Molecular and phenotypic characterization of populations of *Leptosphaeria maculans* and induced resistance to blackleg disease of canola by *Leptosphaeria biglobosa*

BY

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

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Molecular and phenotypic characterization of populations of *Leptosphaeria maculans* and induced Resistance to blackleg disease of canola by *Leptosphaeria biglobosa*

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ABSTRACT

Blackleg of canola (*Brassica napus* L. and *B. rapa L.*) is a disease complex of at least two fungal species: *Leptosphaeria biglobosa* n.sp., weakly virulent and assigned to the pathogenicity group 1 (PG-1) and *L. maculans* (Desmaz.) Ces. & De Not, highly virulent comprising PG-2, PG-3, or PG-4 isolates. In greenhouse tests, disease reduction was observed both on cotyledons of two *Brassica napus* cvs, Westar and Invigor 2153 when PG-1 was either pre- or co-inoculated with PG-2, PG-3, and PG-4 and on six-leaf stage plants of Westar when the lower leaves were treated with either PG-1 or salicylic acid (SA). The activity of defense-related enzymes was greatly enhanced when Westar cotyledons were inoculated first with PG-1 followed by PG-2. Field experiments in 2003 and 2004 showed decreased blackleg severity in plants inoculated with PG-1 alone or prior to PG-2. Pathogenicity tests on a total of 512 isolates collected worldwide or from a single field during a four-year period (2001 to 2004) on three differential cvs. Westar, Glacier and Quinta indicated that PG structure in Western Canada and U.S.A had remained unchanged from 1984 to 2001. They were either PG-1 or PG-2, whereas isolates from Brazil, Australia and UK were mainly PG-3 or PG-4. In 2002 and 2003, the presence of PG-3, PGT and PG-4 were detected for the first time across the Western Prairies of Canada and North Central U.S.A. Co-existence of five PG types in a single field in Manitoba, Canada suggested the possibility of the occurrence of sexual recombination in the pathogen. Genetic diversity and population structure among six geographic populations were determined using the sequence-related amplified polymorphism (SRAP) technique. According to the number of polymorphic loci, values of heterozygosity (*H*) and rate of gene flow, the populations from Western Canada and

North Dakota showed a higher genetic diversity than populations from Australia, United Kingdom. Avirulent PG-1 isolates were clustered into a separate lineage from PG-2, PG-3, PG-4 and PGT populations. The analysis of molecular variance revealed that high genetic variation was found among isolates within populations regardless of the origin and pathogenicity.

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FOREWORD

This thesis follows the manuscript style outlined by the Department of Plant Science, University of Manitoba. The thesis is presented as three manuscripts, each containing an abstract, introduction, materials and methods, results, and discussion. A general review of the literature precedes the manuscripts, and a general discussion follows the manuscripts.

CHAPTER 1

GENERAL INTRODUCTION

Canada is the second largest producer of canola (*Brassica napus* L. and *B. rapa* L.) in the world and the largest exporting country. Canola is the second largest crop after wheat in terms of farm cash receipts in the Western Canada and accounts for about 67 percent of total oilseed production in Canada, of which production in Saskatchewan, Alberta and Manitoba represents 44%, 32% and 22%, respectively (Beckman, 2005). Blackleg of canola, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & De Not, is an economically important fungal disease worldwide and occurs commonly in Canada (Juska et al., 1997a).

In Ontario, 60-100% of winter rape fields and 27-31% of spring rape fields were found affected by blackleg stem cankers in the late 1980s (Hall et al., 1993), whereas in Western Canada, blackleg has been responsible for yield losses estimated to be as much as 50% in individual fields (Gugel and Petrie, 1992). Currently blackleg of canola in Western Canada is controlled by using host resistance and crop rotation (Howlett, 2004). However, care should be taken about the potential risk of disease outbreak caused by new strains recently emerged across the Western Prairies of Canada and the North Central USA (Bradley et al., 2005; Chen and Fernando, 2005; Fernando and Chen, 2003). With the significant increase of canola cultivation around the world, concerns regarding biology, population genetics and disease control of blackleg in canola are increasing.

Induced resistance to disease in plants has been known for many years but little research on mechanisms of induced resistance was done until about a decade ago. There has been an increasing interest in induced resistance as a new, environmentally friendly

means of disease control, as well as a model for the study of the genes involved in host defense and signal pathways that control them. This increased interest has led to a few reports of induced resistance applied to the *Brassica*-blackleg pathosystem. After pre-treatment of the first true leaves of rapeseed (*B. napus*) plants of cv. Bristol with menadione sodium bisilphite (MSB), salicylic acid (SA) or SA-dependent PR-1 induction agent benzothiadiazole (BTH), locally and systemically induced resistance to *Leptosphaeria maculans* was observed (Borges et al., 2003).

In addition to abiotic chemical compounds, biotic elicitors such as necrotrophic fungi and avirulent strains were found to be capable of triggering a hypersensitive reaction (HR) to disease in plants (Conrath et al., 2002). In leaves and stems of *Brassica juncea* and *B. rapa,* phytotoxins brassinin and cyclobrassinin were produced after infection by necrotrophic fungus *L. maculans* (Pedras et al., 2002). After inoculation with an avirulent isolate of *L. maculans*, rapid necrosis of guard cells in the leaves of Indian mustard (*B. juncea*) was observed (Chen and Howlett, 1996). Similarly, a HR reaction took place on cotyledons of *B. napus* with pre-inoculation of an avirulent isolate of *L. maculans* (Roussel et al., 1999).

An avirulent isolate-triggered HR is a localized defense reaction. Systemic acquired resistance (SAR) can provide the plant with longer-lasting protection and act against a broader spectrum of pathogens compared to localized defense HR. It will be more useful if SAR can be activated in canola plants with an avirulent isolate (s) against the infection caused by virulent isolates. Mahuku et al. (1996) first found that lesion size of blackleg on cotyledons and stems of canola was significantly reduced when a weakly virulent

(WV) isolate was introduced 64 h prior to the infection by a highly virulent (HV) isolate. However, the mechanism involved and performance under field conditions were not investigated. Bohman (2001) stated that resistance in *Arabidopsis thaliana* against *L. maculans* is independent of SA, jasmonic acid/ethylene pathway but partially dependent on synthesis of the phytoalexin camalexin, but this needs further confirmation in *Brassica* crops.

The pathogenicity group (PG) test is a quick and reliable method to investigate the population structure with respect to virulence of the blackleg pathogen. According to the gene for gene hypothesis, at least four PGs of the blackleg isolates can be identified on three *B. napus* differential cultivars: Westar (with no known resistance genes), Glacier (*Rlm2* and *Rlm3* resistance genes) and Quinta (*Rlm1*and *Rlm4* linked resistance genes) (Koch et al., 1991; Mengistu et al., 1991). Historically, PG-1 and PG-2 were predominant isolates in North America and PG-3 and PG-4 were prevalent in Australia and Europe (West et al., 2001). However, PG structure in the North American blackleg populations is changing (Chen and Fernando, 2005; Fernando and Chen, 2003) and it is valuable for us to study this change. The importance of PG assessment is to provide useful information for breeders and farmers in combating blackleg epidemics. Monitoring of PGs can provide information on the potential risk of new virulent isolates in specific locations where they might be absent. It also helps with choice of cultivars that condition specific resistance to corresponding virulent isolates. In some tests additional cultivars have been incorporated as differentials in order to combine the genetic correspondence between resistance and avirulence interactions (Badawy et al., 1991; Keri, 1999; Kuswinanti et al.,

1995; Kutcher et al., 1993). The problem is that results obtained in various studies are not always consistent or comparable because of different host cultivars used.

Rational disease control depends on an understanding of population genetics of pathogens because significant crop loss and ecological impact result from disease epidemics caused by infection from an entire pathogen population rather than single lesions on crop plants. Population genetics focuses on the processes that lead to genetic change or evolution over time and space (Hartl and Clark, 1989). Molecular techniques are powerful tools in assessing mutation, genetic drift, gene flow, mating systems and natural selection. Random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) were already applied to analyze phylogenetic distinctions between *L. biglobosa* (avirulent group) and *L. maculans* (virulent groups) (Goodwin and Annis, 1991; Johnson and Lewis, 1990; Koch et al., 1991; Schafer and Wostemeyer, 1992; Taylor and Borgmann, 1994; Voigt et al., 1998). Amplified fragment length polymorphism (AFLP) was also used to construct a phenogram of blackleg populations originated from different regions (Pongam et al., 1999; Purwantara et al., 2000). There is little information on genetic diversity and population structure among and within North American populations, especially genetic relationships of single population with all types of PGs of *L. maculans* detected recently in Western Canada with other populations from different regions.

The objectives of this study were as follows:

1) To investigate induced resistance against virulent pathogenicity groups of blackleg triggered by an avirulent *L. biglobosa* isolate.
2) To determine and monitor the pathogenicity differences of *L. maculans* / *L. biglobosa* isolates from different origins.
3) To analyze and compare the genetic variation and population structure of isolates originating from different locations and pathogenicity groups using molecular markers.

CHAPTER 2

LITERATURE REVIEW

2.1. Host

2.1.1. The *Brassica* genus

The family *Brassicaceae* (*Cruciferae*) encompasses nearly 340 genera and more than 3350 species (Johnston et al., 2005). The genus *Brassica* in *Brassicaceae* contains about 100 species. Among them many are important agricultural plants cultivated as vegetables, fodder or sources of oils and condiments. *Brassica rapa* L. or *B. campesteris* L. sensuato (n=10, AA genome) and *B. napus* L. (n=19, AC genomes) are conventionally grouped as rapeseed/canola (Juska et al., 1997b), cultivated mainly for the extraction of vegetable oil. *Brassica juncea* (L.) Czern (n=18, AB genomes), *B. carinata* (A.) Braun (n=17, BC genomes) and *B. nigra* (L.) Koch (n=8, BB genome) are placed in the mustard group for the production of mustard oil and commercial spices. *Brassica oleracea* L. (n=9, CC genome) is cultivated for vegetable and fodder under the common names of cabbage, cauliflower, broccoli, brussels sprouts and marrowstem kale etc.

In Canada, *B. napus, B. rapa, B. juncea, B. oleracea, and B. nigra* are cultivated either as vegetables, vegetable oil, fodder or condiment (Kimber and McGregor, 1995).

2.1.2. Rapeseed/canola

Rapeseed (*Brassica napus* and *B. rapa*) is among the oldest cultivated plants. Rapeseed production has a long history in China. For example, the Chinese word for rapeseed was first recorded ca. 2500 years ago, and the oldest archaeological discoveries may date back as far as to ca. 5000 BC. Historically, *B. rapa* seems to have the widest distribution, growing wild from the west Mediterranean to central Asian countries such as China and Korea at least 2000 years ago. This widespread distribution allowed domestication to occur in several places independently with the primary center of diversity in the Himalayan region. Wild forms of *Brassica napus* ancestral species are unknown. It is presumed that *B. napus,* an allotetraploid bearing A and C genomes (AACC), originated from crosses between diploid *Brassica oleracea* L. (CC) and *B. rapa* L. (AA) in the Mediterranean area. Since *B. napus* contains doubled chromosome numbers from two parental species, it is called an amphidiploid. Production of oilseed *B. napus* probably started in Europe during the Middle-Ages and then it was introduced to Asia during the 19th century. The present Chinese and Japanese germplasm was developed by crossing European *B. napus* with different indigenous *B. rapa* cultivars (Sovero, 1993).

Brassica rapa is often called turnip rape and *B. napus* is called Swede rape for the differentiation of two species. Both contain spring and winter types that are distinguished by the vernalization requirements. Winter type varieties are mainly grown in Europe and China, and spring type varieties in areas such as Australia and Canada, where *B. napus* is in the medium to high rainfall areas and *B. rapa* is in the drier, shorter season areas. In Canada, *B. rapa* is grown in northern Saskatchewan and Alberta, where it matures in 80

to 85 days while *B. napus* requires a minimum of 100 growing days to mature. As herbicide tolerant *B. rapa* cultivars are not available, the increased cultivation of *B. napus* and the decline in *B. rapa* to near extinction in Canada have taken place since 1995 when herbicide resistant canola variety first released. Rapeseed/canola oil is the third largest global vegetable oil source, after soybean and palm oil (Beckman, 2005). During the past twenty years, it has surpassed peanut, cottonseed, and most recently, sunflower, in worldwide production. This is almost entirely triggered by the plant breeding work initiated in Canada in the 1950s and 1960s which greatly reduced the levels of two anti-nutritional compounds, erucic acid in the oil and glucosinolates in the meal, creating a new, high-value oil and protein crop known as canola in Canada and the United States (Juska et al., 1997b).

Canola, a combination of two words Canadian and oil, is the Canadian Canola Association trademark which refers to any rapeseed with less than 2% erucic acid in the oil and less than 30 μmol of any mix of four major aliphatic glucosinolates per gram of meal. Rapeseed cultivation began in 1942 in Western Canada as a source of marine lubricants for the allied war effort. In 1974, Dr. Baldur Stefansson, a University of Manitoba plant breeder, developed the first canola-quality (double low) cultivar "Tower". Since then canola has been produced extensively in Canada, United States, Australia, Asia and Europe. Canola has gained acceptance worldwide, becoming a global crop.

2.1.3. Economic value of rapeseed/canola

Canola tends to be a higher value crop than cereals, providing 80,000 Canadian farmers with an alternative for market diversification. As the largest cash crop after wheat, canola is mainly grown in Saskatchewan (44% of total growing area), Alberta (32%), and Manitoba (22%) with a total growing area of 4 to 5 million hectares depending on year. Two reasons make canola primarily cultivated in Western Canada: 1) canola is a cool-season crop. The cool night temperatures on the prairies allow it to recover from hot days, and the amount of rainfall is also limited in this region; and 2) growing and harvesting canola utilize the same machinery for growing cereal crops (wheat, oats and barley), which enables farmers to switch to canola production without large cash expenditures.

In 2005 the yield of canola in Western Canada was 8.4 million tons (Mt) and farm cash receipts were $3.2 billion in total. Canola products are sold both domestically and abroad. In the early 1990s, about half of the average 3.9 Mt canola seed crop was crushed domestically and the other half was exported. Starting in 1993, increased production of seed led to increased seed exports which peaked at 4.9 Mt in 1999. Historically, Japan is the major market for canola seed followed from the early 2000s by Mexico, China and the United States (Beckman, 2005).

Over the past decade, canola had positioned itself as a healthy vegetable oil, low in saturated fats, and good for human health. During the early 1990s, production of biofuel began to expand rapidly in Europe and North America as people sought to reduce their dependence on fossil fuels. In 2003, the Biodiesel Association of Canada was formed to promote the development of a biodiesel industry in Canada. Biodiesel is composed of

fatty acid methyl esters (FAME), mainly used as an automotive fuel or as heating oil, pure or as a fuel additive derived from a renewable resource such as plants that can be grown domestically. It can be blended from many different oils and fats such as soy, canola, tallow, mustard, and restaurant greases. In 2004, the production of biodiesel in European Union was nearly 2 million tons, with an increase of about 25% per year. Hence canola/rapeseed production is expected to increase with increasing demand for biodiesel in the European Union and North America.

2.2. Pathogen — *Leptosphaeria maculans*

2.2.1. Taxonomy of *Leptosphaeria maculans*

Leptosphaeria maculans (Desmaz.) Ces. & De Not. (anamorph *Phoma lingam* Tode ex Fr.), an ascomycetic fungus, is a pathogen causing seedling death, stem canker (also termed blackleg) and early senescence on rapeseed/canola plants. This pathogen is a hemibiotrophic parasite, i.e. a facultative necrotroph for short periods at the early and end of the season to produce leaf spot and stem canker by toxins and degradation enzymes, and a biotroph during endophytic latent phase from leaf lesion to stem lesion (cryptic intercellular growth in leaf and stem). Both the sexual and asexual spores of *L. maculans* are pathogenic.

The pathogen was first described by Tode in 1791 on dead stems of red cabbage (*B. oleracea*) and it was named *Sphaeria lingam*. In 1849, Desmazieres found the same fungus on living plants and transferred it to the genus *Phoma* (*Phoma lingam)*. In 1956 H. C. Smith first discovered the perfect (sexual) stage of *P. lingam* and named it

Leptosphaeria napi. In 1964, H. C. Smith and B. C. Sutton changed the name to *L. maculans* (Smith and Sutton, 1964). The complete taxonomy of *L. maculans* is Kingdom Fungi, Phylum Ascomycota, Subphylum Pezizomycotina, Class Dothideomycetes (Loculoascomycetes), Order Pleosporales, Family Leptosphaeriaceae, Genus *Leptosphaeria* and species *maculans* (Rouxel and Balesdent, 2005). In 2001, *Leptosphaeria biglobosa* was assigned for weakly virulent isolates from *L. maculans* (Shoemaker and Brun, 2001).

2.2.2. Host Range

Leptosphaeria maculans has been found to be strictly a pathogen of crucifers, mainly of *Brassica* crops such as *B. napus*, *B. rapa, B. juncea, B. oleracea*, etc. Numerous wild crucifers weed species like *Thlaspi* spp., *Sisymbrium* spp., *Descurainia* spp., *Capsella* spp., *Lepidium* spp., *Raphanus* spp., and *Erysimum* spp. can also be infected by the pathogen (Johnson and Lewis, 1994; William and Fitt, 1999).

2.2.3. Genome of the species

Leptosphaeria maculans is a strictly heterothallic, haploid and outcrossing fungus (Cozijinsen and Howlett, 2003). Heterothallic here means that there are two mating types with alternate forms of mating type (*MAT*) locus required for sexual reproduction (Somda et al., 1997). Haploid here refers to a single set of 16 unpaired chromosomes with a size range from 0.7 to 3.2 Million base pairs (Mb) each, indicating that the genome size of *L. maculans* is smaller (about 34 Mb, thirty times smaller than that of *Brassicas* species)

and predicted to encode ca. 10,000 genes (Howlett, 2004). Some of the chromosomes are of a dispensable "B" type (Leclair et al., 1996). The genome contains two linear plasmids, 9 and 10 kb, respectively but no function of the plasmids has been revealed (Lim and Howlett, 1994). A high level of dispersed repetitive DNA sequences in *L. maculans* genome was observed and chromosomal length polymorphism was found using molecular markers bound to every chromosome and pulsed field gel electrophoresis (Plummer and Howlett, 1995). The linkage groups in the *L. maculans* genome have been investigated with clamped homogeneous electrical field (CHEF) electrophoresis and AFLP molecular markers (Kuhn et al, 2006). According to the analysis of 126 expressed sequence tags (ESTs) isolated from hyphae, eleven protein coding genes such as *znr*1 and *maa*1, etc. have been identified so far in the *L. maculans* genome but only six genes are deposited in the GenBank database (Idnurm et al., 2003; Idnurm and Howlett, 2001). Five of them may have possible roles in infection such as ABC transporters, cellulases and endopolygalacturonases (Howlett et al., 2001).

Genetic and physical maps of *L. maculans* will be useful for genetic and functional genomic studies. Because of the high degree of dispersed repetitive DNA (Cozijnsen et al., 2000), chromosome walking and AFLP markers may be difficult in this fungus because many markers will map to every chromosome (Howlett et al., 2001).

2.3. Blackleg disease

2.3.1. Impact of blackleg on rapeseed/canola industry

Blackleg disease caused by *Leptosphaeria maculans* is of economic importance in the main rapeseed/canola-growing areas except in China. China has the largest rapeseed/canola production area (7.2 million hectares in 2003) in the world. In the early 1970s the most severe epidemics of blackleg limited the expansion of the emerging rapeseed industry in Australia until the recent development of resistant cultivars (West et al., 2001). A virulent strain of the fungus was discovered on spring rapeseed in Saskatchewan, Canada in 1975 and it spread to most canola-growing provinces very quickly, often causing significant economic losses. Several factors helped blackleg to become epidemic across the country during 1980s. For instance, large scale monoculture of highly susceptible cultivars such as "Westar" for high yield lasted at least 8 years (up to 95% of the rapeseed acreage) since 1984 when it was commercially released. In 1987 the cancellation of import quotas of rapeseed into Japan enhanced the growth of canola in Canada. Decline of wheat prices on the world market led to a rapid conversion to rapeseed cultivation. A lack of rapeseed cultivation and experience with its pathogens encouraged farmers to shorten rotations for increased income. All those combined elements made the nationwide outbreak and epidemic of blackleg in canola as a "normal agricultural accident" in Canada (Juska et al., 1997a).

In Ontario, 60-100% of winter rape fields and 27-31% of spring rape fields were found affected by stem cankers in the late 1980s (Hall et al., 1993), whereas in Western Canada, blackleg has been responsible for yield losses estimated to be as much as 50% in

individual fields (Gugel and Petrie, 1992). In the UK, blackleg was first observed on susceptible winter oilseed rape cultivars in 1977 and has become a damaging disease with yield loss in excess of £20 million per season from 1993 to 2001 (Huang et al., 2003). In France, blackleg was reported in the 1950s and has been responsible for mean yield losses of 5-20% (Aubertot et al., 2004). Losses as high as 50% have occurred in Germany (Gugel and Petrie, 1992). Blackleg was first detected in North Dakota (USA) in 1991 and 75-90% losses have been reported (Lamey and Hershman, 1993).

2.3.2. Disease Symptoms

Leptosphaeria maculans can infect all parts of the plant at any stage of crop development. When the disease is seed borne, seedlings of the susceptible cultivars may be killed at emergence or shortly thereafter. However, leaf and stem symptoms are more often observed. Foliar lesions called "phoma leaf spot" are usually the first visible symptoms. The leaf spots are a tan or buff color and round to irregular in shape. The center of leaf spots normally bears tiny black spots (pycnidia) (Guo and Fernando, 2005).

Secondary symptoms are seen either on the upper stem or basal stem. "Phoma stem lesion" is a term normally used to refer to upper stem lesions, which are white to gray in color and shallow with little or no girdling of the stem. Phoma stem lesions usually do not appear until mid season. But if they appear at a relatively early stage, yield losses have been observed in Australia, Canada, Europe, and especially in Poland (Hall et al., 1993; Jedryczka et al., 1999).

Lesions at stem base appear at the end of growing season and are called either blackleg, crown canker, stem (basal) canker or collar rot (West et al., 2001). Stem cankers are dark gray to black in color and have strict brown margins. They usually become sunken and may girdle the stem. Pycnidia can be seen on stem lesions originating from leaf lesions early in the season. Stem cankers cause premature dying of the plant and may also result in lodging. Pods and seeds also may be infected. Infected seeds may be shriveled and gray in color, resulting in seed loss.

2.3.3. Disease cycle

Leptosphaeria maculans overwinters as a saprophyte either on rapeseed/canola stubble in the form of pycnidia and pseudothecia (Williams, 1992), or on seed coat as mycelia or pycnidia (Hall, 1992 and Keri, 1999). Blackleg-infested stubble is a major source of inoculum for subsequent spread from one season to next and from one area to another until it is decomposed. Large pieces of stubble are found to take up to 4 years or longer to break down during a series of dry seasons (Guo et al., 2005).

Two opposite mating types of *L. maculans* isolates colonizing debris can cross to produce sexual fruiting bodies – pseudothecia — at any time (Cozijinsen and Howlett, 2003). Mature pseudothecia produce and release ascospores over an extended period of time under favorable conditions. In Western Canada pseudothecia can be produced on stubble and ascospores released as early as May, nine months after harvest (Petrie, 1995). Released ascospores can be airborne for long distance dispersal (1 to 2 km, maximum up to 10 km) (Gladders and Musa, 1980; Keri, 1999). After landing on cotyledons or leaves,

ascospores germinate and penetrate the plants via stomata or wounds without the aid of specialized infection structures such as appressoria. The fungus then grows into intercellular spaces between mesophyll and causes leaf lesions in a few days. The fungus colonizing young leaves develops an endophytic symptomless growth from the leaf spots down the petiole and finally invades and kills cells of the stem cortex, resulting in basal stem canker (Rouxel and Balesdent, 2005). The earlier the infection of the seedling stage occurs, the greater the likelihood of basal stem canker development and more severe the yield loss (West et al., 1999).

In Western Canada, asexual spores called pycnidiospores from pycnidia (Single: pycnidium) were found to be primary inoculum as well (Guo, 2003). During the growing season, pycnidiospores released from leaf lesions can spread short distances within a field, mainly by rain splash within 1 m, causing secondary infections on nearby plants.

Regional dispersal of the disease occurs through the movement of infected seed. When blackleg-infested seed is sown, the seedlings may develop cotyledon, leaf or stem infections. In Canada the incidence of infected oilseed rape seed at sowing was found to be correlated with the incidence of infected plants with stem canker and the incidence of infected seed at harvest (Hall et al., 1996). Seedling infection arises either from infected seed or from airborne spores. Seedling infection derived from infected seed can be prevented by using a fungicide seed treatment, but this treatment will not protect the seedling from infection by airborne spores. Once leaves are infected, the fungus can move systemically in the plant's vascular tissue to the stem base.

