0) or tap water (pH 6.0). The pH of the distilled 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 plastic pots (7.5 cm in diameter) filled with Metro-Mix 290 soil (Scotts, Columbus, Ohio), at a density of one plant per pot, and maintained in a growth cabinet at 24°C (day) /18°C (night) 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 diluted to approximately $1 \times 10^7$ spores/ml with distilled water. One week-old seedlings, germinated on moistened sterile filter paper, were inoculated by dipping the roots in the spore suspension for a period of 10 sec (Strelkov et al., 2006b). The inoculated seedlings were then immediately planted in 4 cm × 4 cm plastic pots filled with Metro-Mix 290 soil, placed in flat containers without holes at the bottom, and transferred to a greenhouse maintained at 21°C (day) / 18°C (night) 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, 2 = moderate galling and 3 = severe galling. An index of disease (ID) was calculated using the formula 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 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 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 $p \leq 0.05$ 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 (SAS Institute Inc., 1999). 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.

## 2.3. Results

### Single spore isolation

The infection rates obtained with the different populations and inoculation methods (Table 2-1) 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 (1.9%). The hypothesis of equality of proportions was also rejected within the populations SACAN03-1 ($\chi^2$ = 10.5685, 3 $p$<0.0051), AbotJE04-1 ($\chi^2$ = 4.9708, $p$<0.0258), and ORCA04 ($\chi^2$ = 18.6723, $p$<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, 14 $p$>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 (_2 = 18.6723, $p<0.0001$) 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 2-1).

**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 2-2). 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 2-3). 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 2-3). In contrast, one of the isolates, CDCN-ss1, exhibited reduced virulence on 'Jersey Queen,' in addition to its avirulence on 'Wilhemsburger' and 'Badger Shipper' (Table 2-2); 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 2-3). 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 2-3).

The Leduc-1 population also appeared to be heterogeneous in its composition (Table 2-3). 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 2-2 and 2-3). 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 2-2). 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.

## 2.4. 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 (Somé 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., 1982b; 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 (data not shown). 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 to 2 day-old seedling of Chinese cabbage 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%) (data not shown), 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*. The procedure we developed has three advantages: (1) the tendency for resting spores to aggregate was reduced by using 5% (v/v) glycerol (in distilled water) for the preparation of the spore suspension, thereby facilitating the identification of single spores, (2) the

protocol allowed direct contact of the resting spore with the root hairs of the host, resulting in higher infection rates, and (3) fewer technical difficulties were encountered, since agarose pieces 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 2-1). 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 2-1). 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 2-1). 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 different 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., 2006b). 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 (reported in Strelkov et al., 2006b). However, since most isolates examined originated 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 (Buckzacki et al., 1975), with the exception of Chinese cabbage cv. 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 (Toxopeus et al., 1986; Kuginuki et al., 1999; Strelkov et al., 2006b) . Intermediate and fluctuating IDs have been attributed not only to the heterogeneity of *P. brassicae* populations (Tinggal and Webster, 1981; Jones et al., 1982a; Scott, 1985), but also to the heterogeneity of the

differential hosts themselves (Kuginuki et al., 1999). The results from the current study confirm that genetic heterogeneity in some hosts contributes to the development of indistinct reactions (*sensu* 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 2-2). This was particularly evident with the cabbage cv. Jersey Queen, which commonly developed IDs ranging from 30% to 60%. However, in the case of 'Jersey Queen' and other cabbages, 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 et al. (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 ($p \leq 0.05$) used to differentiate resistant from susceptible reactions (Table 2-2). For comparison, we also performed parametric analysis of the average mass of clubbed roots and non-parametric analysis of 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 non-parametric analysis. Given that the mass of the clubs provides a biological measure of susceptibility, this suggests that parametric analysis is more appropriate than non-parametric analysis for study of the *Brassica/P. brassicae* interaction.

Using LSD ($p \leq 0.05$) 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 2-3). Similarly, ANOVA revealed no significant interaction ($p \geq 0.85$) between differential hosts and isolates derived from SACAN03-1, which was previously classified as pathotype 3 (Strelkov et al., 2006b) 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 2-3). 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 2-3). In the case of Leduc-1, a population previously classified as pathotype 3 (Strelkov et

al., 2006b), 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 $P_4$ on the hosts of Williams (1966) and Somé et al. (1996), respectively. The different virulence patterns in isolates derived from Leduc-1 was confirmed by ANOVA, which indicated a significant interaction between differential hosts and single spore isolates, strongly suggesting that heterogeneity exists in this population (Table 2-2).

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., 2006b) 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 2-3). However, Strelkov et al. (2006b) 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., 1982b; 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., 1982b), and indistinct reactions may result from the presence of multiple pathotypes in the same population (Williams, 1966; Toxopeus et al., 1986). On the hosts of Somé et al. (1996), four of the isolates from CDCN04-1 were classified as $P_2$, while the fifth was classified as $P_6$ (Table 2-3).

Similarly, while ORCA04-1 had been classified as pathotype 6 (Strelkov et al., 2006b) on the hosts of Williams (1966), none of the single spore isolates from this population shared that designation (Table 2-3). 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 were designated as $P_2$, and two were classified as $P_3$. The differential classification of three of these 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 2-2) relative to the original population (Strelkov et al., 2006b). The lower virulence of the latter may have been due to its heterogenous composition, since reduced virulence has been previously reported in pathotype mixtures, and may reflect competition between pathogenic and nonpathogenic isolates (Voorrips, 1995). Non-pathogenic isolates may also induce resistance mechanisms in the host, reducing infection by pathogenic isolates. Nevertheless, a reduction in virulence resulting from a pathotype mixture cannot explain the results obtained with isolates ORCA-ss3 and ORCA-ss4, as these isolates (and ORCA-ss2) exhibited greatly reduced virulence on 'Jersey Queen' relative to the original population (Table 2-2). 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., 2006b), and another study found only one (Strelkov et al., 2007b). 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., 2006b; Strelkov et al., 2007b). Pathotype 3 was shown to be highly virulent to all canola cultivars tested from Canada (Strelkov et al., 2006b), and is also highly virulent on the spring oilseed rape differential cv. Brutor (Table 2-2). 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 genotypes are widely 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.

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Table 2-1. Infection rates obtained for 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] | Infection rate (%) |
|---|---|---|---|---|---|
| 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 2-2. 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' | 'Wilhelms-burger' | 'Badger Shipper' | 'Jersey Queen' | |
| SACAN-ss1 | 100 | 99.0 | 81.9 | 65.2 | 4.7$^{R}$ | 17.5$^{R}$ | 41.8 | 12.9 |
| SACAN-ss2 | 90.9 | 96.8 | 86.0 | 62.0 | 12.0$^{R}$ | 23.9$^{R}$ | 48.3 | 22.0 |
| SACAN-ss3[a] | 97.2 | 96.7 | 67.0 | 62.5 | 8.0$^{R}$ | 31.6 | 44.0 | 20.9 |
| SACAN-ss4 | 100 | 96.0 | 80.7 | 69.3 | 9.3$^{R}$ | 25.8$^{R}$ | 46.4 | 17.1 |
| SACAN-ss5 | 100 | 97.7 | 82.0 | 67.7 | 17.8$^{R}$ | 24.3$^{R}$ | 39.3 | 16.6 |
| AbotJE-ss1 | 84.8 | 57.0 | 0.0$^{R}$ | 3.8$^{R}$ | 0.0$^{R}$ | 4.8$^{R}$ | 77.0 | 15.0 |
| AbotJE-ss2 | 83.3 | 65.3 | 0.0$^{R}$ | 0.0$^{R}$ | 6.0$^{R}$ | 8.9$^{R}$ | 82.3 | 18.3 |
| AbotJE-ss3 | 94.4 | 64.1 | 0.0$^{R}$ | 0.0$^{R}$ | 1.0$^{R}$ | 3.9$^{R}$ | 84.3 | 17.7 |
| AbotJE-ss4 | 88.9 | 63.6 | 0.0$^{R}$ | 0.9$^{R}$ | 0.0$^{R}$ | 8.3$^{R}$ | 84.7 | 20.7 |
| AbotJE-ss5 | 88.9 | 62.8 | 0.0$^{R}$ | 1.9$^{R}$ | 0.0$^{R}$ | 5.5$^{R}$ | 85.7 | 12.0 |
| CDCN-ss1 | 72.2 | 17.6$^{R}$ | 37.8 | 68.5 | 8.3$^{R}$ | 10.2$^{R}$ | 21.4$^{R}$ | 29.2 |
| CDCN-ss2 | 100 | 99.0 | 77.0 | 84.0 | 14.0$^{R}$ | 25.7$^{R}$ | 50.1 | 14.4 |
| CDCN-ss3 | 100 | 100 | 62.0 | 68.0 | 13.4$^{R}$ | 15.6$^{R}$ | 49.9$^{R}$ | 40.0 |
| CDCN-ss4 | 100 | 99.1 | 95.3 | 90.1 | 28.0$^{R}$ | 17.0$^{R}$ | 75.2 | 15.6 |
| CDCN-ss5 | 100 | 97.8 | 95.4 | 85.3 | 19.5$^{R}$ | 6.1$^{R}$ | 48.6 | 24.0 |
| Leduc-ss1 | 100 | 99.0 | 68.0 | 83.0 | 7.4$^{R}$ | 17.3$^{R}$ | 56.0 | 29.4 |
| Leduc-ss2 | 91.6 | 16.6$^{R}$ | 0.0$^{R}$ | 0.0$^{R}$ | 0.0$^{R}$ | 3.0$^{R}$ | 81.0 | 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 | 94.0 | 24.0 | 75.0 | 1.0$^{R}$ | 4.6$^{R}$ | 31.0 | 7.4 |
| Leduc-ss5 | 100 | 99.1 | 83.1 | 79.5 | 16.7$^{R}$ | 2.8$^{R}$ | 55.2 | 31.6 |
| ORCA-ss1 | 100 | 100 | 62.0 | 73.0 | 5.3$^{R}$ | 26.0$^{R}$ | 48.0 | 21.7 |
| ORCA-ss2 | 96.7 | 94.0 | 49.0 | 79.0 | 6.0$^{R}$ | 4.0$^{R}$ | 26.8$^{R}$ | 23.3 |
| ORCA-ss3 | 72.7 | 25.0 | 4.0$^{R}$ | 21.3$^{R}$ | 0.0$^{R}$ | 0.0$^{R}$ | 3.0$^{R}$ | 21.8 |
| ORCA-ss4 | 66.7 | 33.0 | 2.0$^{R}$ | 14.0$^{R}$ | 1.0$^{R}$ | 1.0$^{R}$ | 9.0$^{R}$ | 22.3 |
| ORCA-ss5 | 100 | 98.1 | 100 | 93.3 | 21.9$^{R}$ | 13.5$^{R}$ | 71.5 | 16.7 |

