PHENOTYPIC CHARACTERIZATION OF *LEPTOSPHAERIA MACULANS* PATHOGENICITY GROUPS AGGRESSIVENESS ON *BRASSICA NAPUS*

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Julianna Franceschi Fernández

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Plant Pathology

March 2015

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Phenotypic characterization of the aggressiveness of pathogenicity groups of *Leptosphaeria maculans* on *Brassica napus*

By

Julianna Franceschi Fernández

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Luis del Rio Mendoza
Chair

Dr. Gary Secor

Dr. Jared LeBoldus

Dr. Juan Osorno

Approved:

04/14/2015
Date

Jack Rasmussen
Department Chair

ABSTRACT

One of the most destructive pathogens of canola (*Brassica napus* L.) is *Leptosphaeria maculans* (Desm.) Ces. & De Not., which causes blackleg disease. This fungus produces strains with different virulence profiles (pathogenicity groups, PG) which are defined using differential cultivars Westar, Quinta and Glacier. Besides this, little is known about other traits that characterize these groups. The objective of this study was to characterize the aggressiveness of *L. maculans* PG 2, 3, 4, and T. The components of aggressiveness evaluated were disease severity and ability to grow and sporulate in artificial medium. Disease severity was measured at different temperatures on seedlings of cv. Westar inoculated with pycnidiospores of 65 isolates. Highly significant (α=0.05) interactions were detected between colony age and isolates nested within PG's. Warmer temperatures resulted in increased severity by all PG. These studies allowed the identification of aggressive isolates to be used for screen breeding lines for resistance.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Luis E. del Rio Mendoza, whose excellent guidance, logical way of thinking, constructive criticism and support during these years had been of great value to me. I was able to learn and understand the different aspects of plant pathology and blessed to have the opportunity to work with him. I owe my sincere thanks to my committee members, Dr. Jared LeBoldus, Dr. Juan M. Osorno, and Dr. Gary Secor for their constructive comments, valuable guidance, time and efforts throughout the preparation of this dissertation. Also, I wish to thank Mrs. Kasia Kinzer for providing the growth chamber space I needed to complete some of the studies conducted as part of my research and Drs. Kishore Chittem and Marisol Berti and Mr. Alfredo Aponte for their help with the data analyses. Last but not least I wish to thanks the Northern Canola Growers Association for funding this project.

DEDICATION

This dissertation is dedicated first to God, for being there when I need him most and for giving me the patience, strength and wisdom I needed to overcome the difficulties experienced through this journey. To my parents, Julia Awilda Fernández Droz and Abimael Franceschi Casiano, who raised me to be the person I am today and for all the support they gave me during this time; to my dear brother, Abimael “Bebo”, who always believed in me; to my beloved husband, soon to be Dr. Alfredo Aponte Zayas, whose encouragement, love, support, knowledge, and understanding helped me to finish this work and to our daughter Anaelis Julianna and our son to be born Alfredo, for giving me the joy of being a mom. Last, but not least, I want to dedicate this to all my extended family and friends that gave me their support and encouragement during this time. Thank you all for your unconditional love that helped me to be successful and capable of achieving my goals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

LIST OF APPENDIX FIGURES

CHAPTER 1. GENERAL INTRODUCTION

Blackleg is an economically important disease world-wide that affects plants within the family *Brassicaceae*. The disease has been present for more than 85 years in Australia (Sivasithamparam et al., 2005), 70 years in Europe (Aubertot et al., 2004), 35 years in Canada (Petrie, 1978) and 20 years in the United States (Lamey and Hershman, 1993) where it has caused significant yield losses (West et al., 2001; Howlett, 2004). Blackleg incidence and severity differ among growing seasons (Howlett, 2004) and growing regions, as it is influenced by cultivars (Aubertot et al., 2006), agricultural practices (Aubertot et al., 2004) and environmental conditions (Sosnowski et al., 2004).

Leptosphaeria maculans (Desmaz) Ces. & de Not (anamorph = *Phoma lingam* (Tode ex Fr.) the dothideomycete that causes blackleg is a pathogen that produces different virulence profiles and secondary metabolites or phytotoxins. Earlier efforts to distinguish between virulence groups classified *L. maculans* strains as aggressive or highly virulent (group A) which cause stem canker and nonaggressive (group B) or weakly virulent that do not cause stem canker (Howlett et al., 2001), based on the phenotypic reaction on the cotyledons of susceptible canola (*B. napus*) cultivar "Westar". Later on, four highly virulent or aggressive pathogenicity groups (PG) of *L. maculans* were recognized, 2, 3, 4 and T, based on the phenotypic reaction of canola differentials 'Westar', 'Quinta' and 'Glacier' (Keri 1999; Koch et al., 1991; Mengistu et al., 1991). Isolates from group B, weakly virulent or nonaggressive, were considered as PG-1, but more recent studies have classified them as *Leptosphaeria biglobosa* Shoemaker & Brun; strains from this species do not produce phytotoxins and/or sirodesmins in liquid media (Shoemaker and Brun, 2001).

The genetic composition of *L. maculans* populations can change in reaction to the genetic composition of canola cultivars available for infection. For example, in North Dakota highly virulent strains of PG-2 were predominant in years prior to 2004 (Chen and Fernando, 2006). Since 2004, however, isolates from PG-4 isolates have become predominant with PG-3 being in second place and PG-T in third. The near nonappearance of PG-2 isolates in canola fields sampled between 2004 and 2009 indicate they have been replaced by the pathotypes mentioned above (Nepal, 2013).

Perhaps the most evident phenotypic difference between isolates from PG-2 and those from other PGs is their ability to interact with different resistance genes in canola plants. Most canola cultivars planted in North Dakota carry resistance genes *Rlm2* and/or *Rlm3* and thus are resistant to infection by isolates from PG-2. Isolates from PG-3 can infect plants carrying *Rlm2* and/or *Rlm3* but the presence of *Rlm1* provides some protection against it. Isolates from PG-T infect plants carrying *Rlm2* but the presence of *Rlm1* and/or *Rlm3* provide some protection against them. In contrast, to these groups, isolates from PG-4 can infect plants that carry any of the resistance genes listed (Nepal et al., 2014). The absence of effective resistance among commercially available canola cultivars against this group adds a sense of urgency to the identification of sources of effective resistance against these groups. No other phenotypic differences among these PGs have been investigated.

Characterization of different isolates within each of these pathogenicity groups will improve our understanding of this pathogen and will allow us to identify isolates that could help speed up the process of selection of resistant materials. Therefore, our study focuses on the following objectives:

I. To measure the effect of colony age (7, 10, 14 and 21 days old) and temperature (15°C, 20°C, 25°C and 30°C) on the aggressiveness of *L .maculans* pathogenicity groups (PG-2, PG-3, PG-4 and PG-T) on cotyledons of *B. napus* susceptible cultivar 'Westar'.

II. To measure *L. maculans* pathogenicity groups (PG-2, PG-3, PG-4 and PG-T) mycelia growth and pycnidiospores production at different temperature (15°C, 20°C, 25°C, and 30°C) and different artificial culture media.

Literature Cited

Aubertot, J-N., Pinochet, X., and Doré, T. 2004. The effects of sowing date and nitrogen availability during vegetative stages on *Leptosphaeria maculans* development on winter oilseed rape. Crop Prot. 23:635-645.

Aubertot, J-N., West, J. S., Bousset-Vaslin, L., Salam, M. U., Barbetti, M. J., and Diggle, A. J. 2006. Improved resistance management for durable disease control: a case study of phoma stem canker of oilseed rape (*Brassica napus*). Eur. J. Plant Pathol. 114:91-106.

Chen, Y., and Fernando, W. G. D. 2005. First report of canola blackleg caused by pathogenicity group 4 of *Leptosphaeria maculans* in Manitoba. Plant Dis. 89:339.

Chen, Y. and Fernando, W. D. G. 2006. Prevalence of pathogenicity groups of *Leptosphaeria maculans* in Western Canada and North Dakota, USA. Can. J. Plant Pathol. 28:533-539.

Howlett, B. J., Idnurm, A., and Pedras, M. S. C. 2001. *Leptosphaeria maculans*, the causal agent of blackleg disease of Brassicas. Fung. Gen. Bio. 33:1-14.

Howlett, B. J. 2004. Current knowledge of the interaction between *Brassica napus* and *Leptosphaeria maculans*. Can. J. Plant Pathol. 26:245-252.

Kandel, H., and Knodel, J. 2011. Canola Production Field Guide. North Dakota State Univ. Ext. Ser. Fact Sheet No. A-1280.

Keri, M. 1999. Genetic studies of host-pathogen interaction between *Brassica napus* and *Leptosphaeria maculans*. Ph.D. dissertation, University of Manitoba, Winnipeg MB.

Koch, E., Song, K., Osborn, T. C., and Williams, P. H. 1991. Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Mol. Plant-Microbe Interact. 4:341-349.

Markell, S., del Rio-Mendoza, L., Halley, S., Mazurek, S., Mathew, F., and Lamey, A. 2008. Blackleg of canola. NDSU Extension Bulletin PP-1367. 4 p.

Nepal, A. 2013. Genetic structure of *Leptosphaeria maculans* population in North Dakota and identification of genes associated with resistance to *L. maculans* in *Brassica juncea*. Ph.D. dissertation, North Dakota State University, Fargo, ND.

Nepal, A., S. Markell, J. Knodel, and L. E. del Río Mendoza. 2014. Prevalence of blackleg and pathogenicity groups of *Leptosphaeria maculans* in North Dakota. Plant Dis. 98:328-335.

Mengistu, A., Rimmer, S. R., Koch, E., and Williams, P. H. 1991. Pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid–cycling Brassica. Plant Dis. 75:1279-1282.

Sosnowski, M. R., Scott, E. S., and Ramsey, M. D. 2004. Infection of Australian canola cultivars (*Brassica napus*) by *Leptosphaeria maculans* is influenced by cultivar and environmental conditions. Australas Plant Path. 33:401-411.

Shoemaker, R. A., and Brun, H. 2001. The teleomorph of the weakly aggressive segregate *Leptosphaeria maculans*. Can. J. Bot. 79:412-419.

West, J. S. Kharbanda, P. Barbetti, M. J., and Fitt B. D. L. 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) in Australia, Canada and Europe. Plant Pathol. 50:10-27.

CHAPTER 2. LITERATURE REVIEW

The Pathogen: *Leptosphaeria maculans*

Leptosphaeria maculans (Desm.) Ces. & de Not [anamorph = *Phoma lingam* (Tode:Fr.) Desm.], is an economically important plant pathogenic ascomycete that causes blackleg or phoma stem canker disease on cruciferous crops and serious yield losses worldwide. The sexual stage of this fungus produces pseudothecia, whereas the asexual stage produces pycnidia. Independently of their taxonomic status, strains of *L. maculans* have been classified as aggressive or virulent (A) and non-aggressive or avirulent (B), but other characteristics like toxin production, genetic phenotypes, and ITS sequencing analyses have also been used (Howlett et al., 2001). Isolates from pathotype B cause leaf lesions, superficial stem cankers that do not kill plants and have been recently moved into a different species, *L. biglobosa*. In contrast, pathotype A isolates can produce deep stem cankers that could kill plants as well as leaf lesions. Pathotype A isolates have been further divided into pathogenicity groups 2, 3, 4 and T based on the reactions of *Brassica napus* differential canola cultivars “Westar”, “Glacier” and “Quinta” to cotyledon inoculations (Koch et al., 1991). In Europe and North America, *L. maculans* frequently co-exists with *L. biglobosa* (West et al., 2002). These two species are believed to have evolved from a common ancestor (Gudelj et al., 2004). In this thesis, the taxonomy and other characteristics of *L. biglobosa* will not be discussed further.

Classification of *L. maculans*

Kingdom: Fungi

Phylum: Ascomycota

Class: Dothideomycetes

Order: Pleosporales

Family: Leptosphaeriaceae

Genus: *Leptosphaeria*

Species: *maculans* (Desm.) Ces. & De Not.

This fungus was first described in 1791 by Tode (cited in Williams, 1992) on dead red cabbage stems as a saprophytic organism who named it *Sphaeria lingam*. Later, in 1849, Desmazieres collected the same fungus from living *Brassica oleracea* plants and classified it to the genus *Phoma* [*Phoma lingam* (Tode ex Fr.) Desm. (1849)] (Williams, 1992). Over the years various other names were used to refer to this fungus such as *Phoma brassicae, Phoma oleracea* and other generic epithets (Boerema, 1976). The sexual form of *P. lingam* was described in 1957 in New Zealand, and the identity of this fungal organism was confirmed as *Leptosphaeria maculans* (Desm.) Ces. & De Not. with its anamorph or asexual from as *Phoma lingam* (Tode ex Fr.) Desm.

Morphological features of *L. maculans*

L. maculans produces pseudothecia on infected leaves (Fig. 2.1A) and stubble of *Brassica* plants. The pseudothecia are black, globose fruiting bodies with ostioles (Fig. 2.1B) and a diameter that ranges between 300-500 µm; pseudothecia are immersed in the host tissue, and become erumpent when mature (Fig 2.1C). The pseudothecia are composed of hyaline, septate, filiform pseudoparaphyses (Naseri, 2006). These contain cylindrical to clavate, numerous bitunicate asci (80-125 x 15-22 µm) (Fig. 2.1D) with eight ascospores (35-70 x 5-8 µm) per ascus. The ascospores are biseriate and have a cylindrical to ellipsoidal shape with mostly rounded ends. These ascospores are yellow to brown in color, with 5 transverse septa, and each cell contains one to several drops of fat or small oil drops (Fig. 2.1E) (Williams, 1992; Ash, 2000).

The anamorph stage of this fungus is *Phoma lingam*, which forms pycnidia in leaf spots and decaying stems of infected canola plants. Two types of pycnidia are found on infected plant tissues, the type I pycnidia are immersed in the host tissue. These pycnidia lack a definite shape; have narrow ostioles, and have thick-walled cells. While type II pycnidia are globose and black with a wall composed of several layers of cells with a thick-walled outer layer (Ash, 2000). Asexual spores or pycnidiospores (conidia) produced in pycnidia are extruded through the pycnidial ostioles in a pink to amethyst mucilage (Fig. 2.1F and 2.1G) (Boerema, 1976; Williams, 1992). Pycnidiospores (3-6 x 1.5-2 µm) are unicellular, cylindrical and hyaline, although a few may be curved (Smith & Sutton, 1964).

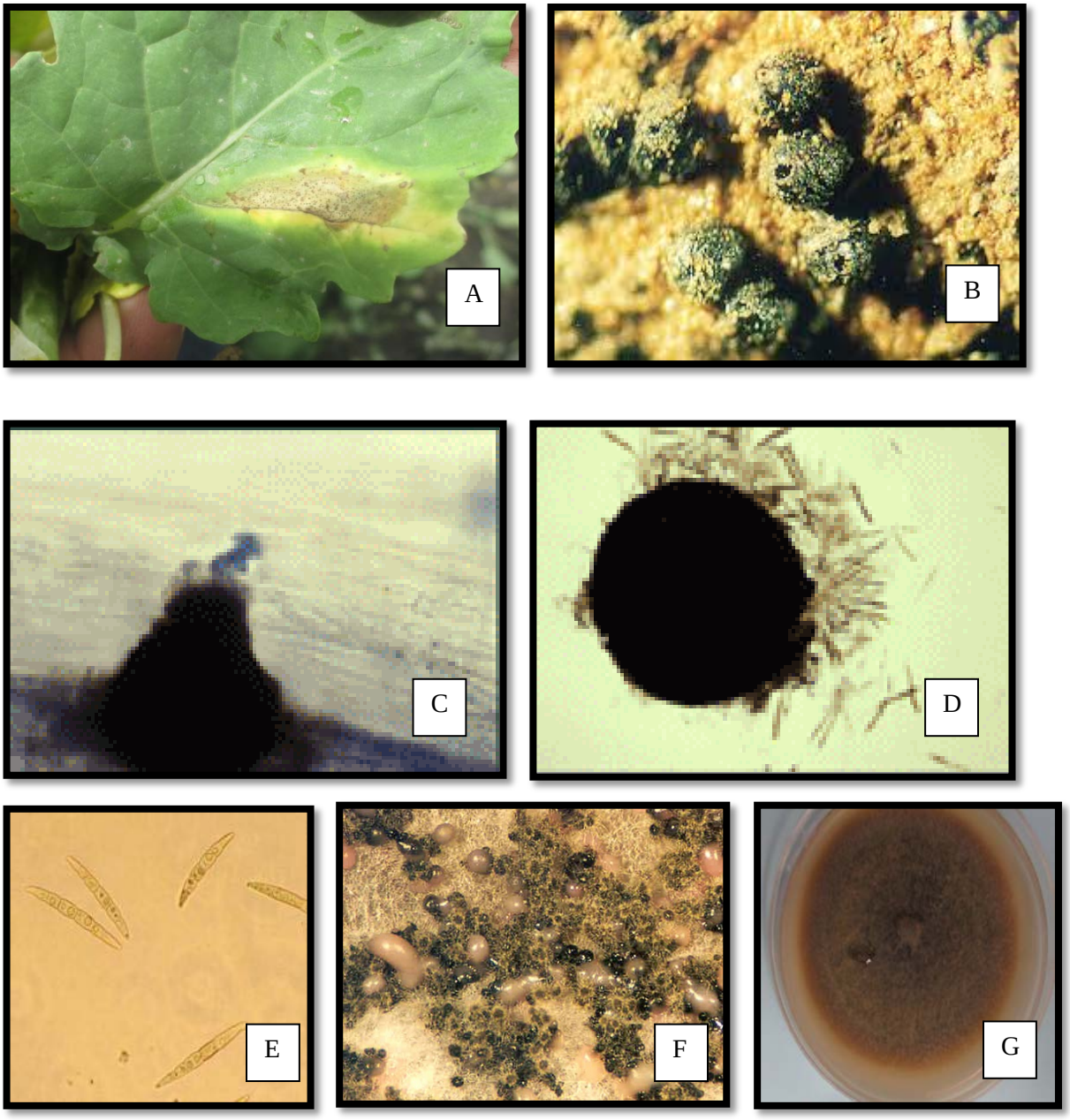

Figure 2.1. *Leptosphaeria maculans* pycnidia on host tissue (A), pseudothecia with ostioles (B), sexual fruiting bodies (C-D) and ascospores (E), asexual fruiting bodies (F), colony growth on artificial medium (G). Figures from Ash, 2000; West et al., 2001; Howlett et al., 2001.

Biology and ecology of *L. maculans*

L. maculans has a complex life cycle, which includes alternating phases of saprophytism on stem residues, then, a long phase of endophytism within plant tissues and eventually necrotrophy on the leaves and at the basis of the stem, killing the plant. *L .maculans* is

heterothallic, i.e. two different mating types must be present for sexual reproduction to occur (Williams, 1992). In artificial medium, V8 juice agar, the fungus is cultured under white light (500 μmol m^2s^{-1}) at 10-25º C to stimulate production of pycnidiospores (Xu et al., 1987; Vanniasingham and Gilligan, 1989).

Sexual recombination is important for the *L. maculans* life cycle because it increases population variability and its ability to overcome resistance on plant host cultivars. To characterize the variability of *L. maculans* populations, genetic studies have been performed utilizing polymorphism at variable number tandem repeat (VNTR) loci using minisatellites consisting of tandemly repeated polymorphic regions in BAC-end sequencing. The single locus minisatellite markers are used to isolate a specific segment of DNA that can be amplified through PCR. A few minisatellites have been characterized in fungi, such as *MinLm1* in *L. maculans* (Attard et al., 2001). By the construction of a genetic map of *L. maculans,* five new minisatellite (*MinLm2-MinLm6)* have been identified and characterized (Eckert et al., 2005).

Blackleg on canola is usually a monocyclic disease (West et al., 2001) and epidemics are initiated by ascospores (Barbetti 1975; McGee, 1977). The disease cycle (Fig.2.2), start with primary signs and symptoms of infection that are visible on the cotyledons and first leaves on canola (Markell et al., 2008; Kandel et al., 2011). After the initial infections in the cotyledons develop, the fungus enters the endophytic phase and moves into the stem and down towards the crown area. Once in the crown area, the fungus enters the necrotrophic phase again producing secondary metabolites (phytotoxin), such as sirodesmin PL, which retards the growth of the plant, and other enzymes that kill cells and contribute to the formation of stem cankers (Ferezou et al., 1977). On more mature leaves, the pathogen produces lesions that are round to irregular and may be surrounded by a yellow halo. These leaf spots enlarge and form pycnidia. Pycnidia

also are produced in the center of sunken lesions in the stem, which disrupt water and nutrient transport between leaves and the root system, reduce yield and vigor, and eventually lead to the death of the plants. After harvest, the fungus survives on the infected crop residues in the form of pycnidia or pseudothecia for several years.