Infections on cotyledons and leaves early in the season produce the most damaging stem cankers. Stem cankers girdle the stem base, preventing the flow of water up the stem and often result in lodging of the crop. Infections occurring beyond the six-leaf stage cause less damage. Pod infection can result in infected seed, which serves as another source of inoculum for future infestation (Hall, 1992).

2.3.4. Disease epidemiology

In Australia, Canada and Europe blackleg disease in rapeseed/canola is generally initiated by airborne ascospores and further transmitted by rain-splashed pycnidiospores. Blackleg disease is usually considered monocyclic because secondary cycles by pycnidiospores produced on infected plants do not affect yield significantly (Hall, 1992; West et al., 2001). Epidemics of this disease result from the interaction among three elements: virulence and amount of *Leptosphaeria maculans* inoculum, cultivar susceptibility and density, and favorable environmental conditions including temperature, humidity and soil type.

In Western Canada blackleg disease varies with growth stage and plant vigor but most yield loss results from stem cankers, which develop principally by infection of young plants from the cotyledon to 6^{th}-leaf stages, rather than secondary infection caused by pycnidiospores during the late season (Hall, 1992). The first six leaves are found to be more susceptible to infections than those produced later, but symptom expression on the first six leaves is less severe than on aging host tissue (Badawy et al., 1991). On the aging leaves, even non-aggressive isolates can cause severe symptoms with heavy sporulation

(Badawy et al., 1992). These differences in symptom expression could explain why correlations between incidence of leaf lesions and severity of stem cankers are not always apparent (Schramm and Hoffmann, 1991).

Temperature influences the germination of spores, pathogen infection, length of incubation period from penetration to appearance of lesions and the rate of growth of the pathogen down the leaf petioles into stems. Under controlled environment conditions, the production of leaf lesions derived from ascospores was found to require 5 days at 20°C and 2 weeks at 8°C (Biddulph et al., 1999), and the time between the appearances from leaf lesions to stem lesions is 77 days at 18°C and 175 days at 3°C (Hammond et al., 1985). With high temperatures, severity of symptom on cotyledons, leaves and stems appears to be promoted. Badawy et al. (1992) noticed that incompatible reactions (small necrotic lesions) on the cotyledons inoculated with avirulent isolates at 18°C changed to completely compatible (enlarged lesions) at 27°C. This supports a theory that resistance genes in young plants may be temperature sensitive, producing more disease at 24°C than at 14°C (West et al., 2001). The symptoms of phoma stem canker were found much less severe at 12°C than at 18°C. In Western Canada, high summer temperatures may lead to severe epidemics of blackleg.

Pseudothecia maturation depends on both temperature and wetness, with an optimum at 14-15°C (Petrie, 1994). In Western Canada, pseudothecia usually form 9-10 months after harvest on stand stubble because subzero winter temperatures delay the pseudothecia maturation (Kharbanda, 1993). The period of ascospore release varies between regions but usually coincides with the presence of young, susceptible plants and

high moisture from rainfall or heavy dews (West et al., 2001). The ascospore release period can last 3-4 months, depending on the environmental conditions, or longer with a peak usually occurring 1-2 months after its onset (Guo and Fernando, 2005). After ascospore release, a minimum wetness period of 8 h at 4-28°C is required for ascospores to germinate and produce hyphae to penetrate plant cells, with optimum of 15-20°C and 48 h of wetness (Biddulph et al., 1999).

Survival of *L. maculans* on rapeseed/canola stubble plays an important role in stem canker epidemics as pycnidiospores and ascospores liberated from infected residues can both serve as primary inoculum for initiating infection of new canola crop in spring (Guo, 2003). Weather conditions and cultural practices affect the persistence of *L. maculans* on infected residues. The rate of degradation of residues depends on wetness and soil temperature, with pathogen survival favored by dry summers and cold winters. In Western Canada infected canola stubble on the soil surface continues to discharge ascospores up to 3-5 years because winters are very cold and summers are dry and hot (Petrie, 1995). Survival of the pathogen on canola stubble for 3-5 years greatly exceeds the mean 3 year length of rotations away from canola followed by farmers. In Western Australia, infected canola debris do not degrade in the hot, dry summers, and *L. maculans* can survive on the debris for up to 4 years (West et al., 2001). In the UK, the climate is mild and wet, and rapeseed residues generally decompose within 2 years (West et al., 1999).

Physical and wind damage could be other factors increasing blackleg severity. Hammond et al. (1985) noticed that pycnidiospores were unlikely to infect intact leaves

in greenhouse unless leaves, petioles and stems were wounded. Therefore wind and hail which are commonly observed in Western Canada could enhance disease infection in the field.

2.3.5. Disease control

Successful disease control requires a thorough knowledge of the causal agent and the disease cycle, host-pathogen interactions in relation to environment factors, and cost. Management practices of blackleg in canola include: cultural, physical and chemical, biological control, etc.

A) Cultural control

1) Crop rotation

Crop rotation is one of the oldest and most effective cultural control strategies. It is a management tool to reduce certain pathogen populations to a level where they will have little or no economic impact when the crop is grown again. The principle of crop rotation is to plan the order of specific crops planted on the same field so that decomposition of infected stubble and a reduction in the viability of pathogen survival structure will take place. The pathogen's ability to produce inoculum as a potential source of a disease will be reduced or eliminated (Bockus and Shroyer, 1998). With blackleg, 3-5 years of crop rotation are usually recommended to decrease the risk of infection by ascospores released from colonized stubble (Guo et al., 2005). However, Morrall et al (1999) found in their

survey, that rotation length did not affect blackleg incidence because other factors, such as the presence of volunteer canola, might contribute to disease incidence.

2) Tillage

Tillage raises the soil temperature and increases soil aeration, leading to faster decomposition of stubble. Tillage systems, on the basis of the amount of surface residues, can broadly be classified into three categories: conventional tillage, reduced tillage and conservation tillage (Workneh and Yang, 2000).

Infested residue is the usual source of primary inoculum (i.e. pycnidiospores and ascospores). Appropriate tillage and crop rotation could effectively reduce stem canker of blackleg (Guo et al., 2005). Appropriate residue management such as burying infected debris by tillage before autumn has proven to be a good control for blackleg (West et al., 2001). The greatest problem with infected residues in Western Canada is zero tillage following canola, which increases the amount of infected stubble remaining on the soil surface.

3) Cultivar resistance

Breeding of resistant cultivars must be given a high priority to cope with the great variability of *Leptosphaeria maculans* populations as it is the most environmentally friendly and reliable method of disease control. The genetic basis of resistance in adult and seedling plants may differ (Rimmer and vandenBerg, 1992) even though in many cases it is identical (Li and Cowling, 2003). Adult resistance, normally conferred by quantitative trait loci, stops the development of stem canker or phoma stem lesions, and seedling resistance, normally conferred by dominant loci, retards the growth of pathogen

in the leaf lamina or spread from the leaves to the petioles, the hypocotyls or to stems. In other words, dominant resistance aims at preventing the fungus from entering the plant. The evaluation of genetic resistance should be done not only by inoculating the wounded cotyledons in greenhouse but by challenging adult plants in the field with high disease pressure. In Canada, *B. napus* cultivars were found to be susceptible to *L. maculans* in the 1980s, but currently grown cultivars are resistant or moderately resistant to *L. maculans.*

4) Other cultural methods

Late sowing dates in Australia and Europe, and early sowing dates in England and Western Australia have been reported to be associated with lower levels of stem canker development. However, sowing date was found to have no overall effect on stem canker development on spring canola in Western Canada because ascospores are released throughout the growing season (Kharbanda and Tewari, 1996).

High nitrogen concentrations are often found to be capable of increasing the susceptibility of plants to disease. However, several Canadian studies reported that the amount of nitrogen fertilizer did not affect the development of stem canker (Aubertot et al., 2004; Hall et al., 1993).

B) Chemical control

Application of fungicides depends on high yield potential of crop, high levels of inoculum, or low resistance of cultivars. Seed treatments or soil or foliar sprays with fungicides are used to control blackleg in different regions. In Canada, seed treatment commenced in 1978 and was recommended even if blackleg was not found in seed lots

(Guo, 2003). The fungicides Cloak (mix of carbathiin:thiram:lindane), Rovral ST (mix of iprodione:lindane) and Vitavax RS powder (mix of carbathiin:thiram:lindane) are often used in Canada for seed dressing. Tilt 250 (propiconazole), registered in 1994 for blackleg control in Canada, has been recommended for application during the canola rosette stage, but it does not completely control the disease. The timing of fungicide sprays in the field is crucial as fungicides are effective for only limited periods of time, generally 2-3 weeks. In addition, once *L. maculans* infects the stem it can no longer be controlled by chemicals. In Western Canada, there is a need to prevent the pathogen from reaching the stem by fungicide application in early spring when possible. In England, control of phoma stem canker depends heavily on the use of fungicide since resistance in current cultivars is not sufficient for complete control (West et al., 2002).

C) Biological control

Biological control is an environmentally-friendly method of plant disease management. In past decades, attempt to develop a biological agent active against *L. maculans* has been conducted in several labs. But no effective agent is available for use at present (Beatty and Jensen, 2002). However, interest in the development of biological control against *L. maculans* is continuously increasing because of public concern for fungicide use and environmental safety. Biological agents under investigation for the control of blackleg include many genera of bacteria and filamentous fungi. Two fungal species, *Cyathus striatus* and *C. olla,* have been found to be capable of biological control because they decrease the stubble food base on which *L. maculans* survives (West et al., 2001).

Pseudophomins B, a cyclic lipodepsipeptide isolated from *Pseudomonas fluorescens* strain BRG100, showed high antifungal activity against *L. maculans* (Pedras et al., 2003). A bacterium *Paenibacillus polymyxa* strain PKB1 was investigated as antifungal agent because it produces two fusaricidin peptides that inhibit the growth of *L. maculans* in culture, on leaves, on stems and on stubble (West et al., 2001). The function of this bacterium was not affected by fungicides and herbicides approved for use on canola in Canada, so it has a great potential to be directly used as a biological agent. Recently, a few *Pseudomonas* and *Bacillus* bacteria strains isolated from canola phyllosphere and rhizosphere were found to be positive in suppression of blackleg due to the production of fungitoxic antibiotics like pyrrolnitrin, phenazine and zwittermicin (Ramarathanam and Fernando, 2006).

Suppression of virulent strains of *L. maculans* was found after inoculating with weakly virulent strains (Mahuku et al., 1996; Petrie, 1982). Li and Barbetti et al. (2006) reported that the collapse of resistance in an Australian cultivar "Surpass 400 could be prevented by co-inoculation of pycnidiospores of an avirulent strain with highly virulent strains. The mechanism involved was due to a hypersensitive response rather than systemic acquired resistance found by Mahuku et al. (1996).

2.4. Species complex and pathogenicity group (PG)

2.4.1. Two species

Populations of *Leptosphaeria maculans* are a complex of at least two genetically distinct groups, described as highly virulent and weakly virulent (Taylor et al., 1991), virulent and avirulent (McGee and Petrie, 1978), aggressive and non-aggressive (Hammond and Lewis, 1987), Tox^{+} and Tox^{0} (Balesdent et al., 1992), or A- and B-group (Johnson and Lewis, 1994). These two groups are now referred to as two different species, *Leptosphaeria maculans* (highly virulent, A-group) and *Leptosphaeria biglobosa* (weakly virulent, B-group) on the basis of differences in pseudothecial morphology and sexual incompatibility (Bonman et al., 1981; Shoemaker and Brun, 2001), culture, symptoms, biochemical factors and molecular genetics. More recently, seven distinct intraspecific groups of the species complex have been elucidated based on the sequence of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) repeat (Mendes-Pereira et al., 2003). This thesis focuses on A- and B-groups as they have been mostly discussed.

2.4.2. Differentiation of pathogen isolates

2.4.2.1. Symptom identification

The characteristics of leaf or stem lesions caused by different groups can be easily differentiated both in the field and greenhouse (Ansan-Melayah et al., 1997; Brun et al., 1997; Johnson and Lewis, 1994). In the field, lesions caused by B-group ascospores on the leaves and stems have few pycnidia and are darker, smaller and less noticeable than the larger, pale grey lesions with many pycnidia produced by A-group ascospores. In

addition, B-group isolates cause only either leaf spots (phoma leaf spot lesions) or superficial upper stem lesions (phoma stem lesions), neither of which causes yield loss; A-group isolates cause severe basal stem cankers, which leads to plant lodging and yield loss. In the greenhouse, many B-group infections remain as pinpoint lesions, suggesting an incompatible reaction (Toscano-Underwood et al., 2001).

In Western Canada, 95% of ascospores released from pseudothecia in early May are A-group isolates. B-group isolates appear late in the season during pod maturation and the phoma stem lesions caused by these isolates are superficial and cause little damage (Kharbanda, 1993; West et al., 2001).

2.4.2.2. Morphological markers

The differences between A- and B-group isolates have been found in rate of mycelial growth, pycnidial production and spore size (Petrie, 1988). A-group (Tox^{+}) isolates are characterized by slow growth *in vitro* and mass production of pycnidia, whereas B-group (Tox^{0}) isolates grow more rapidly *in vitro* with little production of pycnidia. Morphological markers are often limited and may not be accurate in determining genetic relationships in pathogen populations since their heritability may be low, and they may be subject to pleiotropic effects due to the influence of the environment (Keri, 1999).

2.4.2.3. Physiological and biochemical markers

In liquid culture, A-group isolates did not produce water-soluble pigments but B-group isolates accumulated a yellow-brown pigment (McGee and Petrie, 1978). However, Sippell and Hall (1995) reported that A- and B-group isolates may produce pigments in culture. The amount of pigment produced may vary considerably in single-spore isolates of a culture or between isolates, which makes pigmentation an unreliable marker.

Profiles of phytotoxins produced in liquid cultures have been used to differentiate A- and B-group isolates. A-group isolates have ability to produce nonhost-specific phytotoxins named sirodesmin PL, which B-group isolates are unable to produce (Pedras et al., 1995). Phytotoxins are secondary metabolites. At least nine phytotoxins have been found form A-group isolates, including sirodesmin PL, deacylsirodesmin PL, sirodesmin H, sirodesmin J, sirodesmin K, phomalide and phomalizarine, etc. (Pedras and Seguin-Swartz, 1992). High performance liquid chromatography (HPLC) analyses allowed the differentiation of the blackleg isolates into three groups (Soledade et al., 2000): 1). All A-group (Tox^{+}) isolates produce sirodesmin PL, deacetylsirodesmin PL and phomamide; 2) Polish B-group (Tox^{0}) isolates do not produce either sirodesmin PL or phomamide but produce indolyl dioxopiperazines; 3) Canadian (Tox^{0}) isolates produce very low amounts of metabolites such as polyketides (low phytotoxicity), but no indolyl dioxopiperazines, which differs from the profile of Polish isolates. The chemicals produced in culture can be used as biochemical markers to differentiate or group blackleg isolates.

2.4.2.4. Genetic markers

Isozyme differences have been used to classify isolates of A- and B-group (Gall et al., 1995). Isozymes share a common substrate but exhibit different electrophoretic mobility. When different electromorphs are identified by genetic analysis as allelic, they are called allozymes. Polymorphisms of isozymes as genetic markers have been used to estimate heterozygosity originating from dominant, recessive or epistatic alleles or loci. The esterase band profiles generated in native polyacrylamide gel electrophoresis, and soluble protein band patterns obtained by isoelectric focusing showed differences between A- and B-groups (Balesdent et al., 1992). Two monoclonal antibodies were found to be capable of distinguishing A-group isolates from B-group isolates (Stacesmith et al., 1993).

The quantitative measurement of extracellullar enzymes showed elevated activity levels for cellulose, α- and β-glucanases, and polygalacturonase in A-group isolates compared to B-group isolates. These are believed to be important in pathogen infection (Hassan et al., 1991).

Different isolates may vary in the number and size of chromosomes. The separation of whole chromosomes from *L. maculans* by transverse alternating field electrophoresis showed the different karyotypes between the two groups (Taylor et al., 1991). A-group isolates had 6-8 distinct chromosomal bands whereas B-group isolates had 12-14 bands. Chromosome size polymorphisms between two type isolates were confirmed by hybridization of DNA fragments to chromosomes (Morales et al., 1993b).

2.4.2.5. Molecular markers

DNA markers have been used to differentiate or fingerprint A- and B-group isolates. According to RAPD analysis of isolates collected across Canada (Goodwin and Annis, 1991), virulent pathotypes including PG-2 have recently been introduced into Canada and diverged relatively little; but PG-1 isolates have probably been present in Canada for a longer time and diverged with geographic isolation, i.e. PG-1 isolates from Western Canada or Eastern Canada were genetically different. Informative bands for distinguishing A- and B-group isolates could be obtained using four PCR primers (Schafer and Wostemeyer, 1992). After investigating differential RAPD bands, A- and B-group specific PCR primer pairs named RAPD-PCR were designed (Voigt et al., 1998).

DNA polymorphism of two distinct genetic groups was first described using RFLP by Johnson and Lewis (1990). A- and B-group isolates can clearly be characterized by RFLP using molecular probes for actin, ubiquitin, ribosomal RNA and the gene for elongation factor EF1 (TEF) (Hassan et al., 1991). A phylogenetic tree for 28 isolates was constructed using RFLP with two restriction enzymes and 42 probes of cloned nuclear DNA sequences from *L. maculans* (Koch et al., 1991), in which A- and B-group isolates are phylogenetically distant. Genomic DNA of 16 isolates were digested with 6 restriction enzymes and probed with labeled synthetic oligonucleotides, consistently grouping A- and B-group isolates together (Meyer et al., 1992). Phylogenetic analyses of RFLP patterns indicated that A- and B-isolates are not monophyletic (Taylor and Borgmann, 1994). After heat shock responsive genes and cloned genomic DNA sequences from a virulent isolate were used to probe restriction fragments of genomic

DNA of *L. maculans* isolates, the RFLP resulted in distinct banding patterns, clearly separating isolates into two groups (Patterson and Kapoor, 1995).

AFLPs are PCR-based markers for the rapid screening of genetic diversity. Pongam et al. (1999) found that 49 virulent isolates representing PG-2, PG-3 and PG-4 collected from around the world could be clustered into two big groups: AFLP group I comprised the isolates from Western Canada, USA and one from UK, and they were mostly of the same PG group, whereas AFLP group II consisted of the isolates from Ontario, Australia and Europe, which could be further divided into two subgroups and included three PG groups. Similarity between North Dakota isolates (twenty five PG-2 isolates) collected in 1995-96 and Western Canadian isolates (four PG-2 isolates) collected from 1980s was noticed, suggesting that *L. maculans* was introduced into North Dakota from Western Canada and the populations have remained unchanged over the 10 years. The genetic diversity and geographic differentiation of 100 Australian, European and North American isolates of *L. maculans* were analyzed with AFLP and they could be classified into five types (Purwantara et al., 2000). Their results indicated that Australian and European populations were separate adjacent clusters while the North American populations partially overlapped both of these. North American virulent isolates were less diverse than those from Australia and Europe.

Phylogenetic relationships and PG identification of *L. maculans* populations by means of ribosomal DNA (rDNA) molecular assay have been studied since the early 1990s. Based on the DNA sequencing of 17S and 5.8S genes in the ITS1 region of *L. maculans*, the specific PCR primer pairs HV17S/5.8S for A-group isolates and WV17S/5.8S for B-

group isolates were developed (Mahuku et al., 1996; Xue, et al., 1992). The major differences in both ITS1 and ITS2 sequences that correlated with the PG groups of *L. maculans* were confirmed by Morales et al. (1993a). In practice, the amplification and restriction of ITS regions by using just *L. maculans* pycnidia as a PCR template has been achieved (Balesdent et al., 1998).

Besides sequence divergence in ITS sequences, a 5,238-bp repetitive sequence present only in A-group isolates was utilized to develop a set of PCR primers for detection of seed contaminated with A-group isolates (Taylor, 1993).

It should be noted that utility of all investigations mentioned above is restricted to the differentiation of A- from B-group. It seems that only the AFLP method is suitable to characterize phylogenetic relationship within A-group isolates.

2.4.3. PG, distribution and application

Determination of pathogenicity groups (PG) is based on pathogenicity of isolates with different avirulence (*Avr*) genes when inoculated onto cotyledons of three *Brassica napus* differential cultivars Westar (susceptible), Glacier (*Rlm2* and *Rlm3* resistance genes) and Quinta (*Rlm1*and *Rlm4* linked resistance genes) (Koch et al., 1991; Mengistu et al., 1991). According to the interaction phenotype (IP), isolates can be classified into at least four PGs: PG-1 (avirulent on Westar, Glacier and Quinta), PG-2 (virulent on Westar and avirulent on Glacier and Quinta), PG-3 (virulent on Westar and Glacier and avirulent on Quinta) and PG-4 (virulent on Westar, Glacier and Quinta). Recently PGT (virulent on Westar and Quinta, but avirulent on Glacier) was added into PGs (Chen and Fernando,

2005, Rimmer, 2006). PG identification is based on gene-for-gene relationship, where the IP (resistance or susceptibility) depends on the presence of one major gene for resistance in the plant (*Rlm*) and a single corresponding avirulence (*Avr*) gene in the pathogen. For instance, the dominant single resistant allele *Rlm2* in Glacier is in response to avirulent gene *Avrlm2* in PG-2 isolates of *L. maculans*, resulting in "Glacier-PG-2 incompatible interaction. Pair matching between two dominantly linked *Rlm1* and *Rlm4* genes in Quinta and two avirulent unlinked genes *Avrlm1* and *Avrlm4* in *L. maculans* isolates controls the "Quinta-PG-2 interaction. Gene match between *Rlm1* in Quinta and its complementary *Avrlm1* in PG-3 isolates determines the "Quinta-PG-3" incompatible interaction (Ansan-Melayah et al., 1998).

Many additional interaction phenotypes between *B. napus* and *L. maculans* were described in order to combine the genetic correspondence between *Rlm/Avr* interactions. Isolates of *L. maculans*, according to the differential reactions on cvs. Erfurter Zwerg, Glacier, Runde, Quinta and Jet Neuf, were classified as pathotype group A (aggressive) or pathotype group NA (non-aggressive), in which 5 sub-groups A0 — A4 under pathotype group A were determined (Badawy et al., 1991). The variation in pathogenicity of *L. maculans* populations collected from Manitoba, Saskatchewan and Western Australia was investigated on a range of *B. napus* cvs. Glacier, Global, Karat, Marnoo, Quinta, Regent, UM3132 and two DH lines R83-14.DH47 and R83-14.DH26 (Kutcher et al., 1993). The research suggested that isolates from Western Australia were more pathogenic than those from Manitoba and Saskatchewan, isolates from Manitoba and Saskatchewan had a similar pathogenicity spectrum. However, isolates of *L. maculans*

obtained from a single field varied in pathogenicity (Mahuku et al., 1997). An extended differential set (cv. Lirabon, Glacier, Quinta and Jet Neuf) were tested to distinguish six PGs (A1-A6) (Kuswinanti et al., 1995). In 1999, based on six *B. napus* cultivars Westar, Glacier, Quinta, Quantum, Sentry, Sprint, and two lines Val-1 and Dac-1, *L. maculans* PG-2 isolates were subdivided into 15 distinct PG, and PG-3 and PG-4 were subdivided into eight PG (Keri, 1999). It is evident that the various results obtained in these studies may not be consistent or comparable because different isolates of *L. maculans* and different host cultivars were used.

PG-1 isolates (*L. biglobosa*) are present in all blackleg devastating regions, but specifically prevalent in Poland. In Canada, PG-1 isolates were first detected in the 1960s (Vanterpool, 1961). PG-2 has been reported in Western Canada since 1975 (Kutcher et al., 1993; Mahuku et al., 1997; Mengistu et al., 1991). Isolates of PG-3 and PG-4 are widely distributed in Australia and Western European countries like U.K. and France. However, a high proportion of PG-4 (80%) and PG-3 (11%) isolates was found in Ontario, Canada (Mahuku et al., 1997).

Assessment of PG can provide valuable information about the dynamics of *L. maculans* populations and take into account the potential risk of new virulent isolates in specific locations where they might be absent (Sz. Szlávik and Fernando, 2006). It also helps with choice of cultivars because *Avr* genes conditioning pathogenicity of different PGs can be used to predict the corresponding resistance presented in cultivars. Care should be taken in the determination of PGs since variability within batches of

differential seeds, and the effect of temperature and other environmental conditions can alter the phenotype interaction. A more precise method is required.