[R]Indicates a resistant reaction; LSD ($p \leq 0.05$) 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 ($p \geq 0.85$) 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 2-3. 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' | 'Wilhelms-burger' | '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 | - | - | - | - | - | + | $6/P_4$ |
| 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 ($p \leq 0.05$) 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 (33) and Somé et al. (22).

[c] SACAN-ss3 was classified as pathotype 2 on the differentials of Williams based on LSD groupings, although ANOVA indicated no significant interactions ($p \geq 0.85$) between the differential hosts and SACAN-ss1 to SACAN-ss5.

# Chapter 3. Molecular diversity of *Plasmodiophora brassicae* single spore isolates from Canada

## 3.1. Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is one of the most devastating diseases of crucifers worldwide. An understanding of the diversity in pathogen populations is important for the development of cultivars with effective and durable resistance to this disease. Traditionally, such diversity has been assessed using host differential sets, which can be a time consuming and labor intensive process. As such, the development of efficient and reliable molecular markers represents a desirable alternative for the study of variation in *P. brassicae* populations (Manzanares-Dauleux et al., 2000). Specific PCR primers derived from putative repetitive/high-copy number DNA sequences of *P. brassicae* were developed by Buhariwalla et al. (1995), and Möller and Harling (1996) described a correlation between pathotype classification and patterns of random amplified polymorphic DNA (RAPD) for three populations ("field isolates") of the pathogen. However, contamination by host DNA is a major concern with the RAPD method, since *P. brassicae* cannot be grown in pure culture (Buhariwalla et al., 1995). Furthermore, results obtained using RAPD markers may not be consistent among laboratories, because of their sensitivity to variation in the experimental procedures used (Pandey et al., 1996).

Nevertheless, two RAPD markers specific to pathotype $P_1$ of *P. brassicae* were identified by Manzanares-Dauleux et al. (2001), one of which was converted into a sequence characterized amplified region (SCAR) marker that offers reliable identification of this pathotype (Manzanares-Dauleux et al., 2000). Restriction fragment length polymorphism (RFLP) markers were also developed to distinguish four single-spore isolates of *P. brassicae* (Klewer et al., 2001), but the usefulness of RFLPs is limited by the difficulty in extracting sufficient amounts of pathogen DNA for restriction enzyme digestion (Buhariwalla et al., 1995).

Cleaved amplified polymorphic sequence (CAPS) analysis, also known as PCR-RFLP, is a technique that relies on the specific amplification of DNA and the differential digestion of amplified fragments using restriction enzymes (Konieczny and Ausubel, 1993). In contrast to conventional RFLP analysis, only a very small quantity of DNA is required for CAPS, and a Southern hybridization step is not necessary (Saka et al., 2006). Therefore, CAPS is a simple, fast and inexpensive method to evaluate diversity in pathogen populations (Zhang and Stommel, 2001). CAPS markers have been used to characterize populations of *Botrytis cinerea* from Chile (Muñoz et al., 2002), and for the identification of resistance genes to yellow leaf curl disease in tomato (Perez-de-Castro et al., 2007) and green rice leafhopper in rice (Saka et al., 2006). However, to our knowledge, CAPS analysis has not been used to characterize or distinguish between isolates of *P. brassicae.* Given the difficulties in obtaining sufficient quantities of DNA from resting spores of the pathogen for RFLP and other analyses, CAPS markers may be useful to examine diversity in *P. brassicae.* This

chapter describes the development of CAPS markers that can be used to distinguish between two pathotypes of *P. brassicae* found in Canada. The characterization of 25 single spore isolates of the pathogen with seven random decamer primers (Manzanares-Dauleux et al., 2001) and four sequence-generated (SG) primers (Buhariwalla et al., 1995) is also described.

## 3.2. Materials and Methods

### Single spore isolates of *P. brassicae*

Twenty-five single spore isolates of *P. brassicae*, previously characterized on the differential hosts of Williams (1966) and Somé et al. (1996) (Chapter 2), were included in this study and are summarized in Table 3-1. Isolates were maintained on the universal suscept Chinese cabbage cv. Granaat, as previously described (Chapter 2). Galled root material was collected 8 weeks after inoculation with selected isolates (Chapter 2) and stored at −80°C until use.

### Isolation of resting spores

Resting spores of *P. brassicae* were isolated from infected 'Granaat' roots as described by Tewari et al. (2005) with some minor modifications. Briefly, about 6 g of root tissue were homogenized in a blender (Waring, Torrington, CT) in 50 mL sterile distilled water ($sdH_2O$), and filtered through eight layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, N.C.). The homogenate was centrifuged at 1000 × *g* (4°C

for 10 min) and the resulting pellet (consisting of resting spores) washed five times with $sdH_2O$. The pellet was washed twice more with 0.1× TE buffer (1mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.2) and stored at −20°C until used for DNA extraction.

**DNA extraction**

Genomic DNA was extracted from resting spores of *P. brassicae* according to a protocol modified from Klewer et al. (2001). Each spore pellet was re-suspended in 300 µl lysis buffer [50mM Tris-HCl, 50 mM EDTA, 3% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, pH 7.4] in a 1.5 ml Eppendorf tube containing approximately 150 µl of glass beads (0.5mm, BioSpec Producs, Inc., Bartlesville, OK). After agitation on a shaker (3520 Orbit Shaker, Lab-Line Instruments, Melrose Park, IL) at 360 rpm for 10 min, 60 µl of 10% CTAB [10% CTAB (w/v), 0.7 M NaCl] solution was added and the suspension incubated at 65°C for 1 h, followed by extraction with 600 µL of chloroform/isoamyl alcohol (24:1). The upper phase was transferred into a new tube and the DNA precipitated by adding 800 µL of 100% ethanol and 150 µL of 5M NaCl. A DNA pellet was collected by centrifugation (9200 × *g* for 20 min, at room temperature) and washed with 70% ethanol. The pellet was dissolved in 300 µL 0.1× TE buffer, containing 1 µg ribonuclease A, and incubated for 1 h at 37°C. To ensure a very high quality template, the DNA was further purified using a PCR Purification Kit (QIAGEN Inc., Mississauga, ON), according to the manufacturer's instructions, and stored at −20°C in Qiagen EB (elution) buffer until needed. Genomic DNA was also extracted from the roots of 'Granaat' plants according to the CTAB protocol of Rogers and Bendich (1985), and stored at -20 °C in TE buffer. DNA was quantified on a

NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) prior to use.

**RAPD assay**

Seven arbitrary decamer primers and four SG primers reported by Manzanares-Dauleux et al. (2001) and Buhariwalla et al. (1995), respectively, were screened for polymorphisms using DNA from 25 Canadian isolates of *P. brassicae* (Table 3-2). Amplifications were conducted using a GeneAmp PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA) in a 25 $\mu$L reaction volume containing 100 ng host DNA or 20 ng DNA from resting spores of the pathogen, 1 U of *Taq* DNA polymerase (Invitrogen), 1× PCR buffer (20 mM Tris pH 8.4, 50 mM KCl), 1.9 mM $MgCl_2$, 200 $\mu$M each dNTP, and 0.2 $\mu$M of each decamer primer (Manzanares-Dauleux et al., 2001). Reaction cycles consisted of an initial heat denaturing step of 1 min at 94°C, and 45 cycles of 45 s at 93°C, 1 min at 35°C, and 2 min 30 s at 72°C. For analysis using the SG primers (Table 3-2), the PCR protocol was as described by Buhariwalla et al. (1995), except that the $MgCl_2$ concentration was increased from 1 mM to 1.5 mM $MgCl_2$, while the concentration of each dNTP was reduced from 100 mM to 400 µM. Amplification products were separated on 1.8% agarose gels containing ethidium bromide (0.1 µg/ml) in TAE buffer, and photographed using a UV transilluminator with GeneSnap software (Syngene, Frederick, MD).

**Cluster analysis**

The presence or absence of bands was recorded for each isolate/primer combination after amplification of DNA with the random or SG primers (Table 3-2). Genetic distances were calculated using a formula based on Jaccard's distance, $d_{ij} = 1 - p/(p+q+r)$, where $p$ is the number of bands shared by isolate i and j , $q$ is the number of bands found only in isolate i, and $r$ is the number of bands found only in isolate j. The PROC TREE of SAS 9.1 Software (SAS Institute, 1999) was used to construct a dendrogram based on the distance matrices generated by PROC CLUSTER (WARD method), using 1100 data points (25 isolates × 11 primers × 4 random amplified polymorphic DNA loci).