During the spring, under favorable conditions, the pseudothecia produce ascospores that are dispersed by the wind and infect new canola seedlings. After ascospores are released, they can survive dry conditions at 5°C to 20°C for 30 days (Huang et al., 2003). Ascospores enter young leaves and cotyledons through the stomata or wounds to initiate a new cycle of infections. After germination the ascospores, can produce appressorium-like structures to penetrate the leaf tissue (Huang et al., 2003). In addition, *L. maculans* produces a wide range of cell wall degrading enzymes that can help the pathogen penetration into the leaves (Annis & Goodwin, 1996). After the initial infection, the fungus colonizes the intercellular spaces between the mesophyll cells and grows through the xylem parenchyma and cortex (Naseri, 2006) towards the stem crown. On leaves, after colonization, the pathogen becomes necrotrophic and later produces pycnidia in the dead tissue (Hammond et al., 1985). The pycnidia produce millions of pycnidiospores which act as secondary inoculum and are spread by direct contact or by rain. Pycnidiospores infect through stomata or wounds of neighboring leaves and brassica plants. Fungal activity in the stem leads to the death of stem cortex cells causing blackened cankers which provide the basis for the disease name blackleg). In extreme cases the cankers can surround completely the stem of canola plants. Also, the pathogen can survive in infected seeds. Seed infection has epidemiological value as infected seeds can start an epidemic, because the infected plant grows with the pathogen and spreads the disease.

Temperature and moisture are the two most important environmental conditions for the development of *L. maculans* spores. A rainy environment increases disease severity, causing secondary infections by conidia dispersal through rain splash (Toscano-Underwood et al., 2003). Hail storms also increase disease severity. Temperatures below 50° F (10° C) or higher than 80° F (26° C) reduce the ability of the fungus to cause infection, but temperatures in the lower side do not stop them completely. Long periods of rain and 70° F (21° C) temperature are favorable for disease development (Markell et al., 2008) in North America.

The survival structures of the fungus, such as pycnidia (pycnidiospores), pseudothecia (ascospores) and mycelium can survive for up to 5 years on canola stubble or residues (McGee, 1977). On the other hand, pseudothecia naturally is formed on woody remains and begin to appear from 1 to 10 months after harvest as long as the infested crop residue persists (Petrie, 1986). Nevertheless, stem cankering is the major cause of yield loss worldwide in canola growing regions, affecting plant development and seed production (Howlett et al., 2001).

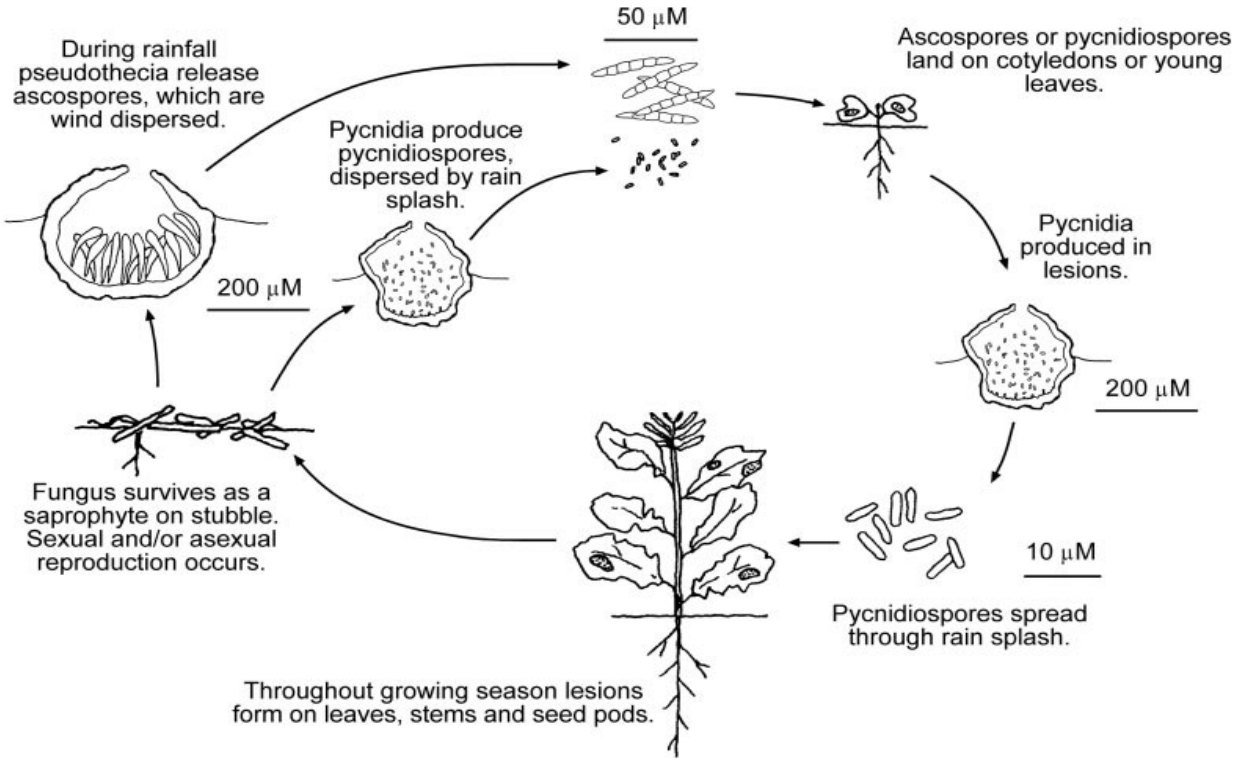

Figure 2.2. *L. maculans* life cycle on *B. napus* (canola). Fig. adapted from Howlett et al., 2001.

Some fungi can produce plant defense responses such as secondary metabolite toxins that play an important role in the pathogenicity. The specie *L. maculans* can produce secondary metabolites such as sirodesmin PL is a non-host-selective phytotoxin that works as part of a multifaceted strategy to obtain nutrients from the host plant (Elliot et al., 2007). The phytotoxins and elicitors are secondary metabolites produced by *L maculans*. The known phytotoxins of this fungus are the sirodesmin PL and the deacetyl-sirodesmin PL, which induced the production of phytoalexin spirobrassinin in both resistant plant species (brown mustard, *Brassica juncea* cv. Cutlass) and susceptible plant species (canola, *B. napus* cv. Westar) (Yu, 2008). New metabolites had been found such as leptomaculins A-E and deacetyl-leptomaculins C-E were the elicitor-phytotoxin active fractions do not display detectable elicitor activity. Also, other metabolites found in *L. maculans* such as maculansins A and B displayed higher phytotoxicity on canola and white mustard (*Sinapis alba* cv. Ochre). Other toxin produced in Minimum media (MM) with

high concentration of NaCl is the metabolite known as bulgarein that is involved in the self-protection of the pathogen.

In response to the presence of toxins produced by the pathogen, the host (*Brassica* spp.) produces phytoalexins which are toxic to the pathogen. These chemicals which are part of the defense system of the plants usually have one indole or indole-related ring system and one sulfur atom in their structure. The phytoalexin brassinin is metabolized and detoxified through different mechanisms by *L. maculans* (Pedras and Taylor, 1993; Pedras, 1998; Pedras et al., 2000). Brassinin which is a precursor of several other phytoalexins inhibits the biosynthesis of sirodesmin PL. The inhibition of this phytotoxin slows down the cellular damage caused by the fungus.

The interactions between *Brassica* spp. and *L. maculans* are complex because of the lack of standardization of homogenous differential cultivars and virulence testing. A lot of genes that confers cotyledon resistance have been mapped in *B. napus* as well as many fungal avirulence genes (*AvrLm1, AvrLm2,* and *AvrLm4*), which confers avirulence to several *B. napus* cultivars (Ansan-Melayah et al., 1995; Balesdent et al., 2002).

Phylogeny of *L. maculans*

Ascomycota is the largest phylum of the kingdom Fungi, with approximately 64,000 known species (Kirk et al., 2008). These species are characterized by the formation of fruiting bodies known as ascomata or ascocarps in which asci are produced. Ascospores are produced in asci, typically eight spores per ascus. Mycelium of these fungi has simple septa with simple pores and Woronin bodies. Molecular phylogenetic analyses of nuclear and mitochondrial ribosomal RNA genes and protein-coding genes support the notion that the Ascomycota phylum is a monophyletic group, with all species descending from a common ancestral species (James et

al., 2006). Basidiomycota is the sister group of Ascomycota, meaning that these terrestrial fungi are close relatives (Berbee, 2001). Using 18S rRNA phylogeny, Berbee and Taylor (1993) estimated that the Ascomycota and Basidiomycota diverged 400 million years ago. Other studies have suggested that the divergence was 1.2 billion years (Heckman et al., 2001). However, Berbee and Taylor (2000) suggest that Ascomycota and Basidiomycota have co-existed for 460 million years ago with land plants, which had been the main nutrient source for these fungal groups.

There are three main subphyla, in Ascomycota, each including saprobes on plant materials, as well animals and human pathogens. The first subphylum to diverge was the Taphrinomycotina (Nishida and Sugiyama, 1994). This subphylum's genera range from fungal obligate plant pathogens to fission yeasts that are pathogenic or saprobicon animals. The next two subphyla are Saccharomycotina and Pezizomycotina. The Saccharomycotina includes more than 40 genera, but only one genus, *Candida*, is pathogenic to human and one, *Emerothecium*, is a plant pathogen (Berbee, 2001). As for the subphylum Pezizomycotina, it includes more than 3000 genera most of which are pathogens and mutualists. Pezizomycotina contains plant pathogenic fungi that are distributed in three classes, Sordariomycetes (=Pyrenomycetes), Dothideomycetes (=Loculoascomycetes, in part), and Leotiomycetes (inoperculate discomycetes) (Berbee, 2001).

The *Pleosporales* is the largest order in the class Dothideomycetes, they are endophytes. The identification of species in this group is mostly based on the ascomatal morphology, ascal arrangement, shape of ostioles, ascospores characteristics and their life cycle habitats, but in recent years phylogenetic analyses have been used to confirm identity and taxonomic classification. Biological characters such as metabolite production, substrate staining reactions,

and host spectrum have been used to distinguish between genera, i.e. *Phaeosphaeria* Miyake and *Leptosphaeria* Ces. & De Not. The family *Leptosphaeriaceae* is likely paraphyletic and comprises two teleomorphs genera *Leptosphaeria* and *Neophaeosphaeria*, but contains several anamorphs such as *Chaetodiplodia, Coniothyrium, Phoma, Plectophomella and Pyrenochaeta* (Wehmeyer 1975, de Gruyter et al. 2009). An important plant pathogenic fungi genus and one of the largest genera in this family is *Leptosphaeria*, which is a dematiaceous (phaeoid, or dark-walled) filamentous fungus found in the soil and grows in a teleomorphic phase. One important species of this genus is *L. maculans* (Desm.) Ces. & De Not. Which has as anamorph *Phoma lingam* (Tode) Desm.. *L. maculans* is a species complex associated with "stem canker" or "blackleg" blackleg disease on crucifers regardless of geographical and host plant considerations (Mendes-Pereira et al., 2003).

Molecular studies have established *Leptosphaeria s. str.* as a putative monophyletic clade to include *L. maculans*, *L. conferta*, *L. congesta*, *L. dryadis*, *L. weimeri* and *L. doliolum* (Camara et al., 2002). This clade contains species that are pathogenic on dicotyledonous plants and have *Phoma* anamorphs. Several techniques have been developed to differentiate between components of the *L. maculans* species complex including virulence studies on *Brassica* species (Koch, Badawy & Hoppe 1989, Badawy, Hoppe & Koch 1991); the host plant from which isolates were obtained from differentiating 'Brassica', 'Sysimbrium', 'Thlaspi', 'Erysimum', 'Lepidium' and 'Descurainia' isolates (Petrie, 1969); RFLP studies differentiating between *L. maculans* groups (A and B) (Johnson and Lewis 1990); production of toxic secondary metabolites leading to the Tox+ / Tox0 terminology (Balesdent et al., 1992); and additional RFLP studies supported by soluble protein and isozyme studies leading to the NA1, NA2 and NA3 subgrouping (Koch et al., 1991; Balesdent et al., 1992; Gall et al., 1995) cited in Mendes-Pereira et al. (2003).

The studies conducted by Mendes-Pereira et al. (2003), used ITS sequencing for phylogenetic species recognition within the *L. maculans* species complex. Their studies established a strongly supported clade encompassing three very closely related species *L. maculans, L. biglobosa, L. conferta*. Within this clade, two very strongly supported clade were determined, the first one encompassing the 'Brassica' Tox^{+} and 'Lepidium' Tox+ isolates, which *L. maculans s. str*. strongly support in this clade. As for the second clade, it includes all the others Tox^{0} isolates, where *L. biglobosa* is strongly supported from various cruciferous hosts. However, these two species show difference in terms of ITS sequence polymorphism, because *L. biglobosa* 'brassicae' is highly monomorphic, whereas *L. maculans* 'brassicae' is non-negligible of ratio polymorphic. The high level of polymorphism in ITS sequence of *L. maculans* 'brassicae' is quite unusual. It could be hypothesized that is currently an expanding species. Also the presence of only one of the two species (*L. maculans* and *L. biglobosa*) in a certain region or area could suggest geographic segregation forcing the species to biological evolution.

***L. maculans* virulence profiles**

As discussed previously, *L. maculans* has been broadly characterized through morphological, physiological, biochemical and genetic studies. Their ability to cause severe cankers on *B. napus* cultivars was used to classify them as pathotypes "A" or virulent and pathotype "B" or avirulent. The differences between pathotypes A and B were supported by isozyme variation and soluble proteins (Gall et al., 1995), as well as by molecular genetic studies of ITA regions of rDNA (Balesdent et al., 1998), RAPD (Goodwin and Annis, 1991) and AFLP analysis (Purwantara et al., 2000). Isolates from pathotype "A" were further classified into pathogenicity groups (PG) 2, 3, T, and 4 based on the reaction of *B. napus* differential cultivars 'Westar', 'Quinta' and 'Glacier' to them (Mengistu et al., 1991; Keri et al., 2001; Chen and

Fernando, 2005) while all pathotype "B" isolates were classified as PG-1. The phenotypic reaction of the *B. napus* differential cultivars to isolates from these pathogenicity groups is presented in table 2.1. Pathotype "B" isolates have been recently classified as a different species, *L. biglobosa* (Shoemaker and Brun, 2001).

Table 2.1. Phenotypic reaction of *B. napus* differentials cultivars to inoculations with *L. maculans* isolates from different pathogenicity groups.

	Differential cultivars [x]		
Pathogenicity Groups	Westar (-)	Quinta (*Rlm1, Rlm3*)	Glacier (*Rlm2, Rlm3*)
1*	R[y]	R	R
2	S	I	R
3	S	I	S
T	S	S	I
4	S	S	S

[x] Resistance genes present in differentials is included in parentheses.
[y] Phenotypic reaction; R= Resistance, I=Intermediate resistance and S=Susceptible.
*PG-1 has been recognized as *L. biglobosa* by Shoemaker and Brun (2001).

Aggressiveness, fitness and virulence of *L. maculans*

Understanding why and how plant pathogens infect and harm their cultivated host plants is fundamental in plant pathology. When a plant pathogen interacts with its host the outcome ranges from disease (susceptibility) to no disease (resistance) with many intermediate levels in between. This range is affected not only by the genetic composition of the plant but also by that of the pathogen. In characterizing the ability of organisms to cause disease, Van der Plank (1963) defined the term aggressiveness as "the non-specific component of pathogenicity". In other words, it describes a quantitative component of pathogenicity that is not dependent on the genetic composition of the host but on that of the pathogen. In this way, to characterize the true aggressiveness of a pathogenic organism one should measure it in a host that does not have resistance genes. This is especially true when one is trying to determine whether a particular race

or in the case of *L. maculans* a particular pathogenicity group is more or less aggressive than other. The presence or absence of resistance genes in the host is less important if one is interested in comparing the aggressiveness of isolates that belong to a single race or pathogenicity group. In this research, we are interested in comparing the aggressiveness of different pathogenicity groups as well as of isolates within the same groups.

Pariaud et al. (2009) provided another definition of aggressiveness; they called it a quantitative measure of pathogenicity and spread, that can be manifested at pathogen population level (rate of spread of a new strain) and at the host plant level (amount of yield loss or infectiousness caused by an organism). Virulence is the ability of the pathogen to cause more or less disease in a host; to an extent, virulence could be considered a synonym of aggressiveness. Fitness is the ability of the pathogen to transmit the disease from host to host. Usually, aggressiveness is measured using quantitative traits such as infection efficacy, duration of latent period, amounts of spore production and lesion size; in some pathogens it is also measured by the amount of mycotoxins produced (Mehta and Zadoks, 1970; Sache, 1997). For instance, the infection efficacy is the ability of a pathogen to cause infection and is usually expressed as percentage, for example an infection efficiency of 60% means that from every 100 spores that land on the plant host, 60 will produce a lesion (del Rio, personal communication). Meanwhile, the latent period is the time between infection and sporulation (Shaner, 1980). The spore production rate is the quantity of spore produced per lesion and unit of time (Clifford and Clothier, 1974; Sache, 1997). As for the infectious period, it is defined as the period in which the pathogen is actively producing spores (Leonard, 1969; Robert et al., 2004).

The aggressiveness of a pathogen can be affected by environmental conditions (temperature and humidity), host physiological stage (tissue age) and the pathogen physiological

state (Pariaud et al., 2009). Thus, whenever a trait that is associated with aggressiveness is measured, one has to provide information regarding the conditions under which the trait was evaluated. For example, studies on *Puccinia triticina* isolates showed that spore production rate is affected by the temperature; cooler temperature, ranging between 2-18°C result in lower spore production than warmer temperatures that range between 10-30°C (Milus and Line 2006). Also it was observed that spore production of two *P. triticina* culture isolates was different when infecting seedlings compared to infecting adult plants. The variability in aggressiveness also depends on the pathogen physiological state.

Some studies suggest that aggressiveness traits are governed by many genes (Blanch et al., 1981; Caten et al., 1984; Hawthorne et al., 1994; Cumagun and Miedaner, 2004). But empirical evidence has found genetic control of only a few aggressiveness components. Nevertheless, if the aggressiveness components are inherited, then the evolution of aggressiveness will be formed by genetic traits. These genetic components, are the variability and heritability for pathogen population's aggressiveness and qualitative virulence (Pariud et al 2009).

The Plant Host: *Brassica napus*

The Brassicaceae (Cruciferae) or mustard family contains approximately 380 genera and 3,200 species. This family includes several crop plants that are grown for oil, condiments, forage, and fodder for animals, or as vegetables (Crisp, 1976; Simmonds, 1986). Some of the genera that belong to this family are *Brassica*, *Erysimum*, *Cakile*, *Cardamine,* and *Raphanus*. The genus *Brassica* contains 37species and include some of the most important crops grown worldwide (Gomez-Campo, 1980). The species in this genus provide edible roots, leaves, stems, buds, flowers, and seeds. Including varieties of *B. oleracea* L. (broccoli, Brussels, sprouts,

cabbage, cauliflower, kale); *B. juncea* (L.) Czern. (Indian mustard); *B. nigra* (L.) W. D. J. Koch (black mustard); *B. rapa* L. (Chinese mustard, and Chinese cabbage); *B. napus* L. var. *napus* (rape) and others (Koch et al., 2003). *Brassica* wild relatives provide nuclear genes that could be used as sources of resistance to certain diseases and could be used to develop hybrid seeds in breeding programs (Rakow, 2004).

Economic importance

Brassica napus known as rape, rapeseed or canola is a crop economically important worldwide. The crop has grown rapidly over the past 40 years, rising from being the sixth largest oilseed crop to being only second to soybean. It is cultivated extensively in India, China, Canada, Europe (France, Germany, and UK), Australia and USA. In North America *B. napus* cultivars are grown in the Northern Plains, in an average of 510,000 ha/year (USDA-NASS, 2014). Canada and Australia are the major rapeseed exporting countries while the production in other countries is locally consumed (Kimber and McGregor, 1995). The major producer of canola in the USA is North Dakota, leading the country with approximately 85 percent of the domestic production in 2014 and amounted approximately 725,747kg with an estimated market value of $440 million (USDA-NASS, 2014).

History of canola

Oilseed rape was cultivated by ancient civilisations in Asia and the Mediterranean as early as 2000 BC and has been grown in Europe since the 13th century for its use as oil for lamps (Colton and Sykes, 1992). Oilseed rape was first grown commercially in Canada in 1942 and its oil was used as lubricant in warships. In Australia, the crop was first grown commercially in 1969. The oil produced by oilseed rape was unsuitable as food source for humans or animals due to the presence of two naturally toxics components, erucic acid and glucosinolates. However, in

the 1970's, intensive plant breeding efforts in Canada, Australia and several other countries, succeeded in producing high quality varieties with ever lower quantities of erucic acid and glucosinolates. Then in 1978, the Western Canadian Oilseed Crushers Association registered these varieties under the denomination of "canola" for marketing reasons. In the following 10 years, European seed producers developed rapeseed lines with lower erucic acid and glucosinolates too and denominated them "double-zero" or "canola-equivalent". The term canola is an acronym for "Canadian Oil, Low Acid that refers to *B. napus* varieties that contain "oil with less than 2% erucic acid and meal low in glucosinolates (less than 30 μ moles/g)" (Canola Council of Canada, 2014 www.canolacouncil.org). The glucosinolates are 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate. In this way, canola oil and the meal left after oil extraction are acceptable as an edible oil and animal protein feed, respectively. Canola oil is considered one with the highest quality edible oils available (Kandel and Knodel, 2011).