2.5. Population genetic diversity and host resistance

2.5.1. Sexual recombination

Population genetic diversity of *L. manculans* that can break down novel resistance sources is mainly derived from three factors: sexual recombination, large population size and a high gene flow through large-scale dissemination of ascospores (Rouxel and Balesdent, 2005). *Leptosphaeria maculans* belongs to the fungal class Dothideomycetes. It has a single mating type (*MAT*) locus with two alternative forms (idiomorphs), which means that two isolates with alternate forms at *MAT* locus are required for sexual reproduction. Sexual reproduction of *L. maculans* is extremely important in the disease cycle for generating genetic variation (Magyar et al., 2006). Any pathogen population with a high evolutionary/variable potential and a high potential for gene flow will pose the greatest risk of breaking down resistance genes (McDonald and Linde, 2002). Molecular studies using RAPD (Mahuku et al., 1997), AFLP (Barrins et al., 2004) and microsatellite markers (Hayden et al., 2004) have shown that the populations of *L. maculans* in Australia and Canada are composed of many different genotypes. A multiplex PCR assay is available to identity the two mating types, *MAT1-1* and *MAT1-2*, in the *L. maculans* population (Cozijinsen and Howlett, 2003). The co-existence of two or more genotypes including two mating types in one lesion (Linde et al., 2002; Mahuku et al., 1996) indicates the genetic diversity in *L. maculans*.

2.5.2 Resistance gene (*Rlm*) and avirulence gene (*Avrlm*)

A resistance (R) gene called *Rlm* in rapeseed/canola against *L. maculans* was first identified in a French winter breeding line (Delwiche, 1980). A total of 14 single resistance gene loci, named *LEM1, LmF1, LmR1, LepR1, LepR2,* and *Rlm1 to Rlm9*, have been identified in the *Brassica* genome. Some of them such as *LEM1* and *LmR1* have been mapped in *B. napus* but none yet has been cloned because of highly duplicated regions in the *Brassica* genomes.

In response to the *Rlm* gene, avirulence (*Avr*) genes such as *alm1* and *Avrlm1* to *Avrlm9* have been genetically characterized in *L. maculans*, but none has been cloned. Identification/mapping of an *Avr* gene is determined by two methods: *in vitro* crosses segregating populations including tetrads, and differential isolates/cultivars phenotypes between *Avr* locus and corresponding *Rlm* (Balesdent et al., 2005).

A dendrogram, generated by neighbor-joining analysis based on avirulence (*Avr*) alleles data (*Avrlm1* to *Avrlm9*), showed that the isolates collected worldwide could be classified into three clusters, which did not correspond to the origin of isolates (Balesdent et al., 2005). For instance, Australian isolates were found in all three branches of the dendrogram, European isolates spanned the two branches and Canadian isolates were split into two groups, with isolates from Ontario being close to European isolates, whereas isolates from Western Canada were placed with the main Australian cluster.

2.5.3. Breakdown of resistance caused by population evolution of *L. maculans*

Strong selection pressures exerted on the pathogen population by major gene resistance can increase potential risk for evolution of new virulent pathotypes of *L. maculans* (Petrie, 1995). There are examples both from Europe and Australia which the pathogen populations' change and breakdown of resistant cultivars occurred.

In France, the *Rlm1* resistance gene existing in cv. Quinta, Doublol, Vivol, Capitol and Columbus was introduced into commercial cultivars in the early 1990s as the ratio of corresponding avirulence races possessing *Avrlm1* allele was higher than 95% in natural populations (Ansan-Melayah et al., 1997). Since they were highly efficient in control of disease, *Rlm1* cultivars composed up to 43.7% of total French acreage at the end of 1990s. However, selection pressure from the host induced rapid shifts in the *L. maculans* population. In 1999 and 2000 isolates with *Avrlm1* allele decreased to less than 13% in the pathogen population, suggesting the loss of efficiency of *Rlm1* resistance in the field and the high evolutionary potential of *L. maculans* (Rouxel et al., 2003a).

In Australia, resistant cultivars are *Rlm4* based (Rouxel et al., 2003b). In 2000 a highly resistant canola cultivar "Surpass400", derived from wild *B. rapa* subsp *Sylvestris* with a single dominant allele (*LepR3*), was commercially released (Li and Cowling, 2003). Three years later (in 2003), 48.8% of isolates showed high virulence on the cultivars containing this single dominant gene, clearly indicating the high variation in *L. maculans* populations in Western Australia (Li and Barbetti et al., 2005). As of 2005, no Australian cultivars that use this form of resistance were being marketed.

2.6. Induced resistance

2.6.1. Induced resistance and types

Induced resistance to disease in plants is a state of enhanced plant defense dependent on the host plant's physical and chemical barriers, activated by biotic or abiotic elicitors (Baker, 2000). Induced resistance is the generation of resistance where there is none, by the activation of latent defense mechanisms (Durrant and Dong, 2004). Induced resistance can be subdivided into two broad categories. The first type is systemic acquired resistance or SAR, which develops locally such as the hypersensitive reaction (HR) or systemically in response to a pathogen that causes a necrotic lesions (Metraux et al., 2002). SAR is associated with the production of pathogenesis-related (PR) proteins and normally mediated via a salicylic acid (SA) signal pathway.

The second type of induced resistance is induced systemic resistance or ISR, which is developed systemically in response to different biotic or abiotic stress such as wounding, plant growth-promoting rhizobacteria (PGPR), or insect feeding. ISR is mediated via a jasmonate/ethylene signal pathway but might have crosstalk with the SA signal pathway (Bostock, 2005).

2.6.2. Avirulent isolates - triggering SAR

SAR has been described in over 30 plant species (Baker, 2000). Elicitors of SAR comprise biotic or abiotic agents, and biotic agents can be avirulent pathogens or other organisms. Avirulent isolates have been found to induce resistance in the host plant against virulent isolates in many pathosystems: two avirulent races triggered systemic

resistance in watermelon against virulent races of *Fusarium oxysporum* f. sp. *niveum* (Martyn et al., 1991), weakly virulent strains of *Phytophthora infestans* induced systemic resistance in potato to late blight (Stromberg, 1995), avirulent isolates activated systemic resistance in broccoli against downy mildew (Monot et al., 2002), induced resistance against Fusarium wilt by nonpathogenic races of *F. oxysporum* f.sp. *ciceris* and *F. oxysporum* f.sp. *lycopersici* was detected in chickpea and tomato (Fuchs et al., 1999; Hervas et al., 1995), respectively, and resistance in susceptible mustard against the highly virulent *Alternaria brassicae* isolate A (AbA) and moderately virulent isolate C (AbC) was induced using an avirulent isolate D (AbD) of *A. brassicae* (Vishwanath et al., 1999).

2.6.3. SAR in Brassica-blackleg system

In the *Brassica* — blackleg system, the hypersensitive reaction (HR) was found on *Brassica* plants pre-treated with avirulent isolates. For instance, rapid necrosis of guard cells in the leaves of Indian mustard (*B. juncea*) to arrest hypha growth was observed after the inoculation of an avirulent isolate *L. maculans* (Chen and Howlett, 1996). The accumulation of phytoalexins in *Brassica* spp. in relation to a HR to *L. maculans* was detected (Pedras et al., 2002). An elicitin (cryptogein) and an avirulent isolate of *L. maculans* were found to be capable of triggering HR on cotyledons of *B. napus* (Roussel et al., 1999). Accumulation of pectin in the lumen of xylem vessels and the production of callose and lignin were confirmed to play a role in inhibiting systemic infection caused by *L. maculans* (Hammond et al., 1985).

All the studies mentioned above were focused on the HR in *Brassica* plants with pre-application of an avirulent isolate of *L. maculans*. However, HR is a localized defense reaction. It will be more useful if avirulent isolates of *L. maculans* can trigger systemic acquired resistance (SAR) against the infection caused by virulent isolates for following reason: SAR rather than HR can provide the plant with longer-lasting protection, and against a broad spectrum of pathogens.

Mahuku et al. (1996) reported SAR in the canola – blackleg system. In this experiment, the size of blackleg lesions on cotyledons of Westar was significantly reduced when a weakly virulent (WV) isolate was introduced 64 h prior to the infection by a highly virulent (HV) isolate. After WV inoculation, the size of lesions caused by the HV pathotype on the same leaf, on neighboring leaves (above and below the inoculated leaf position) and in the stem was reduced. However, the mechanism involved in this SAR still remains unknown and the performance of such SAR under field conditions was not investigated.

In addition to the avirulent isolate, a non-host pathogen, i.e. an isolate of tomato pathogen *Cladosporium fulvum* with *Avr9* gene was found to trigger a temporary induced resistance to *L. maculans* (Hennin et al., 2001). Chemicals other than salicylic acid, such as acibenzolar-S-methyl (BTH) and menadione sodium bisilphite (MSB) were proven to be effective in activating locally or systemically induced resistance to *L. maculans* (Borges et al., 2003).

2.6.4. The mechanisms of induced resistance in the *Brassica*-blackleg system

A few papers have documented the mechanisms of induced resistance involved in *Brassica*-blackleg system. For instance, induction of PR-proteins such as β-1,3-glucanase and chitinase in leaves of resistant and susceptible *Brassica* genotypes has been observed (Dixelius, 1994; Hennin et al., 2001; Rasmussen et al., 1992). A systemic enhancement of ascorbate peroxidase (APX) in MSB-pretreated first true leaves of cv. Bristol was detected by real-time PCR (Borges et al., 2003). The accumulation of five indole phytoalexins, i.e. brassilexin, brassinin, cyclobrassinin, cyclobrassinin sulphoxide and methoxybrassinin, was found in the cotyledons pre-treated with CuC_{12} (Rouxel et al., 1991). At the proteome level, antioxidant enzymes including superoxide dismutase, nitrate reductase, and carbonic anhydrase, were elevated in the incompatible reaction between *Brassicas* and *L. maculans* using two-dimensional electrophoresis followed by tandem mass spectrometry (MS) (Subramanian et al., 2005).

The complexity of defense response at the molecular level was confirmed by sequencing 277 cDNA (ESTs) derived from mRNA of *B. napus* cv. Glacier inoculated with *L. maculans* PG-2, where about 31% of ESTs were sequences homologous to previously-identified defense genes (56 ESTs), disease resistant genes (6 ESTs) and stress-related genes (25 ESTs) (Fristensky et al., 1999).

SAR is normally dependent on salicylic acid (SA) signaling, and ISR is dependent on jasmonic acid/ethylene signaling. But resistance in *A. thaliana* against *L. maculans* was recently found to be partially dependent on phytoalexin camalexin biosynthesis, which is independent of the SA, jasmonic acid/ethylene pathway (Bohman, 2001), suggesting that

the defense response to *L. maculans* might involve complex, multiple, and previously uncharacterized pathways.

PG-1-induced resistance is not observed under field conditions for two reasons: PG-1 isolates appear late in the season during pod maturation (West et al., 2001) and PG-1 isolates may cause compatible interactions with aging host tissue under high temperature (Badawy et al., 1992) and under high humidity (El-Hadrami, Fernando and Daayf, unpublished data).

Recent research programs on the systematics of *L. maculans* using either biochemical or molecular methods have been focused on the distinction between two pathotypes (A- and B-group). However, A-group isolates can be divided into many pathogenicity groups (PGs) based on interaction phenotype (IP). In addition to dominant PG-1 and PG-2 isolates, PG-3, PGT and PG-4 isolates were recently discovered in Western Canada and North Dakota, USA (Bradley et al., 2005; Chen and Fernando, 2005; Fernando and Chen, 2003). Genetic divergence of these isolates from the other ones with different origins should be investigated. A better understanding of the genetic structure and evolution of *L. maculans* populations is essential to manage outbreaks of blackleg disease in canola. Interactions among these isolates may provide information required for alternative controls of this disease.

CHAPTER 3

INDUCED RESISTANCE TO BLACKLEG (*LEPTOSPHAERIA MACULANS*) DISEASE OF CANOLA (*BRASSICA NAPUS*) CAUSED BY A WEAKLY VIRULENT ISOLATE OF *LEPTOSPHAERIA BIGLOBOSA*

3.1. Abstract

Blackleg of canola is a disease complex of at least two fungal species: *Leptosphaeria maculans* and *L. biglobosa*. Isolates of *L. biglobosa* are typically weakly virulent or avirulent and are assigned to pathogenicity group 1 (PG-1). Isolates of *L. maculans* are highly virulent and encompass pathogenicity groups PG-2, PG-3, and PG-4. In greenhouse tests, percent lesion/leaf area (PLLA) on cotyledons of two *Brassica napus* cultivars, Westar and Invigor 2153, was smaller when *L. biglobosa* (PG-1) was either pre- or co-inoculated at 0, 12, 24, and 48 h with virulent isolates of *L. maculans* in PG-2, PG-3, and PG-4. On six-leaf stage plants of Westar, PLLA declined significantly compared to the control when the lower leaves were treated with either PG-1 or salicylic acid (SA) and then challenged with a PG-2 isolate 24 h later. In addition, the activity of four plant enzymes (chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase) was greatly enhanced at 48 and 72 h when cotyledons of Westar were inoculated first with PG-1 followed by PG-2 24 h later, compared to a water control treatment. Field experiments conducted in 2003 and 2004 showed decreased blackleg severity in plants inoculated with PG-1 alone or prior to PG-2 compared to plants inoculated with PG-2 alone or prior to PG-1.

3.2. Introduction

Canola (*Brassica napus* L) is the second largest cash crop in Canada (Beckman, 2004). Blackleg disease, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. [anamorph = *Phoma lingam* (Tode: Fr.) Desm.], is one of most economically important diseases of canola (Howlett, 2004). In the past decade, canola production has expanded significantly worldwide and interest in the blackleg fungus, particularly its biology, pathogenicity, and control, has increased.

Blackleg of canola is a disease complex and consists of at least two types of fungal isolates — *Leptosphaeria biglobosa* (Shoemaker and Brun, 2001), which is weakly virulent and classified as B group or type isolates, and *Leptosphaeria maculans*, which is aggressive, highly virulent and classified as A group or type isolates (Howlett et al., 2001). *Leptosphaeria biglobosa* typically causes phoma leaf spots or superficial stem lesions, but *L. maculans* causes the more serious stem cankers. Based on the pathogenicity of isolates to three *B. napus* cultivars — Westar (susceptible), Glacier (*Rlm2* and *Rlm3* resistance genes), and Quinta (*Rlm1* and *Rlm4* resistance genes), the pathogen complex can be categorized into several pathogenicity groups (PGs) (Mengistu et al., 1991). *Leptosphaeria biglobosa* belongs to PG-1 and *L. maculans* comprises PG-2, PG-3 and PG-4. In addition to isolates of *L. biglobosa*, isolates in the A group causing incompatible reaction on resistant cultivars are also termed weakly virulent isolates of *L. maculans* (Roussel et al., 1999).

Induced resistance to disease in plants is a state of enhanced plant defense capacity dependent on the host plant's physical and chemical barriers, activated by biotic or

abiotic agents (Baker, 2000). Weakly virulent isolates have been shown to induce resistance in the host plant against virulent isolates in many pathosystems. Two avirulent races of *Fusarium oxysporum* f. sp. *niveum* induced resistance to fusarium wilt of watermelon when challenged with a virulent race (Martyn et al., 1991). Weakly virulent strains of *Phytophthora infestans* induced systemic resistance in potato to late blight (Stromberg, 1995) and avirulent isolates activated systemic resistance in broccoli against downy mildew (Monot et al., 2002). Nonpathogenic races of *F. oxysporum* f.sp. *ciceris* and *F. oxysporum* f.sp. *lycopersici* induced resistance to fusarium wilt in chickpea and tomato (Fuchs et al., 1999; Hervas et al., 1995). Similarly, resistance in a susceptible mustard cultivar to the highly virulent *Alternaria brassicae* isolate A and moderately virulent isolate C was induced using the avirulent isolate D (Vishwanath et al., 1999).

In the *Brassica* - blackleg system, a rapid necrosis of guard cells in the leaves of Indian mustard occurred following inoculation of a resistant cultivar with an avirulent isolate of *L. maculans* (Chen and Howlett, 1996). A hypersensitive reaction (HR) and an accumulation of phytoalexins were detected in *Brassica* spp. after inoculation with a weakly virulent isolate of *L. maculans* (Pedras et al., 2002). An elicitin, cryptogein, and a weakly virulent isolate of blackleg pathogen both were capable of triggering HR on cotyledons of *B. napus* (Roussel et al., 1999). Accumulation of pectin in the lumen of xylem vessels and the production of callose and lignin were confirmed to play roles in inhibiting systemic infection caused by *L. maculans* (Hammond et al., 1985). In addition, induction of pathogenesis related (PR)-proteins, β-1,3- glucanase and chitinase, also has been documented (Chen and Seguin-Swartz, 1997).

The above studies were focused on the induction of HR in *Brassica* plants by pre-inoculation with a weakly virulent isolate. It might be beneficial, however, if weakly virulent isolates of *L. biglobosa* (PG-1) could trigger systemic acquired resistance (SAR) instead of the HR, since SAR can provide the plant with longer-lasting protection against a broad spectrum of pathogens.

SAR has been described in over 30 plant species (Baker, 2000) and elicitors of SAR comprise both biotic and abiotic agents. Mahuku et al. (1996) reported SAR in canola to blackleg when a weakly virulent isolate was introduced 64 h prior to infection by a highly virulent isolate. In addition, the size of the lesions on the inoculated leaf and on neighboring leaves (above and below the inoculated leaf) and stem was reduced. However, the mechanism involved in this SAR still remains unknown and the performance of such SAR under field conditions has not been investigated.

It seems like that induced resistance has been found in canola plants after pre-treatment with a weakly virulent isolate. The objectives of this study are to understand the mechanism involved and to investigate this induced resistance under field conditions. In order to do this, we evaluated the ability of a weakly virulent isolate of *L. biglobosa* (PG-1) and the elicitor salicylic acid to induce resistance in *B. napus* against blackleg disease caused by virulent isolates of *L. maculans* (PG-2, PG-3 and PG-4) in both the greenhouse and field. The activity of four PR proteins (chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase) also was measured.

3.3. Materials and methods

3.3.1. Preparation of inoculum.

Isolates PL84-12 (PG-1); PL86-12 (PG-2) were originally obtained from canola fields in Manitoba. Isolates PL89-18 (PG-3) and PL89-12 (PG-4) were from Australia. Each isolate was revived from mineral oil stock cultures where mycelia/pycnidia had been grown on slants of potato dextrose agar (PDA). After reviving the isolates, the cultures were grown on V8 agar medium (200 ml of V8 juice, 0.75g of $CaCO_3$, 15g of agar in 800 ml of distilled H_2O), and then single-spored. The isolates were passed once through canola cv. Westar (very susceptible to blackleg pathogen) to maintain their virulence and then grown on V8 for 14 days under continuous cool-white fluorescent light at 20°C. Sporulating cultures were flooded with 10 15 ml of sterile distilled water and gently scraped with a flamed glass rod to release spores from pycnidia. The pycnidiospores were harvested by filtering through sterilized Miracloth (CALBIOCHEM®) and centrifuged at 9,000 rpm for 20 minutes. The supernatant was decanted and the spore pellet was re-suspended in approximately 1 ml of sterile distilled water and stored as a concentrate at –20°C. Each pycnidiospore suspension was diluted to 1×10^7 spores/ml to be used for inoculations.

3.3.2. Experiments on cotyledons

Brassica napus cultivars Westar (highly susceptible) and Invigor 2153 (Liberty Link, moderately susceptible) were seeded and grown in MetroMix® in a growth chamber (16/21°C night/day and a 16-h photoperiod). Pycnidiospores of each isolate were

inoculated onto wounded cotyledons 7 days after seeding. Central part of each cotyledon was wounded once with a sterile needle and a 10-µl droplet of pycnidiospore suspension was pipetted onto the wound. PL84-12 (PG-1) was co-inoculated, pre-inoculated, and post-inoculated with each of the other three isolates PL86-12 (PG-2), PL89-18 (PG-3) or PL89-12 (PG-4). Cotyledons treated solely with each of the PG isolates mentioned above served as controls. Pre- and post-inoculations were done at 12, 24 or 48 h relative to the initial inoculation. The inoculated cotyledons were allowed to dry for at least 12 h before the next watering. After inoculation, cotyledons were incubated in the same growth chamber. Twelve days after the last inoculation, area of lesion and leaf was first scanned into digital images and percent lesion/leaf area (PLLA) on cotyledons was calculated using the disease analysis computer program "Assess" (ASSESS, 2002). PLLA = (lesion area/leaf area) × 100. Seventy-two cotyledons per treatment were measured. The experiment was repeated once. As the same isolates were used in all greenhouse experiments they will be referred to only as PG-1, PG-2, PG-3, and PG-4 isolates hereafter.

3.3.3. Experiments on adult plants

Brassica napus cv. Westar was seeded and grown in MetroMix® for 1 week. Seedlings were transplanted with one plant/pot (15 cm dia. × 14.5 cm h) containing a mix of soil: sand: peat (2:2:1 in volume). The growth conditions were as described above. At the six leaf stage, separate plants were wound inoculated with one of three reagents: sterile distilled H_2O, salicylic acid (SA, 10 mM) or PG-1 spore suspension on the lowest leaf of

each plant. Twenty four hours later, all plants were wound inoculated with 10µl of PG-2 spore suspension on a leaf just above the pre-inoculated leaf. The plants with initial inoculation of PG-1 were also inoculated with PG-2 on the upper leaves either at 48, 72, 96 or 120 h. Each treatment contained 50 plants with the experiment repeated once. Percent lesion/leaf area (PLLA) on PG2-inoculated leaves was measured 12 days after the inoculation of PG-2.

3.3.4. Assays for pathogenesis-related enzymes

One-week-old cotyledons of Westar were wound inoculated with a 10µl droplet of the following reagents: a) sterile distilled H_2O; b) PG-1 spore suspension; c) PG-2 spore suspension or d) PG-1 pre-inoculation followed by PG-2 24 h later. After PG-2 inoculation, the cotyledons of each treatment were sampled at 0, 6, 12, 24, 48, 72, 96 and 120 h for the estimation of activity of the defense-related enzymes chitinase, β-1,3-glucanase, peroxidase (PO) and phenylalanine ammonia lyase (PAL). Each treatment consisted of 96 plants (two cotyledons/plant) and 12 plants per treatment were sampled each time interval.

3.3.4.1. Chitinase assay

Harvested cotyledons were immediately homogenized in liquid nitrogen using a pestle and mortar. One gram of powdered sample was ground in 1 ml of 0.1M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged at 4°C for 10 min at 10,000 g. Determination of chitinase activity was based upon Boller and Mauch's protocol (Boller

and Mauch, 1988). Since chitinase activity was assayed by following the release of N acetyl glucosamine (GlcNac) from colloidal chitin, the enzyme activity was expressed as nmol of equivalent GlcNac/min/mg protein.

Protein content in each extracted sample was calculated based on a standard curve constructed with bovine serum albumin (EC 232-936-2, Sigma).

3.3.4.2. β-1,3-glucanase assay

Cotyledons were homogenized with liquid nitrogen using a pestle and mortar. One gram of the sample was ground in 1 ml of 0.05 M sodium acetate buffer (pH 5.0) at 4°C. The homogenate was allowed to react with 0.4% laminarin (EC 2327124, Sigma) for 10 min at 40°C. As the β-1,3-glucanase assay was based on the release of reducing glucose equivalent from laminarin as described by McCleary and Shameer (McCleary and Shameer, 1987), specific activity was expressed as μg of equivalent glucose/min/mg protein.

3.3.4.3. Peroxidase assay

One gram of fresh cotyledon tissue was ground in 1 ml of 0.1M phosphate buffer (pH 7.0) using a cold pestle and mortar (4°C). The homogenate was centrifuged at 15,000g at 4° C for 15 min and the supernatant was immediately used for the enzyme assay. Peroxidase activity was determined using pyrogallol (EC 201-762-9, Sigma) as the hydrogen donor and measuring the rate of color development spectrophotometrically at

420 nm at 30 second intervals for 3 minutes (Hammerschmidt et al., 1982). The peroxidase activity was expressed as change in absorbance/min/mg protein.

3.3.4.4. Phenylalanine ammonia lyase (PAL) assay

Five hundred mg of cotyledon tissue was homogenized in 1 ml of cold 0.1 M sodium borate buffer (pH 7.0) containing 0.1 g of insoluble polyvinyl-pyrrolidone (PVP) using a cold pestle and mortar (4°C). The homogenate was centrifuged for 20 min at 15,000g at 4°C. The supernatant was used as the enzyme source for the assay. Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer (pH 8.8) and 0.5 ml of 12 mM L-phenylalanine (EC 200-568-1, Sigma) for 30 min at 30°C. The reaction was terminated by adding 0.5 ml of 1 M trichloroacetic acid. The enzyme blank contained 0.4 ml of crude enzyme extract and 2.7 ml of 0.1 M borate buffer (pH 8.8). Absorbance was measured at 290 nm. A molar extinction coefficient of 9630 per min per cm was used for transcinnamic acid in 0.1 M borate buffer (pH 8.8). PAL activity was determined as the rate of conversion of L-phenylalanine to transcinnamic acid at the absorbance of 290 nm as described by Dickerson et al. (1984). The enzyme activity was expressed as µmol of cinnamic acid/min/mg protein.