**Cloning and sequencing of *P. brassicae* DNA**

A total of 63 primers were initially designed based on the sequences for isopentenyltransferase (IPT) (accession numbers X14410, CAA39647, BAA84213) or an insertion element (accession number M82888) from *Agrobacterium tumefaciens*, and screened for the ability to produce strong, distinctive amplicons from isolates of *P. brassicae* (data not shown). After the initial screening, forward primer M828F1 (5'-CCATGTCGAGAAGCGTATGA-3') and reverse primer M828R1 (5'-GAAGACGCTTTTCCAGTC-3') (based on the insertion element) were selected for differential amplification of pathogen DNA, since they produced strong bands from two pathotypes of *P. brassicae*. All primers were designed using OligoPerfect™ Designer (Invitrogen, Burlington, ON) software.

DNA fragments amplified by M828F1/M828R1 were cloned using a TOPO TA Cloning Kit and pCR® 4-TOPO vector, and transformed into *E. coli* TOP10 competent cells as per the manufacturer's instructions. Plasmids were purified using a QIAprep Spin Miniprep Kit (QIAGEN Inc., Mississauga, ON) and sequenced at the Molecular Biology Service Unit, University of Alberta, Edmonton, Canada. The sequence information obtained was used to design the *P. brassicae*-specific primers A3T3F1 (5'-TCCGGTACTGATCGTGTCTTTAT-3') and A3T3R1 (5'- CACTGTCGTTGACACA GATGG-3'), which were used to amplify DNA from isolates representing pathotypes 3 (SACAN-ss5) and 6 (AbotJE-ss2) of the pathogen. Reaction conditions consisted of 3 min at 94°C; 30 cycles of 30 s at 93°C, 30 s at 55°C, and 2 min at 68°C or 70 °C; and a final extension step of 15 min at 72°C. The primers amplified a single band from each isolate, and each band was cloned and sequenced as above. The sequences obtained were analyzed for the presence of restriction sites using Webcutter 2.0 software (Heiman, 1997), which indicated the presence of restriction sites for *BsbI*/*Bbv*16II/*Bpi*I (gaagacnn/nnnnn) and *XmnI*/*Asp700*I (gaann/nnttc) on all clones.

**CAPS analysis**

Another set of primers, A3T3F2 (5'-GGTACTGATCGTGTCTTTATAGGGCGCG-3') and A3T3R2 (5'- CACTGTCGTTGACACAGATGGCTGTCA-3'), was designed from the *P. brassicae*-sequences obtained with A3T3F1/A3T3R1 and used in CAPS analysis. PCR was carried out in a 25 $\mu$L reaction volume containing 20 ng template DNA, 1 U of *Taq* DNA polymerase (Invitrogen), 1× PCR buffer (20 mM Tris pH 8.4, 50

mM KCl), 1.5 mM $MgCl_2$, 200 $\mu$M of each dNTP, and 0.4 $\mu$M of forward (A3T3F2) and reverse (A3T3R2) primer. PCR parameters consisted of 3 min at 94°C; 10 cycles of 30 s at 93°C, a slow ramp of 1 min to 64°C, and 2 min at 70°C; and 35 cycles of 30 s at 93°C, 30 s at 60°C, and 2 min at 72°C. Based on the restriction site analysis of the amplified sequences, PCR products were digested with *Xmn*I (Fermentas Canada Inc., Burlington, ON), according to the manufacturer's instructions, to detect polymorphisms between the pathotypes. DNA from 'Granaat,' *E. coli* and *A. tumefaciens* was included in all analyses as controls. Digested amplicons were separated by electrophoresis as above, except that 2% agarose gels were used.

## 3.3. Results

### Characterization with RAPD markers

Seven arbitrary decamer primers and 4 SG primers were tested on 25 single spore isolates of *P. brassicae*. Amplifications were also performed on DNA from Chinese cabbage cv. Granaat, which was included as a control. All of the primers produced detectable PCR products from 'Granaat' and the 25 isolates tested. An example of the banding pattern obtained using the primer OPK10 is shown in Fig. 3-1. Some of the arbitrary primers (e.g. OPK10) amplified similar sized bands in certain isolates and the host, but the presence of additional bands in the pathogen samples resulted in distinct banding patterns. Many isolates appeared to have unique multi-locus molecular genotypes, although identical banding patterns were observed in some isolates that originated from the same population and belonged to the same pathotype (Fig. 3-1).

However, none of the primers generated identical banding patterns among all isolates belonging to a particular pathotype. Therefore, although previous research using French isolates indicated that the primers OPA13 and OPL14 gave pathotype-specific profiles (Manzanares-Dauleux et al., 2001), this was not the case with the Canadian isolates used in this study.

Genetic dissimilarity among the 25 *P. brassicae* isolates was analyzed using 1100 data points (25 isolates × 11 primers × 4 random amplified polymorphic DNA loci). The DNA fragments included in the analysis were from 0.2 to over 1.2 kb in size (Table 3-2). Genetic dissimilarity among single spore isolates derived from the same field population ranged from 0.003 to 0.06, whereas genetic dissimilarity among isolates from different populations ranged from 0.05 to 0.27. A dendogram based on genetic dissimilarity between the 25 isolates was constructed and is shown in Fig. 3-2. Five groups were observed, with isolates clustering according to the population from which they were originally obtained. However, there was no clustering according to pathotype.

**Cloning and sequencing**

Sixty three primers, designed based on *A. tumefaciens* sequences available in GenBank, were initially screened for their ability to amplify *P. brassicae* DNA. Most did not give rise to strong bands. However, some primer pairs, including M828F1/M828R1, amplified several strong fragments of DNA (data not shown). Several amplicons obtained with M828F1/M828R1 and ranging up to about 800 bp in

size were cloned and sequenced, including four bands from pathotype 6 isolate AbotJE-ss2 and four bands from pathotype 3 isolate SACAN-ss5. Although some of the amplicons shared a high degree of homology with each other, none had any significant homology to the *A. tumefaciens* sequence on which the M828F1/M828R1 primers were based (data not shown).

Six new pairs of primers were designed using the *P. brassicae* sequence information, and one pair, A3T3F1/A3T3R1, amplified strong, single DNA bands from AbotJE04-ss2 and SACAN-ss5. The amplicons were cloned and sequenced and are compared in Fig. 3-3. The fragments obtained from SACAN-ss5 and AbotJE-ss2 were 727 bp and 724 bp long, respectively, and shared 97% homology. They also shared 97% homology with the amplicon produced using M828F1/M828R1, on which A3T3F1/A3T3R1 was based. Database searches indicated no significant homology to other sequences deposited in GenBank. Sequence analysis revealed the presence of different numbers of *Xmn*I/*Asp700*I and *Bsb*I/*Bbv*16II/*Bpi*I restriction sites in the isolates, suggesting that these enzymes might be appropriate for pathotype differentiation via CAPS analysis.

**CAPS analysis**

For detection of polymorphisms using CAPS, a new pair of primers (A3T3F2/A3T3R2) was designed based on the *P. brassicae* sequence information obtained above. These primers were very similar to A3T3F1/A3T3R1, but longer, in order to increase the stringency of the PCR amplification. The primers amplified a strong, single band about 720 bp in size from each of six isolates of *P. brassicae*

representing two pathotypes, as well as from the 'Granaat' host (Fig. 3-4). No bands were obtained from DNA of *A. tumefaciens* or *E. coli*. The PCR products were digested with three different restriction enzymes in preliminary studies, and it was found that *Xmn*I gave the clearest results. Hence, this enzyme was selected for digestion of the amplicons in the CAPS analysis (Fig. 3-4). After digestion with *Xmn*I, three bands (143 bp, 176 bp and 402 bp in size) were visible for each of the three pathotype 6 isolates tested. In contrast, only two bands (143 bp and 581 bp) were produced after digestion of the amplicons obtained from the three pathotype 3 isolates examined. The size and number of bands corresponded to what was expected based on the location and number of *Xmn*I restriction sites on the sequences amplified from SACAN-ss5 (pathotype 3) and AbotJE-ss2 (pathotype 6) (Fig. 3-3). Four bands were observed after digestion of the host amplicon with *Xmn*I, thus resulting in a unique profile for 'Granaat' (Fig. 3-4). These bands consisted of the 143 bp band observed in pathotypes 3 and 6, as well as the 176 bp and 402 bp bands in pathotype 6, and the 581 bp band in pathotype 3. The banding pattern observed for 'Granaat,' which combined those of pathotypes 3 and 6, suggests that the amplified fragment exists as multiple copies with different numbers of restriction sites (i.e., one or two) in the host genome.

## 3.4. Discussion

Previous research indicated that *P. brassicae* populations from Alberta are relatively homogenous, with pathotype 3 (as per the classification system of Williams) predominant in the province (Strelkov et al., 2006; Strelkov et al., 2007; Chapter 2). However, the current molecular analysis of diversity in single spore isolates of the

pathogen suggests a higher degree of heterogeneity. Manzanares-Dauleux et al. (2001) stated that the use of polymorphic DNA bands can lead to an overestimation of genetic dissimilarity between *P. brassicae* isolates, particularly when compared to the use of differential hosts. This reflects the fact that while differences in DNA sequence may lead to differential amplification patterns with random primers, they are not necessarily related to the virulence or pathotype of specific isolates. Nevertheless, the higher degree of heterogeneity found via molecular genotyping of *P. brassicae* isolates, both in this and other studies, suggests a greater evolutionary potential than that indicated solely by pathotype classification.