Morphology of *B. napus*

Brassica napus L. is the species of canola produced in North Dakota. This species has higher oil content and yield and is more tolerant to diseases than *B. rapa.* Canola has six growth stages: germination, seedling, rosette, bud, flowering and ripening, which are affected by the environment, type of soil and temperature (Kandel and Knodel, 2011). The germination stage involves the absorption of water, seed coat split and emergence of the root tip; then cotyledons are pushed to the soil surface as the seedling emerges from the ground; the germination can occur within four to ten days after planting. During the seedling stage, the two cotyledons emerge and open exposing the growing point. At this stage the plant is very susceptible to soil-borne pathogens and to *L. maculans* and is a poor competitor to weeds. The seedling stage ends

when the plants produce more than three true leaves. The next stage is the rosette when the plant develops four to eight true leaves. During this stage, the leaf area increases rapidly and the plants develop their canopy. Bud formation occurs when the hours with sunlight per day lengthen and temperatures increase. At this time, in the rosette center a cluster of flower buds emerges and secondary branches producing more buds start to develop. The plant reaches the maximum leaf area in the bud stage. In *B. napus* cultivars reach this stage within 40 to 60 days after seedling stage depending on the environmental conditions. Flowering starts with the opening of the lowest bud in the main stem, and then flowers start to open at a rate of three to five flowers per day. Plants will remain in the flowering period for 14 to 21 days. During the ripening stage seeds accumulate dry matter and then start drying, leaves turn yellow and fall from the plant. When 30 to 40 percent of seeds have changed color from green to brown the crop is ready to be swathed in preparation for harvest.

Production and uses

The production of canola is worldwide and China, Australia, India and Canada are the largest producers (Canola Council of Canada, 2014 www.canolacouncil.org). China also is the largest consumer of vegetable oil in the world while Canada is the largest exporter. In the United States canola is produced in the Northern Plains, an area that comprises the states of North Dakota, Minnesota, and Montana. North Dakota is the largest producer in the country with 513,950 ha (1.27 million acres) of canola planted in the 2012 growing season (USDA-NASS, 2013). The main use of canola is as source of oil for human consumption with the remnant seed meal being used as ingredient for concentrate animal food and as forage crop for livestock feed. Other uses include as lubricant and for soaps products, and as biofuel, detergent lubrication oils, resins or vegetable wax substitute.

Insects and diseases

Canola production is affected by environmental conditions, insects, pests, and diseases. Some environmental conditions like hail storms, frost damage, extreme relative humidity and extreme high temperatures cannot be controlled in the field. The insects pests most commonly affecting canola production are; crucifer flea beetles (*Phyllotreta cruciferae* and *Phyllotreta striolata*), lygus buds (*Lygus lineolaris*), cutworms of Lepidoptera species and aphids (Kandel and Knodel, 2011). Usually most of these pests feed on canola leaves, pods and growing points; others insects do not affect the plants directly but by vectoring plant pathogens like *Candidatus* phytoplasma *asteris* (Lee et al., 2004). There are two main diseases that impact canola production in North Dakota: blackleg and sclerotinia stem rot. These fungal diseases cause the major economic losses in canola production every year. Other diseases on canola are *Alternaria* black spot, downy mildews and *Fusarium* wilt. These diseases can be control and manage, using resistant hybrids, crop rotation, seed treated with fungicides, fungicides applications, biological control, and control of weed hosts.

Origin and taxonomy

B. napus is an amphidiploid species with A and C genome (AACC genome, with chromosome n= 19) originated from natural interspecific hybridization between the two diploid species *B. oleracea* (C genome, n = 9) and *B. rapa* (syn. *campestris*, A genome, n = 10). The genetic relationships between these *Brassica* species have been defined by U (1935) (Figure 2.2). The nature, direction and geographic location of the initial hybridization events that led to the generation of *B. napus* remain unclear, although *B. napus* seems to be a relatively new species because the earliest records of its existence are from 500 years ago and not truly wild populations have been recorded. Further, the geographic location where the original hybridization took place,

as well as whether it occurred in a wild or domesticated context also are unknown. Song and Osborn (1992) conducted analyses of chloroplast and mitochondrial DNA that suggested that *B. montana* (n = 9) might be closely related to the prototype that gave rise to cytoplasm for *B. rapa* and *B. oleracea*. It also suggested that *B.napus* may have had multiple origins, and was derived from a cross in which a closely related ancestral species of *B. rapa* and *B. oleracea* was the maternal donor.

Nevertheless, recent studies have discarded species containing the C genome as being the maternal ancestor of the majority of *B. napus* accessions. The presence of multiple chloroplast haplotypes in *B. napus* and *B. rapa* accessions was not correlated with nuclear genetic diversity as determined by AFLPs. While some chloroplast diversity observed within *B. napus* can be explained by introgression from inter-specific crosses, there is evidence that the original hybridization event resulting in to *B. napus* occurred on more than one occasion, and involved different maternal genotypes (Allender and King, 2010).

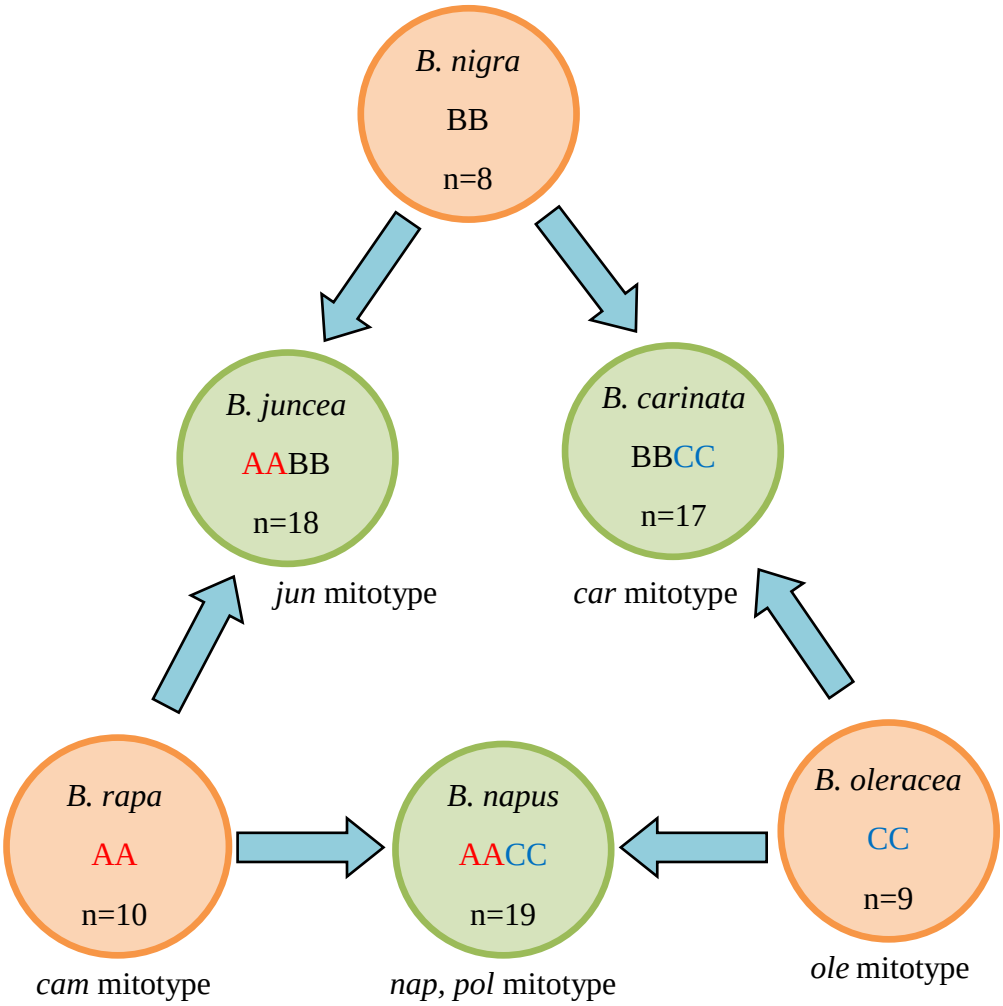

Fig. 2.3. “Triangle of U” displaying genetic relationship between six *Brassica* species (U, 1935).

Resistance to plant diseases

The use of genetic resistance to manage plant diseases is one of the most economical and environmentally friendly methods available to growers. Resistance can be classified as quantitative and qualitative (Poland et al., 2008; Beddington, 2010). Quantitative resistance is controlled by several genes; it is often called ‘partial’ resistance because it does not prevent pathogens from colonization plant tissues but decreases disease severity and/or epidemic progress over time (Chartrain et al., 2004; Brun et al., 2010). As for qualitative resistance, it is usually controlled by single, dominant genes which often are quite effective in preventing pathogens from colonization plant tissues (Balesdent et al., 2001; Rouxel et al., 2003).

Qualitative resistance is also known as ‘complete’ resistance and follows the gene-for-gene interaction. While qualitative resistance is more effective than horizontal resistance, it is generally less durable too because pathogen populations could rapidly evolve to overcome it.

While many diseases affect *B. napus* production, maladies caused by fungal and bacterial pathogens are probably the most common and important. Some important fungal diseases are stem canker (blackleg) caused by *L. maculans*; stem rot (white mold) caused by *Sclerotinia sclerotiorum* (Boland and Hall, 1994); leaf spots caused by *Alternaria* species (Verma and Saharan, 1994); white rust caused by *Albugo candida*; downy mildew caused by *Peronospora parasitica*; club root caused by *Plasmodiophora brassicae*; and *Fusarium* wilt caused by *Fusarium* species (Kandel and Knodel, 2011). In North Dakota the most economically important fungal diseases affecting canola production are *L. maculans* and white mold caused by *Sclerotinia sclerotiorum.*

Resistance to blackleg

There two types of resistance to *L. maculans*, the first is the qualitative resistance and the second is the quantitative resistance. The qualitative resistance is governed by single genes and is also called race specific resistance. The outcome of an inoculation will depend on whether a resistance (R) gene present in the plant matches the avirulence (Avr) gene present in the pathogen (Ansan-Melayah et al. 1998). If the Avr gene does not match the R gene of the host plant, infection occurs, and the host plant becomes susceptible.

The quantitative resistance or partial resistance genes, works in a complex of many pathogen responses genes, and does not provide a strong selective pressure on the fungal pathogen (Zhu et al., 1993; Delourme, et al., 2008). The presence of quantitative resistance increases the durability of the qualitative resistance present in a plant, because it reduces the

probability of a rapid breakdown of resistance. Currently, there are 15 genes in *Brassica* species known to confer resistance against *L. maculans*. Some of these genes are located in chromosomes A10 and A7. Other resistance genes have been introgressed (transferred from a related species via interspecific cross followed by backcrossing. For example, *Rlm10* was introgressed from *B. nigra,*while *Rlm5* and *Rlm6* were introgressed from *B. juncea B. carinata* is another source of highly effective genes against *L. maculans* (Rimmer & van den Berg, 1992; Howlett, 2004). Still this procedure is developing and the breeders are trying to obtain a new cultivar of *B. napus* with these genes inserted in the genome to produce a durable resistance to *L. maculans*.

The mechanisms of resistance of *B. napus* to *L. maculans* are both qualitative and quantitative. Avirulence genes (*AvrLm*) in the pathogen and their corresponding resistance genes in the host (*Rlm*) have been identified in *B. napus* (Ansan-Melaya et al., 1995). Gene-for-gene interactions have been described whereby resistance genes (e.g., *Rlm1*) in the host are matched by complementary avirulence genes (e.g., *AvrLm1*) in the fungus (Ansan-Melayah et al.1998). Lately, five resistance genes (*Rlm1, Rlm3, Rlm4*, *Rlm7*, and *Rlm9)* had been mapped and 10 gene-for-gene interactions have been defined. With this number of interactions, in theory, a total of 29 = 512 races of the pathogen could occur (Balesdent et al. 2001; Delourme et al. 2004). As for the avirulence genes four unlinked genomic region had been mapped *AvrLm1 AvrLm2 AvrLm3*, *AvrLm6,* and one cluster *AvrLm4-AvrLm7*, which may be a single multiallelic locus (Balesdent et al. 2002). Therefore, there are fourteen major resistance genes (*Rlm1-Rlm9* and *LepR1- LepR4*) and polygenic resistance have been identified against specific races of *Leptosphaeria maculans* in *B. napus* (Delourme et al., 2004; Rimmer, 2006; Yu et al., 2005, 2008).

Host (*Brassica napus*) defense responses to *L. maculans* include necrosis of guard cells adjacent to arrested hyphae, phytoalexin, callose, and lignin production. Also the accumulation of pectin in the lumen of xylem vessels when the host is inoculated with avirulent isolates of *L. maculans* producing an hypersensitive response (Roussel et al., 1999), and induction of pathogenesis with proteins such as 1,3-β-glucanase and chitinase (Howlett et al., 2001).

Literature Cited

Allender, C. J., and King, G. J. 2010. Origins of the amphiploid species *Brassica napus* L. investigated by chloroplast and nuclear molecular markers. BMC Plant Biology 10:54-56.

Annis, S. L, and Goodwin, P. H. 1996. Comparison of cell wall degrading enzymes produced by highly and weakly virulent isolates of *Leptosphaeria maculans* in culture. Microbiol Res. 151:401-406.

Ansan-Melayah, D., Balesdent, M. H., Bue´e, M., and Rouxel, T. 1995. Genetic characterization of *AvrLm1*, the first avirulence gene of *Leptosphaeria maculans*. Phytopathology 85:1525–1529.

Ansan-Melayah, D., Balesdent, M. H., Delourme, R., Pilet, M. L., Tanguy, X., Renard, M., and Rouxel, T. 1998. Genes for race specific resistance against blackleg disease in *Brassica napus* L. Plant Breed 117:373-378.

Ash, G. 2000. Blackleg of oilseed rape. The Plant Health Instructor DOI: 10.1094/PHI-I-2000-1111-01.

Attard, A., Gourgues, M., Gout, L., Schmit, J., Roux, J., Narcy, J. P., Balesdent, M. H., and Rouxel, T. 2001. Molecular characterization and polymorphism of *MinLm1*, and minisatellite from the phytopathogenic ascomycete *Leptosphaeria maculans*. Curr. Genet. 40:54-64.

Badaway, H. M. A., Hoppe, H. H., and Koch, E. 1991. Differential reactions between the genus *Brassica* and aggressive single spore isolates of *Leptosphaeria maculans*. J. Phytopathology 131:109-119.

Beddington, J. 2010. Food security: contributions from science to a new and greener revolution. Phil. Trans. Royal Soc. Biol. Sci. 365:61–71. doi: 10.1098/rstb.2009.0201.

Balesdent, M. H., Gall, C., Robin, P. & Rouxel, T. 1992. Intraspecific variation in soluble mycelial protein and esterase patterns of *Leptosphaeria maculans* French isolates. Mycol. Res. 96: 677–684.

Balesdent, M. H., Attard, A., Ansan-Melayah, D., Delourme, R., Renard, M., and Rouxel, T. 2001. Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and

Jet Neuf in *Leptosphaeria maculans*. Phytopathology 91:70–76.

Balesdent, M. H., Attard, A., Kuhn, M. L., and Rouxel, T., 2002. New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. Phytopathology 92:1122–1133.

Barbetti M. J. 1975. Effects of temperature on development and progression in rape of crown canker caused by *Leptosphaeria maculans* Aust. J. Exp. Agric. Animal Husb. 15:705-709.

Berbee M. L., and Taylor, J. W. 1993. Dating the evolutionary radiations of the true fungi. Can. J. Botany 71:1114-1127.

Berbee M. L., and Taylor, J. W. 2000. Fungal molecular evolution gene trees and geological time. In: McLaughlin D, McLaughlin EG, Lemke PA, editors. *The Mycota, a Comprehensive Treatise on fungi as Experimental Systems for Basic and Applied Research*. Vol. VIIB. Systematics and Evolution. NY, U.S.A.: Springer-Verlag, 229-245.

Berbee, M. L. 2001. The phylogeny of plant and animal pathogens in the Ascomycota. Physiol. Mol. Plant Pathol. 59:165-187.

Blanch, P. A., Asher, M. J. C., and Burnett, J. H. 1981. Inheritance of pathogenicity and cultural characters in *Gaeumannomyces graminis* var. *tritici*. Trans. Br. Mycol. Soc. 77:391-399.

Boerema, G. H. 1976. The *Phoma* species studied in culture by Dr. R. W. G. Dennis. Trans. Br. Mycol. Soc. 67:289-319.

Boerema, G. H. 1976. The *Phoma* species studied in culture by Dr. R. W. G. Dennis. Trans. Br. Mycol. Soc. 67:289-319.

Boland, G. J., and Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum.* Can. J. Plant Pathol. 16:93-108.

Brun, H., Chèvre, A. M., Fitt, B. D. L., Powers, F., and Besnard, A. L. 2010. Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. New Phytologist. 185:285–299. doi: 10.1111/j.1469-8137.2009.03049.x.

Canola council of Canada, 2014. http://www.canolacouncil.org/oil-and-meal/what-is-canola/the-history-of-canola: Accessed August 28, 2014.

Caten, C. E., Person, C., Groth, J. V., and Dhahi, S. J. 1984. The genetics of pathogenic aggressiveness in three dikaryons of *Ustilago hordei*. Can. J. Bot. 62:1209-1219.

Chartrain, L., Brading, P. A., Widdowson, J. P., and Brown, J. K. M. 2004. Partial resistance to *Septoria tritici* blotch (*Mycosphaerella graminicola*) in wheat cultivars Arina and Riband. Phytopathology. 94:497–504. doi: 10.1094/phyto.2004.94.5.497.

Chen, Y., and Fernando, W. G. D. 2005. First report of canola blackleg caused by pathogenicity group 4 of *Leptosphaeria maculans* in Manitoba. Plant Dis. 89:339.

Chen, Y., and Fernando, W. G. D. 2006. Prevalence of pathogenicity groups of *Leptosphaeria maculans* in western Canada and North Dakota, USA. Can. J. Plant Pathol. 28:533-539.

Clifford, B. C., and Clothier, R. B. 1974. Physiologic specialization of *Puccinia hordei* on barley hosts with non-hypersensitive resistance. Trans. Br. Mycol. Soc. 63:421-30.

Cumagun, C. J. R., and Miedaner, T. 2004. Segregation for aggressiveness and deoxynivalenol production of a population of *Gibberella zeae* causing head blight of wheat. Eur. J. Plant Pathol.. 110:789-99.

Eckert, M., Gout, L., Rouxel, T., Blaise, F., Jedryczka, M., Fitt, B., and Balesdent, M. 2005. Identification and characterization of polymorphic minisatellites in the phytopathogenic ascomycete *Leptosphaeria maculans.* Curr Genet. 47:37-48.

CODEX . 1999. Codex Standard for Named Vegetable Oils. CODEX-STAN 210-1999:1-16.

Colton, R. T., and Sykes, J. D. 1992. Canola. Agfact P5.2.1.

Crisp, P. 1976. Trends in the breeding and cultivation of cruciferous crops. Pp. 69-118 in J. G. Vaughan, A. J. MacLeod & B. M. G. Jones (editors), The Biology and Chemistry of the Cruciferae. Academic Press, London.

Debrauwere, H., Gendrel, C. G., Lechat, S., and Dutreix, M. 1997. Differences and similarities between various tandem repeat sequences: minisatellites and microsatellites. Biochimie 79:577-586.

Delourme, R., Pilet-Nayel, M. L., Archipiano, M., Horvais, R., Tanguy, X., Rouxel, T., Brun, H., Renard, M., and Balesdent, M. H. 2004. A cluster of major specific resistance genes to *Leptosphaeria maculans* in *Brassica napus*. Phytopathology 94:578–583.

Delourme, R., Piel, N., Horvais, R., Poully, N., Domin, C., Valle´e, P., Falentin, C., Manzanares-Dauleux, M. J., and Renard, M. 2008. Molecular and phenotypic characterization of near isogenic lines at QTL for quantitative resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). Theor. Appl. Genetics 117:1055–1067.

Elliott, C., Gardiner, D. M., Thomas, G., Cozijnsen, A., Van De Wouw, A., and Howlett, B. J. 2007. Production of the toxin sirodesmin PL by *Leptosphaeria maculans* during infection of *Brassica napus*. Mol. Microbiol. 8:791–802.

Ferezou, J. P., Riche, C., Quesneau-Thierry, A., Pascard-Billy, C., Barbier, M., Bousquet, J. F. and Boudart, G. 1977. Structures of two toxins isolated from cultures of the fungus *Phoma lingam* Tode: sirodesmin PL and deacetylsirodesmin PL. Nouv J. Chim. pp. 327–334.

Gall, C., Balesdent, M. H., Desthieux, I., Robin, P., and Rouxel, T. 1995. Polymorphism of Tox^0 *Leptosphaeria maculans* isolates as revealed by soluble protein and isozyme electrophoresis. Mycological Research. 99:221-229.

Gladders, P., and Musa, T. M. 1980. Observations on the epidemiology of *Leptosphaeria maculans* stem canker in winter oilseed rape. Plant Pathol. 29:28-37.

Gomez-Campo, C. 1980. Morphology and morpho-taxonomy of the tribe Brassiceae. In: Tsunoda, S., Hinata, K., Gomez-Campo, C. (editors) Brassica crops and wild allies, biology and breeding. Japan Scientific Societies Press, Tokyo, pp 3-31.

Goodwin, P. H., and Annis, S. L. 1991. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Appl. Environ. Microbiol. 57:2482-2486.

Gruyter J. de, Aveskamp M. M., Woudenberg J. H. C., Groenewald J. Z, Verkley G. J. M., and Crous P. W. (2009). Molecular phylogeny of Phoma and allied anamorph genera: Towards a reclassification of the Phoma complex. Mycological Research. 113:508-519.