3.3.5. Field experiments

Field experiments were conducted at the Carman Research Station, Carman, Manitoba, Canada during the summers of 2003 and 2004. Preparation of blackleg PG-1 and PG-2 inoculum was as described above. The cultivar Westar was used for the field experiments. The plot size was 4×6 m^2 with a 6 m buffer zone. The layout of the plots in the field was a completely random design (CRD). The plots were seeded on 26 May 2003 and 28 May 2004. Seeding density was 6.5 kg/h, i.e. 24 lines (rows) within 4 m width of each plot. Eighty kg/ha of fertilizer 23-23-0 (N-P-K) was applied once at seeding.

PG-1 pycnidiospore suspension (1×10^7 spores/ml) was sprayed at seedling emergence in order to have the weakly virulent isolate present prior to any colonization of the cotyledons by natural blackleg inoculum. PG-2 (1×10^7 spores/ml) was inoculated 24 h later as well as at the 2-3 leaf stage. The eight treatments were as follows: 1) PG1: PG-1 inoculated alone on cotyledons; 2) PG2: PG-2 inoculated alone on cotyledons; 3) PG1+PG2 (1): PG-1 and PG-2 co-inoculated on cotyledons; 4) PG1+PG2 (2): PG-1 and PG-2 co-inoculated at 2-3 leaf stage; 5) PG1-24h-PG2 (1): PG-1 inoculated first followed by PG-2 24 h later on cotyledons;; 6) PG1-24h-PG2 (2): PG-1 inoculated first followed by PG-2 24 h later at 2-3 leaf stage; 7) PG2-24h-PG1 (1): PG-2 inoculated first followed by PG-1 24 h later on cotyledons; 8) PG2-24h-PG1 (2): PG-2 inoculated first followed by PG-1 24 h later at 2-3 leaf stage. In 2004 the treatment PG2-24h-PG1 (2) was changed to a non-inoculated blank control (background field inoculum). Each treatment was replicated three times.

Blackleg disease severity (DS) was assessed on 100 plants per plot in 2003 (August 22) and 200 plants per plot in 2004 (August 30) before the Westar plants were fully ripe using a 0-5 disease severity rating scale where: 0 = no diseased tissue visible in the cross-section of the stem base; 1 = diseased tissue occupies 1 to 25% of cross-section; 2 = diseased tissue occupies 26 to 50% of cross-section; 3 = diseased tissue occupies 51 to 75% of cross-section; 4 = diseased tissue occupies more than 75% of cross-section with little or no constriction of affected tissues; and 5 = diseased tissue occupies 100% of cross-section with significant constriction of affected tissues; tissue dry and brittle; plant dead.

The experimental data were analyzed using SAS (Ver. 8, Cary, NC, USA) statistical program and differences among treatments were evaluated by the linear model of ANOVA (Fisher's LSD).

3.4. Results

3.4.1. Greenhouse experiments on cotyledons

In order to determine whether data variances between two repetitions were homogeneous, Levene's test was employed. For both cultivars (Westar and Invigor2153) the test was not significant at $P < 0.05$, indicating that variances were homogeneous across repetitions. Therefore data from the two trials were combined.

Treatments with blackleg isolates PG-2, PG-3 and PG-4 inoculated alone produced larger lesions than that of isolate PG-1 inoculated alone on cultivars Westar and Invigor 2153 (Table 3.1). Lesion size was smaller in all PG-1 pre-inoculated treatments compared

to PG-2, PG-3, and PG-4 inoculated alone. The lesion sizes on Westar plants treated with PG-1 preceding PG-2, PG-3, or PG-4 at different time intervals, or PG-1 co-inoculated with each of virulent isolates were not significantly different from PG-1 inoculated alone (Table 3.1). However, lesion sizes on Invigor 2153 plants inoculated with PG-1 preceding PG-4 at 24 h were significantly different from PG-1 isolate inoculated alone (Table 3.1). The lesion sizes in all PG-1 pre-inoculated treatments on Westar were always < 1%. Lesion sizes on Westar plants treated with PG-2, PG-3 and PG-4 inoculated alone was 5 to 17 fold larger than the PG-1 pre-inoculated treatments. When PG-2, PG-3 or PG-4 were inoculated first, followed by PG-1, lesion size on both cultivars Westar and Invigor 2153 was generally equal to or larger than the corresponding virulent isolates inoculated alone. The lesion size of the treatment with PG-4 inoculated prior to PG-1 on Westar plants became larger with time (8.5%, 11.8%, and 14.1% at 12, 24 and 48 h, respectively). Similar results were obtained on cultivar Invigor 2153.

3.4.2. Greenhouse experiments on adult plants

The PLLA in the H_2O pre-application treatment followed by PG-2 on upper leaves was significantly higher than that of the treatments with salicylic acid and/or PG-1 pre-application (Fig. 3.1). The lesion size of the salicylic acid pre-treatment was not significantly different from the lesion size in PG-1 pre-treatments. The PLLAs of PG-1 pre-inoculated followed by PG-2 on the upper leaves at different time intervals (up to 120 h) were not different from each other.

3.4.3. Pathogenesis-related enzyme activity

All four treatments - H_20, PG-1, PG-2 inoculated alone and PG-1 pre-inoculated followed by PG-2 24 h later (PG1-24h-PG2) showed an increase in chitinase activity that declined by 96 h after inoculation (Fig. 3.2A). The increase in chitinase activity in treatment PG1-24h-PG2 was much higher after inoculation compared to the other three treatments. Peak chitinase activity was at 96 h, and reached 1270 nmol of GlcNac /min/mg protein, which was 6 fold higher than that of the H_20 treatment (70 nmol of GlcNac/min/mg protein).

During the first 48 h the activity of β-1,3-glucanase was at low levels in all four treatments (Fig. 3.2B). Thereafter the activity of β-1,3-glucanase in the PG1-24h-PG2 treatment began to increase and reached a peak of 6,200 μg equivalent of glucose/min/mg protein by 120 h after inoculation, which was 5 fold higher than that of the H_20 treatment (1,250 μg equivalent of glucose/min/mg protein) on the same day.

Peroxidase activity increased within 24 h in all treatments (Fig. 3.2C). It was greatest in the PG1-24h-PG2 treatment and peaked at 96 h with a change of 6.9 absorbance/min/mg protein at 420nm as compared to 2.7 absorbance/min/mg protein in cotyledons treated with H_20.

PG1-24h-PG2-treated plants had an increased accumulation of PAL compared to those of the H_20, PG1 or PG-2 treatments (Fig. 3.2D). Enzyme activity in PG1-24h-PG2 treatment increased within 6 h of inoculation, and maintained a higher level until 72 h with 110 μmol of cinnamic acid/min/mg protein, which was 1.8 fold higher than that of

the cotyledons treated with H_20 (60 µmol of cinnamic acid/min/mg protein). PAL activity started to decline after 72 h post- inoculation in all treatments.

3.4.4. Field experiments

In 2003, blackleg disease severity in plots treated with PG-1, PG1+PG2 (1) and PG1+PG2 (2) or PG1-24h-PG2 (1) and PG1-24h-PG2 (2) was significantly reduced compared to the plots treated with PG-2 or PG2-24h-PG1 (1) and PG2-24h-PG1 (2) (Fig. 3.3A). The results indicated that the PG-1 isolate, whether pre-inoculated (PG1-24h-PG2 (1) and PG1-24h-PG2 (2)) or co-inoculated (PG1+PG2 (1) and PG1+PG2 (2)) at early stages (cotyledon and 2-3 leaf stage), can reduce the disease severity caused by virulent isolate PG-2 under field conditions.

In 2004, blackleg was more severe in all treatments than in 2003. As in 2003, treatments with PG-2 alone or PG2-24h-PG1 (1) had significantly higher disease severity than all other treatments (Fig. 3.3B). PG-2 inoculation produced much higher disease pressure in the field than the natural field inoculum (non-inoculated blank control). Disease severity with PG-1 alone, PG1-24h-PG2 (1) and PG1-24h-PG2 (2) was reduced compared to the plots treated with PG-2 alone; PG2-24h-PG1 (1); and the non-inoculated blank control. Disease severity of treatments with PG1+PG2 (1) and PG1+PG2 (2) was significantly lower than that of treatments with PG-2 alone, or with PG2-24h-PG1 (1) (similar to 2003 results), but higher than that of background field inoculum.

3.5. Discussion

PG-1-induced resistance to blackleg against PG-2, PG-3 and PG-4 isolates was proven on the cotyledons of Westar in this study. Also, effective PG-2 control by PG-1 was observed under field conditions in 2003 and 2004.

The co-infection and co-existence of highly virulent and weakly virulent strains in a single blackleg leaf lesion were confirmed both by culture morphology and PCR detection (Mahuku et al., 1996). However, the time it takes for the defense response during the interaction between the highly virulent and weakly virulent isolates is important. On cotyledons, disease reduction was noticed only in treatments when PG-1 was pre- or co-inoculated with PG-2, PG-3 and PG-4 isolates. When PG-2, PG-3 and PG-4 were inoculated first followed by PG-1, an increased lesion size occurred in some treatments. This might have resulted from virulent isolates weakening the plant's defense and then facilitating PG-1 colonization, causing further infection. This also may occur under natural field conditions; however, the weakly virulent isolate is known to cause infections later in the season (West et al., 2001).

According to Baker (2000), SAR is a general, non-specific defense response in plants that develops after localized attack by viral, bacterial or fungal pathogens. PG-1 isolate of *L. biglobosa* used in this study is considered weakly virulent because it causes superficial lesions on canola plants. Roussel et al. (1999) observed that wound inoculation with an incompatible isolate on a resistant cultivar induces lignifications in leaf vascular bundles, resulting in a hypersensitive reaction (HR). In the greenhouse, PG-1-induced resistance on the cotyledons showed characteristics similar to HR as inoculation was on the same

wounding site. Wounding may be a potential causal factor of HR, however, from our experiment it was clearly evident that reduced disease was seen in co-inoculated plants compared to PG-2, PG-3 or PG-4 inoculated individually with wounding. Sequeira (1983) noted that the role of wounding in induced resistance has not been determined with precision. Felton et al. (1999) reported that a separate signaling pathway involving jasmonic acid is involved in systemic responses to wounding and insect herbivory. Working with weakly aggressive isolates of *Bipolaris sorokiniana*, Peltonen (1998) was able to show that wounding alone did not induce PAL.

Inoculations with PG-1 at the 6-leaf stage provided some evidence for SAR. When plants were pre-treated with the PG-1 isolate on lower leaves and then treated with PG-2 on the upper leaves, resistance was observed. Salicylic acid, a well known elicitor of SAR, provided a similar defense response to that of PG-1 treatment, indicating that PG-1 may be involved in inducing the salicylic acid (SA) pathway.

If SAR occurs in the plants, defense-related enzymes will be activated (Van Loon, 1997). Biles and Martyn (1993) found that peroxidase isozymes were greatly enhanced in watermelon seedlings inoculated with an avirulent race of *Fusarium oxysporum* f. sp. *niveum*. The increase in activity of chitinase, β-1,3-glucanase, peroxidase and PAL in plants treated with PG-1 (pre-application) indicated that the defense genes coding for these enzymes are induced by the stimulus of a PG-1 isolate through a signal pathway, probably the SA pathway. Chitinase and β-1,3-glucanase are lytic enzymes potentially acting on cell walls of organisms that have chitin and glucan as their cell wall component and they can be induced in the plant system (McCleary and Shameer, 1987). Peroxidase

represents a component of early response in plants to pathogen attack and plays a key role in the biosynthesis of lignin which limits the extent of pathogen spread (Bernards et al., 2004). PAL is the principal enzyme involved in the phenyl propanoid pathway and promotes the production of phytoalexins, terpenes and phenolic substances leading to formation of lignin with the help of peroxidases (Cools and Ishii, 2002).

Two years of field trials suggested that PG-1 inoculated alone or 24 h prior to PG-2 inoculation significantly reduced disease severity by *L. maculans*.

Both ascospores and pycnidiospores of *L. maculans* can be the primary inoculum source of blackleg disease, but ascospores are more important in causing yield loss through stem cankers during the growth period from cotyledons to the 8-leaf stage (Hall, 1992). Both weakly virulent and highly virulent isolates exist in Western Canada (West et al., 2001). However, weakly virulent isolates typically appear late in the season during pod maturation (West et al., 2001) and they can have compatible interactions on aging host tissue, causing infections under high temperatures (Badawy et al., 1992). This may be why PG-1-induced resistance has not been observed in canola fields. The application of PG-1 pycnidiospores prior to the natural infection by PG-2 might effectively induce resistance and significantly decrease infection and disease caused by the virulent isolates.

Induced resistance is a manageable trait and is unlikely to break down with a change in pathogen populations. This is important particularly at a time when the blackleg pathogen population seems to be changing in Western Canada and in the North Central United States (Bradley et al., 2005; Chen and Fernando, 2005; Fernando and Chen, 2003). Moreover, induced resistance to blackleg can be functional even in susceptible varieties

of canola (Chen and Fernando, 2006a) and this could greatly benefit both farmers and canola breeders since many currently used Canadian cultivars lack resistance to the highly virulent PG-3 and PG-4 isolates (W.G.D. Fernando and Y. Chen, unpublished data).

Table 3.1. Relative size[w] of leaf lesions caused by *Leptosphaeria maculans* PG-2, PG-3, PG-4 isolates reduced by pre- or co-inoculation with *L. biglobosa* (PG-1) isolate in canola cultivars Westar and Invigor 2153[x]

Cultivars	Treatment[y]	Time (h)	PG-1	PG-2	PG-3	PG-4
Westar	Single isolate		0.4 ij[z]	1.9 gh	3.6 rf	6.6 d
	Co-inoculated			0.3 ij	1.5 ghi	0.4 ij
	Pre-inoculated	12		0.4 ij	0.3 ij	0.3 ij
		24		0.4 ij	0.4 ij	0.8 hij
		48		0.5 ij	0.2 ij	0.1 j
	Post-inoculated	12		2.6 fg	3.2 ef	8.5 c
		24		4.0 e	6.8 d	11.8 b
		48		6.0 d	6.2 d	14.1 a
Invigor 2153	Single isolate		0.5 k	2.1 fgh	2.5 efg	5.4 c
	Co-inoculated			0.1 k	1.1 ijk	0.5 jk
	Pre-inoculated	12		0.2 k	0.2 k	0.5 jk
		24		0.3 k	0.5 jk	1.8 ghi
		48		0.3 k	0.1 k	0.2 k
	Post-inoculated	12		1.5 hij	2.3 fgh	4.5 d
		24		2.8 ef	3.3 e	8.2 b
		48		2.2 fgh	2.7 efg	11.3 a

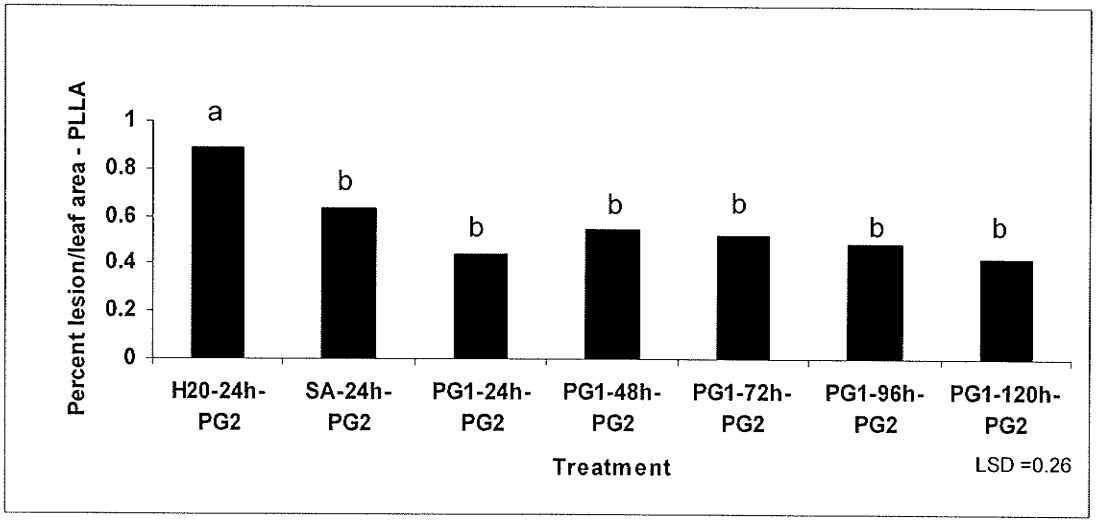
[w] Relative lesion size was defined as % lesion/leaf area (PLLA), PLLA values are the means of two repetitions involving 144 cotyledons/treatment.

[x] Cultivar Westar is highly susceptible and Invigor 2153 is moderately susceptible to blackleg.

[y] Pycnidiospores (1×10^7 spores/ml) of *L. biglobosa* isolate PG-1 (PL84-12) and *L. maculans* isolates PG-2 (PL86-12), PG-3 (PL89-18) or PG-4 (PL89-12) were inoculated onto cotyledons. PG-1 was co-, pre- and post-inoculated with each of the other three isolates. Pre- and post-inoculations were done either at 12, 24 or 48 h following the initial treatment.

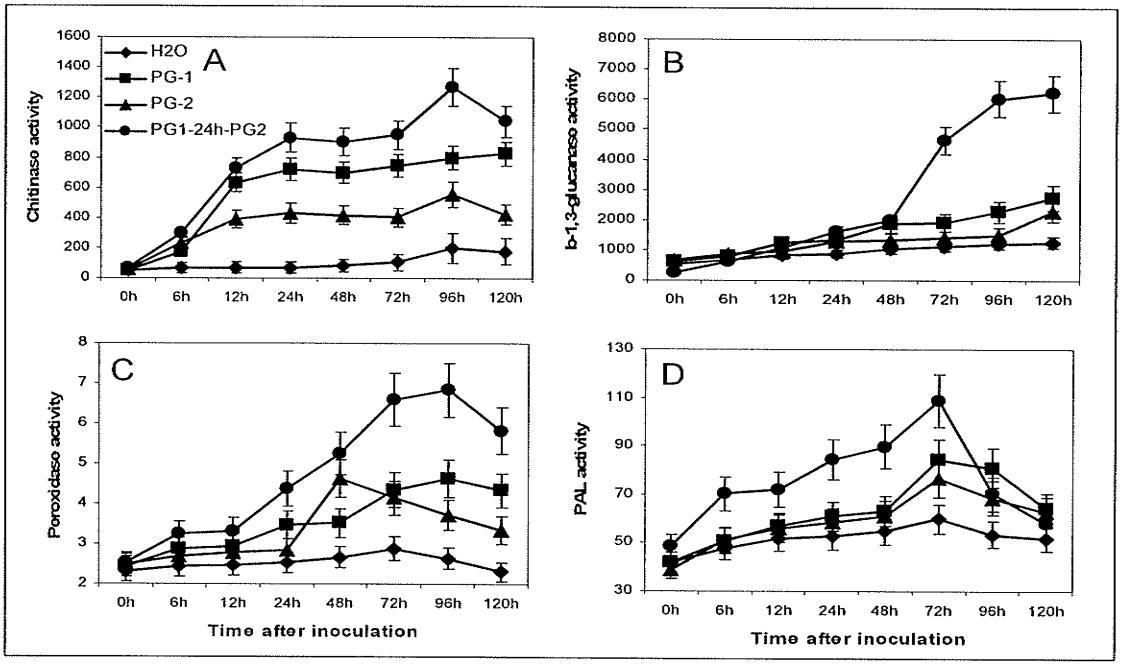
[z] All means for the same cultivar followed by same letter are not significantly different (Duncan's multiple range test, $P < 0.01$). Critical value is 1.37 - 1.43 in Westar and 1.07 - 1.11 in Invigor 2153.

Fig. 3.1. Relative lesion size at six-leaf stage of canola plants pre-inoculated with salicylic acid (SA) or *Leptosphaeria biglobosa* isolate PL84-12 (PG-1) on lower leaves, and followed by inoculation of *L. maculans* isolate PL86-12 (PG-2) either at 24, 48, 72, 96 and 120 h on upper leaves. Each mean was derived from 100 leaves*



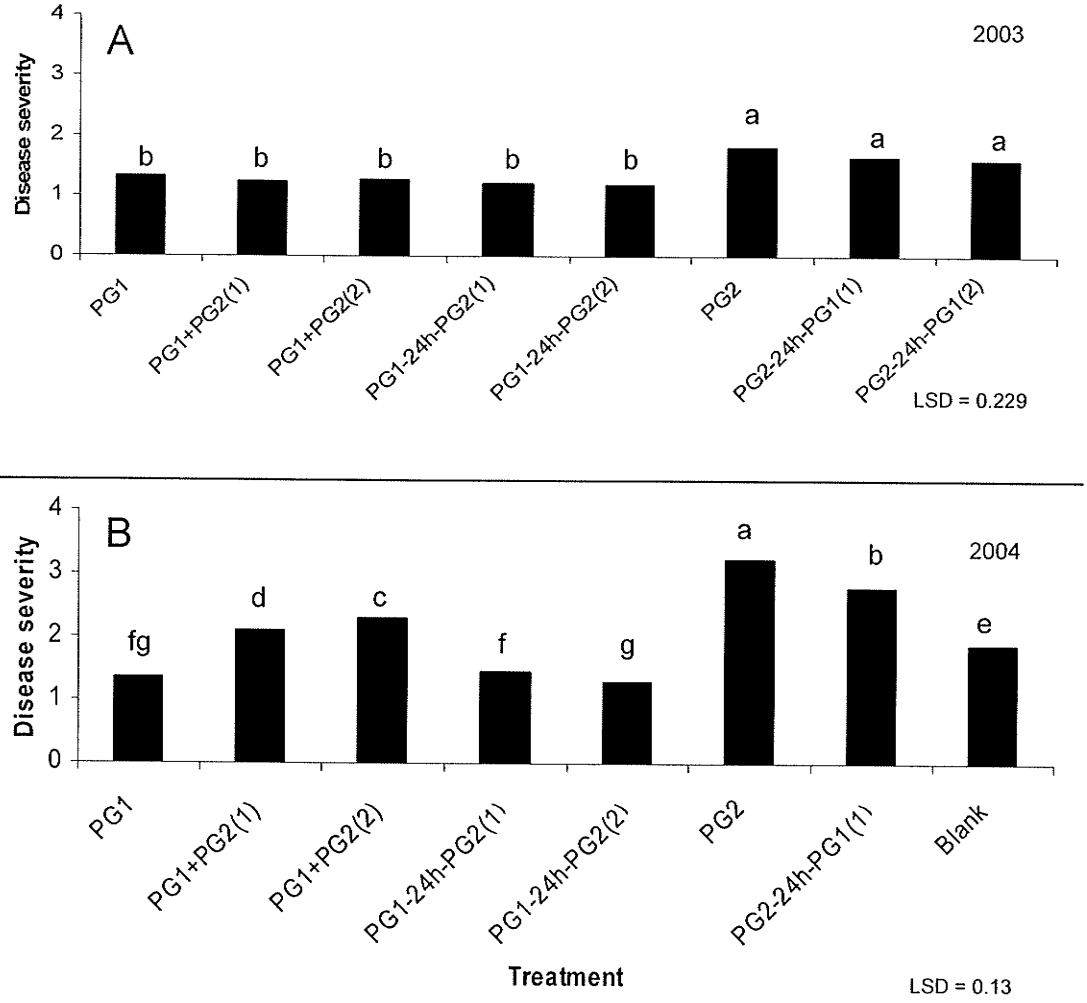
*Treatments with the same letter are not significantly different ($P < 0.05$).

Fig. 3.2. Changes of defense enzyme activity in canola cultivar Westar cotyledons treated with H_2O, or inoculated with *Leptosphaeria biglobosa* isolate PL84-12 (PG-1), *L. maculans* isolate PL86-12 (PG-2) or PG-1 24 h prior to PG-2 at time interval 0, 6, 12, 24, 48, 72, 96 and 120 h*



*A. Chitinase activity (nmol of GlcNac/min/mg protein); B. β-1,3-glucanase activity (μg of glucose/min/mg protein); C. Peroxidase activity (change in absorbance/min/mg protein); and D. Phenylalanine ammonia lyase (PAL) activity (μmol of cinnamic acid/min/mg protein). Error bars in each curve represent standard deviation.