None of the eleven arbitrary and SG primers used to characterize the 25 isolates of *P. brassicae* produced pathotype-specific markers. In contrast, Manzanares-Dauleux et al. (2000) reported that the RAPD marker $OPL14_{1200}$, amplified by the primer OPL14, was found in the molecular pattern of all isolates belonging to pathotype $P_1$ (classified as per the system of Somé et al., 1996). Although OPL14 was included in this study, it did not amplify any pathotype-specific DNA fragments (Fig. 3-1). However, no isolates belonging to pathotype $P_1$ were tested (Table 3-1), nor have any been reported from Canada (Strelkov et al., 2007; Chapter 2). No clear correlation between pathotype and amplified DNA fragment patterns was observed, likely because the evolution of pathotypes is independent of the evolution of neutral DNA markers (Manzanares-Dauleux et al., 2000), a phenomenon also found with *Puccinia striiformis* (Chen et al., 1993). While Buhariwalla et al. (1995) found that their SG primers did not produce highly amplified DNA bands from host ('Granaat') DNA samples, we consistently

observed PCR products using four of the same primers and the same host. However, Buhariwalla et al. (1995) also noted frequent amplification of host DNA at suboptimal annealing temperatures, and our results may reflect the sensitivity of the PCR fingerprinting technique.

Cluster analysis of the DNA polymorphisms generated with the eleven arbitrary and SG primers revealed that genetic dissimilarity was greater between populations than within populations of the pathogen (Fig. 3-2). This tight clustering of single spore isolates according to population suggests a low level of genetic recombination, at least on a very small scale (i.e. single spores derived from the same gall), which would support a significant role for asexual reproduction in the life cycle of *P. brassicae* (Buczacki, 1983; Naiki et al, 1984; Mithen and Magrath, 1992). In contrast, Manzanares-Dauleux et al. (2001) observed similar mean genetic dissimilarities between and within populations ("field isolates") of the pathogen, and suggested that the high level of genetic diversity in single spore isolates from the same population was indicative of an important role for recombination in the cycle of *P. brassicae.* However, the pathogen life cycle is still not completely understood, and the differences observed in diversity within populations may reflect variation related to regional or climatic conditions. Thus, sexual and asexual propagation may both be significant processes in *P. brassicae* (Buczacki, 1983; Ingram and Tommerup, 1972; Naiki et al, 1984; Mithen and Magrath,1992).

As noted above, the extent of variability found in the current study was greater between populations than within populations. However, the extent of genetic

dissimilarity between populations (no greater than 0.27) was still less than reported in other studies, which found levels as high as 0.7 (Manzanares-Dauleux et al., 2001) or nearly 0.8 (Buhariwalla et al., 1995). However, the previous studies used *P. brassicae* populations from Europe, whereas in this study we examined populations from Canada, where the pathogen is presumed to have been introduced more recently by settlers to the country. Hence, it would be reasonable to assume that Canadian populations of *P. brassicae* might be less diverse. Moreover, this discrepancy may also reflect the use of different statistical methodologies; the distance matrices used to construct the dendogram in the present study (Fig. 3-2) were calculated using the Ward method, which reduces the increase in total within-cluster error (Brian et al., 2001).

In addition to providing information on genetic diversity, molecular markers can also be useful for the identification of particular isolates or pathotypes. Manzanares-Dauleux et al. (2000) developed a SCAR marker specific for pathotype $P_1$ of *P. brassicae*. However, as this pathotype has not been reported in Canada, different markers are required to distinguish isolates representing pathotypes common in Alberta and other parts of the country. Of particular interest are pathotypes 3 and 6 [as classified on the differentials of Williams (1966), and corresponding to $P_2$ and $P_3$ on the system of Somé et al. (1996)], which have been reported most frequently from Canada (Reyes et al., 1974; Hildebrand and Delbridge, 1995; Strelkov et al., 2006; Strelkov et al., 2007; Chapter 2). We chose CAPS analysis as a fast and inexpensive method to determine variation at specific loci in different pathotypes. However, since no pathotype-specific RAPD markers were identified, primers were designed based on *A. tumefaciens*

sequences for IPT (involved in the synthesis of cytokinins) or an insertion element. Isopentenyltransferase was selected because cytokinin production has been detected in plasmodia of *P. brassicae* (Müller and Hilgenberg, 1986), suggesting that IPT homologues may be present in the pathogen, whereas we hypothesized that there would be a greater chance of identifying pathogen-specific sequence differences in an insertion element.

However, the primers amplified numerous fragments, and the bands that were cloned and sequenced (chosen based on intensity and size and initially amplified using M828F1/M828R1) bore no significant homology to the sequence (insertion element M82888) used to design the original primers, nor to other sequences available in GenBank. Hence, the sequence selected for CAPS analysis (Fig. 3-3) was, in effect, arbitrary, although the presence of differential restriction sites in amplicons from different isolates indicated that this locus was suitable for use as a CAPS marker. Digestion with the enzyme *Xmn*I revealed consistent DNA fragment patterns on agarose gels, which were specific to three pathotype 3 isolates, three pathotype 6 isolates, and host DNA (Fig. 3-4). However, because all isolates representing each pathotype were obtained from the same population, it is not clear whether the CAPS marker identified is pathotype-specific or population-specific. Further testing, with pathotypes 3 and 6 isolates originating from different populations and/or different geographic origins, is necessary to resolve this issue. Similarly, it will also be desirable to clone and sequence the DNA fragment(s) amplified from 'Granaat,' in order to enable comparison with the

pathogen. Nevertheless, the utility of CAPS analysis for the detection of specific populations or pathotypes of *P. brassicae* is clear.

Molecular analyses of diversity, along with the characterization of virulence on host differential sets, will be important for monitoring genetic variation in populations of *P. brassicae* in Canada. This information will be useful to track changes in pathogen populations, as well as to develop crucifer varieties with durable genetic resistance, ultimately facilitating successful management of clubroot in Canada and elsewhere.

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Table 3-1. Pathotype designation of single spore isolates derived from populations of *Plasmodiophora brassicae* collected in Canada.

| Population[a] | Origin | Single Sporeb | Pathotype[c] |
|---|---|---|---|
| SACAN03-1 | St. Albert, Alberta (canola) | SACAN-ss1 | $3/P_2$ |
| | | SACAN-ss2 | $3/P_2$ |
| | | SACAN-ss3 | $2/P_2$[d] |
| | | SACAN-ss4 | $3/P_2$ |
| | | SACAN-ss5 | $3/P_2$ |
| AbotJE04-1 | Abbottsford, British Columbia (soil) | AbotJE-ss1 | $6/P_3$ |
| | | AbotJE-ss2 | $6/P_3$ |
| | | AbotJE-ss3 | $6/P_3$ |
| | | AbotJE-ss4 | $6/P_3$ |
| | | AbotJE-ss5 | $6/P_3$ |
| CDCN04-1 | Edmonton, Alberta (canola) | 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-1 | Leduc, Alberta (soil) | Leduc-ss1 | $3/P_2$ |
| | | Leduc-ss2 | $6/P_4$ |
| | | Leduc-ss3 | N/A |
| | | Leduc-ss4 | $3/P_2$ |
| | | Leduc-ss5 | $3/P_2$ |
| ORCA04 | Orton, Ontario (cabbage) | 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]As described in Strelkov et al. (2006).

[b]Isolation and pathotype classification of single spore isolates was described in Chapter 2.

[c]As determined on the differential hosts of Williams (1966)/Somé et al. (1996).

[d]SACAN-ss3 was classified as pathotype 2 on the differentials of Williams (1966) based on LSD groupings, although ANOVA indicated no significant interactions ($p \geq 0.85$) between the differential hosts and SACAN-ss1 to SACAN-ss5.

Table 3-2. Primers used for the molecular characterization of single spore isolates of *Plasmodiophora brassicae* from Canada and DNA bands analyzed.

| Primer | Sequence | DNA bands (bp) analyzed |
|---|---|---|
| Random[a] | | |
| OPA13 | CAGCACCCAC | 400, 500, 600, 700 |
| OPJ20 | AAGCGGCCTC | 600, 730, 880, 1100 |
| OPK10 | GTGCAACGTG | 720, 900, 1000, 1500 |
| OPK20 | GTGTCGCGAG | 420, 600, 900, 1100 |
| OPL12 | GGGCGGTACT | 200, 270, 360, 500 |
| OPL14 | GTGACAGGCT | 420, 700, 760, 1000 |
| OPL14LP | CACTGTCCGA | 700, 1000, 1100, 1200 |
| Sequence-generated[b] | | |
| HKB17/33 | GCATCGTCTG | 400, 650, 700, 1200 |
| RFM8 | GGAACAAGCTCGAAGCCATG | 240, 320, 400, 420 |
| RFM9 | AGGTGAGGAGAAATGTCTC | 400, 600, 800, 840 |
| RFM11 | TGTACGTGTCCTTCGTGGGA | 300, 360, 430, 530 |

[a]Random decamer primers are as reported by Manzanares-Dauleux et al. (2001).

[b]Sequence-generated (SG) primers were developed by Buhariwalla et al. (1995).