Hawthorne, B.T., Ball, R.D., and Reesgeorge, J. 1994. Genetic-analysis of variation of pathogenicity in *Nectria haematococca* (*Fusarium solani*) on *Cucurbita sp*. Mycological Research 98:1183-1191.

Hayward, A., Mclanders, J., Campbell, E., Edwards, D., and Batley, J. 2012. Genomics advances will herald new insights into the *Brassica*: *Leptosphaeria maculans* pathosystem. Plant Biol. 14: 1-14.

Heckman, D. S., Geiser, D. M., Eidell, B. R., Stauffer, R. L., Kardos, N. L., and Hedges, S. B. 2001. Molecular evidence for the early colonization of land by fungi and plants. Science 293:1129-1133.

Howlett, B. J., Idnurm, A., and Pedras, M. S. C. 2001. *Leptosphaeria maculans*, the causal agent of blackleg disease of Brassicas. Fung. Gen. Biol. 33:1-14.

Howlett, B. J. 2004. Current knowledge of the interaction between *Brassica napus* and *Leptosphaeria maculans*. Can. J. Plant Pathol. 26:245–252.

Huang Y. J. Toscano-Underwood, C., Fitt, B. D. L., Hu, X. J., and Hall, A. M. 2003. Effects of temperature on ascospore germination and penetration of oilseed rape (*Brassica napus*) leaves by A- or B-group *Leptosphaeria maculans* (phoma stem canker). Plant Pathol. 52:245-255.

James, T.Y., Kauff, F., Schoch, C., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H.T., Rauhut, A., Reeb, V., Arnold, A.E., Amtoft, A., Stajich, J.E., Hosaka, K., Sung, G. H., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J.M., Slot, J.C., Wang, Z., Wilson, A.W., Schußler, A., Longcore, J.E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P.M., Powell, M.J., Taylor, J.W., White, M.M.,

Griffith, G.W., Davies, D.R., Humber, R.A., Morton, J.B., Sugiyama, J., Rossman, A.Y., Rogers, J.D., Pfister, D.H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R.A., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Spotts, R.A., Serdani, M., Crous, P.W., Hughes, K.W., Matsuura, K., Langer, E., Langer, G., Untereiner, W.A., Lucking, R., Budel, B., Geiser, D.M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D.S., Lutzoni, F., McLaughlin, D.J., Spatafora, J.W., and Vilgalys, R. 2006. Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature 443:818-822.

Jeffrey, S. I. B., Jinks, J. L., and Grindle, M. 1962. Interracial variation in *Phytophthora infestans* and field resistance to potato blight. Genetica 32:323-38.

Jinks, J. L., and Grindle, M. 1963. Changes induced by training in *Phytophthora infestans*. Heredity 18:245-64.

Johnson, R. D., and Lewis, B. G. (1990). DNA polymorphism in *Leptosphaeria maculans*. Physiol. Mol. Plant Pathol. 37:417–424.

Kandel, H., and Knodel, J. 2011. Canola Production Field Guide. North Dakota State Univ. Ext. Ser. Fact Sheet No. A-1280.

Kirk, P. M., Cannon, P. F., Minter, D. W., and Stalpers J. A. 2008. Ainsworth and Bisby's dictionary of the Fungi. 10th ed. Wallingford (UK): CAB International.

Koch, E., Badawy, H. M. A. and Hoppe, H. H. 1989. Differences between aggressive and non-aggressive single spore lines of *Leptosphaeria maculans* in cultural characteristics and phytotoxin production. J. Phytopathology 124:52–62.

Koch, E., Song, K., Osborn, T. C., and Williams, P. H. 1991. Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Mol. Plant-Microbe Interact. 4:341-349.

Koch, M., Al-Shenbaz, I. A., and Mummenhoff, K. 2003. Molecular systematics, evolution, and population biology in the mustard family (Brassicaceae). Annals of Missouri Botanical Garden, Vol. 90, No. 2. Pp. 155-171.

Lee, I-M., Gundersen-Rindal, D. E., Davis, R. E. Bottner, K. D., Marcone, C., and Seemu¨ller, E. 2004. '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. Int. J. Syst. Evol. Microbiol. 54:1037-1048.

Leonard, K. J. 1969. Selection in heterogeneous populations of *Puccinia graminis* f.sp. *avenae*. Phytopathology 59:1851-7.

Lumbsch, H. T. and Huhndorf S. M. 2007. Outline of Ascomycota. Myconet. 13:1-58.

Markell, S., Del Rio, L., Halley, S., Mazurek, S., Mathew, F., and Lamey, A. 2008. Blackleg of Canola. NDSU Extension Bulletin PP-1367. 4 p.

McGee, D. C. 1977. Blackleg *Leptosphaeria maculans* (Desm.) Ces. & De Not. of rapeseed in Victoria: sources of infection and relationships between inoculum, environmental factors and disease severity. Aust. J. Agric. Res. 28:53-62.

Mehta, Y. R., and Zadoks, J. C. 1970. Uredospore production and sporulation period of *Puccinia recondita* f.sp. *triticina* on primary leaves of wheat. Netherlands Journal of Plant Pathology 76: 267-76.

Mendes-Pereira, E., Balesdent, M. H., Brun, H., and Rouxel, T. 2003. Molecular phylogeny of the Leptosphaeria maculans - L. biglobosa species complex. Brit. Mycol. Soc. 107:1287-1304.

Mengistu, A., Rimmer, S. R., Koch, E., and Williams, P. H. 1991. Pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid-cycling brassicas. Plant Dis. 75:1279-1282.

Milus, E. A., Seyran, E., and McNew, R. 2006. Aggressiveness of *Puccinia striiformis* f.sp. *tritici* isolates in the south-central States. Plant Dis. 90:847–52.

Naseri, B. 2006. Epidemiology of blackleg disease of canola, caused by *Leptosphaeria maculans*. School of Agriculture, food and wine. University of Adelaide, Australia. Pp.2-8.

Nishida, H., and Sugiyama, J. 1994. Archiascomycetes: detection of a major new lineage within the Ascomycota. Mycoscience 35:361-366.

Pedras, M. S. C., and Taylor, J. L. 1993. Metabolism of the phytoalexin *brassinin* by the 'blackleg fungus'. J. Natural Prod. 56:731-738.

Pedras, M. S. C. 1998. Towards an understanding and control of plant fungal diseases in Brassicaceae. Recent Research Developments in Agricultural and Food Chemistry Pp.513–531

Pedras, M. S. C., Okanga, F. I., Zaharia, I. L., and Khan, A. Q. 2000. Phytoalexins from crucifers: synthesis, biosynthesis, and biotransformation. Phytochemistry 53:161–176.

Smith, H. C., and Sutton, B. C. 1964. *Leptosphaeria maculans* the ascogenous state of *Phoma lingam*. Transactions of the British Mycological Society 47:159–65.

Petrie, G. A. 1969. Variability in *Leptosphaeria maculans* (Desm.) Ces. & De Not., the cause of blackleg of rape. PhD dissertation, University of Saskatchewan.

Poland J. A., Balint-Kurti P. J., Wisser R. J., Pratt R. C., and Nelson R. J. 2008. Shades of gray: the world of quantitative disease resistance. Trends in Plant Sci. 14:21–29.

Pongam, P., Osborn, T. C., and Williams, P. H. 1999. Assessment of genetic variation among *Leptosphaeria maculans* isolates using pathogenicity data and AFLP analysis. Plant Dis. 83: 149-154.

Pringle, A., and Taylor, J. W. 2002. The fitness of filamentous fungi. Trends Microbiol. 10:474–81.

Purwantara, A., Barrins, J. M., Cozijnsen, A. J., Ades, P. K., and Howlett, B. J. 2000. Genetic diversity of isolates of the *Leptosphaeria maculans* species complex from Australia, Europe and North America using amplified fragment length polymorphism analysis. Mycol. Res. 104:772-781.

Rakow, G. 2004. Species origin and economic importance. In: Pua, E. C. and Douglas, C. J. (editors) *Brassica*. Springer-Verlag Berlin Heidelberg, Germany. Pp. 3-11.

Rimmer, S. R., van den Berg, C. G. J. 1992. Resistance of oilseed *Brassica spp*. to blackleg caused by *Leptosphaeria maculans*. Can. J. Plant Pathol. 14:56–66.

Robert, C., Bancal, M. O., and Lannou, C. 2004. Wheat leaf rust uredospore production on adult plants: influence of leaf nitrogen content and *Septoria tritici* blotch. Phytopathology 94:712-21.

Roussel, S., Nicole, M., Lopez, F., Ricci, P., Geige, J. P., Renard, M., and Brun, H. 1995. *Leptosphaeria maculans* and cryptogein induce similar vascular responses in tissues undergoing the hypersensitive reaction in *Brassica napus.* Plant Sci. 144:17-28.

Rouxel, T., Penaud, A., Pinochet, X., Brun, H., Gout, L., et al. 2003. A 10-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the *Rlm1* resistance gene of oilseed rape. Eur. J. Plant Path. 109:871–881.

Sache, I. 1997. Effect of density and age of lesions on sporulation capacity and infection efficiency in wheat leaf rust (*Puccinia recondita* f.sp. *tritici*). Plant Pathol. 46:581-589.

Shaner, G. E. 1980. Probits for analyzing latent period data in studies of slow rusting resistance. Phytopathology 70:1179-1182.

Shoemaker, R. A. and Brun, H. 2001. The teleomorph of the weakly aggressive segregate *Leptosphaeria maculans*. Can. J. Bot. 79:412-419.

Simmonds, N. W. (editor). 1986. Evolution of Crop Plants. Longman, Harlow.

Toscano-Underwood, C., Hall, A., Fitt, B., and Huang, Y. 2003. Effects of temperature on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape stem debris. Plant Pathol. 52:726-736.

Vanniasingham, V. M., and Gilligan. C. A. 1989. Effects of host, pathogen and environmental factors on latent period and production of pycnidia of *Leptosphaeria maculans* on oilseed rape leaves in controlled environments. Mycol. Res. 93:167-174.

Van der Plank, J. E. 1963. Plant Diseases: Epidemics and Control. New York, USA: Academic Press.

Van der Plank, J. E. 1968. Disease Resistance in Plants. New York, USA: Academic Press

Verma, P. R. and Saharan, G. S. 1994. Monograph on alternaria diseases of crucifers, Saskatoon Research Center Technical Bulletin 1994-6E, Agric Agri-Food Canada, Saskatoon, 162 pp.

Wehmeyer L. E. (1975). The pyrenomycetous fungi. Mycologia Memoir. 6:1-50.

West, J. S. Kharbanda, P. Barbetti, M. J. and Fitt B. D. L. 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) in Australia, Canada and Europe. Plant Pathol. 50:10-27.

Williams, PH. 1992. Biology of *Leptosphaeria maculans*. Can. J. Plant Pathol. 14:30-5.

Xu, X. H., Hill, C. B., and Williams, P. H. 1987. Environmental conditions for the production of pseudothecia of *Leptosphaeria maculans.* Acta Mycologia Sinica. 6:236-241.

Yu F. Q., Lydiate D. J., Rimmer S. R. 2008. Identification and mapping of a third blackleg resistance locus in *Brassica napus* derived from *B-rapa* subsp. *sylvestris*. Genome 51:64-72.

Zhu, J. S., Struss, D., Robbelen, G. 1993. Studies on resistance to *Phoma lingam* in *Brassica napus–Brassica nigra* addition lines. Plant Breeding 111:192–197.

CHAPTER 3. EVALUATION OF AGGRESSIVENESS OF *L. MACULANS* PATHOGENICITY GROUPS ON COTYLEDONS

Abstract

Blackleg disease, caused by *Leptosphaeria maculans* is one of the most destructive diseases of canola (*Brassica napus*) production worldwide. In North Dakota, yield losses due to this disease could amount up to 50%. However in 2002 PG-2 was the predominant pathotype while in 2003 and 2004, PG-3, PG-T, and PG-4 were reported for the first time. The first objective of these experiments are to evaluate the effect of temperature (15, 20, 25 and 30 ^{o}C) and *L. maculans* isolates spore age colonies (7, 10, 14 and 21 day old) on the phenotypic reaction of *B. napus* susceptible cv. Westar. The plants were inoculated at on the cotyledons seedling stage with 10µl droplet spore suspension ($1x10^{5}ml^{-1}$). Results shown AUDPC values were higher for all PGs isolates when the spore age was between 10-14 days old colonies. The second objective are to evaluate the effect of different artificial culture (PDA and V8 agar) and temperature (15, 20, 25 and 30^{o}C) on *L. maculans* isolates. Results shown that *L. maculans* pathogenicity groups (PGs) produce more spores and colony growth on V8 agar and 25-30^{o}C. This will helps us to identify the most proliferative and aggressive isolates to be used to select resistant materials.

Introduction

Blackleg disease is caused by *Leptosphaeria maculans* (Desm.) Ces. & De Not. (anamorph *Phoma lingam* (Tode ex Fr.) Desm.), and is one of the most important diseases affecting oilseed rape (*Brassica napus*) production worldwide (West et al., 2001). The disease causes yield losses estimated at $76.6 million annually in Australia (Elliott et al., 2011) and $72 million in the United Kingdom (Fitt et al., 2006). In Europe yield losses were estimated at 30%

with a value of $200 million (Hall et al., 1993; Zhou et al., 1999; Barbetti and Khangura, 1999) and at > 50% in Canada. In the United States, more than 70% of the area dedicated to production of canola is located in North Dakota (NASS, 2014) and yield losses due to blackleg can be as high as 45-50% (del Rio Mendoza et al., 2012; Markell et al., 2008)

Blackleg is making a comeback as a serious threat to the canola industry in North Dakota. The first blackleg outbreaks were detected in 1991 in the northeastern area of the state with incidences that ranged between 10 to 60%. During that epidemic and in subsequent years the most prevalent strains belonged to PG-1 and PG-2 (Lamey and Hershman, 1993). By 1993, the seed industry had abandoned production of cv. Westar because it was very susceptible to pathogenicity group (PG) 2 strains of *L. maculans*, the most prevalent group in North Dakota at that time (del Rio Mendoza et al., 2012). By the end of the 1990s most cultivars planted in the region were resistance to PG-2. In 2003, strains belonging to PG-3 and PG-4 were detected for the first time (Bradley et al., 2005). In that same year, PG-T was detected in Western Canada and a year later in North Dakota (Chen and Fernando, 2006). A study conducted by Nepal et al. (2014) indicated that 56% isolates retrieved from infected stubbles between 2004 and 2009 belonged to PG-4, 7% and 8% as PG-3 and PG-T respectively and < 1% belonged to PG-2. In addition, approximately 21% of isolates could not be classified in any of these groups. This shift in prevalence has important implications for the canola seed industry as well as for researchers and extension specialists working with canola and canola diseases. Blackleg outbreaks caused by strains of PG-4 are becoming more and more frequent and the intensities of these epidemics seem to be increasing too. The intensity of epidemics is affected not only by environmental factors but also by attributes of the pathogen like their ability to interact with specific resistance genes in the host and their aggressiveness among others. The interaction between specific

resistance and avirulence genes has been documented well using sets of differential cultivars and the results can be applied to almost any place in the world where the crop and the pathogen interact. Studies of aggressiveness, however, have a more localized impact as isolates that are prevalent in one region may not necessarily be present in others. Nevertheless, characterizing the aggressiveness of different pathogenicity groups would help understand and predict the development of epidemics while comparing the relative aggressiveness of isolates within each group could allow researchers to identify a set of isolates that could be used to more effectively identify sources of resistance against them. , Van der Plank (1963) defined the term aggressiveness as "the non-specific component of pathogenicity". In other words, it describes a quantitative component of pathogenicity that is not dependent on the genetic composition of the host but on that of the pathogen. In this way, to characterize the true aggressiveness of a pathogenic organism one should measure it in a host that does not have resistance genes that may affect its performance. This is especially true when one is trying to determine whether a particular race or in the case of *L. maculans* a particular pathogenicity group is more or less aggressive than other. The presence of resistance genes in the host would invalidate any conclusion reached if one of the groups is incapable of infecting the host due to an incompatible gene for gene interaction. Aggressiveness is defined by the Illustrated Glossary of Plant Pathology (D'Arcy et al., 2001) as the differential ability of a pathogen to colonize and cause damage to plants and can be expressed in different forms, i.e. the size of lesions (severity) caused by the pathogen or its the ability to attack plants at different temperatures or even its ability to produce spores whether *in vitro* or *in planta*. The objectives of this study are to compare the aggressiveness of different pathogenicity groups of *L. maculans* and to identify the most aggressive isolates within each groups.

Materials and Methods

In this study the aggressiveness of PG 2, 3, 4, and T of *L. maculans* were compared by inoculating them on cotyledons of two weeks-old seedlings of cv. Westar. This cultivar was selected because it does not contain genes for resistance against *L. maculans.* Four studies were conducted: in the first, spores from 65 isolates representing four PGs and collected from colonies of four different ages, 7, 10, 14, and 21 days, were inoculated and incubated at 22^{o}C; in the second study, the aggressiveness of these PGs, represented by the four most aggressive isolates from each group, were compared at four different temperatures, 15, 20, 25, and 30^{o}C; in the third study, mycelial growth and spore production of all isolates was measured at 22^{o}C in two culturing media, potato dextrose agar and V8 agar; in the fourth study, PGs were compared for their ability to produce spores in V8 medium at 15, 20, 25, and 30^{o}C. Each PG was represented by the four most aggressive isolates identified in the first study. All experiments were conducted under controlled environment conditions at North Dakota State University.

Inoculum production

A total of 65 *L. maculans* isolates retrieved from samples collected from 15 North Dakota counties (Table 3.1) were used for the first and third studies, while only 16 isolates were used in the second study (Table 3.2). Single spore cultures of all these isolates were prepared as described by Del Rio Mendoza et al. (2012). Once purified, isolates were kept in deep freeze storage (-80°C) until used. Two weeks before starting the experiments the isolates were retrieved from storage and inoculated on V8 medium in 9 mm Falcon Disposable Petri Dishes (BD Biosciences, Northbrook, Illinois).

Table 3.1. Origin of North Dakota isolates of *L. maculans* inoculated on canola cv. Westar to evaluate their aggressiveness under greenhouse conditions.

		Pathogenicity Groups			
Counties	Isolates	2	3	4	T
Benson	1	-[y]	-	-	1
Bottineau	8	3	1	1	3
Cavalier	19	8	5	3	3
Divide	1	-	1	-	-
Grand Forks	1	-	-	1	-
McHenry	6	-	-	3	3
McLean	6	1	1	2	2
Mountrail	1	-	-		1
Nelson	1	-	-	1	-
Renville	5	1	1	1	2
Rolette	2	1	-	-	1
Towner	11	3	4	2	2
Ward	1	-	-	1	-
Wells	2	1	-	1	-
Total	65	18	13	16	18

[y] Short dash line = No isolate were selected from the county for these studies.

Evaluation of colony age on aggressiveness at 22ºC

For this study, all 65 isolates were grown on V8 medium. Each liter of medium was prepared by combining 837 ml distilled water, 163 ml V8 juice (Campbell Soup Co., Camden, NJ), 15 g agar (Bacto-Agar, Becton Dickinson and Co., Sparks, MD), and 3 g CaCO3. The pH of the medium was adjusted to 7.2 by adding 1M NaOH before autoclaving. The medium was sterilized by autoclaving at 120°C and 103.4 kPa for 20 min. The inoculated plates were incubated at 22° C under constant soft white light for 7, 10, 14 and 21 days before the spores were harvested. Spores were collected by pouring 1000 µl of sterile distilled water on each dish and gently rubbing the colonies with a sterile glass rod. The resulting spore suspensions were transferred using pipettes into 2.5 ml plastic vials. The spore concentrations were estimated with

help of a hemocytometer and adjusted to 10^7 spores·ml^{-1}. These suspensions were placed on ice and taken to the greenhouse were they were inoculated on 12 day old seedlings.

Table 3.2. Origin of *L. maculans* isolates from four pathogenicity groups used to evaluate the effect of temperature on disease progress on cotyledons of cv. Westar growth chamber conditions.

		Pathogenicity Groups			
Counties	Isolates	2	3	4	T
Bottineau	3	2	-	-	1
Cavalier	4	1	2	-	1
McHenry	1	- [y]	-	1	-
McLean	3	-	-	1	2
Nelson	1	-	-	1	-
Renville	1	1	-	-	-
Towner	3	-	2	1	-
Total	16	4	4	4	4

[y] Pathogenicity group isolate no used for these studies.

Evaluation of aggressiveness at 15, 20, 25, and 30°C

Spores from four isolates considered the most aggressive within each of the four PG evaluated were produced in V8 medium at 22°C but the spores were harvested from 10 days-old colonies. The procedures followed to collect the spores and to adjust the concentrations of the resulting spores suspensions to 10^7 spores·ml^{-1} were similar to that described above.

Mycelial growth and spore production at 22°C

Hyphal tips of the 65 isolates used in this study were obtained by cutting agar plugs 3 mm in diameter from the edges of 12 days-old colonies growing on V8 medium. These plugs were transferred into dishes containing potato dextrose agar (PDA) or V8 agar. The spore suspensions of the 65 isolates were harvested from 10 day-old colonies growing on V8 as described. One ml of each of these suspensions was spread on individual dishes containing V8 medium using a glass rod as described.