Fig. 3.3 Disease severity caused by a virulent isolate of *Leptosphaeria maculans* [PL86-12 (PG-2)] on canola cultivar Westar in the presence or absence of a weakly virulent isolate of *L. biglobosa* [PL84-12 (PG-1)] in field trials conducted in Carman, Manitoba in 2003 (Fig. 2.3A) and 2004 (Fig. 2.3B)*



*PG1 = PG-1 inoculated alone on cotyledon; PG1+PG2 (1) = PG-1 and PG-2 co-inoculated on cotyledon; PG1+PG2 (2) = PG-1+ PG-2 co-inoculated at 2-3 leaf stage; PG1-24h-PG2 (1) = PG-1 preceded PG-2 by 24 h on cotyledon; PG1-24h-PG2 (2) = PG-1 preceded PG-2 by 24 h at 2-3 leaf stage; PG2 = PG-2 inoculated alone on cotyledon; PG2-24h-PG1 (1) = PG-2 preceded PG-1 by 24 h on cotyledon; PG2-24h-PG1 (2) = PG-2 preceded PG-1 by 24 h at 2-3 leaf stage. Treatments with the same letter are not significantly different ($P<0.05$). Fig.3.3B treatments are same as in Fig.3.3A except blank = Non-inoculated control. Each mean is based 100 plants in 2003 and 200 plants in 2004.

CHAPTER 4

CHARACTERIZATION OF ISOLATES OF *LEPTOSPHAERIA MACULANS* BASED ON TEST OF PATHOGENICITY GROUPS (PGs)

4.1. Abstract

During a four-year period (2001 to 2004), a total of 512 isolates of *Leptosphaeria maculans* / *L. biglobosa* either from different geographic locations (50 in total) across Northern America, Australia and Europe or from single field were characterized into pathogenicity groups (PGs) based on host-pathogen interactions using canola (*Brassica napus* L.) host differentials. Collection date of some isolates used in this study could be traced back to 1984. Seven–day–old cotyledons of the canola cultivars Westar, Glacier and Quinta were wound inoculated with each isolate, and interaction phenotypes (IP) were classified on a 0-9 scale, 12 days after inoculation. At least five PGs named PG-1, PG-2, PG-3, PGT or PG-4 were assigned to each isolate according to resistant or susceptible reactions. Isolates collected before 2001 in Western Canada and U.S.A were either in weakly virulent PG-1 or in virulent PG-2, whereas isolates from Brazil, Australia and UK were mainly in PG-3 or occasionally in PG-4 or PG-1, suggesting that virulence structure of isolates between these regions were different. In Western Canada and North Dakota, USA, the high similarity in pathogenicity of isolates collected in 1980s and in the early 21st century, implied that *L. maculans* populations had remained relatively unchanged (PG-2 or PG-1) over the past one and a half decades. In 2002 and 2003, the presence of PG-3, PGT and PG-4 were detected across the Western Prairies of

Canada and North Central U.S.A, indicating that new pathogenicity groups had surfaced for the first time in these regions. The origin of these new PGs was unknown. However, the co-existence of five PG types in a single field in Manitoba, Canada suggested the possibility of the occurrence of sexual recombination of *L. maculans* under natural environmental conditions, or introduction of new strains by human activities through seed-borne import, leading to the generation of new virulent isolates.

4.2. Introduction

The fungus *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. (anamorph *Phoma lingam* Tode ex Fr.) causes major economic losses in rapeseed/canola crops (*Brassica napus* L. and *B. rapa* L.) worldwide; especially in Europe (West et al., 2001), Australia (Sivasithamparam et al., 2005) and Canada (Gugel and Petrie, 1992), despite the fact that each of those countries has different growing seasons, cultivars, agricultural practices and climates. *Leptosphaeria maculans* has been known to be present for over 60 years in Europe (Aubertot et al., 2004), at least 80 years in Australia (Sivasthamparam et al., 2005) and 30 years in Canada (Petrie, 1978). Variation in the pathogen population has been noticed and efforts have been made to characterize different isolates, through morphological (Petrie, 1988), physiological (McGee and Petrie, 1978; Sippell and Hall 1995), biochemical (Pedras et al.,1995; Soledade et al., 2000), genetic (Balesdent et al., 1992; Gall et al. 1995; Taylor et al., 1991) and molecular markers (Goodwin and Annis, 1991; Johnson and Lewis, 1990; Pongam et al., 1999; Purwantara et al., 2000; Schafer and Wostemeyer, 1992). Among them, the virulence marker (pathogenicity group — PG) is the most widely used for characterizing the pathotype (Pongam et al., 1999). The pathogenicity test is used to investigate the interaction phenotype (IP) of the isolates on a limited number of differential cultivars (Keri, 1999; Koch et al., 1991; Kuswinanti et al., 1995; Kutcher et al., 1993; Mengistu et al., 1991). Variation in virulence of isolates is displayed as physiological or host specificity on differential cultivars in the form of resistance or susceptibility.

Usually isolates of *L. maculans* are grouped as either non-aggressive (B-group) or aggressive types (A-group), based primarily on cotyledon reactions of susceptible cultivars such as Westar. To date, four PGs have been recognized using *Brassica napus* cultivars Westar, Glacier and Quinta (Keri, 1999; Koch et al., 1991; Mengistu et al., 1991). The PG-1 (B-group) isolates are non-aggressive on Westar, Glacier and Quinta, and now classified as a new species *L. biglobosa* (Shoemaker and Brun, 2001). A-group isolates are quite diverse in aggressiveness and are classified as PG-2, PG-3 and PG-4 in response to their virulence spectrum on the differentials.

Breakdown of rapeseed/canola resistance to blackleg disease, resulting from a shift in *L. maculans* populations, has been reported in Europe (Rouxel et al., 2003a) and Australia (Li and Sivasithamparam et al., 2003). Cultivars with moderate to high levels of resistance to *L. maculans* were developed in Canada (Rimmer, 2006). Whether these cultivars will provide long-lasting protection against the natural populations of *L. maculans* is unknown. In 2002 and 2003, a resistant cultivar (to PG-2) Q_2 was found severely infected in Roland and Morden, Manitoba, Canada. If there is a pathogen population change and breakdown of resistance, the canola industry could be affected. The working hypothesis for this study was that there has been a shift in the strain structure of *L. maculans* population in Western Canada and North Dakota. In this chapter we describe the application of the pathogenicity test to evaluate variability for virulence in *L. maculans* population using isolates collected from North America especially Western Canada and North Dakota from 2002 through 2004, which was then compared to

a collection from 1984 through 2001. We were also interested in doing an extensive study on the *L. maculans* population in a single field (Chen and Fernando, 2006b).

The studies on the population biology and structure are relevant to the development of rational control strategies to manage the entire pathogen population rather than a single isolate or a race. An epidemic of plant disease that causes significant crop loss involves thousands if not millions of infection events. Recognition and understanding of the population-at-large are important for the management strategies to be effective. This is even more imperative in the blackleg pathogen-complex. It is vital for us to understand the population structure and evolution of *L. maculans* with emphasis of pathogenicity analysis so that we can find answers to the following questions: why and how the *L. maculans* populations are changing in Western Canada and North Dakota.

4.3. Materials and methods

4.3.1. Isolation, preparation and storage of isolates

The isolates of *L. maculans* used for test of pathogenicity groups came mainly from three sources: 1) the *L. maculans* collection maintained in Dr. D. Fernando's Plant Pathology Lab, Dept. of Plant Science, University of Manitoba; 2) the isolates provided by Dr. Barbara J. Howlett (the University of Melbourne, Australia), and Dr. Bruce Fitt (IACR-Roth Amsted, UK); and 3) isolates obtained either from different locations across Canada and North Dakota from 2001 to 2004, or from a single field (La Riviere, Manitoba) in 2004.

4.3.1.1. Re-culture of isolates from UM collection

The *Leptosphaeria maculans* collection at the University of Manitoba encompasses a total of 235 isolates collected from 1983 to 1989, with origins from Canada (Alberta, Manitoba, Ontario and Saskatchewan), USA, Australia and Europe (France, Germany and Netherlands). The mycelia/pycnidia are grown on a slant of potato dextrose agar (PDA) and fully covered with a thick layer of sterile mineral oil for long term storage in 23×85 mm (dia × ht) bottle and kept at room temperature.

The following method was followed to re-culture the isolates. Using a sterile spatula, a small piece of agar was cut from the slant of bottle and transferred onto an autoclaved filter paper. The oil on the agar piece was gently blotted on to the sterile filter paper. The agar piece, free of oil, was placed on the V8-juice agar (200 ml of V8-juice, 0.75 g of $CaCO_3$, 15 g of agar in 800 ml distilled H_2O) and the plate was sealed with parafilm and incubated under continuous cool white fluorescent light at room temperature (20 °C).

Sporulating cultures (pycnidia) that formed on V8-juice agar plates were harvested and kept in a highly concentrated state in a microcentrifuge tube (2.0 ml) at - 20 °C for short term storage (one year viability at least, see 4.3.1.5). All retrieved isolates were passaged through *Brassica napus* cv. Westar (very susceptible to blackleg) once to revive and maintain pathogenicity. Single sporing was done for each isolate.

4.3.1.2. Re-culture of isolates obtained from Australia and UK

Isolates from Australia or UK were sent either after growing on V8 or PDA agar in small tubes or as pycnidiospores-coated filter paper disks. Under aseptic conditions, a piece of agar culture from a tube was cut with a sterile pick or a paper disk was picked up using a sterile medical tweezers, and then sub-cultured on fresh V8-juice agar plates under continuous cool white fluorescent light at room temperature. Cultures were passaged through Westar once and stored at -20°C as mentioned above.

4.3.1.3. Isolation of blackleg pathogen from leaf lesions or stubble collected in Canada and North Dakota, USA

Surface sterilization and pathogen isolation from residue were performed as follows.

Small pieces of stubble bearing pseudothecia, or small pieces of infected leaf tissue were surface sterilized in 1% sodium hypochlorite (NaOCl) solution for 3-5 min (stubble) or 30 seconds (leaf tissue). Stubble and leaf pieces were rinsed in sterile distilled water three times, dried on autoclaved filter paper and placed onto V8-juice agar, and incubated under continuous cool-white fluorescent light for 5-7 days at room temperature. V8-juice agar was amended with 0.03% chloramphenicol (EC200-287-4, Sigma) to inhibit bacterial growth. The fungi were sub-cultured again until pure colonies resembling *L. maculans* were obtained. Pure cultures of the pathogen were isolated and characterized as *L. maculans* by means of colony morphology, pycnidia, and pycnidiospores. As mentioned before, pycnidiospores of all isolates were passaged once through cv. Westar to maintain their virulence and then kept at –20°C for single spore purification.

4.3.1.4. Single spore isolation

One µl of concentrated pycnidiospore suspension was placed on 2% water agar and 10-15µl of sterile distilled H_2O was added to the plate for dilution. Diluted spore suspension was spread over the surface of the agar using a flamed "L"- shaped glass rod. The plates were wrapped in the foil to block the light and incubated at room temperature for 36-48 h. Single germinating pycnidiospores were transferred to V8-juice agar at 100X using a sterile needle. Single spored isolates were grown on V8-juice agar and allowed to fully sporulate before harvesting pycnidisopres.

4.3.1.5. Pycnidiospore harvesting and storage

After growing on V8-juice agar for 14 days at room temperature, sporulating cultures were flooded with 10 ml of sterile distilled water, and the surface of the plates scraped gently with a flamed glass rod to release pycnidiospores. The pycnidiospores were harvested by filtering through sterilized "Miracloth" (CALBIOCHEM, La Jolla, CA) and centrifuged at 9,000 rpm for 20 min. The supernatant was decanted and the spore pellet re-suspended in approximately 1 ml of sterile distilled water. Concentrated pycnidiospore suspension was transferred into a microcentrifuge tube (2.0 ml) and stored at - 20 °C. Pycnidiospores in this short term storage can be kept viable for over a year.

4.3.1.6. Inoculum preparation

The isolates kept at - 20°C were thawed to room temperature, and then vortexed. A few drops of concentrated suspension were mixed into a sterile tube containing about 10 ml of sterile distilled water under aseptic conditions. The number of spores was determined using a hemacytometer (Hausser Scientific Company, Horsham, PA, US) and the concentration of inoculum was prepared at 1×10^7 spores/ml for use in inoculations.

4.3.2. Host cultivars

Three differential cultivars Westar, Glacier and Quinta were used in this study (Table 4.1). Blackleg resistant cv. Q2 and Defender were also used to determine the interaction phenotypes of new highly virulent isolates recently found in Manitoba.

Twelve seedlings of each cultivar, two cotyledons/seedling, twenty four cotyledons in total, were seeded and grown in flats with soil-less mix (MetroMix®, W. R. Grace and Co. Ltd, Ajax, Ontario). All seedlings were watered daily and kept in a growth chamber (16/21°C night/day and a 16-h photoperiod).

4.3.3 Cotyledon inoculation and IP evaluation

Seven days after seeding, fully expanded 24 cotyledons of each differential cultivar were inoculated. Each cotyledon was wounded once with a sterile needle and a 10-µl droplet of pycnidiospore suspension pipetted onto each wound. The inoculated cotyledons were allowed to dry at room temperature at least 12 h before the next application of watering. After inoculation, cotyledons were moved back and incubated in

the same growth chamber. The inoculation experiment for each testing isolate was repeated at least once.

Interaction phenotype (IP) on cotyledons was assessed 12 days post-inoculation by using a 0-9 scale modified from Williams (1985), where 0: no darkening around the wounds; 1: limited blackening around the wound, lesion diameter is 0.5 – 1.5 mm, faint chlorotic halo may be present, sporulation absent; 2: limited brown necrotic lesions, 0.5-1.5 mm, light brown halo may be present, sporulation absent; 3: dark necrotic lesions, 1.5 – 3.0 mm, chlorotic halo may be present, sporulation absent; 4: necrotic lesions limited by dark margin, 1.5-3.0 mm, no tissue collapse; 5: non sporulating 3- 5 mm lesions, sharply limited by dark necrotic margin, may show gray-green tissue collapse; 6: lesions with dark necrotic margin, 3-5 mm with a little sporulation in center of lesion 7: gray-green tissue collapse, 3 -5 mm diameter, sharply delimited, non-darkened margin; 8: lesions with rapid tissue collapse, accompanied by sporulation in center of lesion, more than 5 mm; 9: rapid tissue collapse at about 10 days, accompanied by profuse sporulation in large, more than 5 mm, lesions with diffuse margins.

In this 0-9 scale, 0 to 2 = resistant, 3 to 6 = intermediate, and 7-9 = susceptible.

4.3.4. PG classification

At least five pathogenicity groups (PGs) named PG-1, PG-2, PG-3, PGT and PG-4 were found among isolates according to interaction phenotype (IP) on three differential cultivars (Table 4.2). Isolates in PG-1 are avirulent on three cultivars (resistant IP); PG-2 are virulent on Westar (susceptible IP) but avirulent on Glacier and Quinta (resistant IP);

PG-3 are virulent on Westar and Glacier (susceptible IP) but avirulent on Quinta (resistant IP); PGT are virulent on Westar and Quinta (susceptible IP) but avirulent on Glacier (resistant IP); PG-4 are virulent on three cultivars (susceptible IP).

4.4. Results

A total of 24 isolates obtained in the 1980s (15 from Manitoba, 3 from USA and 6 from Australia) were re-cultured from UM collection (see Table 4.3). All isolates collected in 1980s from Manitoba and North Dakota were either PG-1 or PG-2. No PG-3 or PG-4 isolates were detected. However, 5 of the six Australian isolates collected in 1980s were classified as PG-3 (Table 4.3).

Regarding the isolates collected from 1980s, 1999, 2000 and 20001, pathogenicity group (PG) test indicated that 39 isolates both from Western Canada and U.S.A were either in weakly virulent PG-1 (15% of isolates) or in virulent PG-2 (85% of isolates), whereas isolates from Brazil (7 isolates), UK (5 isolates) and Australia (6 isolates) were mainly in PG-3 (78% of isolates), occasionally in PG-4 (5.6%) or PG-1 (11.1%) (Table 4.3), suggesting that pathogenicity structure of *L. maculans* isolates from Western Canada, Europe and Australia was different ($\chi^2 > 54.86, p < 0.00001$). In Western Canada and U.S.A, similarity of pathogenicity between isolates collected in 1980s and in the early 21st century, grouping either in PG-1 or PG-2, revealed that *L. maculans* populations have remained relatively unchanged over the past one and half decades (1984 to 2001).

In 2002, a total of 47 isolates obtained from three locations in Manitoba, three locations in Saskatchewan and six locations in North Dakota, U.S.A. were evaluated for their pathogenicity (Table 4.4). PG-2 was represented by up to 84% of isolates and PG-1 by 14 % of isolates, very similar to the results of 2001 (Table 4.3, $\chi^2=0.558 < 3.84$, $p > 0.05$). One isolate PL02-02 from Vanaert farm located at east Selkirk, 60 km northeast of Winnipeg, Manitoba was identified as PG-3. This is the first time PG-3 has been detected from isolates in Western Canada. This field was grown with canola (cv. Hyola 401) in 2000 and then planted with barley (2001) and flax (2002).

In 2003, 22 isolates from 8 locations in Manitoba, 17 isolates from 3 locations in Saskatchewan, 12 isolates from 5 locations in Alberta and 107 isolates from at least 13 locations in North Dakota (U.S.A.) were screened for PGs on the canola differentials (Table 4.5). Roland, Carman and La Riviere in Manitoba had PG-3 isolates. PG-3 isolates from Roland and Carman were obtained from co-op blackleg nursery fields where blackleg-resistant canola cv Q_2 was severely infected. Alvin farm, at La Riviere, 200 km southwest of Winnipeg, was reported to be severely affected by blackleg during the growing season 2003. PG-3 was not isolated from Saskatchewan samples but PGT, a new pathotype, was recognized to be present in Saskatchewan's collection (65% of isolates). In North Dakota six PG-3 isolates from two locations (Cavalier and Ward) were isolated and confirmed through the differential test. In addition, PG-4 isolates PL03-54-01 from Camrose, Alberta and PL03-53-31 from Cavalier, North Dakota were first detected. The field where PG-4 was found at Camrose, Alberta was also a co-op blackleg nursery field.

In order to investigate the pathogenicity profile of *L. maculans* population from a single field in 2004, 108 isolates were isolated from a canola field on Alvin farm in La Riviere (Table 4.6), the same field where PG-3 isolates were found in 2003. All five pathotypes were identified within this *L. maculans* population. One percent of isolates was PG-1, 75% of isolates PG-2, 9.3 % PG-3, 7.4 % PGT and 7.4 % PG-4. This is first observed of PG-4 isolates in Manitoba. Several other locations were found in 2004 to have the new pathotype PG-3 or PGT, such as Roland, Selkirk and Morden in Manitoba, Antler in Saskatchewan, Camrose in Alberta, and Ramsey, Towner, Cavalier and Ward in North Dakota (Table 4.6). Again, one isolate PL04-25-01 from Camrose, Alberta was confirmed to be in PG-4 in the 2004 test.

Pathogenicity profiles of *L. maculans* isolates obtained from Western Canada and North Dakota, U.S.A. during period of 1984 to 2004 are reported in Fig 4.1. Data in this figure shows that only PG-1 (15.3%) and PG-2 (84.5%) were detected before or in 2001. PG-1 isolates were found to be present each year but at a low level. Highest level of PG-1 was found appeared in 2002 (21.3%). PG-2 was a dominant pathotype in this region as its proportion of the population was always over 70% of isolates in all four years of investigation. PG-3 was a new pathotype in this area, first found in 2002 with a frequency of 2.1%. It increased across this region to 9.0% in 2003 and 9.3% in 2004. PGT was first shown to be present in 2003 with a frequency of 12.3%, a little higher than that of PG-3 and maintained a similar population size (11.4%) in 2004. PG-4 was discovered in 2003, a year after PG-3 was discovered. However, the occurrence of PG-4 in this area is still low (1.3 and 4.1 % in 2003 and 2004).

4.5 Discussion

Interaction phenotype with artificial inoculation is subject to variation for environmental factors such as temperature. A temperature range of 16/21°C night/day and a 16-h photoperiod were shown to be favorable to the growth of *L. maculans* on *Brassica* species (Nathaniels and Taylor, 1983). We used these environmental parameters for all experiments. Increase of temperature may change interaction phenotypes. For instance, incompatible reaction of weakly virulent isolate PG-1 on a resistant cultivar might switch to a compatible reaction with an increase of temperature (Badawy et al., 1992).

Wound inoculation is vital in achieving constant reactions under controlled growth chamber (Rimmer and vandenBerg, 1992) conditions, because unwounded spray inoculation with pycnidiospores is often unreliable (Moreno-Rico et al., 2001). This method using wound inoculation ensured us to obtain reliable data in our experiments.

Our results show that weakly virulent isolates PG-1, now classified into a separate species *L. biglobosa* (Shoemaker and Brun, 2001), and highly virulent pathogenicity complex PG-2, PG-3, PGT and PG-4 in the species *L. maculans*, are present in canola fields of Western Canada (Chen and Fernando, 2005). No correlation between locations and pathogenicity groups was found. PG-1 occurred at all sites surveyed but at low levels. Isolates of PG-1 are also found in Europe (Jedryczka et al., 1999; Kuusk et al., 2002) and Australia (Plummer et al., 1994), although the genetic origin of PG-1 isolates is unknown. According to AFLP phylogenic analysis, it seems that PG-1 isolates from Europe have a different phylogenetic origin than North American PG-1 because North American PG-1

had a greater genetic diversity and no common bands were found between these isolates (Purwantara et al., 2000). A more thorough genetic analysis of PG-1 is needed to determine the extent of variation. Nevertheless, PG-1 isolates are weakly virulent and do not cause stem canker. Therefore, the implication of PG-1 in the blackleg epidemics is not significant in North America.

PG-2 isolates are prevalent in all locations of Western Canada. They were the predominant population each year in all locations. Actually, PG-2 has been initially reported in Western Canada since 1975 (Kutcher et al., 1993; Mengistu et al., 1991; Mahuku et al., 1997). PG-2 virulent populations in Western Canada have been quite stable in the last one and half decades and thought to be much less diverse (fewer virulent pathotypes) than those from Europe and Australia (Balesdent et al., 2005; Purwantara et al., 2000).

Although PG-3 and PG-4 isolates have been detected previously in Ontario, Eastern Canada (Mahuku et al., 1997) and Georgia, Southeastern USA (Phillips et al., 1999), it was the first time in 2002 and 2003 that PG-3, PGT and PG-4 isolates were found to be present across Western Prairies of Canada and North Central U.S.A. Pongam (1999) indicated that isolates of *L. maculans* in Ontario were genetically linked to those in Australia, France and Germany, whereas isolates of *L. maculans* in Western Canada, North Dakota and Georgia were genetically similar to those in the UK. If that is correct, the origin of virulent isolates found in Ontario and those in Western Canada could be different. PG-2 isolates are rare in Western Europe and Australia, whereas PG-3 and PG-

4 are most abundant (Williams, 1992). The question is why and how are new strains recently appearing in Western Canada?

A few possible scenarios could explain this. First, it could be through introduction of new strains into Western Canada from other regions or continents through seed (McGee and Petrie, 1978; Purwantara et al., 2000). The Canadian seed growers association (CSGA) has a strict regulation of crop seed trades. *Leptosphaeria maculans* is a disease quarantine objective in the importing/exporting of canola seeds. In addition, seed treatment with fungicides against the blackleg pathogen is required in Canada. However, the contribution of seedlot to variation among the isolates was still found in Canada even with such regulations (Mahuku et al., 1997).

Secondly population shifts associated with the sexual recombination capacity of this fungus are well known across the world. *Leptosphaeria maculans* is a heterothallic and outcrossing fungus. Sexual reproduction takes place at least once per year in a life cycle of *L. maculans*, which is a critical process for the production of new strains. PG tests for the isolates from a single field at La Riviere in 2004 showed the co-existence of all PG types, suggesting that sexual recombination occurs in the natural environment. The study of Mahuku et al. (1997) verified the variation among isolates of *L. maculans* caused by sexual reproduction in Canadian canola fields.

Finally, selection pressure exerted by monoculture of similar host resistance (Balesdent et al., 2005) favors the variation of *L. maculans* populations. In France, the large-scale use of single *Rlm1* gene within ten years (1990 to 2000) shifted the population isolates from *Avlm1* to others (Rouxel et al., 2003a). Similarly in Australia, a single dominant

resistance gene derived from *B. rapa* subsp. *sylvestris* collapsed within a period of 3 years of commercial use due to the change of population structure (Li and Barbetti et al., 2005).