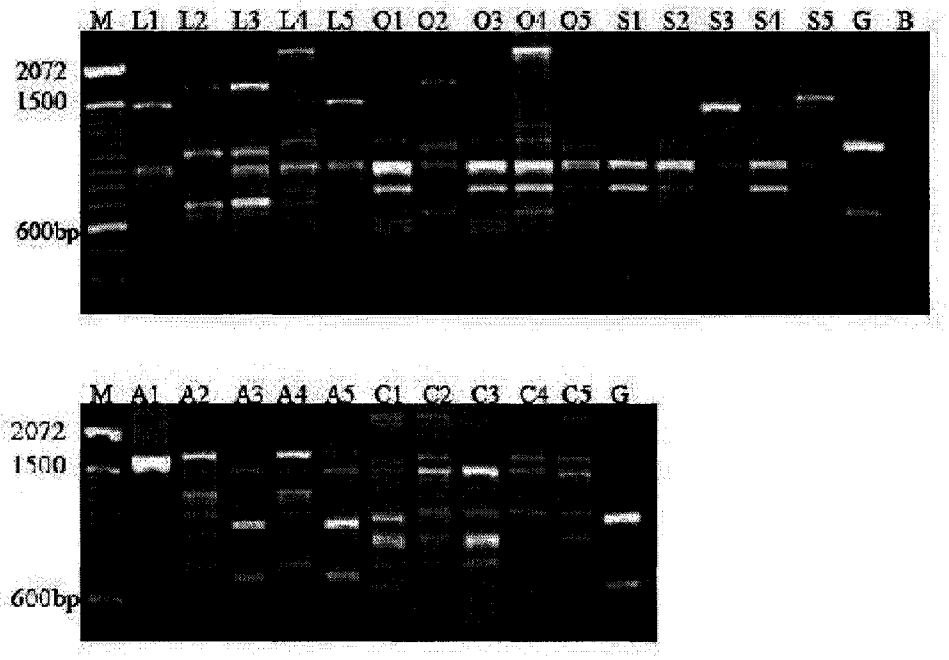


Fig. 3-1. Amplicons produced with primer OPK10 using genomic DNA from different single spore isolates of *Plasmodiophora brassicae* as a template. **Top gel**, Lane M: DNA ladder (100 bp DNA Ladder, Invitrogen, Burlington, ON), L1: Leduc-ss1, L2: Leduc-ss2, L3: Leduc-ss3, L4: Leduc-ss4, L5: Leduc-ss5, O1: ORCA-ss1, O2: ORCA-ss2, O3: ORCA-ss3, O4: ORCA, O5: ORCA-ss5, S1: SACAN-ss1, S2: SACAN-ss2, S3: SACAN-ss3, S4: SACAN-ss4, S5: SACAN-ss5, G: *Brassica rapa* var. *pekinenses* (cv. Granaat), and B: sterile distilled $H_2O$. **Bottom gel**, Lane M: DNA ladder (100 bp DNA Ladder, Invitrogen, Burlington, ON), A1: AbotJE-ss1, A2: AbotJE-ss2, A3: AbotJE-ss3, A4: AbotJE-ss4, A5: AbotJE-ss5, C1: CDCN-ss1, C2: CDCN-ss2, C3: CDCN-ss3, C4: CDCN-ss4, C5: CDCN-ss5, and G: *Brassica rapa* var. *pekinenses* (cv. Granaat).

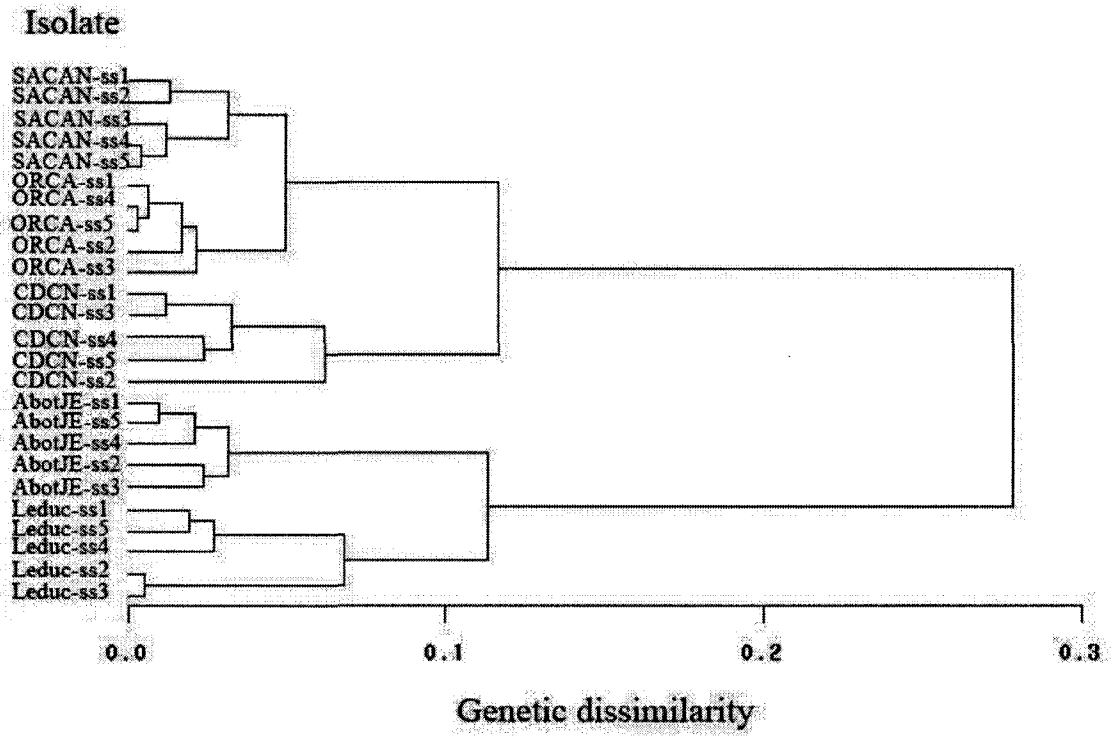


Fig. 3-2. Dendrogram showing genetic dissimilarity among 25 single spore isolates of *Plasmodiophora brassicae* from Canada. The tree was constructed using the PROC TREE of SAS 9.1 Software (SAS Institute, 1999) based on the distance matrices generated by PROC CLUSTER (WARD method), using 1100 data points (25 isolates × 11 random or sequence-generated primers × 4 DNA bands).