Mycelial growth and spore production at 15, 20, 25, and 30ºC

Spore suspensions of the four most aggressive isolates from each PG were produced as described and inoculated on dishes containing V8 medium as described. The newly inoculated dishes were incubated for 10 days at 15, 20, 25, or 30ºC before harvesting. To evaluate the aggressiveness of *L. maculans* isolates at different temperatures (15, 20, 25, and 30°C) we used a total of 16 isolates retrieved from samples collected from 15 counties North Dakota (Table 3.2). In this study we used the same methods describe above for inoculum production.

Seedling production and inoculation

The seeds used in all studies involving inoculation of *L. maculans* on seedlings of cv. Westar were planted in individual plastic cells containing soilless Professional Growing Mix 1 (SunGro Horticulture Canada, Seba Beach, AB, Canada). At planting time, two seeds of cv. Westar were deposited in each cell and after emergence a single seedling was allowed to continue growing. To study the effect of colony age on aggressiveness, the seedlings were grown and maintained in greenhouse rooms at 22° C with 16 hours light daily. To study the effect of different temperatures on aggressiveness, the seedlings were grown and maintained in growth chambers (CMP4030 Conviron; Manitoba Canada) set to provide 16 hours with fluorescent soft white light and 60% relatively humidity at either 15, 20, 25, or 30° C.

To inoculate seedlings a wound was made on each cotyledon leaf using a pointy tweezers and 10 µl of a spore suspension with $1x10^5$ spores ml^{-1} were deposited on each wound (Figure 3.1). Immediately after inoculation, the seedlings were transferred to misting chambers set at 22º C where Vicks cool mist humidifiers (V5100NS Vicks® Ultrasonic Humidifier; Southborough, Massachusetts) delivered fog-like mist for 30 seconds every 12 minutes. After 24 h, the seedlings

were returned to the greenhouse room at 22^{o} C or to their respective growth chambers. Disease progress was recorded at 4, 7, 11 and 15 days after the inoculation.

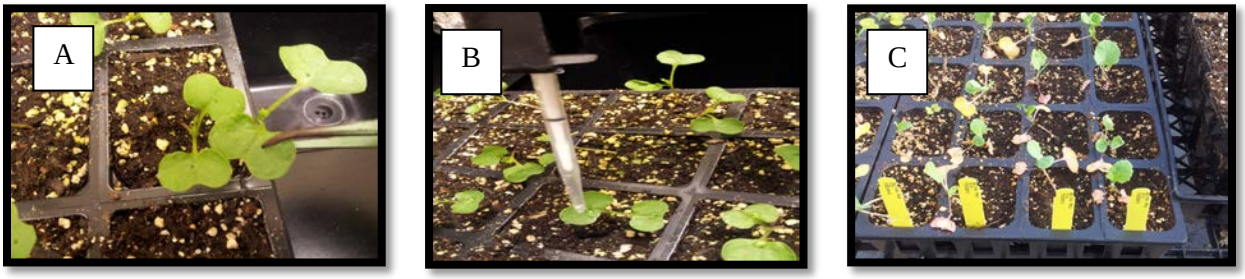

Figure 3.1. Inoculation method of *L. maculans* isolates on seedlings of cv. 'Westar' under greenhouse condition in trials conducted in 2012-2013 in Fargo, ND. (A) tweezers are used to wound the cotyledons; (B) 10µl of spore suspension are delivered onto each wound using a pipette; (C) reaction to inoculation is recorded 11 days after inoculation.

Response scoring

For the studies that compared aggressiveness of PGs via inoculation of seedlings of cv Westar, the reaction of the plants to the fungus was recorded 4, 7, 11 and 15 days after the inoculation in two ways: by measuring the area directly affected by the pathogen and by visually estimating the area indirectly affected. To estimate the direct effect, the diameter of the area visibly affected by the pathogen was measured in two perpendicular directions. To estimate the indirect effect, changes in cotyledon color were monitored and recorded using a 0 to 2 scale; in the scale, 0=no change in color, both cotyledons remain green; 1= > 50% of the cotyledons tissues have turned yellow; and 2= the entire cotyledon tissues have turned yellow and/or have been dropped from the plant. During this time, information on the number of seedlings that dropped its cotyledons because they were destroyed by the pathogen was recorded too.

For studies that compared PGs for their ability to grow and produce spores in artificial media, data was collected as follow: To compare mycelial growth the colony diameters were measured in two directions one perpendicular to the other. Colony sizes were expressed as diameter length in mm. To estimate spore production, after 10 days of incubation, 2 ml of sterile

water were poured on the colonies and a glass rod was gently rubbed on its surface to suspend spores that were oozing from pycnidia. This procedure was repeated three times. The spore suspensions were collected and their volume measured. A dilution of $1x10^5$ spores $\cdot$ ml^{-1} from the original spore suspension was used to estimate the amount of spores produced by each isolate in a dish. Spore concentrations were estimated using a hemocytometer with a light microscope set at 40x (ZEISS Axiostar plus, Focus Precision Instruments, Thornwood, New York). Spore production was expressed as number of spores per ml.

Experimental designs

For all studies a randomized complete block design (RCBD) with nested arrangement was used. To compare aggressiveness of PG as affected by colony age, the factors evaluated were PG, isolates nested within PG, and colony age. Each treatment was applied to six seedlings and each seedling was considered a replication. This study was conducted twice. To compare the aggressiveness of each PG at different temperatures the factors evaluated were PG and isolates nested within PG. Due to limitations in the number of growth chambers available, the trials for each temperature were conducted separately. In each study treatments were applied to two seedlings and each seedling was considered a replication. Each trial was conducted twice. To compare PGs for their ability to grow and produce spores in artificial media incubated at 22^o C each trait was evaluated in separate studies although in each study the factors were the same: PG, isolates nested within PG, and media. Treatments in each study had three replications and each study was conducted two times. To compare PG for their ability to produce spores in artificial media incubated at different temperatures, the factors were: PG and isolates nested within PG. Due to limitations in the number of growth chambers available, trials for each temperature were

conducted separately. Treatments in each study had three replications and each study was conducted two times.

Data processing and statistical analyses

Trials and replications were considered random effects while colony age, pathogenicity groups (PG) and isolates nested within PG were considered fixed effects. Prior to the analyses that compared aggressiveness of PG as affected by colony age, the means of cotyledon readings were estimated per replication. The quantitative summary of disease intensity over time was achieved by calculating the area under the disease progress curve (AUDPC) using (PROC MEANS) from SAS version 9.3 (SAS Institute, Inc., Cary, NC). To further characterize the isolates within each PG, arbitrary thresholds were used to denominate isolates as non-aggressive, lesions smaller than 2mm in size; moderately aggressive, lesions 3-6 mm in size; and aggressive if lesions were greater than 7 mm after 15 days of inoculation (Fig.3.2). Also, the number of days until the cotyledons appearance changed from green to yellow was estimated.

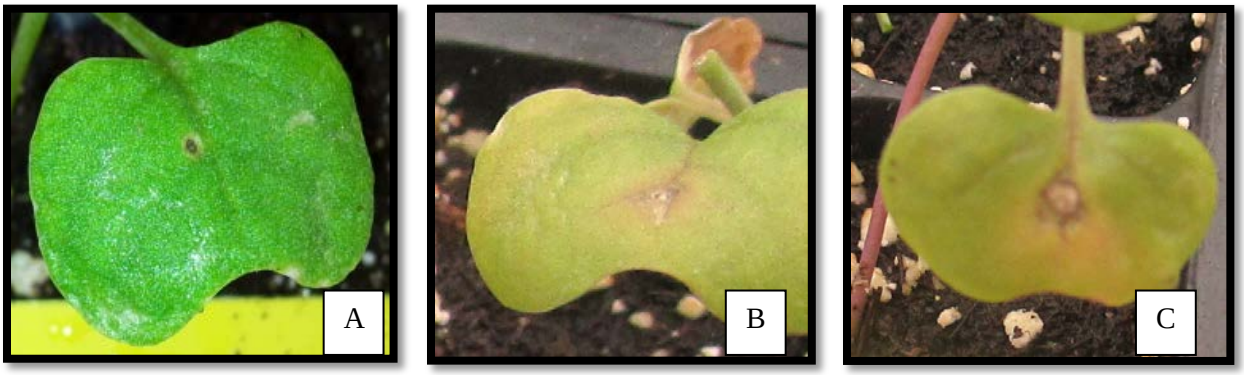

Figure 3.2. Scale used to measure aggressiveness of *L. maculans* isolates on seedlings of cv. 'Westar'. (A) 0-2 mm: non aggressive; (B) 3-6 mm: moderately aggressive; (C) 7-9 mm: aggressive.

The AUDPC is a quantitative summary of disease intensity over time, for comparison across years, locations, or management strategies. The most commonly used method for calculate the AUDPC, is the trapezoidal. This method approximates the time variable (hours,

days, weeks, months, or years) and calculates the average disease intensity between each pair of adjacent time points (Madden et al., 2007). The area is calculated as follow

$$\mathrm{A}_k = \sum_{i=1}^{Ni-1} \frac{y_i + y_{i+1}}{2} \left(t_{i+1} - t_i\right)$$

Where, $\{t_i\}$= time data points in a sequence, where the time interval between two time points may be consistent or differ. Also, $\{y_i\}$= measures of the disease level. Then we define $y(0) = y_0$ as the initial infection at $t = 0$ (the first disease severity observation in our experiment). $A(t_k)$, the AUDPC at $t = t_k$, is the total accumulated disease until $t = t_k$.

To further characterize the isolates within each PG, arbitrary thresholds were used to denominate isolates as non-aggressive if the lesions they caused were on average smaller than 2 mm in size; moderately aggressive if the lesions were 3-6 mm in size; and aggressive if the lesions were greater than 7 mm after 15 days of inoculation (Fig.3.2). Also, the number of days until the cotyledons appearance changed from green to yellow was estimated. Levene's test for homogeneity of variances was conducted to determine whether the trials could be combined for analysis. Upon confirmation of homogeneity a combined ANOVA was performed using a mixed linear model (PROC MIXED) from SAS version 9.3 (SAS Institute, Inc., Cary, NC). The sources of variation used in the combined analysis are listed on table 3.3. Treatments means separations were determined by *F*-protected least significant difference (LSD) comparisons at $\alpha \leq 0.05$. Also, single-degree-of-freedom contrasts were conducted to compare different PGs and also different colony ages. Linear regression analyses were conducted to quantify the rate of change in aggressiveness as a function of colony age for each isolate and PG. The models helped visualize the interactions between isolates nested within pathogenicity groups and colony ages. Non-significant slopes ($\alpha \leq 0.05$) were interpreted as lack of interaction.

To analyze data that compared PGs for their aggressiveness of PGs at different temperatures, data preparation was similar to that described for the previous study. The average lesion diameter was used to calculate the AUDPC. Since a direct comparison between temperatures was not possible, the effect of temperature on aggressiveness of each PG was measured using a linear regression analysis. Also, 95% confidence intervals for the mean of each temperature were calculated and plotted in figures.

Results

Effect of colony age on PG aggressiveness

Levene's test indicated the variances of the trials for aggressiveness were homogenous at $\alpha \leq 0.05$and thus a combined analysis of variance could be conducted. The combined analysis indicated statistically significant ($\alpha<0.0001$) interactions for *Colony-age x isolate* and for the main effects *PG* ($\alpha<0.01$) and *isolate(PG)* ($\alpha<0.0001$) for AUDPC (Table 3.3).

Table 3.3. Combined analysis of variance for the area under the disease progress curve (AUDPC) produced by *Leptosphaeria maculans* isolates inoculated on seedlings of cv. Westar under greenhouse environmental conditions in trials conducted in 2012 and 2013.

Source of Variation[1]	Df[2]	Mean Square	F value	Pr>F
PG	3	225002.00	44.16	0.01
Isolate(PG)	64	5898.90	9.69	<0.0001
Colony-age (CA)	3	7076.35	4.64	0.1295
CA x isolate(PG)	192	1116.11	5.08	<0.0001
CV (%)		49.76		

[1]Only fixed effects: pathogenicity groups (PG), colony-age (CA), Isolates, and their interactions.
[2]Df=degrees of freedom.

On average the cotyledon leaves remained green until 4 days after inoculation for all PG's except T. The cotyledons of seedlings inoculated with strains of PG-T remained green in appearance for 7 days after inoculation. In general, cotyledons turned yellow during 7-15 days after inoculation for PG-2, PG-3 and PG-4 (Figure 3.3).

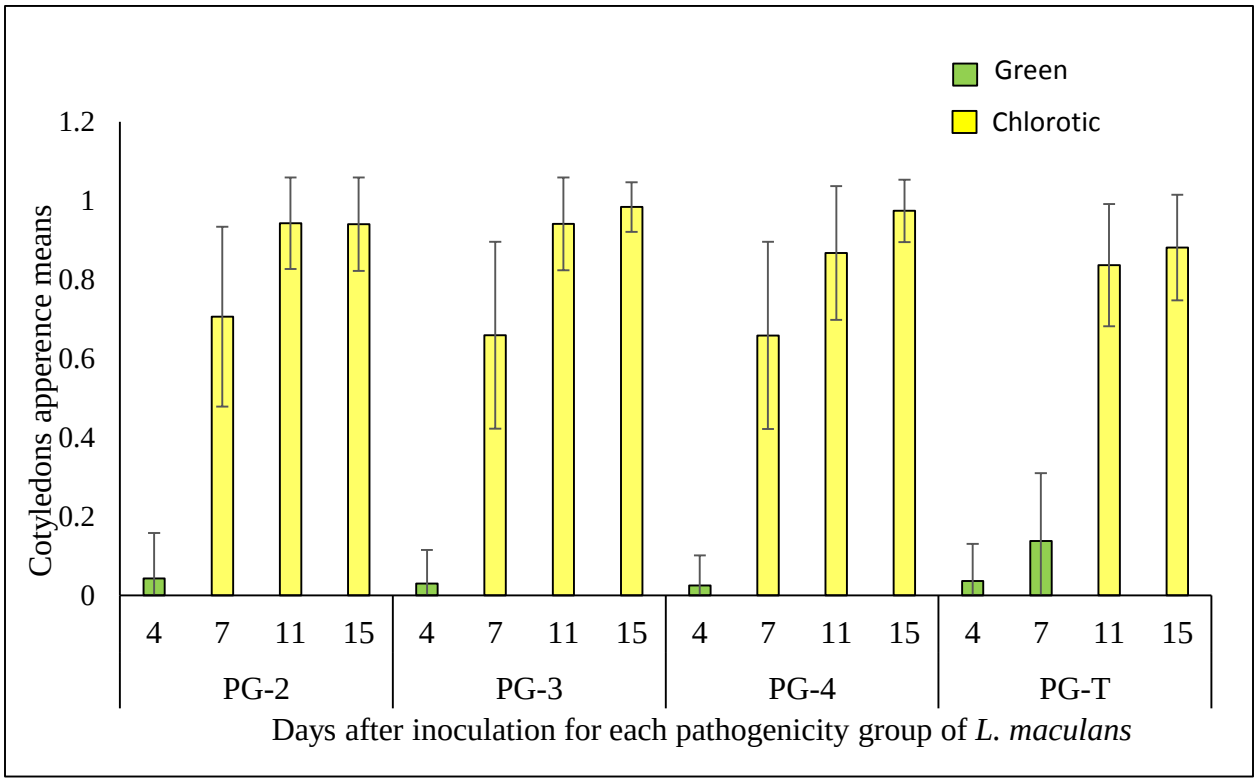

Figure 3.3. Changes in color of cotyledons leaves of cv. 'Westar' over time after being inoculated with *L. maculans* isolates from different pathogenicity groups. Bars on columns represent standard error.

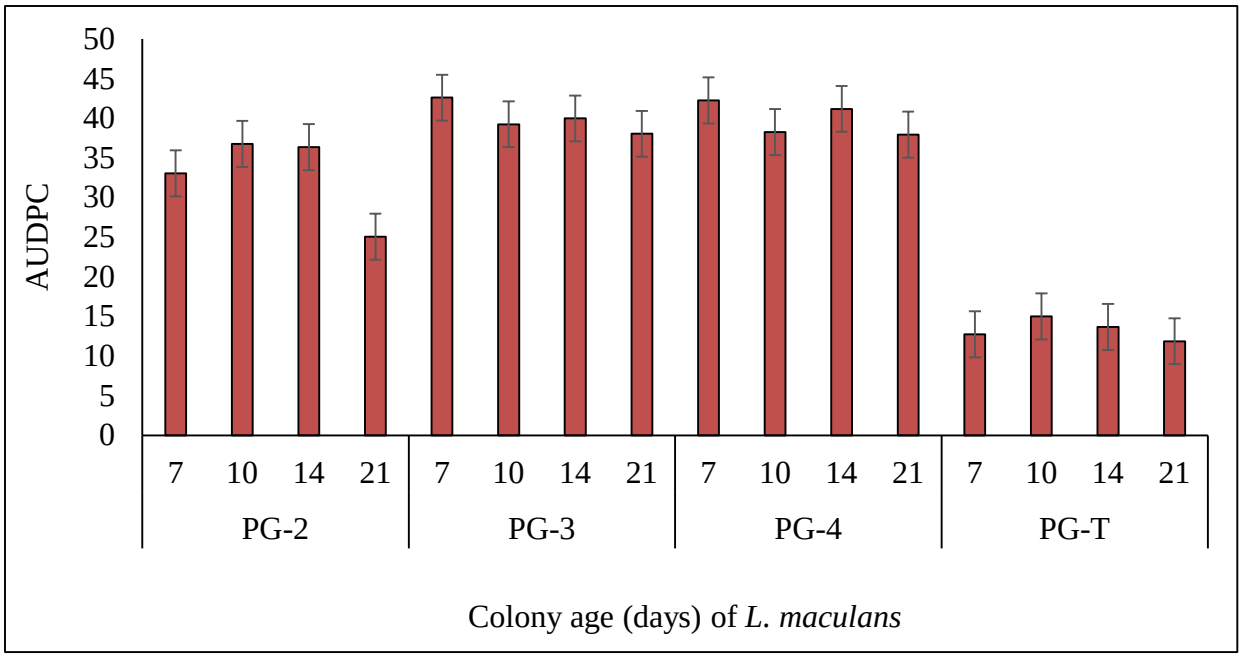

Figure 3.4. Effect of colony age and pathogenicity groups on *L. maculans* isolates aggressiveness measured on seedlings cotyledons of cv. 'Westar' under greenhouse conditions. Bars on the columns represent the standard error.

The figure 3.4 summarizes the interaction between colony age and *L. maculans* isolates from different pathogenicity groups on canola cv. Westar cotyledon leaves. PG-2 was the only group that experienced a significant reduction in aggressiveness when the spores used were produced by colonies older than 14 days. Overall, however, isolates were more aggressive on the canola cotyledons when the colonies ranged between 7-14 days old. When the mean of aggressiveness for each PG was calculated, significant differences (α<0.05) were observed (Figure 3.5). It was observed that PG-3 and PG-4 were the most aggressive groups but they were not significantly different from each other (LSD=2.72). PG-2 was significantly different from PG-3, PG-4 and PG-T. As for PG-T, it was the less aggressive group. These results were confirmed by the contrast analyses conducted (Table 3.4).

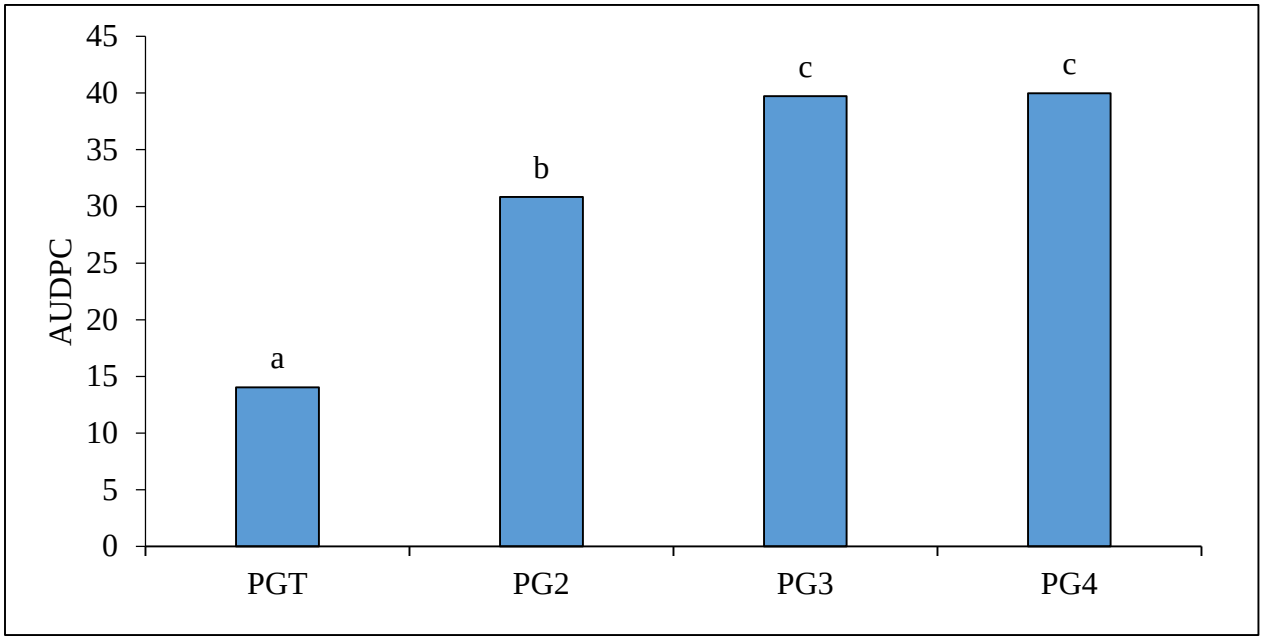

Figure 3.5. Aggressivenes of four pathogenicity groups of *L. maculans* expressed as the area under the disease progress curve produced within 21 days from inoculation of seedings of cultivar Westar. Columns with different letters are statistically different at *P*<0.05.