Isolates in each PG group have their own virulence spectrum, and cause incompatible reactions on different resistance genes in host species. Differential cv. Glacier was known to have two resistance genes *Rlm2*, *Rlm3* and Quinta has linked resistance genes *Rlm1* and *Rlm4* (Balesdent et al., 2005). In response to those genes, avirulent genes in the isolates are designated as *Avrlm1, Avrlm2, Avrlm3 and Avrlm4*, which condition incompatible reactions with the matching resistance genes. Ansa-Melayah et al. (1998) suggested that resistance of Glacier to PG-2 isolates was conditioned by a gene pair *Rlm2/Avrlm2*; resistance of Quinta to PG-2 was conditioned by two dominant loci *Rlm1* and *Rlm4*, which matched genes *Avrlm1* and *Avrlm4* in the isolates. Since PG-2 isolates are a dominant population in Canada, it can be assumed that *Avrlm1*, *Avrlm2* or *Avrlm4* are ubiquitously present in Canadian isolates, which is in agreement with the results of Balesdent et al. (2005). Hence corresponding resistance genes *Rlm1*, *Rlm2*, *Rlm3* or *Rlm9* can still be useful to help manage the disease in Canada. Actually, recessive and polygenic control of adult plant resistance in Canadian cultivars may play a role in stabilizing the pathogen population (Dion et al., 1995; Rimmer, 2006). Initial source of monogenic resistance to blackleg in Canadian cultivars is either from French cv. Jet Neuf (*Rlm4*) and Cresor (*LmFr1*) or from Japanese cv. Norin 20 and Mutu (unknown resistance genes). More recent utilization may be from spring-type materials from Australia such as cv. Maluka (*LmR1*) (Rimmer and vandenBerg., 1992; Rimmer, 2006).

Resistance of Quinta to PG-3 should be determined by incompatible interaction between *Rlm1/Avrlm1*. If it is the case, Canadian resistant cultivars with *Rlm1* gene to PG-2 should be effective against PG-3 isolates. However, cv. Q_2 and Defender in Canada were found resistant to PG-2 isolates but susceptible to newly detected Canadian PG-3 isolates (Table 4.7). Therefore, *Avr* allele(s) of PG-3 isolates recently identified from North America need to be precisely defined. Resistant background of Glacier to PGT is unknown but could be postulated to the incompatible reaction governed by the gene pair *Rlm2/Avrlm2* like Glacier-PG2 reaction, or *Rlm3/Avrlm3* or both linked together, which needs further study.

Alteration of differential cultivars will definitely give different terminology to the testing isolates even through they may be synonymous. For instance, if cv. Jet Neuf was added as an additional differential host, each PG could be further subdivided into two groups, in total A1 to A6 identified (Badawy et al., 1991; Kuswinanti et al., 1999). Further discrimination of each PG was achieved using five additional *B. napus* lines Sentry, Val-1, Quantum, Sprint and Dac-1 (Keri, 1999), in which PG-2 isolates could be subdivided into 15 groups. Lack of differential cultivar uniformity prevents comparison of population structure in different countries or continents. Also, heterogeneity or more than one R gene in differential cultivars may cause a problem in the identification of PGs. Despite these problems, the differential set used in this study has been demonstrated as a reliable, quick and effective method in the identification of PGs (Koch et al., 1991; Mengistu et al., 1991; Williams, 1992). Many researchers have failed in attempts to correlate the pathogenicity of the fungus with molecular tools because frequent

recombination between isolates in different PGs may allow occurrence of virulence polymorphisms independent of molecular polymorphisms (Mahuku et al., 1997).

In general, *L. maculans* populations in North America were found relatively unchanged over the period of 1980s' to early 21st century. However, PG3 and PG4 were first observed to a great extent in Western Canada and North Dakota, USA recently. The origin of those strains was unknown but sexual recombination or introduction from other regions is possible.

Table 4.1. Cultivar name, year of release, species, pedigree and R gene of three *Brassica* cultivars used as host differentials to characterize interaction phenotype of isolates of *Leptosphaeria maculans.*

Cultivar name	Year	Species	Pedigree	Resistance gene	Reference
Westar	1982	*B. napus*	/Boronowski x Tower/Zephyr/568-2895 /Midas/Tower, Canada	none	Keri, 1999
Glacier	1987	*B. napus*	Ledos/Rapol x Hector, USA	*Rlm2,Rlm3*	Ansan-Melayah et al., 1998; Balesdent et al., 2002
Quinta	1976	*B. napus*	ORO x Rapol*3, Germany	*Rlm1,Rlm4*	Balesdent et al., 2001

Table 4.2. Pathogenicity groups of *Leptosphaeria maculans* / *L. biglobosa* and their interaction phenotype (IP) on three canola cultivars*

	Westar (no *R* genes)	Glacier (*Rlm2, 3*)	Quinta (*Rlm1,4*)
PG-1	0 (R)	0 (R)	0 (R)
PG-2	7-9 (S)	0-2 (R)	3-6 (R)
PG-3	7-9 (S)	7-9 (S)	3-6 (R)
PGT	7-9 (S)	3-6 (R)	7-9 (S)
PG-4	7-9 (S)	7-9 (S)	7-9 (S)

* IP was rated with a scale of 0-9, where 0 = no visible symptoms and 9 = collapse of the tissue with abundant pycnidia formation. S in bracket stands for susceptible and R for resistant.

Table 4.3. Pathogenicity groups of *Leptosphaeria maculans / L. biglobosa* and number of isolates collected up to 2001

Origin	Location[a]	Year[b]	No. of Isolate	PG-1	PG-2	PG-3	PGT	PG-4
Canada	Manitoba	1984	4	3	1			
		1985	3		3			
		1986	4		4			
		1987	4	1	3			
		1999	5		5			
		2000	2		2			
		2001	6	2	4			
U.S.A.	n/a	1987	3		3			
	North Dakota	2001	8		8			
Brazil	n/a	2001	7	1		6		
UK	Roth Amsted	2001	5	1		3		1
Poland	Poznan	2001	5	5				
Australia	Mountbarker	1989	6		1	5		

[a] n/a – not available.
[b]The time when the isolate was collected from field.

Table 4.4. Pathogenicity groups of *Leptosphaeria maculans / L. biglobosa* and number of isolates collected in 2002

Origin	Location	Year[a]	No. of Isolate	PG-1	PG-2	PG-3	PGT	PG-4
Canada	Selkirk (MB)	2002	13	6	6	1		
	Grosse isle (MB)	2002	3		3			
	Winnipeg (MB)	2002	1		1			
	Yorktown (SK)	2002	1		1			
	Melfort (SK)	2002	1		1			
	Tadmore (SK)	2002	1		1			
U.S.A.	Cavalier (ND)	2002	13	2	11			
	Bottineau (ND)	2002	1		1			
	Mclean (ND)	2002	2		2			
	Ward (ND)	2002	4	2	2			
	Benson (ND)	2002	2		2			
	Towner (ND)	2002	5		5			

[a]The time when the isolate was collected from field.
MB – Manitoba, SK – Saskatchewan, ND – North Dakota.

Table 4.5. Pathogenicity groups of *Leptosphaeria maculans* / *L. biglobosa* and number of isolates collected in 2003

Origin	Location	Year[a]	No. of Isolate	PG-1	PG-2	PG-3	PGT	PG-4
Canada	Roland (MB)	2003	4			4		
	Darlingford (MB)	2003	3		3			
	La Riviere (MB)	2003	3		1	2		
	Plum Coulee (MB)	2003	5		1		4	
	Carman (MB)	2003	2			2		
	Gretna (MB)	2003	2		2			
	Dauphin (MB)	2003	1	1				
	Brandon (MB)	2003	2		1		1	
	Kenaston (SK)	2003	5		1		4	
	Tisdale (SK)	2003	9		4		5	
	Arboorfield (SK)	2003	3		1		2	
	Viking (AB)	2003	3		1		2	
	Vermilion (AB)	2003	3	3				
	Camrose (AB)	2003	2				1	1
	Killam (AB)	2003	1	1				
	Westlock (AB)	2003	3	3				
U.S.A.	Ramsey (ND)	2003	2		2			
	Towner (ND)	2003	8		8			
	Foster (ND)	2003	5		5			
	Cavalier (ND)	2003	6		4	1		1
	Ward (ND)	2003	24	1	20	3		
	Eddy (ND)	2003	1		1			
	Mclean (ND)	2003	7		7			
	Mountrail (ND)	2003	4	1	3			
	Bottineau (ND)	2003	10		10			
	Renville (ND)	2003	5		5			
	Ward (ND)	2003	2		2			
	Osnabrock (ND)	2003	1		1			
	n/a (ND)	2003	32		28	2	2	

[a]The time when the isolate was collected from field.
MB – Manitoba, SK – Saskatchewan, AB – Alberta, ND – North Dakota.

Table 4.6. Pathogenicity groups of *Leptosphaeria maculans* / *L. biglobosa* and number of isolates collected in 2004

Origin	Location	Year[a]	No. of Isolate	PG-1	PG-2	PG-3	PGT	PG-4
Canada	Roland (MB)	2004	3		2	1		
	Darlingford (MB)	2004	4		4			
	La Riviere (MB)	2004	108	1	81	10	8	8
	Selkirk (MB)	2004	37		36		1	
	Morden (MB)	2004	7		5	2		
	N. Battlefield (SK)	2004	1		1			
	Antler (SK)	2004	5		4		1	
	n/a (SK)	2004	3		3			
	Camrose (AB)	2004	8				7	1
	n/a (AB)	2004	3		2	1		
U.S.A.	Ramsey (ND)	2004	4		3		1	
	Towner (ND)	2004	9		6	1	2	
	Burke (ND)	2004	2		2			
	Cavalier (ND)	2004	12	2	8		2	
	Ward (ND)	2004	7		6		1	
	Nelson (ND)	2004	4		4			
	Mclean (ND)	2004	4		4			
	Mountrail (ND)	2004	3		3			
	Bottineau (ND)	2004	4		4			
	Renville (ND)	2004	5	1	4			
	Pierce (ND)	2004	6		5		1	
	Benson (ND)	2004	3		3			
	Divide (ND)	2004	1		1			
	Williams (ND)	2004	1		1			
	McHenry (ND)	2004	1		1			

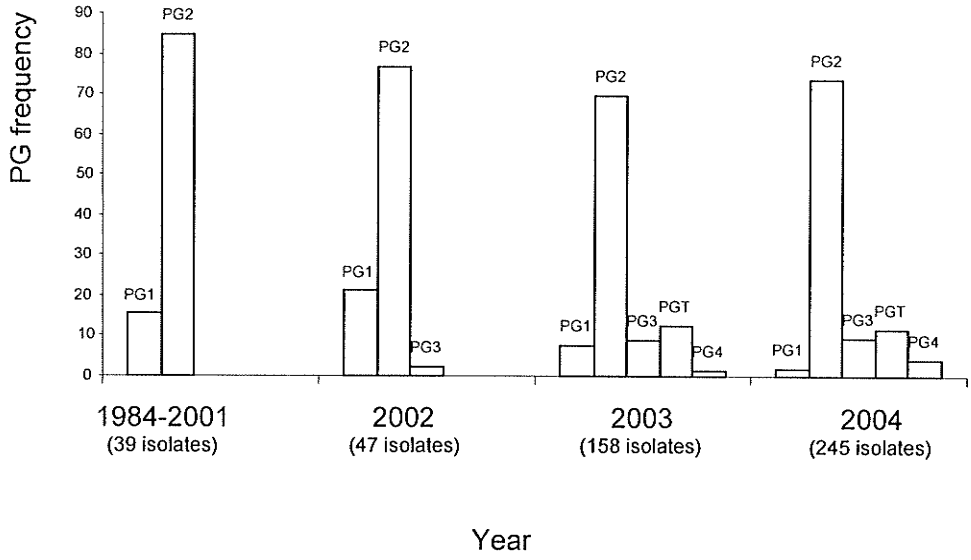
[a]The time when the isolate was collected from field.
MB – Manitoba, SK – Saskatchewan, AB – Alberta, ND – North Dakota.

Table 4.7. Interaction phenotype reaction ratings of *Leptosphaeria maculans* PG-3 isolates from Manitoba on canola cultivar Q_2 and Defender *

Blackleg isolate	Westar	Glacier	Quinta	PG	Q_2	Defender
PL02-02	9.0	6.4	7.5	3	9.0	8.7
PL03-01	8.0	7.6	4.3	3	7.3	7.8
PL03-02-01	8.5	7.5	4.5	3	8.3	7.8
PL03-02-02	8.0	7.3	4.0	3	8.5	7.3
PL03-02-03	8.4	7.7	3.0	3	8.2	7.5
means	8.4	7.3	4.7		8.3	7.8

*Scale of 0-9, where 0 = no visible symptoms and 9 = collapse of the tissue with abundant pycnidia formation. Range of 0-2 is highly resistant, 3-6 intermediate and 7-9 susceptible.

Fig. 4.1. Frequency of pathogenicity groups of *Leptosphaeria maculans / L. biglobosa* detected in Western Canada and North Dakota from 1984 - 2004



Appendix 4.1.

First Report on the Presence of *Leptosphaeria maculans* Pathogenicity Group-3, the Causal Agent of Blackleg of Canola in Manitoba. W. G. D. Fernando and Y. Chen, Department of Plant Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. Plant Dis.87:1268, 2003; published on-line as D-2003-0808-02N, 2003. Accepted for publication 23 July 2003.

Blackleg, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. (anamorph = *Phoma lingam*) (Tode:Fr.) Desmaz.), is an economically important and serious disease of canola (*Brassica napus* L.) in Australia, Europe, and Canada. *L. maculans* isolates can be categorized into four pathogenicity groups (PGs) on the basis of the interaction phenotypes (IP) on the differential canola cvs. Westar, Glacier, and Quinta (1) by using a standard screening protocol in the greenhouse. PG1 isolates are weakly virulent and PG2, PG3, and PG4 isolates are highly virulent. In Manitoba, *L. maculans* population consists mainly of PG2 (virulent on cv. Westar; avirulent on cvs. Glacier and Quinta) and a few PG1 isolates (avirulent on all three differentials). The Oilseed Pathology Lab in the Department of Plant Science, University of Manitoba examines the pathogenic variability of blackleg isolates obtained from Manitoba each year. In 2002, the blackleg-resistant cv. Q2, was found to be severely infected in Roland, Manitoba. The canola stubble collected from a coop trial plot (Roland, Manitoba) and a farm in East Selkirk (60 km northeast of Winnipeg, Manitoba) was isolated for the blackleg fungus. Small pieces of stubble were cut from the pseudothecia forming section and surface sterilized with 1% sodium hypochlorite solution for 3 to 5 min and then rinsed in sterile distilled water. V8 agar medium containing 1% streptomycin sulphate was used to culture the isolates under continuous cool-white fluorescent light for 14 days. Pure cultures of the pathogen were isolated and characterized as *L. maculans* by means of colony morphology, pycnidia, and microscopic observations of pycnidiospores. Pycnidiospores that formed on V8 plates were flooded with 10 ml of sterile distilled water and then harvested by filtering through sterilized Miracloth and kept at –20°C. The isolates were passed once through cv. Westar to maintain their virulence. The PG test was performed with the three differential cultivars. Two additional cultivars, Q2 (resistant to PG2 isolates) and Defender (moderately resistant to PG2 isolates), were included for comparisons. Twelve 7-day-old cotyledons of each differential cultivar grown in Metro Mix were wound inoculated with a 10-µl droplet of pycnidiospore suspension (1 × 107 pycnidiospores per ml). Inoculated cotyledons were maintained in the greenhouse (16/21°C night/day and a 16-h photoperiod). The experiment was repeated twice. Disease severity on cotyledons was assessed 12 days postinoculation by using a 0 to 9 scale (2). All five isolates from Roland and East Selkirk were highly virulent on Glacier (6.4 to 7.7), Q2 (7.1 to 8.2), and Defender (7.2 to 8.4), but intermediately virulent on Quinta (4.5 to 5.4). This clearly indicated that these isolates were of PG3. Isolates of PG2 have been predominant in Manitoba for the past 25 years, and highly virulent isolates belonging to PG3 had not been detected previously. To our knowledge, this is the first report of the presence of PG3 in *L. maculans* in Manitoba.

References: (1) A. Mengistu et al. Plant Dis. 75:1279, 1991. (2) P. H. Williams. Crucifer Genetics Cooperatives (CrGC) Resource Book, University of Wisconsin—Madison, 1985.

Plant Dis.87:1268, 2003; published on-line as D-2003-0808-02N, 2003.

Appendix 4.2.

First Report of Canola Blackleg Caused by Pathogenicity Group 4 of *Leptosphaeria maculans* in Manitoba. Y. Chen and W. G. D. Fernando, Department of Plant Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. Plant Dis. 89:339, 2005; published on-line as DOI: 10.1094/PD-89-0339B. Accepted for publication 13 December 2004.

Leptosphaeria maculans (Desmaz.) Ces. & de Not., causal agent of blackleg of canola (*Brassica napus* L.), was initially placed in several pathogenicity groups (PG) on the basis of the interaction phenotypes (IP) of *L. maculans* isolates on the differential canola cvs. Westar (W), Glacier (G), and Quinta (Q) (4). PG1 isolates are weakly virulent and PG2, PG3, and PG4 isolates are highly virulent. In Manitoba, the *L. maculans* population consists mainly of PG2 isolates (virulent on W and avirulent on G and Q), a few PG1 isolates (avirulent on W, G, and Q), and PGT (virulent on W and Q, but avirulent on G) (3). Since the blackleg fungus is known to have a high level of evolutionary potential, the Oilseed Pathology Laboratory at the University of Manitoba, Winnipeg, Canada, examines the pathogenic variability of *L. maculans* isolates from the Canadian Prairies and North Dakota each year. During 2002, the presence of PG3 (virulent on W and G and avirulent on Q) was reported in Manitoba (1). During 2003, a canola field located at La Riviere, Manitoba, 200 km southwest of Winnipeg, was found to be severely affected by blackleg. Stubble from this field was arbitrarily collected in mid-April 2004, and 98 single-pycnidia pure cultures were obtained by isolating fungi from surface-sterilized (2% sodium hypochlorite), infested residue, cultured on V8 agar at room temperature under cool-white florescent light for 24 h. Pycnidiospores were harvested after 14 days of incubation using the Miracloth filtering method (1). PG testing was performed using the three differential cultivars in the greenhouse. Known PG2, 3, and 4 isolates, 86-12, Liffole-6, and PL30.2, respectively, were included as positive controls. For each of the 98 isolates, 12 7-day-old cotyledons of each differential cultivar grown in Metro Mix were wound-inoculated with 10 µl of a pycnidiospore suspension (1 × 10(^7) per ml) (1). Inoculated plants were maintained in the greenhouse (16/21°C night/day and a 16-h photoperiod with cool-white florescent light). The experiment was repeated three times. Disease severity on cotyledons was assessed 12 days after inoculation with a 0 to 9 scale (0 to 2 = resistant; 3 to 6 = intermediate; and 7 to 9 = susceptible). Of the 98 isolates tested, five were PG1, 51 were PG2, 24 were PG3, 13 were PGT, and five were PG4. The isolates classified as PG4 gave IP reactions of 7 to 9, 7 to 9, and 6.6 to 8.2, on W, G, and Q, respectively. PG3 was reported one year ago, but highly virulent isolates belonging to PG4 have not been previously detected in Manitoba. To our knowledge, this is the first report of the occurrence of PG4 isolates of *L. maculans*, and the first report of PG4 causing canola blackleg in Manitoba. The appearance of PG4 may be evidence of pathogen population changes occurring under high-selection-pressure exerted by resistance genes in commercial cultivars (2), or through importation of PG4 isolates with canola seed.

References: (1) W. G. D. Fernando and Y. Chen. Plant Dis. 87:1268, 2003. (2) B. J. Howlett. Can. J. Plant Pathol. 26:245, 2004. (3) M. Keri et al. Can. J. Plant Pathol. 23:199, 2001. (4) A. Mengistu et al. Plant Dis. 75:1279, 1991.

Plant Dis. 89:339, 2005; published on-line as DOI: 10.1094/PD-89-0339B.

Appendix 4.3.

First Report of Pathogenicity Groups 3 and 4 of *Leptosphaeria maculans* on Canola in North Dakota. C. A. Bradley, Department of Plant Pathology, North Dakota State University, Fargo 58105; and P. S. Parks, Y. Chen, and W. G. D. Fernando, Department of Plant Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. Plant Dis. 89:776, 2005; published on-line as DOI: 10.1094/PD-89-0776C. Accepted for publication 18 April 2005.

Blackleg, caused by *Leptosphaeria maculans* (Desmaz) Ces. & de Not (anamorph = *Phoma lingam*), is an economically important disease of canola (*Brassica napus* L.) worldwide and was first detected in North Dakota in 1991 (3). *L. maculans* can be categorized into one of several pathogenicity groups (PGs) on the basis of the interaction phenotypes in differential canola cvs. Westar, Glacier, and Quinta by using a standard screening protocol in the greenhouse (4). With this system, PG1 strains are weakly virulent and PG2, PG3, and PG4 are highly virulent. The predominant strains of *L. maculans* in North Dakota are PG1 and PG2 (3). In cooperation with the Oilseed Pathology Lab in the Department of Plant Science, University of Manitoba, blackleg infested canola stubble was collected arbitrarily from fields in North Dakota during August and September of 2003. Isolates of the pathogen were obtained by plating surface-sterilized (2% NaOCl), collected stubble on V8 agar containing 0.03% chloramphenicol at 22°C under continuous cool-white fluorescent light. Pycnidiospores were harvested from single pycnidia after 14 days of incubation with the Miracloth filtering method (2) and stored at –20°C. Each isolate was passed once through cv. Westar to maintain virulence. Isolates were confirmed as being *L. maculans* by the presence of characteristic pink pycnidia formed on V8 agar and the characteristic symptoms caused on inoculated cotyledons of cv. Westar. The PG test was performed using a standard screening protocol (4) and was repeated three times for each isolate. For each isolate, 12 7-day-old cotyledons of each differential cultivar were wound inoculated with 10 µl of a pycnidiospore suspension (1 × 10(^7) per ml). Disease severity on cotyledons was assessed 12 days after inoculation with a 0 to 9 scale (0 to 2 = resistant; 3 to 6 = intermediate; and 7 to 9 = susceptible). A total of 106 isolates were obtained from the stubble collected from 47 fields. Of these isolates, three were characterized as PG1, 94 as PG2, six as PG3, and one as PG4; two isolates could not be characterized according to the PG system as described (4). PG3 isolates originated from two fields in Cavalier County and one field in Ward County. The PG4 isolate was from Cavalier County. To our knowledge, this is the first time highly virulent strains of PG3 and PG4 have been detected in North Dakota. PG3 and PG4 strains of *L. maculans* were found only recently in western Canada (1,2). The discovery of these PGs in North Dakota and western Canada has immense implication to canola breeding programs and blackleg control, since these PGs may cause greater levels of blackleg severity on canola cultivars that are resistant to only PG2 type isolates.

References: (1) Y. Chen and W. G. D. Fernando. Plant Dis. 89:339, 2005. (2) W. G. D. Fernando and Y. Chen. Plant Dis. 87:1268, 2003. (3) H. A. Lamey and D. E. Hershman. Plant Dis. 77:1263, 1993. (4) A. Mengistu et al. Plant Dis. 75:1279, 1991.

Plant Dis. 89:776, 2005; published on-line as DOI: 10.1094/PD-89-0776C.

CHAPTER 5

GENETIC VARIATION, POPULATION STRUCTURE AND PHYLOGENETIC RELATIONSHIPS BETWEEN *LEPTOSPHAERIA MACULANS* AND *L. BIGLOBOSA* ISOLATES FROM CANOLA

5.1. Abstract

Populations of *Leptosphaeria maculans* (Desmaz.) Ces. & De Not and *L. biglobosa* Shoemaker & Brun collected from six geographic locations worldwide were evaluated using the sequence-related amplified polymorphism (SRAP) technique to determine their genetic diversity and population differentiation. Extensive diversity was detected in the *L. maculans / L. biglobosa* populations including 85 isolates from Manitoba, Canada, and 12 isolates from North Dakota, USA, 6 isolates from Brazil, 7 isolates from Australia and 6 isolates from United Kingdom. The number of polymorphic loci varied from 54.95 to 91.21% in the geographic populations based on 91 polymorphic fragments. Seventy one to 100% of isolates in the geographic populations and 100% in pathogenicity group (PG) populations sampled from six geographic regions represented unique haplotypes. Values of heterozygosity (*H*) varied from 22.51 to 32.60% in the geographic populations and 13.03 to 27.98% in the PG populations. In general, the *L. maculans / L. biglobosa* populations isolated from Western Canada and North Dakota showed a higher genetic diversity than those populations collected from Australia and United Kingdom, whereas the avirulent *L. biglobosa* PG-1 population showed a lower genetic diversity compared to the highly virulent *L. maculans* PG-2, PG-3, PG-4 and PGT populations sampled from

different regions. However, no significant population differentiation was found among the geographic populations and almost 91% of isolates from different populations were clustered into one lineage. Four highly virulent PG populations (PG-2 to PGT) didn't show a significant difference and they were grouped together in the dendrogram. But avirulent PG-1 was clustered in a different lineage from PG-2, PG-3, PG-4 and PGT populations as it is a different species. Indirect estimation of gene flow showed that high levels of gene flow existed between all populations ($Nm > 2.76$). Sexual recombination was proposed as cause of genetic diversity in the populations and human activities were suggested to lead to high gene flow between the populations. Population genetic similarity between PG-3 isolates surfaced recently in North America and ones collected from Australia supported this assumption. The analysis of molecular variance revealed that a major genetic variance source was the genetic variation among isolates within populations regardless of the origin and pathogenicity. Although some locations had a limited number of isolates, the results of this study clearly showed that the genetic diversity and population differentiation of *L. maculans* / *L. biglobosa* were not closely associated with geographic locations and pathogenicity groups.