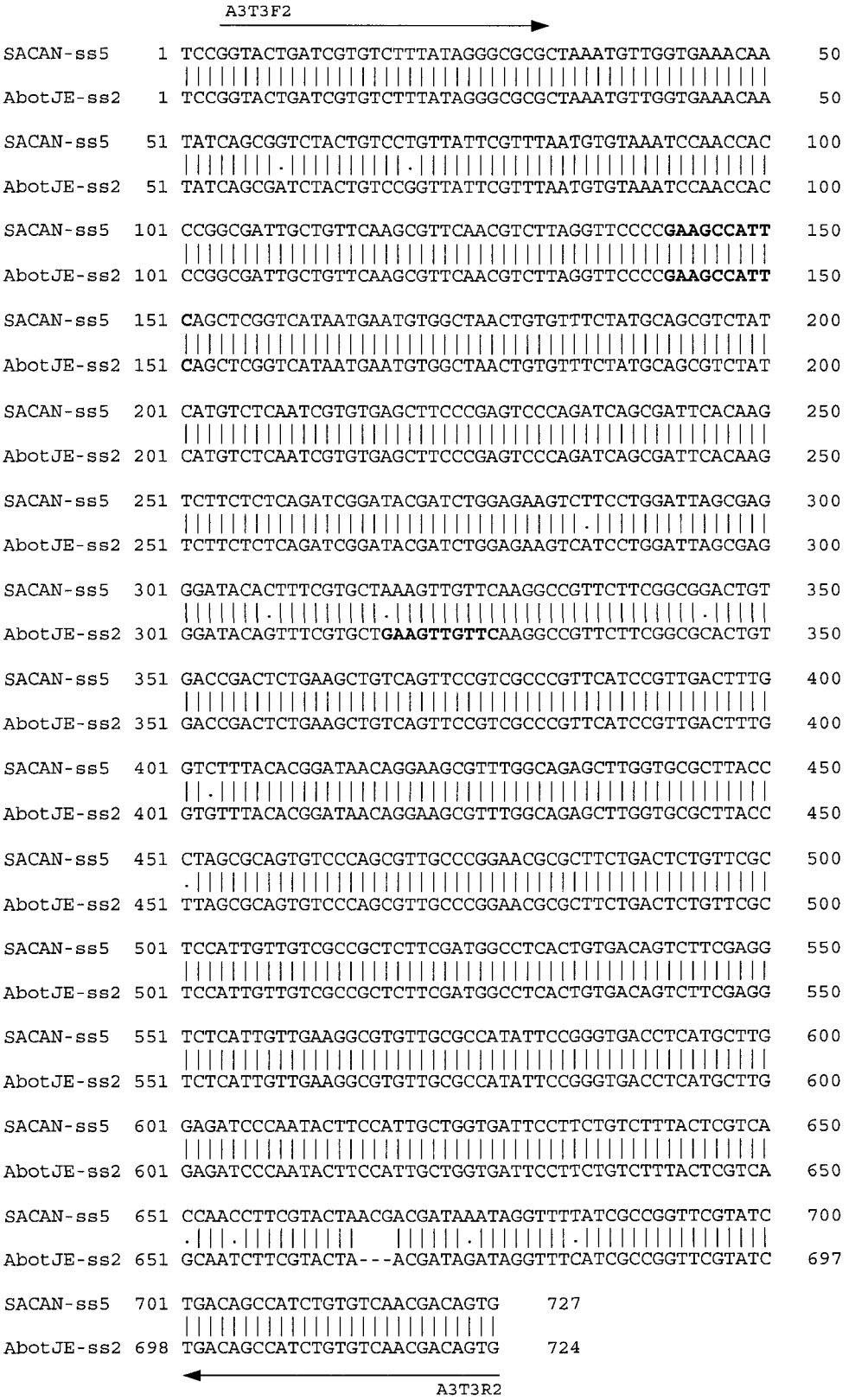
A3T3F2
SACAN-ss5 1 TCCGGTACTGATCGTGTCTTTATAGGGCGCGCTAAATGTTGGTGAAACAA 50
AbotJE-ss2 1 TCCGGTACTGATCGTGTCTTTATAGGGCGCGCTAAATGTTGGTGAAACAA 50
SACAN-ss5 51 TATCAGCGGTCTACTGTCCTGTTATTCGTTTAATGTGTAAATCCAACCAC 100
AbotJE-ss2 51 TATCAGCGATCTACTGTCCGGTTATTCGTTTAATGTGTAAATCCAACCAC 100
SACAN-ss5 101 CCGGCGATTGCTGTTCAAGCGTTCAACGTCTTAGGTTCCCCGAAGCCATT 150
AbotJE-ss2 101 CCGGCGATTGCTGTTCAAGCGTTCAACGTCTTAGGTTCCCCGAAGCCATT 150
SACAN-ss5 151 CAGCTCGGTCATAATGAATGTGGCTAACTGTGTTTCTATGCAGCGTCTAT 200
AbotJE-ss2 151 CAGCTCGGTCATAATGAATGTGGCTAACTGTGTTTCTATGCAGCGTCTAT 200
SACAN-ss5 201 CATGTCTCAATCGTGTGAGCTTCCCGAGTCCCAGATCAGCGATTCACAAG 250
AbotJE-ss2 201 CATGTCTCAATCGTGTGAGCTTCCCGAGTCCCAGATCAGCGATTCACAAG 250
SACAN-ss5 251 TCTTCTCTCAGATCGGATACGATCTGGAGAAGTCTTCCTGGATTAGCGAG 300
AbotJE-ss2 251 TCTTCTCTCAGATCGGATACGATCTGGAGAAGTCATCCTGGATTAGCGAG 300
SACAN-ss5 301 GGATACACTTTCGTGCTAAAGTTGTTCAAGGCCGTTCTTCGGCGGACTGT 350
AbotJE-ss2 301 GGATACAGTTTCGTGCTGAAGTTGTTCAAGGCCGTTCTTCGGCGCACTGT 350
SACAN-ss5 351 GACCGACTCTGAAGCTGTCAGTTCCGTCGCCCGTTCATCCGTTGACTTTG 400
AbotJE-ss2 351 GACCGACTCTGAAGCTGTCAGTTCCGTCGCCCGTTCATCCGTTGACTTTG 400
SACAN-ss5 401 GTCTTTACACGGATAACAGGAAGCGTTTGGCAGAGCTTGGTGCGCTTACC 450
AbotJE-ss2 401 GTGTTTACACGGATAACAGGAAGCGTTTGGCAGAGCTTGGTGCGCTTACC 450
SACAN-ss5 451 CTAGCGCAGTGTCCCAGCGTTGCCCGGAACGCGCTTCTGACTCTGTTCGC 500
AbotJE-ss2 451 TTAGCGCAGTGTCCCAGCGTTGCCCGGAACGCGCTTCTGACTCTGTTCGC 500
SACAN-ss5 501 TCCATTGTTGTCGCCGCTCTTCGATGGCCTCACTGTGACAGTCTTCGAGG 550
AbotJE-ss2 501 TCCATTGTTGTCGCCGCTCTTCGATGGCCTCACTGTGACAGTCTTCGAGG 550
SACAN-ss5 551 TCTCATTGTTGAAGGCGTGTTGCGCCATATTCCGGGTGACCTCATGCTTG 600
AbotJE-ss2 551 TCTCATTGTTGAAGGCGTGTTGCGCCATATTCCGGGTGACCTCATGCTTG 600
SACAN-ss5 601 GAGATCCCAATACTTCCATTGCTGGTGATTCCTTCTGTCTTTACTCGTCA 650
AbotJE-ss2 601 GAGATCCCAATACTTCCATTGCTGGTGATTCCTTCTGTCTTTACTCGTCA 650
SACAN-ss5 651 CCAACCTTCGTACTAACGACGATAAATAGGTTTTATCGCCGGTTCGTATC 700
AbotJE-ss2 651 GCAATCTTCGTACTA---ACGATAGATAGGTTTCATCGCCGGTTCGTATC 697
SACAN-ss5 701 TGACAGCCATCTGTGTCAACGACAGTG 727
AbotJE-ss2 698 TGACAGCCATCTGTGTCAACGACAGTG 724
A3T3R2

Fig. 3-3. Alignment of the nucleotide sequences obtained from single spore isolates SACAN-ss5 (pathotype 3) and AbotJE-ss2 (pathotype 6) of *Plasmodiophora brassicae* and used in cleaved amplified polymorphic sequence (CAPS) analysis. The arrows indicate the position of the gene-specific primers (A3T3F2/A3T3R2) that were used in this analysis. The locations of *Xmn*I restriction sites (gaann/nnttc) are indicated in the bold font.

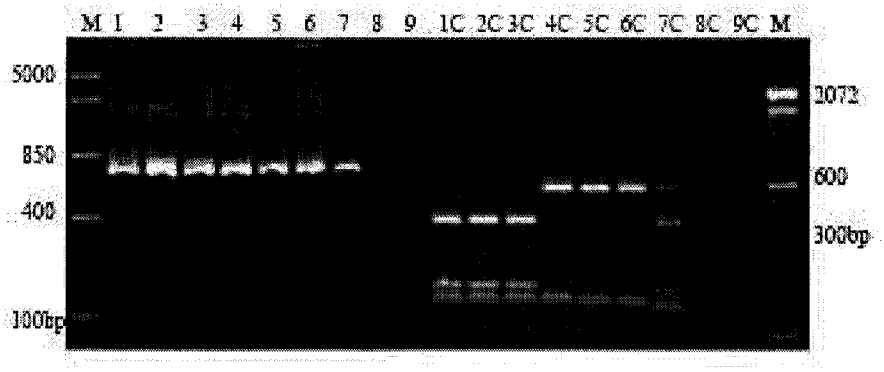


Fig. 3-4. Cleaved amplified polymorphic sequence (CAPS) analysis of single spore isolates representing pathotypes 3 and 6 of *Plasmodiophora brassicae*. Genomic DNA was amplified with forward primer A3T3F2 and reverse primer A3T3R2 (lanes 1 to 9), followed by digestion with the restriction enzyme *Xmn*I (lanes 1C to 9C). Lane M: DNA ladder (100 bp DNA Ladder, Invitrogen, Burlington, ON), lane 1: AbotJE-ss1, lane 2: AbotJE-ss2, lane 3: AbotJE-ss3, lane 4: SACAN-ss1, lane 5: SACAN-ss2, lane 6: SACAN-ss5, lane 7: *Brassica rapa* var. *pekinenses* (cv. Granaat), lane 8: *Escherichia coli*, lane 9: *Agrobacterium tumefaciens*, lane 1C: AbotJE-ss1, lane 2C: AbotJE-ss2, lane 3C: AbotJE-ss3, lane 4C: SACAN-ss1, lane 5C: SACAN-ss2, lane 6C: SACAN-ss5, lane 7C: *B. rapa* var. *pekinenses* (cv. Granaat), lane 8C: *E. coli*, lane 9C: *A. tumefaciens*, and lane M: DNA ladder (Fast Ruler DNA Ladder, Middle Range, Fermentas, Inc., Burlington, ON). Single spore isolates AbotJE-ss1, AbotJE-ss2 and AbotJE–ss3 represent pathotype 6, while isolates SACAN-ss1, SACAN-ss2 and SACAN–ss5 represent pathotype 3.

# Chapter 4. General discussion

Clubroot is emerging as an important new disease of canola in Alberta (Tewari et al., 2005; Strelkov et al., 2007a; Strelkov et al., 2007b). Information regarding the virulence and evolutionary potential of local populations of *Plasmodiophora brassicae* is critical for the development of durable genetic resistance and effective clubroot management strategies. Recent reports suggested that local populations of the pathogen are fairly homogenous, with pathotype 3 [as classified on the differential hosts of Williams (1966)] predominant in Alberta (Strelkov et al., 2006a; Strelkov et al., 2007b). However, those studies used field populations of *P. brassicae*, which may consist of a complex mixture of pathogen genotypes (Tinggal and Webster, 1981; Jones, et al., 1982; Somé et al., 1996). The principal goals of the current research included development of an effective method to isolate single resting spores of the pathogen, characterization of the virulence of single spore-derived isolates on host differentials, and analysis of molecular diversity in these isolates.

The isolation of single resting spores of *P. brassicae* and their successful use in the inoculation of host roots is a difficult process, given the very small size of the spores and the fact that the pathogen is an obligate parasite. However, we developed a relatively simple and efficient method to isolate single resting spores of *P. brassicae* (Chapter 2). Using this method, which was based on serial dilution of spore suspensions, we isolated 25 isolates representing five populations of the pathogen from Alberta, Ontario and British Columbia. The virulence patterns of these isolates were compared on the differentials of Williams (1966) and Somé et al. (1996). Symptoms were

evaluated six weeks after inoculation and Fisher's least significant difference (LSD) ($p \leq 0.05$) was used to distinguish susceptible from resistant host reactions.

The pathotype composition of *P. brassicae* in Canada and Alberta appeared more complex when single spore isolates rather than populations of the pathogen were examined. In Alberta, at least three and possibly four pathotypes were identified among the 14 isolates tested (Chapter 2). These included pathotypes 3, 6, 8 and possibly 2, as classified on the differentials of Williams (1966), or $P_2$, $P_4$ and $P_6$ as classified on the differentials of Somé et al. (1996). In contrast, a maximum of only two pathotypes had been previously reported when populations of the pathogen were examined (Strelkov et al., 2006b; Strelkov et al., 2007b). Nevertheless, pathotype 3 or $P_2$, as classified on the differentials of Williams and Somé et al., respectively, was found to be predominant in tests of both single spore isolates and populations (Strelkov et al., 2006b; Strelkov et al., 2007b; Chapter 2). This seems to confirm previous reports that the host reaction to infrequently occurring pathotypes within a population is masked by the reaction to the predominant pathotypes (Jones et al., 1982).