Table 3.4. Comparison of the aggressiveness of different pathogenicity groups of *L. maculans* using single-degree-of-freedom contrasts. Means represent the area under the disease progress curve calculated 21 days after inoculation of seedlings of canola cv. Westar under greenhouse conditions.

Contrast[1]	Mean 1[2]	Mean 2[3]	Pr>F[4]
PG-2 vs. PG-3	32.33	39.98	<0.0001
PG-2 vs. PG-4	32.33	39.98	<0.0001
PG-2 vs. PG-T	32.33	14.05	<0.0001
PG-3 vs. PG-4	39.98	39.98	0.6022
PG-3 vs. PG-T	39.98	14.05	<0.0001
PG-4 vs. PG-T	39.98	14.05	<0.0001

[1]PG's=Pathogenicity groups of *L. maculans.*

[2]Mean 1= the mean of the PG in first term of each contrast.

[3]Mean 2= the mean of the PG in second term of each contrast.

[4]Significant at $P<0.05$ and $P<0.01$ probability levels.

The regression models produced to quantify the change in aggressiveness as a function of the age of the colonies were statistically significant only for eight of the 65 isolates evaluated. Each PG had two isolates with significant models (Table 3.5). This means these isolates were more sensible to the aging of spores. These models allowed estimating the reduction in aggressiveness, for example, the two isolates from PG-2 experienced a reduction of 1.25 and 2.82 units of AUDPC, respectively, for every day the colonies aged in the medium. Lesions on seedlings developed in almost linear fashion during the 11 days following inoculation; after that, not significant increase in lesion size was observed (Fig.3.6). In general, PG-3 and PG-4 developed lesions faster than PG-T and PG-2.

Table 3.5. Linear regression analysis interaction between isolates and spore age of *L. maculans* on the cv. Westar under a greenhouse environment in Fargo, ND 2012 to 2013.

PG's[1]	Isolate[2]	Intercept	Slope	R-Square	Pr>F[3]
PG-2	BL 1060	51.54	-1.25	0.92	0.03
PG-2	BL 1803	9.85	-2.82	0.90	0.04
PG-3	BL 1062	47.61	-0.88	0.94	0.03
PG-3	BL 1855	25.07	-1.29	0.91	0.02
PG-4	BL 64	50.62	-1.43	0.98	0.01
PG-4	BL 1868	73.15	-2.47	0.91	0.04
PG-T	BL 1905	14.97	-0.37	0.94	0.01
PG-T	BL 1912	-2.61	0.68	0.92	0.04

[1] PG's=Pathogenicity groups of *L. maculans.*
[2] Isolates of *L. maculans.*
[3] Significant at $P < 0.05$.

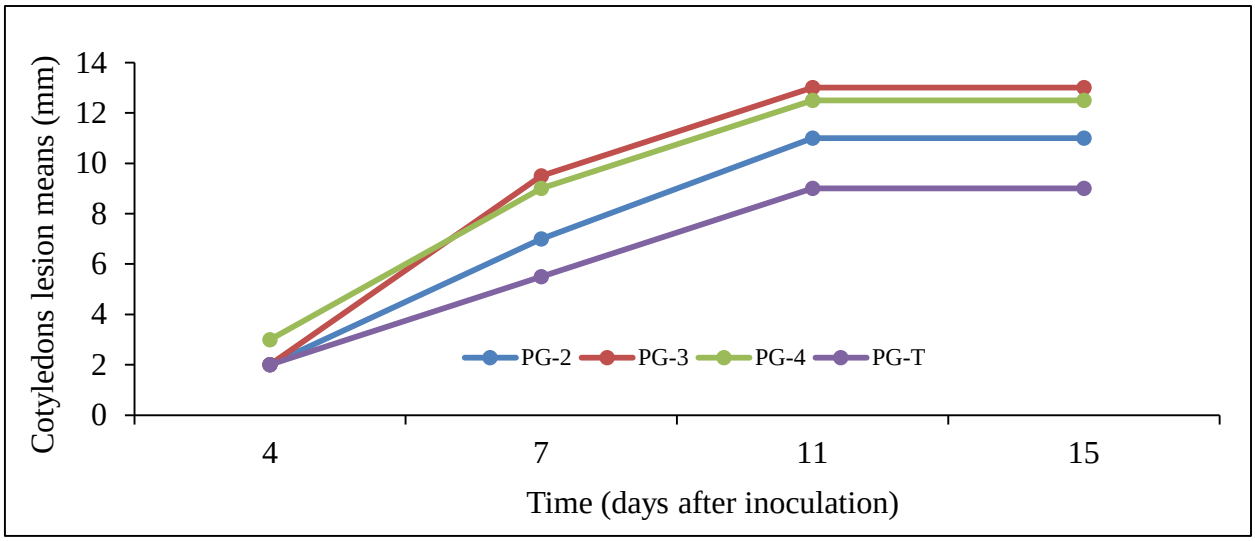

Figure 3.6. Disease development on cotyledons of canola cv. Westar after inoculation with isolates from four pathogenicity groups of *L. maculans* under greenhouse conditions.

Effect of temperature on PG aggressiveness

The combined analysis of variance for AUDPC shows PGs were significantly different ($\alpha < 0.01$) at temperatures of 15 and 30° C, but not at 20 or 25° C (Table 3.6). In contrast, significant differences among isolates nested within PGs were detected for all temperatures evaluated (Figure 3.7). At 15°C, PG-4 produced the largest AUDPC value, 9.71, and therefore it is considered the most aggressive group. At this temperature, PG-2 and PG-3 produced AUDPC

that were not significantly different from each other. Also, the AUDPC produced by PG-T, 2.25, was significantly smaller than the one produced by all other PGs. As incubation temperatures increased, each PGs became more aggressive (Figure 3.8). Interestingly, however, at 30°C, the AUDPC produced by PG-2 was 15% greater ($\alpha < 0.05$) than the ones produced by PG-3 and PG-4 and almost 39% greater than that of PG-T.

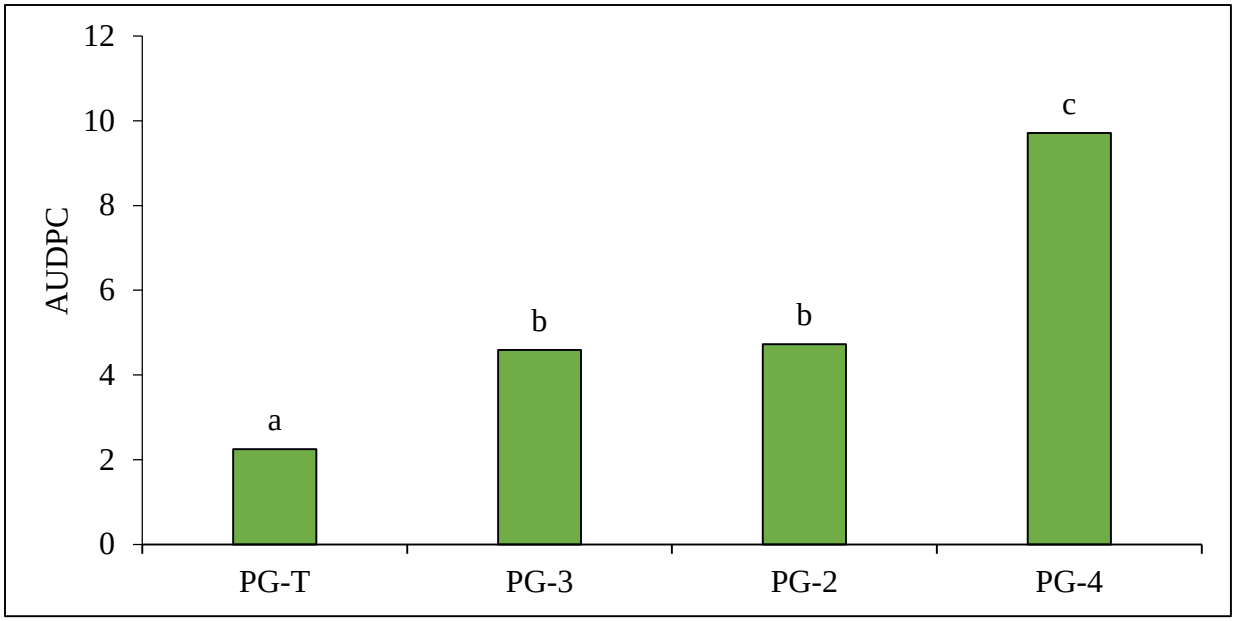

Figure 3.7. Effect of temperature AUDPC values at 15°C on the pathogenicity group's isolates of *L. maculans* aggressiveness under growth chamber conditions measure on seedlings cotyledons of canola cv. Westar in the year 2013. Columns with different letters are different at $P<0.05$.

Table 3.6. Combined mean square values for AUDPC produced by *L. maculans* isolates inoculated on seedlings of canola cv. Westar a and incubated at different temperatures (°C) on growth chamber environmental conditions in trials conducted in 2013.

		Incubation temperatures (^{o} C)											
		15			20			25			30		
Source of Variance[1]	Df[2]	MSE[3]	F-value	Pr<F	MSE	F-value	Pr<F	MSE	F-value	Pr<F	MSE	F-value	Pr<F
PG	3	473.1	390.27	<0.0001	332.76	1.18	0.45	53.32	0.38	0.78	173.71	32.03	0.01
Isolate(PG)	15	37.43	24.54	0.02	437.61	4.47	0.01	179.65	2.58	0.05	397.48	30.54	<0.0001
CV (%)		81.01			89.54			82.25			73.86		

[1] Fixed effects PG= pathogenicity groups; Isolates(PG)= isolates nested within pathogenicity groups

[2]Df=degrees of freedom.

[3]MSE= mean square error

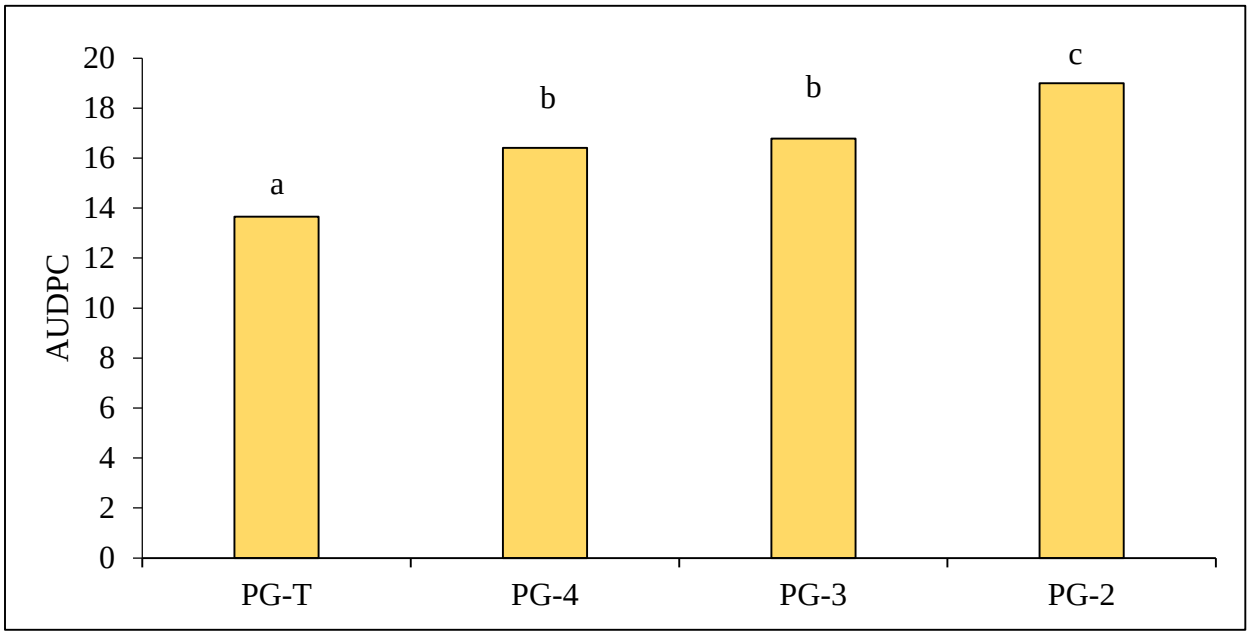

Figure 3.8. Effect of temperature AUDPC values at 30°C on the pathogenicity group's isolates of *L. maculans* aggressiveness under growth chamber conditions measure on seedlings cotyledons of canola cv. Westar in the year 2013. Columns with different letters are different at $P<0.05$.

Table 3.7 shows the effect of isolates nested within PGs for each incubation temperature. In the data analysis the most aggressive isolates at 15°C were BL295 and BL428 from PG-4 11.25 and 11.04 units, respectively. At 20°C, PG-4 isolate BL295 and PG-3 isolate BL1034 were the most aggressive with 20.15 and 17.94 units, respectively. Unlikely, at 25°C the highest AUDPC value was produced by an isolate from PG-T BL803 with 20.19 AUDPC units. However, the isolates most and least aggressive at 30°C were PG-4 isolates BL295 and BL1864 with 23.72 and 7.04 units, respectively. Overall, all isolates within PG's were more aggressive on the cotyledons, when temperature rise between 25 and 30°C. Also we can observe more clearly in the figure 3.9, that the isolates within PG's have higher values for AUDPC at 30°C.

Table 3.7. Comparison of AUDPC for isolates within PG's of *L. maculans* among different temperatures on canola cv. Westar cotyledons under growth chamber environment for 2013.

		AUDPC[3]			
Pathogenicity Groups[1]	Isolate[2]	15	20	25	30
		----------°C----------			
PG-2	BL942	3.88	15.52	14.23	21.69
PG-2	BL951	5.23	12.56	17.58	12.17
PG-2	BL1100	6.88	8.17	12.50	17.19
PG-2	BL1773	2.94	6.88	13.60	21.77
PG-3	BL732	4.42	11.23	17.81	9.92
PG-3	BL935	1.67	2.63	6.77	14.60
PG-3	BL1034	5.54	17.94	15.73	20.15
PG-3	BL1065	6.73	14.65	12.73	9.96
PG-4	BL295	11.25	20.15	17.19	23.73
PG-4	BL428	11.04	15.54	18.00	17.00
PG-4	BL1027	7.06	13.42	17.94	19.38
PG-4	BL1864	9.48	12.19	8.79	7.04
PG-T	BL803	3.42	19.44	20.19	20.21
PG-T	BL1094	0.92	0.92	12.23	21.73
PG-T	BL1205	2.67	12.83	15.85	7.96
PG-T	BL1923	2.00	3.17	13.65	15.77
AUDPC means[4]		5.32	11.70	14.67	16.27
LSD (0.05)†		**0.99**	**8.85**	**7.24**	**2.81**

[1]Pathogenicity groups of *L. maculans.*

[2]Isolates of *L. maculans.*

[3]AUDPC (area under disease progress curve) at four different temperatures.

[4]AUDPC means among isolates.
†Compare means among isolates for each temperature.

Effect of culture medium on colony growth and spore production

Levene's test indicated the variances of the two trials that evaluated the ability of PGs to grow on two artificial media were homogenous ($\alpha<0.01$) and therefore could be analyzed in a combined ANOVA. The combined analysis indicated the interaction between *media* and

isolate(PG) as well as the main effects of *isolate*, *media* and *PG* were statistically significant at $P < 0.02$ for spore production and mycelial growth (Table 3.8).

Colonies produced by PG-4 isolates as a group were larger and significantly different than that of other PGs ($P < 0.05$) when growing on PDA medium (Fig. 3.9) followed closely by isolates from PG-T and PG-3, which were not significantly different from each other. As a group, isolates from PG-2 produced colonies that were almost 22% smaller than those from PG-4 and 9% smaller than those from PG-3 and PG-T growing on the same medium, PDA. When growing on V8, PG-4 as a group still produced the largest colonies ($P<0.05$), but those were not significantly different from the colonies produced by PG-3 (Fig. 3.10). The colonies produced by PG-2 were not the smallest ones but they still were 20% smaller than those from PG-4 and PG-3. The colonies produced by isolates from PG-T were as a group 34% smaller than those from PG-4 and PG-3 and 12% smaller than those produced by isolates from PG-2.

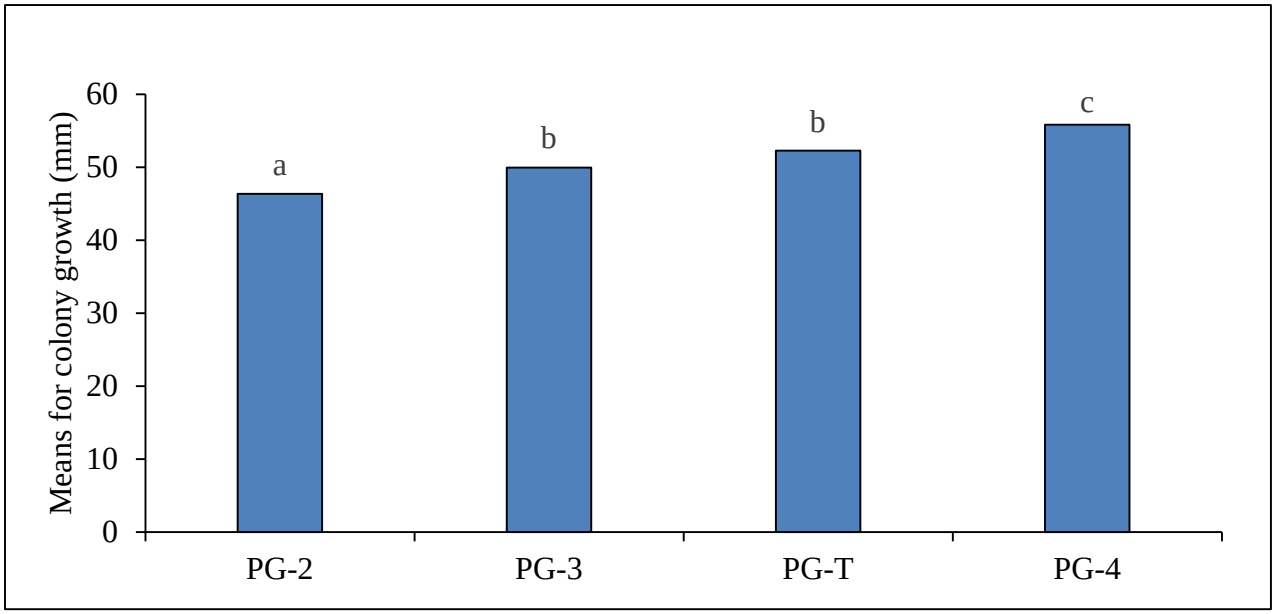

Figure 3.9. Average colony diameters for isolates from four pathogenicity groups of *L. maculans* incubated on potato dextrose agar for 12 days at 22° C. Columns with different letters are statistically different at $P<0.05$.

Table 3.8. Combined mean square values for spore production and colony growth of *L. maculans* isolates on two different artificial media conditions in trials conducted in Fargo, ND 2013 to 2014.

		Spore			Mycelium		
Source of Variation[1]	Df[2]	MSE[3]	F-value	Pr<F	MSE	F-value	Pr<F
PG	3	536989.02	244376	0.001	8729.43	7322.68	<0.0006
Isolate(PG)	64	112094.90	375461	<0.0001	425.23	1109.56	<0.0001
Media	1	17643814.77	6856471	0.01	58758.05	637945	0.02
Media x isolate(PG)	64	89588.20	244992	<0.0001	305.61	791.59	<0.0001
CV (%)		128.15			38.6		

[1]Only fixed effects: Media= potato dextrose agar and V8 juice artificial medium; Isolates(PG)=isolates nested within pathogenicity groups.
[2] Df= degrees of freedom.
[3]MSE= mean square error.