5.2. Introduction

Blackleg, caused by the fungus *Leptosphaeria maculans* (Desmaz.) Ces. & De Not, is economically important in the canola/rapeseed (*Brassica napus* L.) industry worldwide (Howlett, 2004 and Juska et al., 1997a). Ascospores arising from pseudothecia on stubble or pycnidiospores from over-wintering sources primarily infect cotyledons or true leaves of canola during spring, initiating leaf lesions. The pycnidiospores produced in leaf lesions disseminate and spread via rain-splash or through wind to new leaves, causing secondary infection. The pathogen develops from leaves to stem and produces stem canker, which causes yield loss of canola (Howlett et al., 2001). After canola is harvested *L. maculans* lives on stubble of canola and forms pseudothecia as the over-wintering structure (Petrie, 1986).

Isolates of the pathogen can originally be classified into at least four pathogenicity groups (PGs), ranging from PG-1, PG-2, PG-3 to PG-4. Determination of PGs is based on interaction phenotype (IP) on the cotyledons of three *B. napus* cultivars: Westar (susceptible with no known resistance genes), Glacier (*Rlm2* and *Rlm3* resistance genes) and Quinta (*Rlm1*and *Rlm4* linked resistance genes) (Koch et al., 1991 and Mengistu et al., 1991). Recently PGT was added as a new group (Rimmer, 2006). PG-1 is weakly virulent and has been designated recently as the new species *L. biglobosa* using morphological characteristics (Shoemaker and Brun, 2001). PG-2, PG-3, PG-4 and PGT are virulent groups and belong to *L. maculans*. Pathogenicity groups show the gene-for-gene relationship between the pathogen and host and indicate the differences between isolates in pathogenic ability.

Previous studies have specified that populations of *L. maculans* were quite diverse worldwide. Based on pathogenicity of blackleg isolates, Mengistu et al. (1991) found that the predominant population of Canadian isolates consisted of PG-2, whereas isolates from Australia were more varied and classed in groups PG-2, PG-3 and PG-4. At present ten avirulence genes (*alm1* and *AvrLm1* to *AvrLm9*) have been characterized in the pathogen population (Balesdent et al., 2005). Among them *AvrLm2*, *AvrLm3* and *AvrLm9* are absent but *AvrLm6* and *AvrLm7* are consistently present among European isolates. In the Canadian populations *AvrLm7* and *AvrLm8* are absent but *AvrLm1*, *AvrLm2*, *AvrLm3* and *AvrLm9* are present. In Australia, *Avr* allele combinations are extremely diverse because many Australian resistance genes have originated from *B. juncea*, *B. nigra* and *B. carinata* (Ballinger and Salisbury, 1996; Purwantara et al., 1998). At the race level, a larger number of races was observed in Australia (n, number of race, =18) than Europe (n=8), whereas isolates in Western Canada tended to be more related to Australian races, and isolates in Eastern Canada (Ontario) were more similar to European races (Balesdent et al., 2005).

A number of molecular techniques have been used to investigate genetic diversity of pathogen populations (Goodwin and Annis, 1991; Johnson and Lewis, 1990; Koch et al., 1991; Patterson and Kapoor, 1995; Schafer and Wostemeyer, 1992; Taylor and Borgmann, 1994; Voigt et al., 1998). RAPD or RFLP techniques were applied to analyze phylogenetic distinctions between avirulent and virulent groups. The amplified fragment length polymorphism (AFLP) seems to be a more efficient PCR-based technique than others (Pongam et al., 1999; Purwantara et al., 2000), because it produces many more

polymorphic fragments. The AFLP method was used to investigate the genetic diversity of populations obtained from either two different virulent groups or the same virulent group with different origins. The results of Pongam et al (1999) indicated that PG-2 isolates from Western Canada, USA and UK were quite similar but isolates from Eastern Canada, Australia and Europe, consisting of different PG groups, were quite diverse. Purwantara et al (2000) noticed that the isolates collected from different geographic regions could be classified into five types, with genetic separation between Australian and European populations, and North America populations partially overlapping with either Australian or European populations.

The sequence-related amplified polymorphism (SRAP) molecular marker technique, which has been applied to gene tagging in *Brassica* plants, is a relatively new and highly efficient marker system (Li and Quiros, 2001). In SRAP two 16-22-mer primers are used to amplify genomic DNA in one step PCR to produce about 50-100 highly reproducible potentially polymorphic bands similar to those generated by the AFLP technique (Budak et al., 2004; Ferriol et al., 2003; Li and Gao et al., 2003). SRAP PCR runs at low annealing temperatures during the first 5 cycles and then increases to high annealing temperatures for 35 cycles to ensure that the PCR products from first 5 cycles are efficiently and specifically amplified. SRAP PCR products are separated with a polyacrylamide gel or a capillary DNA analyzer. Compared to AFLP, SRAP is simpler and less costly with the omission of enzyme restriction, ligation of primer adapters, and pre-amplification. Although SRAP technique was initially developed for gene tagging in *Brassica* plants, it has been used to determine the genetic diversity and population

structure of plant pathogens of field crops and shade trees (Fernando et al. 2005; Fernando et al. 2006; Zhang, et. al. 2005a; and Zhang et al. 2005b).

Although studies have been conducted on phylogenetic distinction between avirulent and virulent populations and genetic diversity among populations with different geographic origins, there are few previous reports on genetic variation and population differentiation of isolates belonging to different PGs or isolates obtained from a single field. In 2003, the isolates collected from a single field located in La Riviere, Manitoba, 200 km southwest of Winnipeg, were found to be diverse in PGs, and all PGs of *L. maculans* were detected in a single field (Chen and Fernando, 2006b). In order to obtain valuable information on genetic variation of the isolates in this field and also to test the genetic structure between different PG populations, we used the SRAP technique to develop molecular markers. The objectives of this study were to characterize the population structure of *L. maculans/L. biglobosa* from different geographic regions and to determine genetic variation within a single population and between different PG-groups.

5.3. Materials and methods

5.3.1. Isolate collection

To analyze genetic diversity and differentiation of *L. maculans* / *L. biglobosa* populations, a total of 116 isolates were collected from six geographic regions in Canada (La Riviere and Roland), USA, Brazil, Australia and UK (Table 5.1). Canadian, American and Brazilian isolates were mostly obtained from stubble through surface sterilization, whereas Australian and UK isolates were provided by Dr. Barbara J. Howlett (the University of Melbourne, Australia), Dr. Bruce Fitt (IACR-Rothamsted, UK) or from the stock culture at the University of Manitoba. All geographic populations were isolated from *B. napus*. In addition, 116 isolates consisting of five PGs were used to investigate genetic diversity and population differentiation among populations defined by pathogenicity phenotypes (Table 5.1, 5.4).

Small pieces of stubble were cut from the pseudothecia forming section and then surface sterilized with 1% sodium hypochlorite (NaOCl) solution for 3-5 min. After rinsing in sterile distilled water three times and drying on an autoclaved filter paper, disinfected stubble was plated onto V8-juice (Campbell Soup Company Ltd, Toronto, Canada) agar (200 ml of V8 juice, 0.75g of $CaCO_3$, 15g of agar in 800 ml of distilled H_2O) under continuous cool-white fluorescent light for 5-7 days of incubation. V8-juice agar was amended with 0.03% chloramphenicol (EC200-287-4, Sigma) to inhibit bacterial growth. The fungi were sub-cultured again until uniform colonies were obtained.

The isolates from Australia or UK were sent either after growing on V8 or potato dextrose agar (PDA) in a small tube or in the form of pycnidiospores-coated filter paper

disks, which were re-grown on V8 agar directly. The isolates from the UM collection had been stored in specimen bottles (23×85 mm) with mineral oil, and were revived on V8 agar after removing mineral oil from sample pieces.

5.3.2. Single spore isolation

A piece of sporulating culture on V8 was cut and transferred into distilled water to make a pycnidiospore suspension by vortexing. The dilute spore suspension was spread over the surface of a 2% water agar (Agar Technical, DIFCO, Becton, Dickinson and Company, Sparks, MD, USA) plate and incubated at room temperature for 36-48 h. Single germinating pycnidiospores clearly separated from others were marked on plates under the microscope, and then transferred to V8-juice agar plates, and incubated at room temperature (20-24°C) to obtain pure single spore isolates.

5.3.3. Pathogenicity group (PG) test

Single spored isolates were sub-cultured on V8 agar at room temperature under continuous cool-white fluorescent light for 14 days. Sporulating cultures were flooded with 10 ml of sterile distilled water; filtered through sterilized Miracloth (Calbiochem, CN Biosciences, Inc. La Jolla, CA) and centrifuged at 9,000 rpm for 20 min. Concentrated pycnidiospore suspension was stored in a micro centrifuge tube (2.0 ml) at -20 °C. Inoculum was diluted to 1×10^7 spores/ml in water before inoculation.

Brassica napus cv. Westar, Glacier and Quinta were grown in MetroMix® (W. R. Grace and Co. Ltd, Ajax, Ontario) in a growth chamber with 16/21°C (night/day) and a

16-h photoperiod. Seven-day old cotyledons were wound inoculated (10 µl spore suspension/wound) and incubated for 12 days under the same growth condition mentioned above. Interaction phenotype (IP) on cotyledons was assessed by using a 0-9 scale (Williams, 1985) and PG identification was determined as follows: PG-1 (avirulent on three cultivars); PG-2 (virulent on Westar and avirulent on Glacier and Quinta); PG-3 (virulent on Westar and Glacier but avirulent on Quinta); PGT (virulent on Westar and Quinta but avirulent on Glacier); PG-4 (virulent on three cultivars).

5.3.4. DNA extraction

Pycnidiospores produced on V8 plates from 2-wk-old single spore cultures were scraped gently with a sterile glass rod and inoculated into a 250 ml flask containing Czapek-Dox Broth ((75ml water; 2.625 g Czapek-Dox (C1551, Sigma); 0.15 g yeast)). The flasks were incubated at room temperature (20-24°C) for 12 days with agitation (100 rpm). Once a mycelium pad was formed, it was harvested by vacuum filtration through two layers of sterilized Miracloth. Mycelia were rinsed twice with sterile water, and stored at –80°C until lyophilized. DNA of each isolate was extracted using the following steps:

Lyophilized mycelia were ground in liquid nitrogen using mortar and pestle and transferred into centrifuge tubes. The extraction buffer (100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 500 mM NaCl and 1.25 % SDS) was added to the powder and the mixture vortexed thoroughly. After adding 2% CTAB and 1% β-mercaptoethanol, the contents of the tubes were incubated at 60°C for 30-60 min and placed on ice for 20 min. The

solutions were mixed with equal volume of phenol/chloroform/isoamyle alcohol (25:24:1), followed by centrifugation at 10,000 prm for 15 min. The supernatant was transferred to new tubes and extracted with equal volume of chloroform/isoamyle alcohol (24:1). After another centrifugation at 10,000 prm for 15 min, the DNA in the supernatant was precipitated in two volumes of ice-cold 100% ethanol. DNA pellets were dissolved in TE buffer. DNA solution can be cleaned by adding RNase (10 mg/ml) and incubating at 37 °C, 30 min. The solution should be extracted again using chloroform/isoamyle alcohol (24:1) in order to get clean DNA. DNA was precipitated by ice-cold ethanol and DNA pellets were suspended into TE buffer. DNA concentration and purity were determined using a spectrophotometer and adjusted to a final concentration of 5 ng μl^{-1} for PCR analysis.

5.3.5. Amplification of DNA

The PCR-based SRAP technique was used to analyze DNA of blackleg isolates for genetic diversity and population differentiation among geographic, single field and PG populations. PCR amplification reaction with the SRAP primers was performed in a 15 µl reaction volume containing 15 ng template DNA, 0.4 µM of each primer, 0.75 unit of Taq polymerase (Invitrogen Life Technologies, ON, Canada), 100 mM Tris-HCl (pH 8.0), 500 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM each of dNTPs. The fragments were amplified in a Thermal Cycler (PTC-200, MJ Research, Inc. Waltham, MA, USA). The first five cycles were run at 94°C for 1 min, 35°C for 50 s, and 72°C for 1 min, for denaturing, annealing and extension, respectively. The remainder of the amplification

consisted of 35 cycles at 94°C for 50 s, 50°C for 50 s, and 72°C for 1 min. PCR repetitions using the same set of primers and isolates and DNA preparations were conducted to check the reproducibility of results. To select effective primer combinations, thirty pairs of the SRAP primers were screened for the PCR amplification. The amplified PCR products were separated first by electrophoresis, using a denaturing 5% (wt/vol) polyacrylamide DNA sequencing gel containing 7.5 M urea. The silver staining kit (Promega, Madison, WI, USA) was used for visualizing DNA bands. Manufacturer's instructions were followed for the fixation, staining and development of the polyacrylamide gel. Based on efficacy of primers producing polymorphic bands on polyacrylamide gels, four pairs out of 30 primer combinations were chosen for generating the SRAP data: DC1 (5 -TAA ACA ATG GCT ACT CAA G-3) and ODD30 (5 -GCG ATC ACA GAA GGA AGG T-3), FC1 (5 -TCA AGG GCA GGT AAG AAC AA-3) and ODD30, EM1 (5 -GAC TGC GTA CGA ATT CAA T-3) and ODD30, SA12 (5 -TTC TAG GTA ATC CAA CAA CA--3) and ODD30. The PCR products produced with these selected primer combinations were then separated in an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems and Hitachi, Ltd., Japan) to perform the fragment analysis. The protocols of the company were employed to fill the polymer gel fluid, load PCR products, set the preferences of the data collection software, monitor the analysis run, and view and analyze raw data in the ABI Prism® 3100 Genetic Analyzer. The fragment analysis data were collected using the data collection software based on sizes of PCR products. Raw data from a completed run were first viewed and analyzed using the GeneScan software. To visually view band patterns and screen the polymorphic bands,

the fragment analysis data were then reconstructed into a gel image using the software Genographer (Version: 1.6), developed by Montana State University (http://hordeum.msu.montana.edu/genographer). The range of fragment sizes and visual intensity of bands for screening were defined easily in the Genographer program, and the fragment analysis data were viewed as a gel image, along with thumbnails, and graphs, which allowed for a quick and easy way of scoring a gel. The presence and absence of all fragments between molecular sizes of 100 and 600 bp were visually scored for each isolate in the Genographer program.

5.3.6. Statistical analysis

Populations were defined according to their geographic locations and PG phenotype. Amplified fragments were scored manually as putative loci with two alleles, one allele indicating the presence of a fragment (designated as 1) and the other the absence (0) of homologous bands to create a binary matrix of isolates and molecular fragments of the different SRAP phenotypes. The analysis of molecular variance (AMOVA) was used to partition the total genetic variance within and among populations from different geographic regions and PGs (Excoffier et al., 1992). AMOVA was performed by treating a SRAP profile as a haplotype, using Arlequin version 3.0 software provided by L. Excoffier (Institute of Zoology, University of Berne, Switzerland). A molecular evolutionary and phylogeny program, V_MLDIST (Drummond and Strimmer, 2001), was used to compute maximum likelihood distances between SRAP discrete data of all 116 isolates and a consensus tree was constructed using Neighbor-Joining method with

100 bootstrap replicates. The software programs, Population Genetic Analysis (POPGEN32, Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada), and Tools for Population Genetic Analyses (TFPGA, version 1.3; Northern Arizona University, USA) were used for statistical analysis by the standard methods of population genetics. The data were processed as a haploid model with two alleles per locus. Genetic diversity analysis was measured among six geographic populations (Table 5.3) and five populations of different PGs (Table 5.4). Heterozygosity (H) and percent polymorphic loci (99% criterion) were estimated for all populations. Genotypic diversity was calculated using Shannon's information index (Shannon and Weaver, 1949). Population structure was analyzed using F statistics (Weir and Cockerham, 1984) in order to test the significance for the null hypothesis of no differentiation at the corresponding hierarchical level. Differentiation among populations from geographical locations and PGs was also estimated using an exact test (Raymond and Rousset, 1995) and by indirect estimation of gene flow using $Nm = ½ (1\text{-}G_{st})/G_{st}$ (Nei, 1973; Slatkin, 1987), where N is the effective population size, m is the migration rate of gene flow, and Nm is the average number of migrants among populations per generation (Slatkin, 1987; Slatkin and Barton, 1989). Cluster analysis of multilocus SRAP genotypes was based on allele frequencies observed for each population. A phenogram was constructed for PG populations using the unweighted pair-group method with arithmetic average (UPGMA) from a Nei's genetic distance matrix (Nei, 1978) in the TFPGA 1.3 software package. Bootstrap sampling (1000 replicates) was performed for statistical support of branches of the constructed phenogram (Felsenstein, 1985).

5.4. Results

5.4.1. PG testing

PG testing results of the isolates from different regions are listed in Table 5.1. The isolates from La Riviere, Manitoba, Canada, comprised all types of PGs except PG-1. The isolates inform Roland, Manitoba, Canada also had all types of PGs except PGT. Actually PGT isolates were only detected in the La Riviere population. The North Dakota population comprised of PG-1, PG-2 and PG-3. Brazilian isolates were mostly PG-3. Australian populations belonged to only PG-3, and the isolates in UK population belonged to PG-2, PG-3 and PG-4.

5.4.2. SRAP variation

A total of 91 polymorphic loci were generated from the 116 isolates of *L. maculans* / *L. biglobosa* included in this research using four pairs of SRAP primers (Fig. 5.1). AMOVA analysis (Table 5.2) revealed that only 4.12% of variation was caused by differences among different geographic populations ($P < 0.001$), and 95.88 % was due to molecular diversity within population. AMOVA was performed by partitioning the data among and within populations from pathogenicity groups. The results showed that 10.82 % of total variance was due to differences among pathogenicity groups, and 89.18 % of total genetic variance was caused by differences within pathogenicity groups. The major source of the genetic variation was differences within populations regardless of whether a population was based on geographic location or pathogenicity group (Table 5.2).

Because AMOVA indicated high genetic variability within geographic and pathogenicity populations, genetic diversity was assessed on the populations of *L. maculans* / *L. biglobosa* collected from different regions in Canada, USA, Brazil, Australia, and United Kingdom (Table 5.3). The number of polymorphic loci (based on the 99% criterion) varied from 54.95% of the populations collected from UK and Australia to 86.81% and 91.21 of the populations from La Riviere, Roland in Manitoba, Canada and North Dakota, USA. Based on the 91 polymorphic loci, the percentage of unique genotypes (g/n) in geographic populations varied from 71.43 to 100% (Table 5.3). Every single isolate represented a unique genotype in Roland (*g/n* = 8/8), North Dakota (*g/n*= 12/12), Brazil (*g/n*= 6/6), Australia (*g/n*= 7/7), UK (*g/n*= 6/6) population but not in La Riviere (*g/n*= 55/77) (Table 5.3). Similarly, the values of the Shannon index (*s*) of geographic populations also revealed variation in genotypic diversity of the isolates ranging from 0.46 (Roland) to 0.31 (Australia) (Table 5.3). The average unbiased Heterozygosity (*H*) obtained by estimates of allele frequency according to Nei's (1978) formula varied from 22.51 % (Australia) to 32.6% (Roland) in the six geographic populations. In general, all populations showed high levels of heterozygosity (Table 5.3).

Heterozygosity and genotypic diversity were also compared among five PG populations sampled from different geographic populations (Table 5.4). A high level of polymorphic loci (*r*) was shown in the isolates of PG-2 (86.81%), PG-3 (83.52%), PG-4 (72.53%) and PGT (58.24%) but not PG-1 (29.67%). Shannon index (*s*) indicated that the populations of PG-2 (0.42), PG-3(0.40), PG-4 (0.36) and PGT (0.29) were more diverse than those of PG-1 (0.17). With *H* values, a uniform level of heterozygosity and

genotypic diversity was observed in the populations of PG-2, PG-3, PG-4 and PGT ($H >$ 20.0%), but PG-1 (H = 13.0%) was much lower. However, all isolates in the different PGs showed high levels of unique genotypes with g/n values of 100% in all PGs.

5.4.3. Population structure

SRAP data did not show a high level of discrimination between the geographic populations but PG populations. Genetic identity and distances were not significantly different among 15 pair combinations of six geographic populations of *L. maculans and L. biglobosa* (Table 5.5). Genetic identity, as presented by Nei's genetic identity index (Nei, 1978), ranged from the maximum value 0.99 (between the La Riviere and North Dakota populations) to the minimum value 0.90 (between the Australian and Roland populations) (Table 5.5). Nei's (1978) genetic distance coefficients were ranged from the maximum value 0.11 to the minimum value 0.01 (Table 5.5) and no big difference was observed.

A program V_MLDIST was applied to construct a dendrogram (Fig. 5.2) for combined SRAP data from four primer pair sets using Neighbor Joining method with 100 bootstrap replicates of maximum likelihood distances in order to assess relatedness among 116 isolates. Ninety one percent of isolates, no matter of geographic or PG origin, was clustered together and no distinct subgroups was observed capable of differentiating six geographic populations. However, four PG-1 isolates (ND1, Ro12, Ro11 and Br11) was classified together as a distinct subgroup.

Exact tests (Raymond and Rousset, 1995) were used for analyzing population differentiation. The results didn't show significant differentiation between the

populations as all p values > 0.05, indicating an absence of differentiation (Table 5.7). Indirect estimation of gene flow between geographic populations using the number of migrants (*Nm*) as a measurement of population differentiation showed the rates of gene flow between populations ranged from 2.77 to 15.96 migrants per generation, meaning that gene migration was quite different between geographic population pairs. The lowest gene flow occurred between Australian and other populations (*Nm* = 2.77 to 5.46). Populations between Australia and United Kingdom also had lower gene flow (*Nm* = 3.82). Higher probabilities of gene flow seem to have taken place among La Riviere, Roland, North Dakota, Brazil, and UK populations (*Nm* = 6.42 to 15.96). In general, when higher gene flow was observed, lower differentiation among geographic population pairs from each other was observed (Table 5.7). For instance, lowest differentiation and greatest gene flow were measured between the La Riviere and North Dakota populations (*Nm* = 15.96, $P = 0.30$).

Population structure was analyzed for populations from different pathogenicity groups. Nei's (978) genetic distances among five PG populations sampled from different geographic populations ranged from 1.17 to 35.70% (Table 5.6). Based on the genetic identity and distances, the populations from the PG-2 and PG-3, PG-4 and PGT (Nei's distance < 5.0%) were more closely related to each other than to PG-1 population (Nei's distance > 28.1%) (Table 5.6). A UPGMA dendrogram was generated for PG populations. UPGMA cluster analysis using Nei's' (1978) unbiased genetic distances indicated that PG-1 isolates were closely clustered into one subgroup, it was obviously different from

other PG isolates which were fallen into another distinct subgroup with statistically well supported branches by 1000 bootstrappings (Fig. 5.3).

Significant differentiation was also detected between population pairs from different PG populations based on the exact tests (Table 5.8). The rate of gene flow for populations from different PGs was highly different. PG-1 population had very low gene flow to other PG populations ($Nm < 1$). But high gene flow was observed among PG-2, PG-3, PG-4 and PGT ($Nm > 5.2$). The greatest gene flow was between PG-2 and PG-3 ($Nm = 14.6$) (Table 5.8).

5.5. Discussion

The first goal of this study was to use SRAP markers to characterize the genetic structure of different PG populations belonging to *L. maculans* / *L. biglobosa* with emphasis on the genetic background of PG-3 that surfaced in Western Canada recently (Fernando and Chen, 2003). In 2003, a canola field located in La Riviere, Manitoba, Canada was severely infected by blackleg and all types of *L. maculans* PGs were observed from this single field (Table 5.1). Its genetic structure and difference compared to other populations from around the world was examined. This study indicated genetic similarity among the populations consisting of *L. maculans* and *L. biglobosa* collected from different geographic regions. However, the genetic differentiation was strongly associated with the pathogenicity populations that were sampled from different geographic populations. To our knowledge, this is the first report of genetic diversity and

population structure in a single blackleg population composed of all types of *L. maculans* pathogenicity group isolates in Western Canada.