As pathotype 3 was shown to be highly virulent on all canola cultivars tested from Canada (Strelkov et al., 2006b), and is also highly virulent on the spring oilseed rape differential 'Brutor' (Chapter 2), this pathotype appears to be a reasonable choice to use in clubroot-resistance screening in Alberta. However, given the occurrence of additional pathotypes of *P. brassicae* at lower frequencies, caution should be used in the development of a breeding strategy, since rare pathotypes of *P. brassicae* may quickly become predominant if susceptible host genotypes are continuously grown (Seaman et

al., 1963). Indeed, it may be desirable to introgress resistance to multiple pathotypes into canola germplasm, although this may not be practical because of space and time constraints. Nonetheless, genetically resistant cultivars will have to be utilized together with other management strategies, including crop rotation and proper sanitation, to ensure their durability.

Although very important, virulence patterns in *P. brassicae* and other pathogens do not necessarily reflect the entire extent of diversity in the pathogen populations. Indeed, the molecular analysis of diversity that we conducted as part of this research suggested that populations of *P. brassicae* from Alberta and other parts of Canada are more heterogeneous than indicated solely by their pathotype designations (Chapter 3). Analysis of DNA amplification patterns produced using arbitrary and sequence-specific (SG) primers revealed that many isolates had unique multi-locus molecular genotypes. Cluster analysis indicated that isolates obtained from the same population were more closely related than isolates obtained from different populations. This similarity between isolates from the same population suggests an important role for asexual propagation in *P. brassicae* (Buczacki, 1983; Naiki et al., 1984; Mithen and Magrath, 1992), at least under Canadian conditions. Interestingly, there was no clustering according to pathotype, which also suggests that minor changes in the pathogen genotype are sufficient to produce a new virulence pattern.

In addition, we used cleaved amplified polymorphic sequence (CAPS) analysis to compare isolates of *P. brassicae* representing two different pathotypes (specifically, pathotypes 3 and 6) (Chapter 3). A DNA fragment was amplified and sequenced, and

although it bore no significant homology to any sequences in GenBank, the presence of differential restriction sites in amplicons from the different isolates indicated that this locus was suitable for use as a CAPS marker. Digestion with the enzyme *Xmn*I revealed consistent DNA fragment patterns on agarose gels, which were specific to three pathotype 3 isolates, three pathotype 6 isolates, and host DNA. However, because all isolates representing each pathotype were obtained from the same population, it is not clear whether the CAPS marker identified is pathotype-specific or population-specific. This question will have to be resolved by testing additional isolates of pathotypes 3 and 6 that originate from different populations. Nevertheless, the usefulness of CAPS analysis for detection of specific populations or pathotypes of *P. brassicae* was demonstrated.

The current project not only provided important new information on *P. brassicae* populations from Canada, it also served to highlight additional areas for further research. The availability of single spore isolates that represent a single genotype of the pathogen will facilitate studies into the genetics of the Brassica/*P. brassicae* interaction. As summarized at the beginning of this thesis (Chapter 1), numerous models have been proposed to interpret interactions between *P. brassicae* and different Brassica hosts. Given that clubroot now appears to be endemic on canola in central Alberta (Strelkov et al., 2007a; Strelkov et al., 2007b), the interaction between the pathogen and *B. napus* canola genotypes is of particular interest. Similarly, additional work on the development of CAPS markers for the detection of specific pathotypes will be useful for monitoring the disease and spread of particular genotypes. As noted above, this would

include further validation of the pathotype-specificity of the locus identified in this study, but could also include development of additional markers, including markers effective for the identification of other pathotypes.

Collectively, the results from the current study indicate a fairly heterogeneous population of *P. brassicae* in Alberta, consisting of numerous pathotypes with varying levels of virulence on *B. napus* genotypes. This will make management of clubroot through the development of genetically resistant cultivars a challenging task. Knowledge of the pathogen populations prevalent in this region will facilitate breeding efforts. Nevertheless, successful clubroot control will require an integrated approach that combines genetic resistance with cultural, chemical and biological management strategies.

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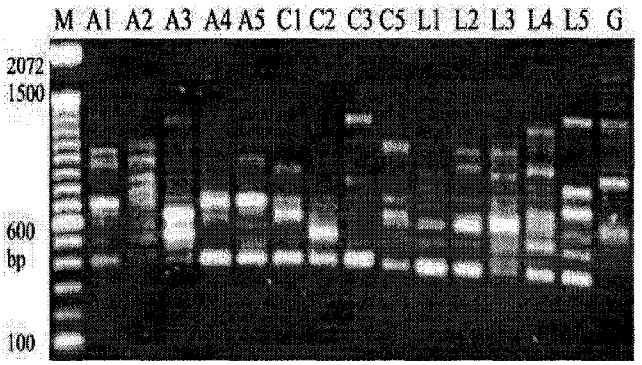
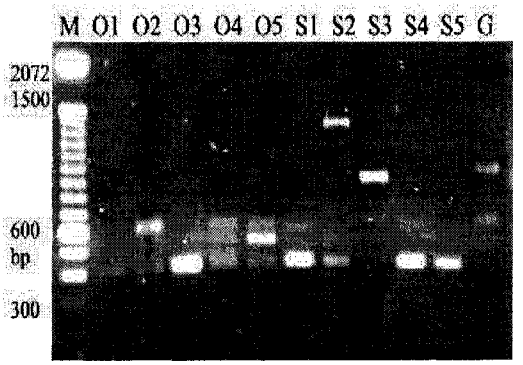
# Appendix

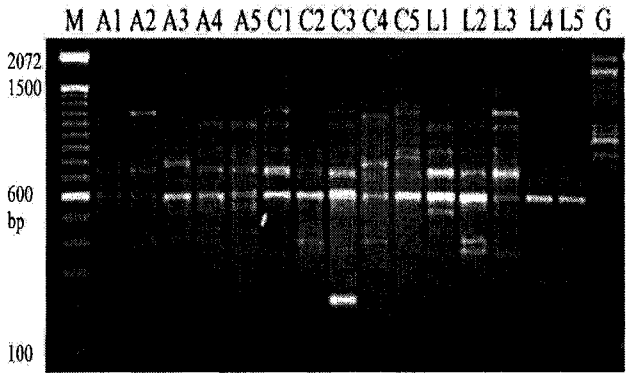
The following images show the amplicons produced with various random and sequence-generated primers (listed in Table 3-2) using genomic DNA from different single spore isolates of *Plasmodiophora brassicae* as a template. Lane M: DNA ladder (100 bp DNA Ladder, Invitrogen, Burlington, ON), A1: AbotJE-ss1, A2: AbotJE-ss2, A3: AbotJE-ss3, A4: AbotJE-ss4, A5: AbotJE-ss5, C1: CDCN-ss1, C2: CDCN-ss2, C3: CDCN-ss3, C4: CDCN-ss4, C5: CDCN-ss5, L1: Leduc-ss1, L2: Leduc-ss2, L3: Leduc-ss3, L4: Leduc-ss4, L5: Leduc-ss5, O1: ORCA-ss1, O2: ORCA-ss2, O3: ORCA-ss3, O4: ORCA, O5: ORCA-ss5, S1: SACAN-ss1, S2: SACAN-ss2, S3: SACAN-ss3, S4: SACAN-ss4, S5: SACAN-ss5, G: *Brassica rapa* var. *pekinenses* (cv. Granaat), and B: sterile distilled $H_2O$.