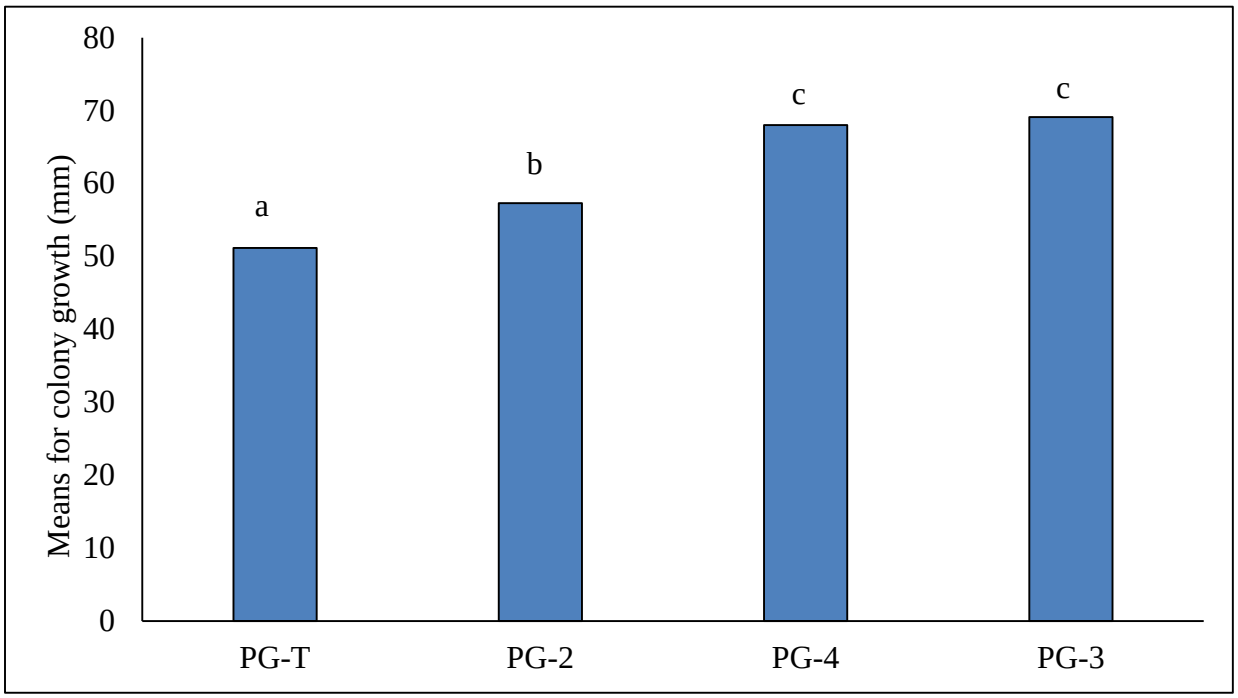

Figure 3.10. Average colony diameters of isolates from four pathogenicity groups of *L. maculans* incubated on V8 culture medium for 12 days at 22º C. Columns with different letters are statistically different at $P<0.05$.

Significant ($P<0.05$) differences in spore production were detected among the pathogenicity groups evaluated (Fig. 311). PG-4 isolates produced on average 44% more spores than PG-3 and almost 2.5 times more spores than PG-2 and PG-T when cultured on PDA medium. Spore production of PG-2 and PG-T isolates were not significantly different from each other ($P<0.05$). When grown on V8 medium, all groups produced significant greater amounts of spores (Fig. 3.12). In general, PG-3 and PG-4 doubled their spore production on V8, while PG-T increased it almost five-fold and PG-2 tripled it. Also, PG-4 and PG-2 remained as the largest and lowest spore producers, respectively, while PG-T increased its spore production dramatically when compared to PDA. As a group, PG-4 produced 51% more spores than PG-2 and PG-3, although its spore production was not different than that of PG-3.

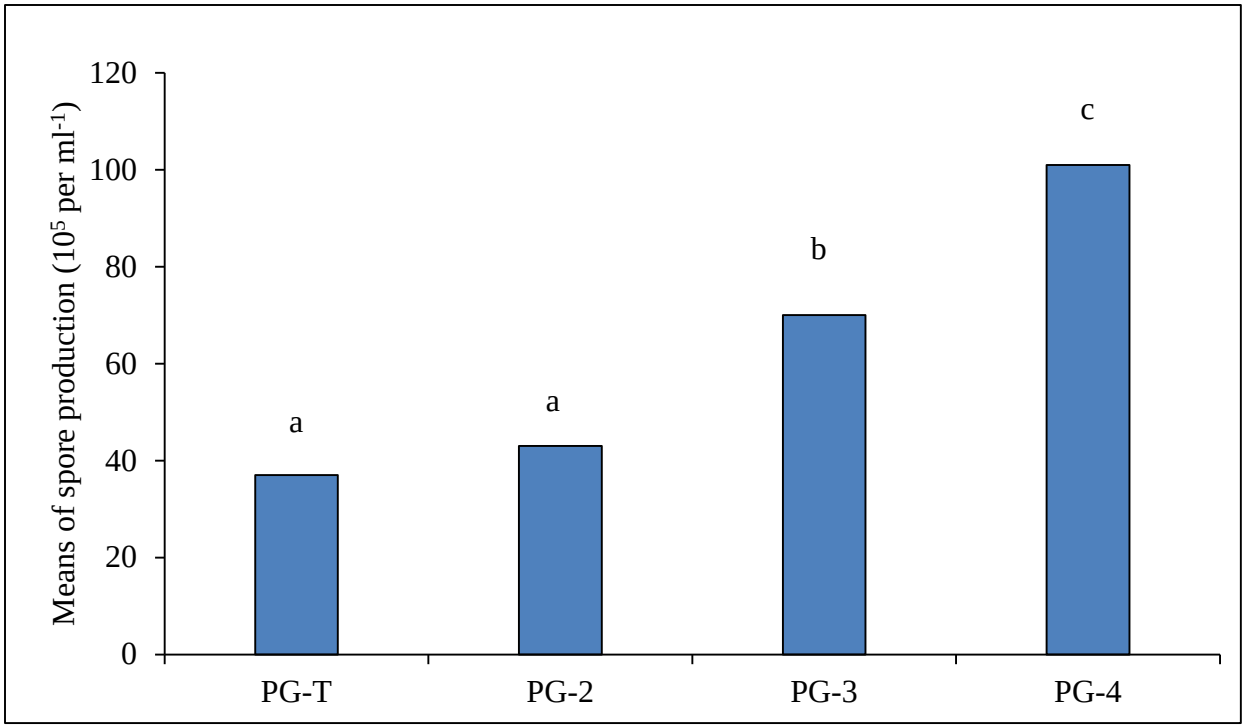

Figure 3.11. Average spore production of four pathogenicity groups of *L. maculans* incubated on PDA culture medium for 12 days at 22° C. Columns with different letters are statistically different at $P<0.05$.

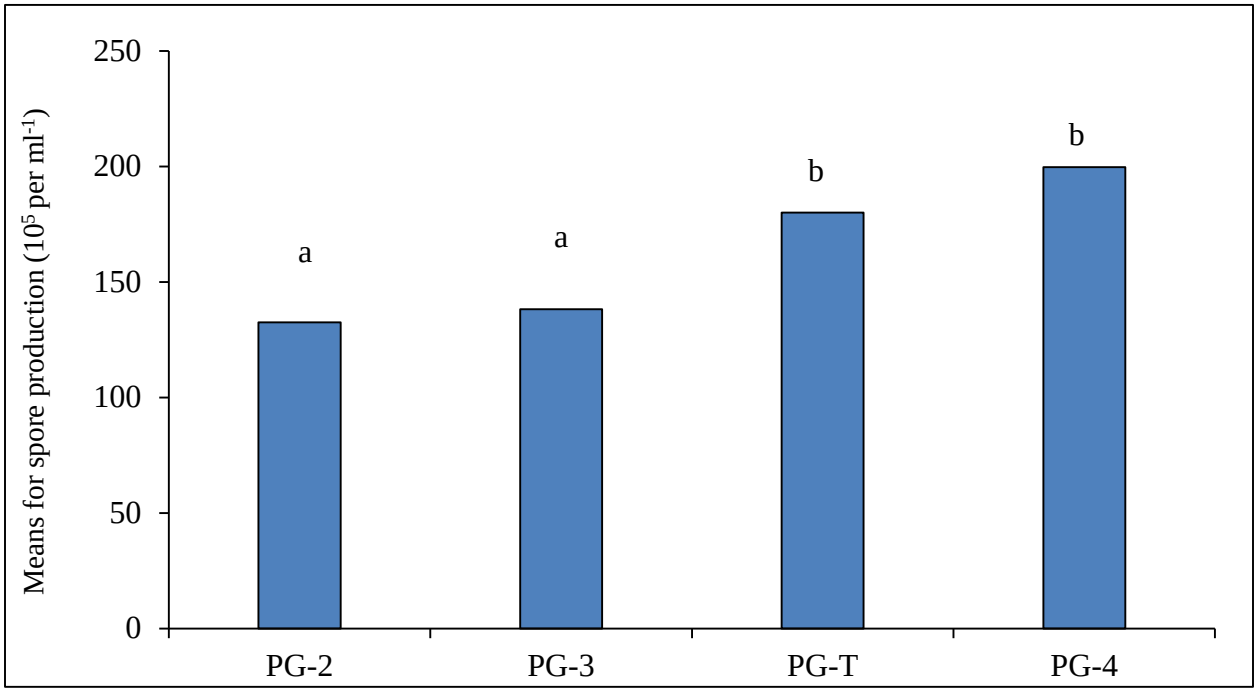

Figure 3.12. Average spore production of four pathogenicity groups of *L. maculans* incubated on V8 culture medium for 12 days at 22° C. Columns with different letters are statistically different at $P<0.05$.

Effect of temperature on colony growth and spore production

Significant differences ($P < 0.001$) were detected at each temperature among PGs as well as among isolates evaluated in each of the trials conducted (Tables 3.9 and 3.10). However, the ranking of PGs did not remain the same as incubation temperatures increased. At 15^{o} C, the average colony diameter for PG-4 isolates was 20 mm after 12 days of incubation, while the average diameter for the other three groups was 13 mm (Fig.3.13). This difference in diameter made PG-4 colonies 2.4 times larger than colonies of the other three groups. At 20^{o}C, the average colony diameter for PG-4 isolates was 40 mm after 12 days of incubation while the average diameter for PG-3 and PG-2 was 35 mm, a 15% difference only.

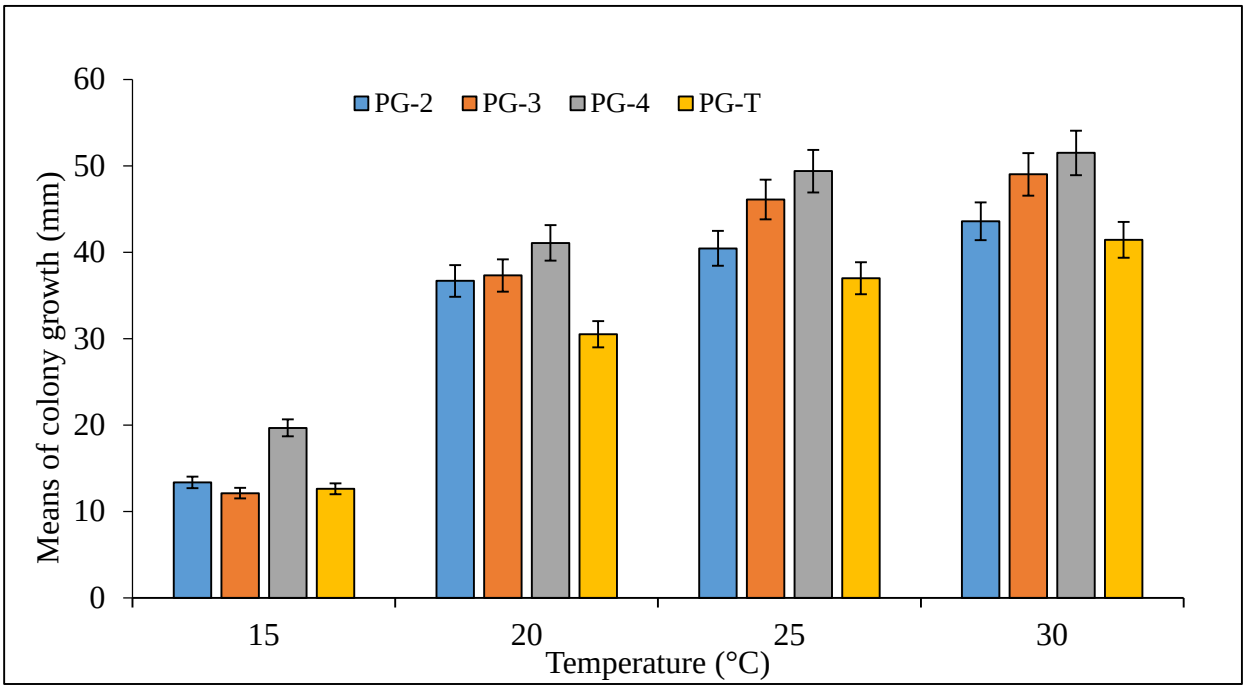

Figure 3.13. Temperature effect on the colony growth of *L. maculans* pathogenicity groups in V8 culture medium. Bars on the columns represent the standard error of the means.

Table 3.9. Combined mean square values for colony growth of *L. maculans* isolates on V8 juice medium at different temperatures (°C) of incubation of *L. maculans* isolates on a laboratory incubator conditions in trials conducted on 2014.

		Incubation Temperatures (°C)											
		15			20			25			30		
Source of Variance[1]	Df[2]	MSE[3]	F-value	Pr>F	MSE	F-value	Pr>F	MSE	F-value	Pr>F	MSE	F-value	Pr>F
PG	3	792	69526	<0.0001	1227	35240	<0.0001	1981	103153	<0.0001	1394	8317	<0.0001
Isolate(PG)	15	276	14393	<0.0001	372	25431	<0.0001	4085	2057	<0.0001	11.2	353	<0.0001
CV (%)			58.17			56.14			68.17			62.9	

[1] Rep= replications; ; PG= pathogenicity groups; Isolates(PG)= isolates nested within pathogenicity groups

[2]Df=degrees of freedom.

[3]MSE= mean square error.

Table 3.10. Combined mean square values for spore production of *L. maculans* isolates on V8 juice medium at different temperatures (°C) of incubation of *L. maculans* isolates on a laboratory incubator conditions in trials conducted on 2014.

		Incubation Temperatures (°C)											
		15			20			25			30		
Source of Variance[1]	Df[2]	MSE[3]	F-value	Pr>F	MSE	F-value	Pr>F	MSE	F-value	Pr>F	MSE	F-value	Pr>F
PG	3	887	56806	<0.0001	688	26429	<0.0001	2223	6804	<0.0001	2364	90806	<0.0001
Isolate(PG)	15	157	2751	<0.0001	1344	24579	<0.0001	683	1667	<0.0001	577	24648	<0.0001
CV (%)			53.75			58.17			31.75			28.54	

[1] Rep= replications; PG= pathogenicity groups; Isolates(PG)= isolates nested within pathogenicity groups

[2]Df=degrees of freedom.

[3]MSE= mean square error.

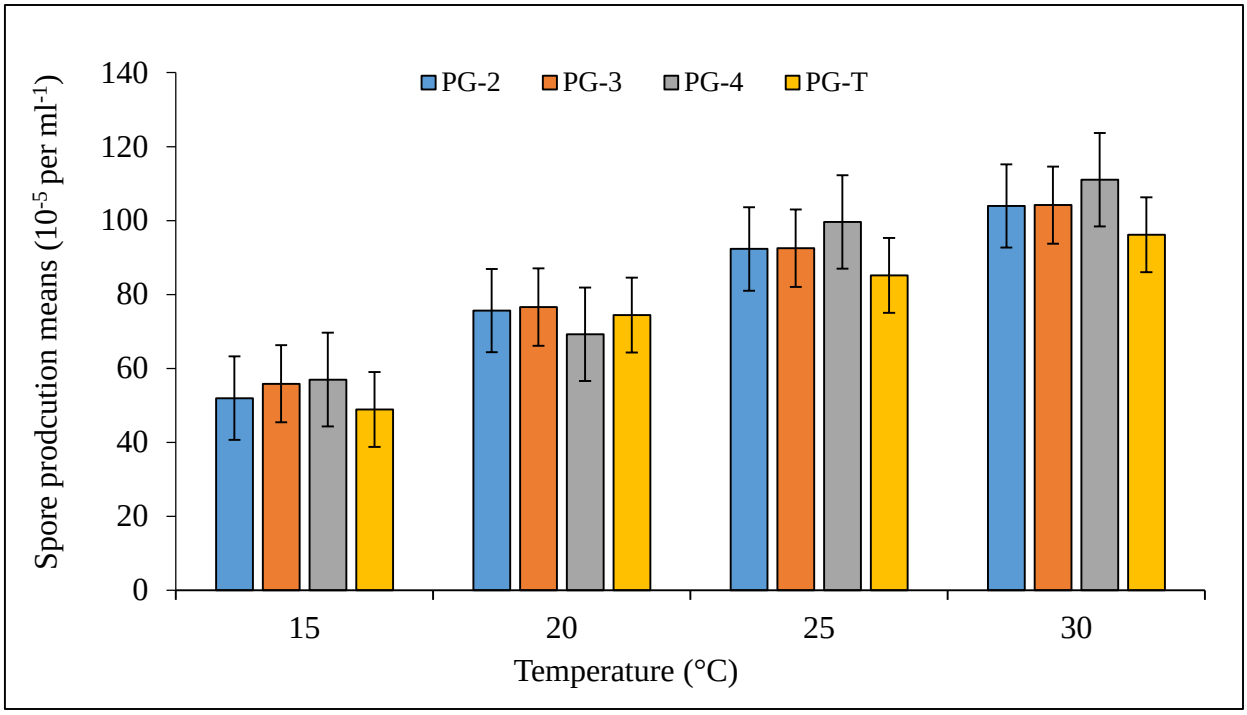

Figure 3.14. Temperature effect on spore production by *L. maculans* pathogenicity groups in V8 culture medium. The bars on the columns represent the standard error.

At 25° C, the average colony diameter for PG-4 isolates was 49 mm after 12 days of incubation while the average diameter for PG-2 was 40 mm, a 22% difference. At 30°C, the difference in colony diameter was 18% greater for PG-4 compared to PG-2 (Fig 3.13). When looking at individual isolates (Table 3.11), isolate BL1864, from PG-4, was the one that produced the largest colonies at all temperatures; at 15°C, it produced colonies with a diameter of 29 mm; at 25°C, the diameter was 50 mm, and at 30°C, it was 52 mm. Meanwhile at 20°C, the PG-3 isolate BL1034 produced the largest colony with a diameter of 45 mm.

Spore production followed a similar trend (Fig. 3.14) to that observed for mycelial growth. At 15° C, the average spore production for PG-4 and PG-3 isolates was 56.5 x 10^5 spores per ml after 12 days of incubation. PG-4 as a group produced almost 10% more spores at this temperature than PG-2. However, when incubated at 20°C, the production of spores by PG-2 was almost 10% greater than that of PG-4. As the incubation temperatures increased to 25 and 30° C,

the differences in spore production became non-significant. When looking at individual isolates (Table 3.12), it was clear that PG-4 isolate BL428 was among the most prolific isolates at all temperatures evaluated. At 15°C, it produced 59.13 x 10^5 spores per ml; at 25°C, it produced 101.63 x 10^5 spores per ml; while at 30°C, it produced 114.38 x 10^5 spores per ml. At 20°C, the PG-T isolate BL1094 was the most prolific with 85 x 10^5 spores per ml.

Table 3.11. Means values for colony growth interaction between pathogenicity groups, isolates and temperature of *L. maculans* on incubator laboratory conditions in Fargo, ND 2014.

Leptosphaeria maculans pathogenicity Groups	Isolate	Temperature (C°)			
		15	20	25	30
		----------diameter (mm)[1]----------			
PG-2	BL942	13	35	36	43
PG-2	BL951	9	32	42	43
PG-2	BL1100	14	38	41	43
PG-2	BL1773	18	41	42	45
PG-3	BL732	13	35	47	50
PG-3	BL935	9	27	45	48
PG-3	BL1034	16	45	47	49
PG-3	BL1065	11	43	46	49
PG-4	BL295	13	41	49	51
PG-4	BL428	18	42	48	51
PG-4	BL1027	19	41	50	51
PG-4	BL1864	29	41	50	52
PG-T	BL803	11	28	36	42
PG-T	BL1094	15	31	37	41
PG-T	BL1205	10	28	38	42
PG-T	BL1923	14	34	37	41
Diameter means[1]		14	36	43	46
LSD (0.05)†		**0.10**	**0.18**	**0.10**	**0.15**

[1]Diameters are means of four observations.

†Compare means among isolates for each temperature.

Table 3.12. Means values for spore production interaction between pathogenicity groups, isolates and temperature of *L. maculans* on laboratory incubator conditions in Fargo, ND 2014.

		Temperature (C°)[2]			
Pathogenicity Groups[1]	Isolate	15	20	25	30
		----------Spore (10^5)----------			
PG-2	BL942	54.56	81.37	97.00	109.13
PG-2	BL951	44.25	59.88	78.76	92.19
PG-2	BL1100	51.25	76.38	94.00	106.00
PG-2	BL1773	57.81	85.00	99.50	108.50
PG-3	BL732	54.37	65.88	84.38	95.94
PG-3	BL935	57.06	87.25	101.38	112.50
PG-3	BL1034	56.44	79.00	92.00	103.25
PG-3	BL1065	55.63	74.19	92.31	105.00
PG-4	BL295	55.19	66.06	98.75	110.25
PG-4	BL428	59.13	79.75	101.63	114.38
PG-4	BL1027	56.81	65.50	97.88	109.38
PG-4	BL1864	56.88	65.63	100.25	110.25
PG-T	BL803	47.38	65.25	78.75	89.88
PG-T	BL1094	49.88	85.00	92.00	100.75
PG-T	BL1205	48.13	68.50	82.63	93.38
PG-T	BL1923	50.13	78.88	87.38	100.75
Spore means[4]		53.43	73.97	92.41	103.84
LSD (0.05)†		**0.16**	**0.15**	**0.44**	**0.11**

[1]Pathogenicity groups of *L. maculans.*

[2]Spore production at four different temperatures.

[3]Spore means among isolates.

†Compare means among isolates for each temperature.