The frequency of recombination through the sexual stage is an important genetic diversity source in fungal populations. Asexual reproduction by pycnidiospores results in clonal population structures that have distinctive features such as widespread occurrence of identical genotypes and linkage between independent sets of genetic markers (Milgroom, 1996). However, a high level of diversity within a population, coupled with a lack of linkage among loci, is indicative of a genetically recombining population (Hartl and Clark, 1989). In this study, we isolated the pathogen from single pycnidiospores, and found that a high level of genetic diversity existed in the *L. maculans* and *L. biglobosa* populations. In all our populations, i.e. La Riviere, Roland, ND, Brazil, Australia and UK (Table 5.3), nearly every isolate has unique genotype, suggesting that a high level of recombination had occurred in these geographic populations, thus causing a high level of genetic diversity.

Populations of *L. maculans* from Australia and United Kingdom had lower genetic diversity compared to the populations from North America and Brazil consisting of *L. maculans* and *L. biglobosa* (Tables 5.1 and 5.3). For example, the percentage of polymorphic loci among isolates in the United Kingdom and Australian populations (r = 54.95%) was notably lower than that observed among the La Riviere, Roland, ND and Brazil populations (r = 67.03 to 91.21%). However, this didn't result in different genetic clusters (Fig 5.2). We did not find specific evidence to explain this reduction among *L. maculans* isolates collected from Australia and United Kingdom. One of the factors

responsible for this may be that populations from Australia and United Kingdom were geographically and genetically distant, but the North American populations shared markers with both regions (Purwantara et al., 2000). Disparity of PG structure in the populations could be another reason for differences (Balesdent et al., 2005) because the populations collected from Australia and United Kingdom were less diverse in the structure of PGs (absence of PG-1 isolates, Table 5.1). Diversity of host genotypes from which pathogens were isolated has been suggested to play a role in genetic diversity of pathogens in other host and pathogen systems (González et al., 1998; Woo et al, 1998). In our study, although all populations were isolated from *Brassica napus,* genetic background of cultivars and resistance genotype from which the isolates were obtained was not available.

Corresponding to the genetic diversity, our results showed that among six populations based on geographic locations, there existed high levels of gene flow (Table 5.7) so that most isolates from different locations were clustered together (Fig. 5.2). However, the rate of gene flow among the populations was different. It seems that populations from closer geographic locations had higher possibility of gene exchange. For instance, gene flow between La Riviere, MB and North Dakota populations isolated from the closest geographic locations in this study had the greatest rate (*Nm*= 15.96) of gene flow compared with populations from other geographic locations, indicating that genetic similarity was present between the populations at these two locations. This result supported the conclusion by Pongam et al (1999) that *L. maculans* was introduced into North Dakota from Western Canada. This can be easily explained. According to Gout et

al. (2006), the reason of a high level of gene flow between the populations with short distance could be proposed as either natural long distance dispersal of propagules within a country or a rapid countrywide diffusion of novel virulence alleles whenever novel resistance sources are used. Relative lower level of gene flow was found among populations from Australia and United Kingdom (Nm = 3.82), as they were separated in geographic locations, which supported Purwantara's results (2000). However, gene flow did occur between these two populations as $Nm > 1$. This could be proposed as human activities leading to genetic exchange between populations located on different continents. This is why Gout et al. (2006) suggested that the structure of blackleg population from West Europe is similar to that in Australia.

The single population from La Riviere was a good example to demonstrate that sexual recombination and human activities may have caused genetic alternation. It was found that this population had the most divergence in PG constitution (PG2 to PGT, Table 5.1), high number of genotype (r = 86.81), high proportion of heterozygosity (H = 0.27) (Table 5.3), and gene flow to the Australian (Nm = 5.45) and United Kingdom (Nm = 8.85) populations (Table 5.7). The isolates in this population was not clustered into different groups with ones from Australia and United Kingdom populations (Fig. 5.2), indicating that global gene flow existed in *L. maculans* populations.

Analysis of the genetic structure of *L. maculans* and *L. biglobosa* showed that epidemics of blackleg disease are caused by genetically diverse populations of the pathogen. Regardless of geographic location, the major source of genetic variation was differences among isolates within populations (Table 5.2, 5.3). These findings have

implications for quarantine and disease management. Resistant cultivars are one of the most effective means of disease control. Because of high levels of genetic diversity within populations, our results indicated that the expression of disease resistance to *L. maculans* may be dependent on the strain of the pathogen. When screening breeding lines for resistance to blackleg disease, it is advisable that several strains, or a broad selection of isolates, be used to effectively screen germplasm for resistance in a region. Based on population differentiation, we also suggest that representative strains from each geographic region should be used for inoculating hybrids or lines obtained from breeding programs to find blackleg resistance corresponding to different geographic regions.

Pathogenicity groups (PGs) are an important index of the ability of pathogen aggressiveness (Koch et al., 1991 and Mengistu et al., 1991). Among the PG populations sampled from the global populations across six geographical locations, non-aggressive PG-1 had the lowest genotype (r = 29.67%) and heterozygosity (H = 0.13) compared with other PG populations (Table 5.4), and also had a much greater genetic distance (> 28.13%) from other PG populations (Table 5.6), which made it fall into a different cluster from the rest of the virulent PG-groups (Fig. 5.3). This result showed that the PG-1 population was different from other PG populations in genetics and also resulted in PG-1 being assigned as a new species *L. biglobosa* (Shoemaker and Brun, 2001) different from *L. maculans* which consists PG-2, PG-3, PG-4 and PGT. No significant genetic difference among PG-2, PG-3, PG-4 and PGT populations were observed (Fig. 5.3). The rate of gene flow among these PG groups was high (Nm > 5.21, Table 5.8), which decreased the genetic diversity among them. The gene flow between PG-1 and other PGs

was very low ($Nm < 1$, Table 5.8) that led to large genetic diversity (Nei's genetic distance > 0.28, Table 5.6) between them. In general, population variation was found between two species *L. maculans* and *L. biglobosa* but not strongly associated with difference among PG populations that belong to *L. maculans*. However, high genetic diversity was noticed within each of PG-2, PG-3, PG-4 and PGT populations ($r > 58\%$, Table 5.4). The new North American PG-3 population, which was recently observed in Western Canada and North Dakota, was compared with PG-3 populations from Australia and United Kingdom. The result indicated that the North American PG-3 population was genetically similar to Australia's and United Kingdom's PG-3 ($P > 0.05$, Table 5.9), suggesting that PG-3 found in Western Canada and North Dakota had most likely evolved through either sexual recombination but with a high possibility being imported from Europe or from eastern Canada because the isolates between eastern Canada (Ontario) and Europe were similar (Balesdent et al., 2005). However, more PG-3 isolates in the United Kingdom and Australian populations would need to be examined to improve the significance of this hypothesis. Additional research is needed before stronger conclusions can be made with respect to the origin of PG-3, PG-4 and PGT recently found in Western Canada and North Dakota. As an initial study on the genetic diversity and population structure of *L. maculans* from canola fields in Western Canada, this research has provided some valuable information for future research on this disease and may serve as a baseline for monitoring population evolution of *L. maculans*.

TABLE 5.1. Pathogenicity group (PG) of *Leptosphaeria maculans / L. biglobosa* isolates collected from different regions according to phenotype interaction on three *Brassica napus* cvs. Westar, Glacier and Quinta

Population	Location	# of isolates	PG-1	PG-2	PG-3	PG-4	PGT
La Riviere	MB, Canada, 2004	77	0	47	13	8	9
Roland	Winnipeg, MB, Canada, 2002-2004	8	2	1	3	2	0
ND	North Dakota, USA, 2003-2004	12	1	4	7	0	0
Brazil	Brazil, 2001	6	1	0	5	0	0
Australia	Australia, 1989-2004	7	0	0	7	0	0
UK	United Kingdom, 2002-2003	6	0	3	2	1	0
Total		116	4	55	37	11	9

TABLE 5.2. Analysis of molecular variance (AMOVA) for populations from six geographic populations and five pathogenicity group (PG) populations of *Leptosphaeria maculans / L. biglobosa* using sequence-related amplified polymorphism (SRAP) markers[a]

Source of variation	d.f.	Sum of squares	Variance components	*P*	Total variance (%)
Among regions	5	94.304	0.53979	< 0.001	4.12
Within regions	88	1105.706	12.56484	< 0.001	95.88
Among PGs	4	161.177	1.47574	< 0.001	10.82
Within PGs	111	1350.427	12.16601	< 0.001	89.18

[a] The total population of *Leptosphaeria maculans / L. biglobosa* was partitioned into hierarchical components: among the six geographic regions (locations), within regions (isolates from a location), among pathogenicity phenotype (isolates from five pathogenicity groups), and within a PG phenotype (isolates from a PG).

TABLE 5.3. Genetic diversity among six *Leptosphaeria maculans/L. biglobosa* populations collected from different regions based on sequence-related amplified polymorphism (SRAP) fingerprinting[a]

Population	SRAP data[b]				
	n	*g*	*s*	*r(%)*	*H*
La Riviere, MB	77	55	0.4198	86.8132	0.2760
Roland, MB	8	8	0.4610	86.8132	0.3260
ND	12	12	0.4468	91.2088	0.3042
Brazil	6	6	0.3608	67.0330	0.2611
Australia	7	7	0.3096	54.9451	0.2251
UK	6	6	0.3104	54.9451	0.2284

[a]Population ND = North Dakota and UK = United Kingdom.
[b]Number of polymorphic loci = 91, *n* = population size, *g* = number of genotypes in populations, *s* = Shannon index, *r* = percentage of polymorphic loci (99% criterion), *H* = average unbiased proportion heterozygosity.

TABLE 5.4. Genetic diversity of five pathogenicity group (PG) populations of *Leptosphaeria maculans / L. biglobosa* based on sequence-related amplified polymorphism (SRAP) fingerprinting

Population	SRAP data[a]				
	n	*g*	*s*	*r(%)*	*H*
PG-1	4	4	0.1697	29.6703	0.1303
PG-2	55	55	0.4229	86.8132	0.2798
PG-3	37	37	0.4039	83.5165	0.2661
PG-4	11	11	0.3596	72.5275	0.2462
PGT	9	9	0.2867	58.2418	0.1971

[a]Number of polymorphic loci = 91, *n* = population size, *g* = number of genotypes in populations, *s* = Shannon index, *r* = percentage of polymorphic loci (99% criterion), *H* = average unbiased proportion heterozygosity.

TABLE 5.5. Pairwise comparison of the genetic identity and distance among six geographic populations of *Leptosphaeria maculans / L. biglobosa*[a]

Population	La Riviere	Roland	ND	Brazil	Australia	UK
La Riviere	****	0.9645	0.9854	0.9741	0.9534	0.9779
Roland	0.0362	****	0.9772	0.9771	0.8988	0.9740
ND	0.0147	0.0231	****	0.9800	0.9539	0.9807
Brazil	0.0263	0.0232	0.0202	****	0.9301	0.9816
Australia	0.0477	0.1067	0.0472	0.0724	****	0.9519
UK	0.0223	0.0263	0.0195	0.0186	0.0493	****

[a]Population ND = North Dakota and UK = United Kingdom.
Nei's (1978) genetic identity based on 91 sequence-related amplified polymorphism loci is above the diagonal, Nei's (1978) genetic distance coefficients are below the diagonal.

TABLE 5.6. Pairwise comparison of the genetic identity and distance among five pathogenicity group (PG) populations from *Leptosphaeria maculans* and *L. biglobosa*[a]

Population	PG-1	PG-2	PG-3	PG-4	PGT
PG-1	****	0.7042	0.6998	0.7548	0.7258
PG-2	0.3508	****	0.9790	0.9671	0.9519
PG-3	0.3570	0.0212	****	0.9851	0.9807
PG-4	0.2813	0.0334	0.0150	****	0.9884
PGT	0.3204	0.0493	0.0195	0.0117	****

[a]Nei's (1978) genetic identity based on 91 sequence-related amplified polymorphism loci is above the diagonal, Nei's (1978) genetic distance coefficients are below the diagonal.

TABLE 5. 7 Pairwise comparison of gene flow and probability of population differentiation among six geographic populations of *Leptosphaeria maculans / L. biglobosa*[a]

Population	La Riviere	Roland	ND	Brazil	Australia	UK
La Riviere	****	7.9753	15.9618	8.3400	5.4572	8.8541
Roland	0.0000	****	9.2604	7.1769	2.7625	6.4215
ND	0.3016	1.0000	****	8.3206	5.0747	8.0837
Brazil	0.9954	1.0000	1.0000	****	3.1128	6.5044
Australia	0.3451	0.4558	0.9997	0.9979	****	3.8184
UK	1.0000	1.0000	1.0000	1.0000	1.0000	****

[a]Population ND = North Dakota and UK = United Kingdom. Estimates of the number of migrants (*Nm*) between populations are above the diagonal; probabilities of each pairwise comparison using the exact test are below the diagonal.

TABLE 5.8. Pairwise comparison of gene flow and probability of population differentiation among five pathogenicity group (PG) populations from *Leptosphaeria maculans* and *L. biglobosa*[a]

Population	PG-1	PG-2	PG-3	PG-4	PGT
PG-1	****	0.7917	0.7451	0.8143	0.8143
PG-2	0.0000	****	14.6929	8.1846	5.2120
PG-3	0.0000	0.0000	****	13.4430	9.7899
PG-4	0.0000	0.2460	0.9993	****	10.2645
PGT	0.0000	0.0356	0.9999	1.0000	****

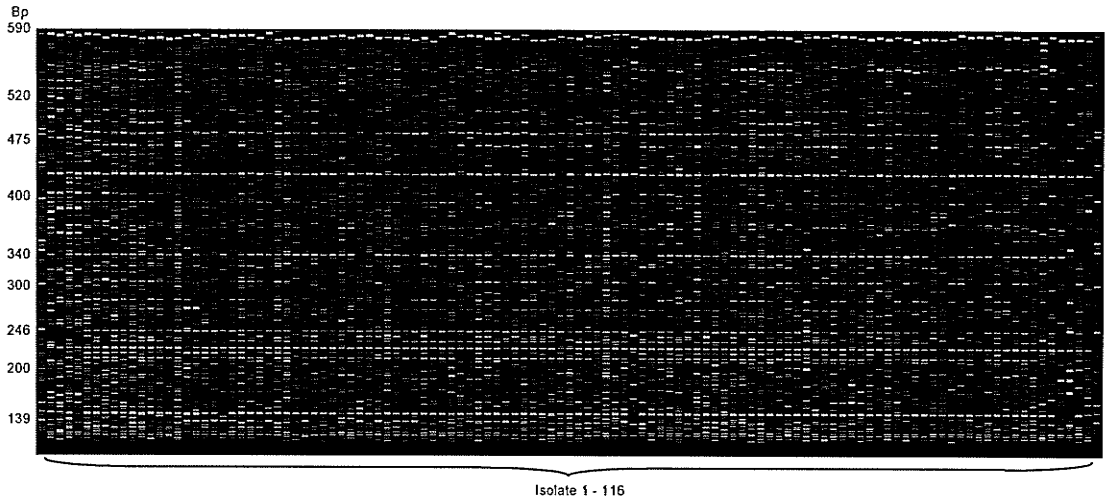
[a]Estimates of the number of migrants (*Nm*) between the pair of populations are above the diagonal; probabilities of each pair-wise comparison using the exact test are below the diagonal.

TABLE 5.9. Pairwise comparison of probability of population differentiation and the genetic distance among four pathogenicity group-3 (PG-3) populations of *Leptosphaeria maculans*[a]

PG-3 population	North America	Brazil	Australia	UK
North America	****	1.0000	0.9999	1.0000
Brazil	0.0256	****	1.0000	1.0000
Australia	0.0358	0.0666	****	1.0000
UK	0.0396	0.0462	0.0409	****

[a]Population North America includes PG-3 isolates from La Riviere, Roland, Manitoba, Canada and North Dakota, USA. UK = United Kingdom. Probabilities of each pairwise comparison using the exact test based on 91 sequence-related amplified polymorphism loci are above the diagonal, Nei's (1978) genetic distance coefficients are below the diagonal.

Fig. 5.1 A gel separating the PCR products amplified with the sequence-related amplified polymorphism (SRAP) primers EM1 and ODD30 against DNA samples of *Leptosphaeria maculans* and *L. biglobosa* isolates.

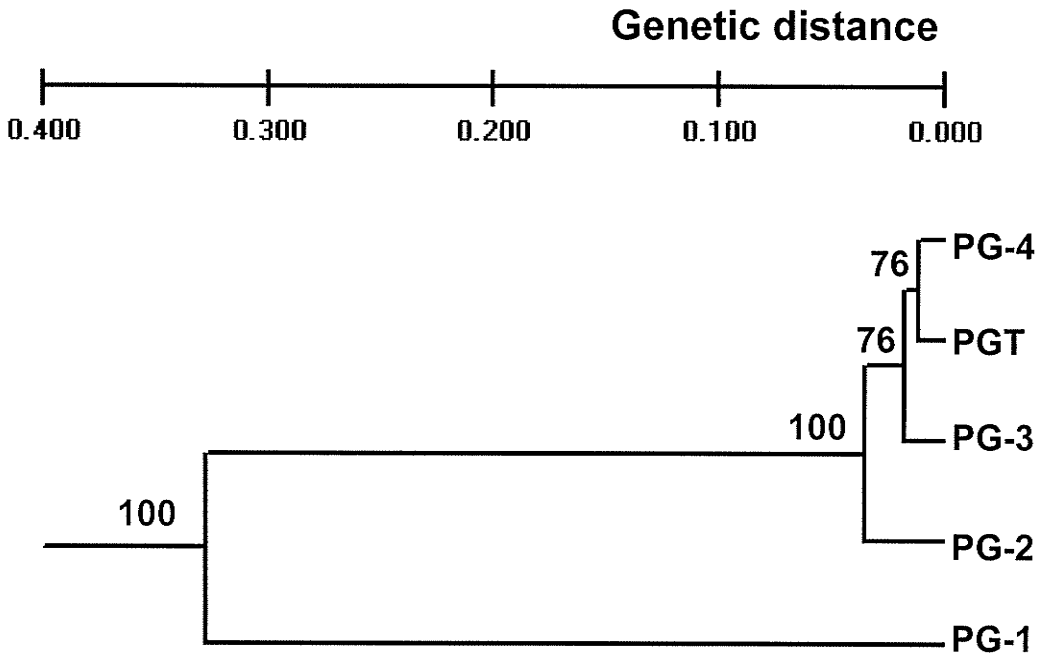


The gel was reconstructed by the Genographer program using the fragment analysis data generated by the ABI Prism® 3100 Genetic Analyzer, and showed genetic diversity within the blackleg geographical populations and blackleg PG populations.

Fig. 5.2 Estimates of genetic similarity among *Leptosphaeria maculans* and *L. biglobosa* isolates collected from La Riviere (LR), Roland (Ro), North Dakota (ND), Brazil (Br), Australia (Au) and United Kingdom (UK) based on SRAP polymorphisms.

Dendrogram was constructed by Neighbor Joining with maximum likelihood distances (100 bootstrapping)

Fig. 5.3 Phenogram of Nei's (1978) genetic distance between pathogenicity group (PG) populations sampled from six geographic regions. Numbers at branches indicate the percentage of occurrence of the cluster in 1000 bootstrapped phenograms.



CHAPTER 6

GENERAL DISCUSSION

Studying the induced resistance to blackleg (*Leptosphaeria maculans*) disease of canola (*B. napus*) caused by a weakly virulent isolate of *L. biglobosa* is important for formulating management strategies of the disease in addition to the management of resistance, rotation and fungicides. We showed, in this study, that the disease on cotyledons and true leaves caused by highly virulent PG-2, PG-3 and PG-4 isolates on canola cv. Westar was significantly reduced when a weakly virulent PG-1 isolate was either pre- or co-inoculated at different time intervals in the greenhouse. With pre-inoculation of PG-1 for 24 h followed by PG-2 challenge on the cotyledons of Westar, activity of defense-related enzymes (chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase) was enhanced at 48 and 72 h. Pre-application of salicylic acid (SA) on six-leaf plants produced induced resistance similar to that of the PG-1 isolate, suggesting that the SA signal pathway may be involved in PG-1-triggered induced resistance. These results were consistent with previous studies that weakly virulent isolates can elicit locally or systemically induced resistance against the infection caused by highly virulent isolates (Chen et al., 1996; Hammond et al., 1985; Mahuku et al., 1996; Pedras et al., 2002; Roussel et al., 1999). However, our study demonstrated that the elicitor (PG-1 isolate) can induce resistance against not only PG-2, but also PG-3 and PG-4 isolates that have different pathogenicities on canola. The activation of defense-related enzymes was also investigated for the first time in this study. The demonstration of disease reduction resulting from pre-inoculation of PG-1 under field conditions will provide us with a novel alternative option in the management of blackleg, especially at a time when resistance of the cultivars may be lost due to pathogen population shifts. We

need further research on the mechanism of PG-1-initiated induced resistance as many other potential signal pathways may be involved in this system.

Investigating the pathogenicity groups (PGs) of blackleg isolates is important for the monitoring of virulence evolution among the *L. maculans* populations and the generation of valuable information regarding potential risk of new virulent isolates to canola production. A total of 512 isolates collected from different locations and times were classified into different PGs on three differential *B. napus* cv.: Westar, Glacier and Quinta. The results showed that PG structure in North American populations had remained unchanged from 1980s (predominant PG-2) until the early 21st century when new PG-3, PGT and PG-4 isolates were detected for the first time.

Many factors could be responsible for the appearance of new strains across the Western Prairies of Canada and North Central U.S.A, however, sexual recombination and seed borne introduction can be potential reasons for the report here. The genetic relationship between newfound isolates in North America and ones from Australia and Europe need to be investigated to confirm these conclusions.

The four-year program of monitoring PG (2001 to 2004) in this study indicated that virulence structure of isolates from different regions was different. Therefore reasonable layout of available resistance is important for the blackleg control. The genetic background of Canadian cultivars of canola is not known and it is necessary to determine the resistant spectra of those cultivars against different PG isolates including newly isolated virulent isolates. Addition of new cultivars into the differentials will definitely change the terminology of PG isolates (Badawy et al., 1991; Keri, 1999; Kuswinanti et al., 1995; Kutcher et al., 1993), which makes the PG testing inconsistent and incomparable

among different researchers. Assessment of avirulence (*Avr*) genes in blackleg populations might be another good option in the population monitoring and blackleg-resistant breeding (Balesdent et al., 2005).

Leptosphaeria maculans is heterothallic, outcrosses prolifically on canola stubble during winter in North America and then undergoes several cycles of asexual reproduction during the summer as the disease epidemic progress, which makes this pathogen a high risk of overcoming resistance genes with a high potential of genetic diversity. Studying genetic diversity and population structure of the blackleg pathogen is important for us to understand the biology of this pathogen, and to develop optimum management strategies. Molecular techniques such as RAPD, RFLP and microsatellite markers facilitate gene diversity analysis by allowing unambiguous assignment of alleles to individual loci. The sequence-related amplified polymorphism (SRAP) marker technique was selected in this study because it is quick and a cost effective tool compared to others. Six geographic populations of *L. maculans* and *L. biglobosa* collected from around the world showed extensive genetic diversity. Virtually most isolates were unique haplotypes, suggesting that a high level of sexual recombination had occurred and caused a high level of genetic diversity. A single population from La Riviere, Manitoba, Canada was a good example to confirm this assumption as it was found to be highly divergent in PG constitution: genotype ($r = 86.81$), heterozygosity ($H = 0.276$). Significant gene flows were found between Western Canada and Australian populations or Western Canada and United Kingdom populations, suggesting that human activity in seed trade may be another factor leading to the appearance of new strains recently detected in Western Canada and North Central USA. It was interesting that populations collected from

Western Canada and North Dakota had a higher genetic diversity than populations from Australia and United Kingdom according to the percentage of polymorphic loci, values of heterozygosity (*H*) and gene flow. However, no significant population differentiation was found among the geographic populations and almost 91% of isolates from different populations were clustered into one lineage because of global gene flow. Compared to other populations, higher gene flow values were observed among North American populations, indicating that populations from closer geographic locations had a higher possibility of genetic exchange. The *L. biglobosa* population showed a reduction in genetic diversity and clustered in a different lineage compared to *L. maculans* PG-groups and as a result it was recently classified into different species (Shoemaker and Brun, 2001). However, no significant diversity was detected among different PG populations of *L. maculans*. AMOVA indicated that high genetic variation was found among isolates within populations regardless of the origin and pathogenicity.

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