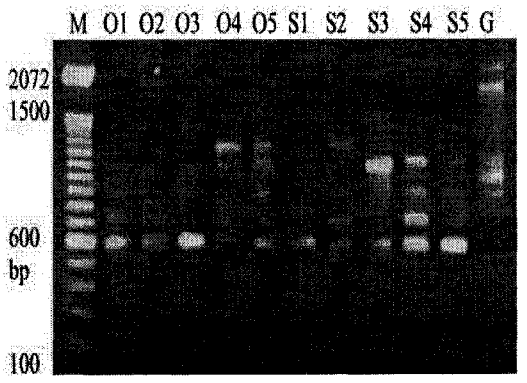
**Primer: OPA13 (CAGCACCCAC)**



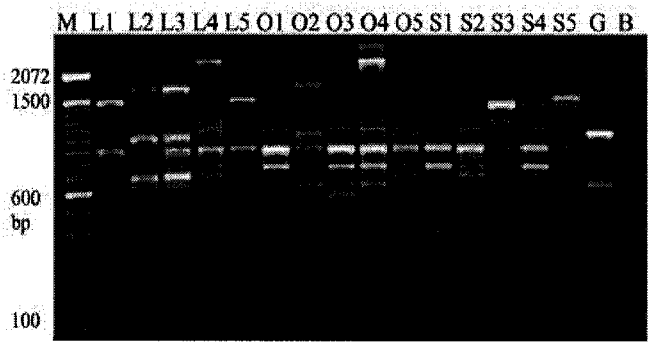



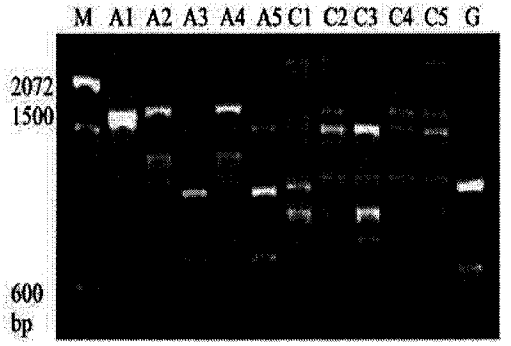

**Primer: OPJ20 (AAGCGGCCTC)**



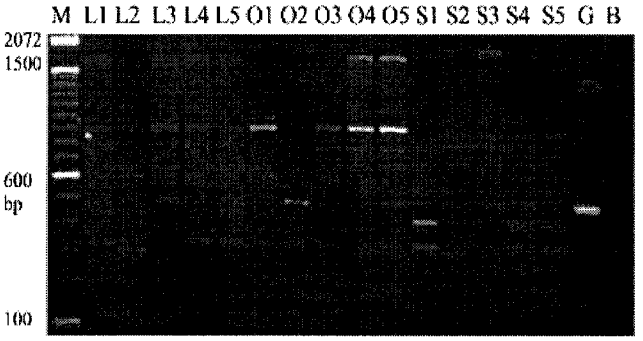



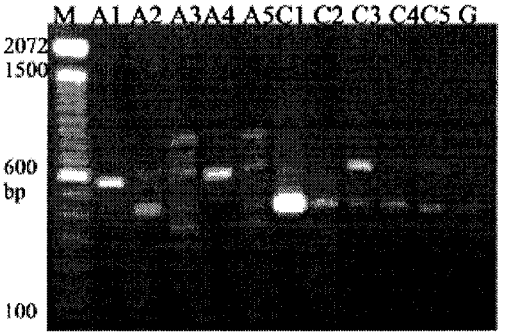
**Primer: OPK10 (GTGCAACGTG)**



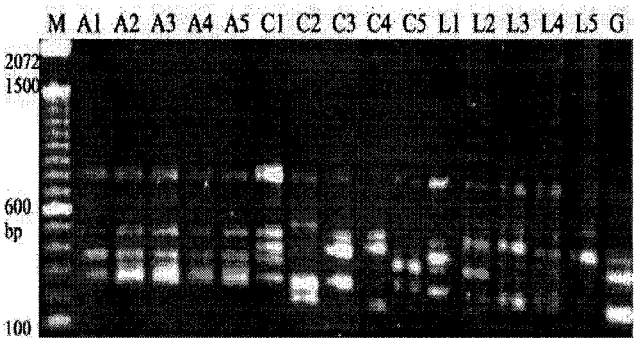



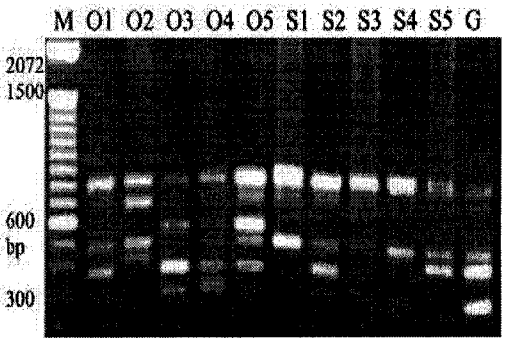

**Primer: OPK20 (GTGTCGCGAG)**



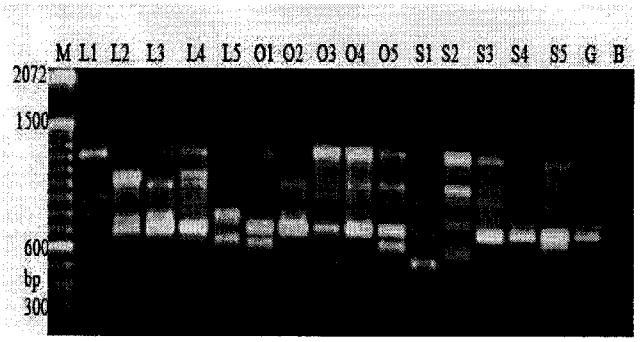



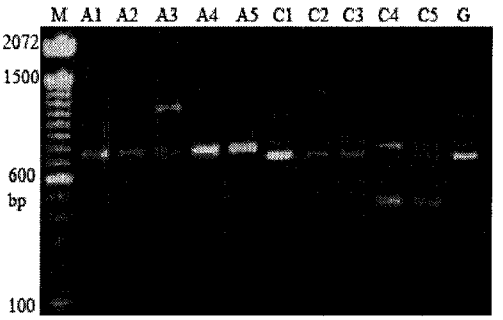

**Primer: OPL12 (GGGCGGTACT)**



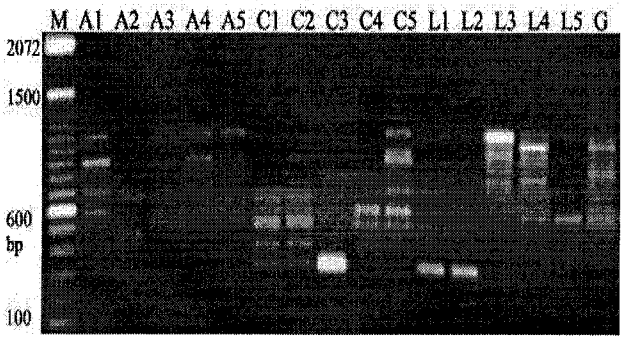



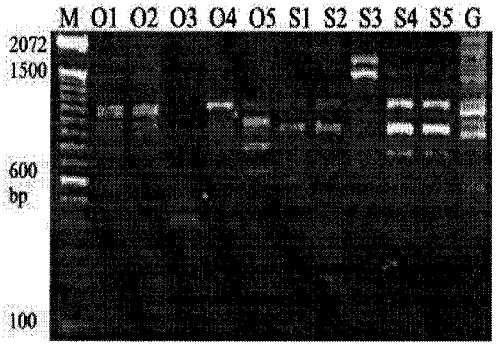
**Primer: OPL14 (GTGACAGGCT)**



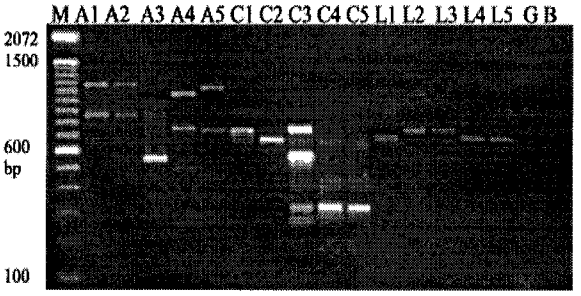



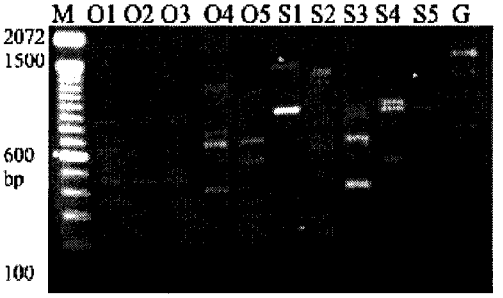

**Primer: OPL14LP (CACTGTCCGA)**



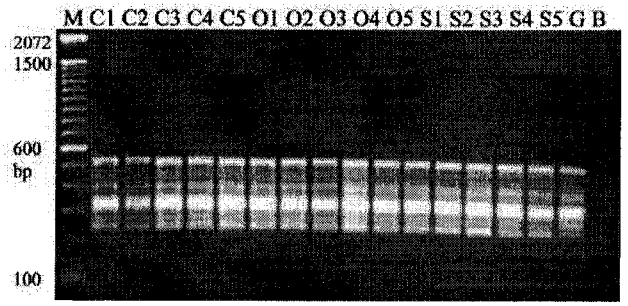



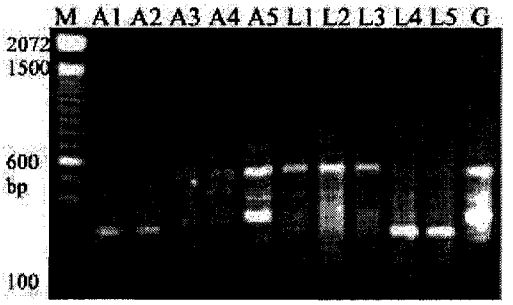

**Primer: HKB17/33 (GCATCGTCTG)**



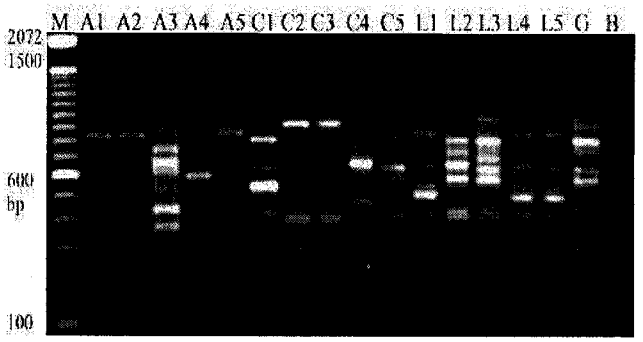



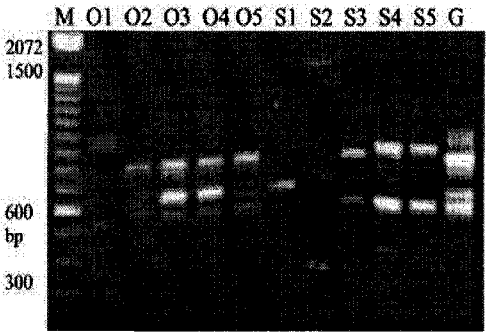
**Primer: RFM8 (GGAACAAGCTCGAAGCCATG)**




**Primer: RFM9 (AGGTGAGGAGAAATGTCTC)**




**Primer: RFM11 (TGTACGTGTCCTTCGTGGGA)**