Discussion

In these studies, *Brassica napus* cv. Westar was used to compare the aggressiveness of isolates from different pathogenicity groups of *Leptosphaeria maculans.* Since most commercial canola cultivars planted in North Dakota are either resistant or moderately resistant to isolates belonging to PG-2 but not to isolates from PG-3 or PG-4 (del Rio et al, 2012, Nepal et al., 2014) using these cultivars would not allow us to determine whether the PGs differ in aggressiveness;

in contrast, cv. Westar does not carry any resistance gene. These studies showed that all isolates were capable of infecting the cotyledons, although some differences were noted in the production of chlorotic margins as well as in the production of pycnidia.

Differences in aggressiveness among PG were easier to see when using spores produced by three weeks-old colonies compared to those produced by younger colonies. The age of the colonies is a factor that affects the overall aggressiveness including spore germination and penetration into plant tissues which leads to reduced infection efficiency (Lalancette et al., 1998). It is possible that by the time the colonies are reaching the three weeks of age, the nutrients in the medium are being exhausted and the concentrations of byproducts of fungal growth and secondary metabolites are reaching levels that are toxic to them; after all, all isolates from all PGs had reduced activity when the spores were produced by older colonies. This situation is expected especially if the organism is being cultured in an artificial medium; however, no attempts were made in this study to measure changes in the quality of the incubation medium. On the other hand, spores produced by 10-14 days-old colonies were better than those produced by 7 days-old colonies. This could happen because spores collected this early may not be mature enough. Overall, results of this study suggest that 10-14 day old colonies should be used when producing inoculum that will be used to screen materials for resistance. Spores of these colonies will be at the peak of their aggressiveness and will have the ability to present an effective challenge to the lines being screened.

It was also observed that cotyledons of seedlings inoculated with isolates from all PGs but PG-T were yellowing by the 7th day after inoculation. This could indicate that isolates from PG-2, PG-3 and PG-4 may produce metabolites that trigger senescence of cotyledons that were not present in isolates from PG-T. Spores from 21 days-old colonies produced in general 35%

smaller lesions; some isolates were so affected by colony age that did not even developed lesions on the cotyledons. This was most noticeable on PG-T. The spores from younger colonies (7-14 day) of isolates from PG-4 and PG-3 were more aggressive than those from PG-2 or PG-T.

The disappearance of PG-2 from North Dakota fields may have been due to a combination of factors. In North Dakota isolates from PG-2 were the most prevalent during the 1990s. By mid-2000s, prevalence of PG-2 isolates was declining and by 2009, they had almost disappeared (Nepal et al., 2014). Soon after blackleg epidemics threatened to be wiped out the canola industry of the region, seed companies introduced cultivars with genetic resistance against PG-2. However, not all materials were resistant but "moderately resistant". This means, these cultivars did not have the necessary set of vertical genes to protect the plants from infection. This provided aggressive isolates that already existed in the population the opportunity to overcome the newly incorporated resistance and be able to multiply. Over time, the frequency of this isolates has increased slowly and are becoming the most prevalent. This study provides support to this idea because it is showing that isolates from PG-3 and PG-4 are more aggressive than PG-2. If these PG-3 and PG-4 isolates were produced by mutations they would not necessarily be more aggressive than PG-2, and would likely become more prevalent by attrition because the other isolates would not be able to reproduce (Fundal et al., 2009). Mutations that are more aggressive and are capable of defeating newly introduced resistance genes usually reproduced and become established much faster.

Little information is available about the effect of incubation temperatures on the aggressiveness of PGs of *L. maculans.* The advantage of PG-4 over other PGs at the coolest temperature evaluated, 15^{o}C, is of epidemiological importance because in North Dakota those are the air temperatures that prevail when canola is at the seedling stage. Infections that start at

the seedling stage and before the canola plants reach the 5th leaf growth stage usually cause the greatest economic damage (Marcroft et al., 2005). In this way, PG-4 has the chance to establish itself in fields before other groups.

Temperature affects not only the activity of the pathogen but also that of the host. This study showed that PG-4 isolates have the advantage at 15ºC, but as temperature increase the other PGs catch up. Badawy et al. (2008) conducted experiments to observe the effect of temperature on infection by *L. maculans* on canola cultivars Quinta, Lirasol and Jet Neuf and observed that lesions not only increased in size when the incubation temperature increased from 18°C to 27°C but at high temperatures the plants suffer heat stress and make them more susceptible to blackleg infections. Similar studies were conducted by Huang et al. (2006) who investigated the effects of temperature and leaf wetness duration on phenotypic expression of *Rlm6*-mediated resistance on *B. napus* lines Darmor (lacking *Rlm6*) and DamorMX (carriying *Rlm6*). In this study the canola leaves were inoculated with *L. maculans* ascospores or pycnidiospores (conidia) carrying the effector gene *AvrLm6*. Then the plants were incubated at different temperatures 5 to 25°C. The researches observe that *L. maculans* produced large grey lesions on Darmor at 5–25°C and DarmorMX at 25°C. But 5–20°C small dark spots on DarmorMX. With the increasing temperature/wetness duration, numbers of lesions or spots generally increased. *L. maculans* grew from leaf lesions down leaf petioles to stems on DarmorMX at 25°C but not at 15°C. They conclude that temperature and leaf wetness duration affects the phenotypic expression of Rlm6-mediated resistance in canola leaves.

The *L. maculans* isolates investigated displayed variability in aggressiveness, colony growth and spore production on different artificial media and temperature. Overall, PGs isolates were more aggressive when the colony age was 7-14 days. It was noted as well that isolates were

more aggressive on seedlings growing at 30°C than at cooler temperatures. Also the colony growth of *L. maculans* isolates cultured on PDA medium was slower compare to that on V8 juice medium. Fewer spores were produced when *L. maculans* isolates were cultured on PDA than on V8 medium. Overall the PG's isolates has more spore production and mycelium growth when they are culture on V8 juice medium. This make sense because the V8 juice contains a widely variety of vegetables such as beets, carrots, spinach, and tomatoes, while the PDA agar only contains potato starches and dextrose.PG-4 isolates had more mycelial growth and produced more spores than the others PGs.

Other factor that was studied on this research was the effect of temperature on the mycelium growth and spore production of *L. maculans* isolates. In the study we observed that PG's isolates had less mycelial growth when the temperatures were between 15-20°C and when the temperatures increased, mycelial growth also increased. A similar pattern was observed for spore production; when the isolates were exposed at higher temperature 25-30°C, the spore production increased rapidly, but when the temperature were lower the spore production decreased. Still we observe that PG-4 isolates has higher mycelium growth and spore production when the temperatures were 15, 25 and 30°C compare to other PG's isolates.

In our studies, overall PG-4 isolates are the most aggressive compare to the other PGs isolates. In North Dakota the incidence of blackleg disease has been increasing since 2004. Were PG-2 isolates was the most prevalence, until 2009 that *L. maculans* population shift and the PG-4 become more prevalence (Nepal, 2013). Several factors may had contribute to the prevalence of PG-4 isolates such as susceptibility of most commercially available canola cultivars, shortening of crop rotations, limited fungicides that eventually the pathogen develop resistance (Marino, 2011). But still there is a higher probability that in the future PG-3 and PG-T become more prevalent. I would expect

that these results could be reproducible in plant tissue studies. Meaning that I would expect that the PG-4 isolates will produce more spores than PG-2 isolates.

Effects of temperature on colony growth and spores production can affect the aggressiveness of *L. maculans* pathogenicity groups. Other studies shows that *L. maculans* spores isolates divided on two groups A and B were germinated on water agar at 5-20ºC after 24 hour of incubation (Huang et al., 2001). The results shows that A-group spores germinated at 10-20ºC and some B-group spores had germinated at 5-20ºC. As well they observed that when temperature increases from 5-20ºC, the spore production increase for both A-group an B-group.. But at 15ºC and 20ºC, A-group spores had larger mean diameter (1.8 µm) than B-group spores (1.2 µm).

Related studies on *Leptosphaeria lindquistii* Frezzi (anamorph: *Phoma macdonaldii*), causal agent of sunflower black stem. In this study 17 isolates of *P. macdonaldii* were used to observe the effect of temperature (5, 10, 20, 25, 30 and 35°C) on the asexual spore (pycnidiospores) production and mycelium growth (Roustaee et al., 2001). The results presented that 20-30°C was the optimum temperature for mycelium radial growth. Meanwhile, when the temperature ranges 15-30°C, pycnidiospores production increases. Similarly results we observe in our study, were *L. maculans* isolates across all pathogenicity groups has optimum mycelium radial growth and pycnidiospores production at 25-30°C. This explains that the *L. maculans* isolates are more aggressive when temperature increases, this may be due to toxins production or heat stress that makes the pathogen to produce more spores or mycelium to perpetuate their survival.

To summarize this work, we have demonstrated that PG-4 isolates are more aggressive than PG-2 although PG-3 was closely behind it and PG-T was the less aggressive. Within each

PG, the most aggressive isolates were identified. These isolates were characterized for causing greater levels of disease and because they produced the highest amounts of spores in artificial medium. These isolates can be used from now on to evaluate the resistance of breeding materials to these PG and will contribute in this way to the development of resistant materials against this important disease.

Literature Cited

Ansan-Melayah, D., Balesdent, M. H., Delourme, R., Pilet, M. L., Tanguy, X., Renard, M. and Rouxel, T. 1998. Genes for race specific resistance against blackleg disease in *Brassica napus* L. Plant Breed 117:373-378.

Badawy, H. M. A., Kakau, J., and Hope, H. H. 2008. Temperature and Ageing of Host Tissue Affect the Interactions between Different Oilseed Rape Cultivars and Pathotype Groups of *Leptosphaeria maculans.* J. Phythopathology 134:255-263.

Balesdent, M. H., Barbetti, M. J., Li, H., Sivasithamparam, K., Gout, L., and Rouxel, T. 2005. Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. Phytopathology 95:1061-1071.

Barbetti, M. J., and Khangura, R .K. 1999. Managing blackleg in the disease prone environment of Western Australia. Proceeding of the 10th Int. Rapeseed Congress, 1999, Canberra, Australia.

Biddulph, J. E., Fitt, B. D. L., Gladders, P., Jedryczka, M., West, J. S., and Welham, S. J. 1999. Conditions for infection of oilseed rape leaves by ascospores of UK (A group) and Polish (B group) *Leptosphaeria maculans* (stem canker). Groupe Consultatif International de Recherche Sur le Colza Bulletin 16:82-3.

Chen, Y., and Fernando, W. G. D. 2005. First report of canola blackleg caused by pathogenicity group 4 of *Leptosphaeria maculans* in Manitoba. Plant Dis. 89:339.

del Rio-Mendoza, L. E., Bradley, C. A., Henson, R. A., Endres, G.J., Hanson, B. K., McKay, K., Halvorson, M., Le Gare, P. M., D. G., and Lamey, H. A. 2007. Impact of *Sclerotinia* stem rot on yield of canola. Plant Dis. 91:191-194.

del Rio-Mendoza, L. E., Nepal, A., Bjerke, J. M., Boyles, M., and Peeper, T. 2011. Identification of *Leptosphaeria maculans* pathogenicity group 4 causing blackleg on winter canola in Oklahoma. Plant Dis. 95:614.

del Rio Mendoza, L. E., Nepal, A., and Markell, S. 2012. Outbreak of blackleg in canola in North Dakota is caused by new pathogenicity groups. Online. Plant Health Progress doi: 10.1094/PHP-2012-0410-01-RS.

Elliott C. E., Fox E. M., Jarvis, R., and Howlett, B. J. 2011. Sirodesmin production is mediated through a cross pathway control gene, cpcA, in the blackleg fungus. BMC Microbiology 11:169.

Fernando, W. G. D., Parks, P. S., Tomm, G., and Viau, L. V. 2003. First report of blackleg disease caused by *Leptosphaeria maculans* on canola in Brazil. Plant Dis. 87:314.

Fitt B. L., N. Evans, B. J., Howlett, B., and Cooke, M. 2006. Sustainable strategies for managing Brassica *napus* (oilseed rape) resistance to *Leptosphaeria maculans* (phoma stem canker). Eur. J. Plant Path. 114:3-15.

Fudal, I., Ross, S., Brun, H., Besnard, A. L., Ermel, M., Kuhn, M. L., and Rouxel, T. 2009. Repeat-induced point mutation (RIP) as an alternative mechanism of evolution toward virulence in Leptosphaeria maculans. Mol. Plant Microbe Interact. 22:932-941.

Gladders P, Musa T M. 1980. Observations on the epidemiology of Leptosphaeria maculans stem canker in winter oilseed rape. Plant Pathol. 29:28-37.

Hall, R. 1992. Epidemiology of blackleg of oilseed rape. Can. J. Plant Pathol. 14:46-55.

Hall, R, Peters, R. D., Assabgui, R. A., 1993. Occurrence and impact of blackleg of oilseed rape in Ontario. Can. J. Plant Pathol. 15:305.

Huang Y. J. Toscano-Underwood, C., Fitt, B. D. L., Todd, A. D., West, J. S., Koopmann, B., and Balesdent, M. H. 2001. Effects of temperature on germination and hyphal growth from ascospores of A-group and B-group *Leptosphaeria maculans* (phoma stem canker of oilseed rape). Ann. Appl. Biol. 139:193-207.

Huang, Y. J., Evans, N., Li, Z-Q. Eckert, M., Chevre, A-M., Renard, M., and Fitt, B. D. L. 2006. Temperature and leaf wetness duration affect phenotypic expression of *Rlm6*-mediated resistance to *Leptosphaeria maculans* in *Brassica napus.* New Phytologist. 170:129-141.

Lalancette, N., Ellis, M. A., and Madden, L. V. 1998. Development of an infection efficiency model for *Plasmopara viticola* on American grape based on temperature and duration of leaf wetness. Phytopathology 78:794-800.

Lamey, H. A., and Hershman, D. E. 1993. Canola (*Brassica napus* L.) blackleg caused by *Leptosphaeria maculans* in North Dakota. Plant Dis. 77:1263.

Madden, L.V., Hughes, G., and van den Bosch, F. 2007. The Study of plant disease epidemics. The American Phytopathological Society, APS Press St. Paul, Minnesota.

Marcroft, S. J., Sprague, S. J. Pymer, S. J., Salisbury, P. A., and Howlett, B. J. 2005. *Brassica napus* plants infected by *Leptosphaeria maculans* after the third to fifth leaf growth stage in south-eastern Australia do not develop blackleg stem canker. Eur. J. Plant Pathol. 112:289-292.

Marino, D. 2011. Screening of germplasm accessions from *Brassica* species for resistance against PG3 an PG4 isolates of blackleg. M.S. Thesis. North Dakota State University. Fargo, ND 126 p.

McGee D C. 1977. Blackleg (*Leptosphaeria maculans* (Desm.) Ces. et de Not.) of rapeseed in Victoria: sources of infection and relationships between inoculum, environmental factors and disease severity. Aust. J. Agric. Res. 28:53-62.

McGee D C, Petrie G A. 1978. Variability of *Leptosphaeria maculans* in relation to blackleg of oilseed rape. Phytopathology 68:625-630.

Nepal, A. 2013. Genetic structure of *Leptosphaeria maculans* population in North Dakota and identification of genes associated with resistance to *L. maculans* in *Brassica juncea*. Ph.D. dissertation, North Dakota State University, Fargo, ND.

Nepal, A., Markell, S., Knodel, J., Bradley, C. A., and del Rio-Mendoza, L. E. 2014. Prevalence of blackleg and Pathogenicity groups of *Leptosphaeria maculans* in North Dakota. Plant Dis. 98:328-325.

Pariaud, B., Ravigné, V., Halkett, F., Goyeau, H., Carlier, J., and Lannou, C. 2009. Aggressiveness and its role in the adaptation of plant pathogens. Plant Pathol. 58:409–424.

Roustaee, A., Costes, S., Dechmp-Guillaume, G., and Barrault, G. 2001. Phenotypic variability of *Leptosphaeria lindquistii* (anamorph: *Phoma macdonaldii*), a fungal pathogen of sunflower. *Plant Pathology* 49:227-234.

Schramm H, Hoffmann G H. 1991. Biologische grundlagen zur integrierten bekampfung von Phoma lingam (teleomorph *Leptosphaeria maculans* (Desm.) Ces & de Not.), dem erreger der wurzelhals und stengefäule and winterraps. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 98:586-596.

Toscano-Underwood, C., West, J. S., Fitt, B. D. L., Todd, A. D., and Jedryckzka M. 2001. Development of phoma lesions on oilseed rape leaves inoculated with ascospores of A-group and B-group *Leptosphaeria maculans* (stem canker) at different temperatures and wetness durations. Plant Pathol. 50:28–41.

West, J. S., Kharbanda, P. D., Barbetti, M. J., and Fitt, B. D. L. 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. Plant Pathol. 50:10-27.

Williams, R. H., and Fitt, B. D. L. 1999. Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. Plant Pathol. 48:161-175.

Zhou, Y., Fitt, B. D. L., Welham, S. J., Gladders, P., Sansford, C. E., and West, J. S. 1999. Effects of severity and timing of stem canker (*Leptosphaeria maculans*) symptoms on yield of winter oilseed rape (*Brassica napus*) in the UK. Eur. J. Plant Path. 105:71.

CHAPTER 4. GENERAL CONCLUSIONS

This work allowed the identification and characterization of the most aggressive isolates within each of four pathogenicity groups of *Leptosphaeria maculans,* the causal agent of blackleg on canola. It also provided evidence that indicates PG-4 and PG-3 are more aggressive than PG-2, information that helps explain why strains of PG-2 have disappeared almost completely from canola fields in North Dakota and why these two strains are now the most prevalent. It was noted that aggressiveness was negatively affected by colony age with higher severities being produced by spores from 10-14 days-old colonies. Results from these studies also verified that independently of pathogenicity groups, this pathogen can cause high levels of disease when incubated between 15 and 30^{o}C. In general, greatest severities were observed at warmer temperatures, but one interesting fact was that PG-4 is statistically more aggressive at 15^{o}C than PG-2. This gives an advantage to PG-4 in field conditions. For inoculum production it was evident that V8 was a better medium than PDA. As well the higher temperatures (25-30°C) affect positively the spore production and the radial growth of the *L. maculans* isolates. Giving us an idea that the optimum temperatures for the pathogen to developed caused higher disease severity are between 25-30°C.

With this research we can developed resistance materials to produced resistance canola varieties to these PG's, taking in consideration the most aggressive isolates. However more studies should be done to makes us understand more the pathogen-host interactions. For example, evaluate these PG's isolates on the field to observe differences in aggressiveness. Further characterization of these PG isolates should be done by toxins production. Also molecular assessments could help us to identify the gene that confers aggressiveness.

APPENDIX A. EFFECT OF TEMPERATURE ON DISEASE PROGRESS

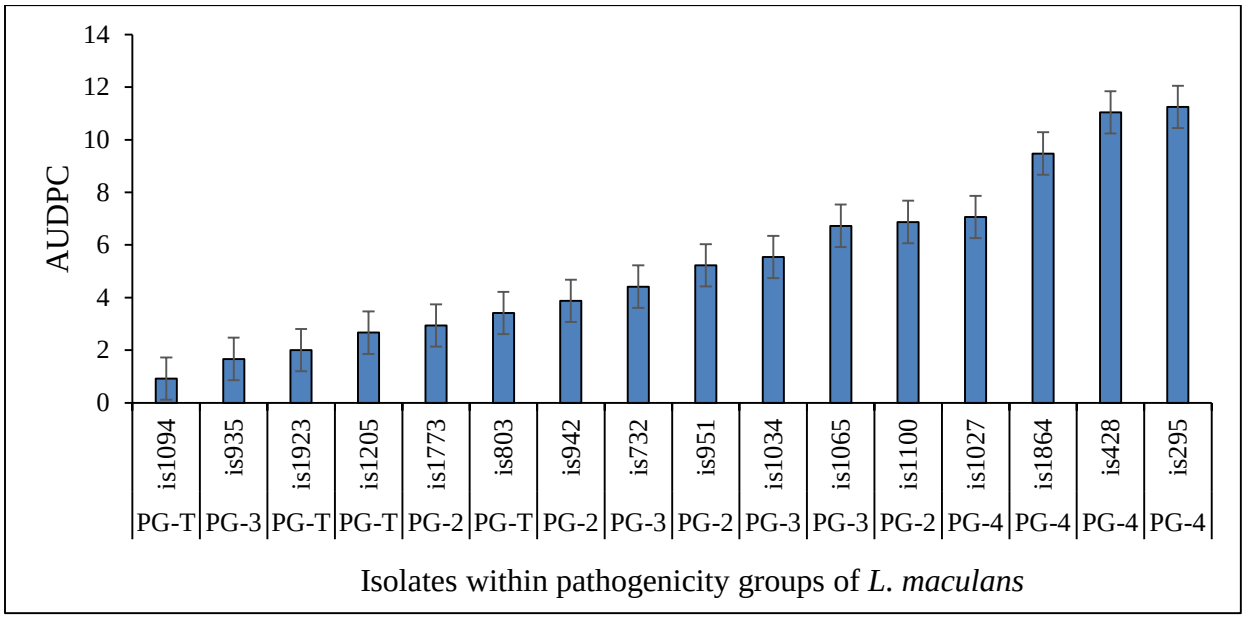

Figure A.1. Effect of temperature AUDPC values at 15°C on the isolates within pathogenicity groups of *L. maculans* aggressiveness under growth chamber conditions measure on seedlings cotyledons of canola cv. 'Westar' in the year 2013. The bars on the columns represent the standard error.

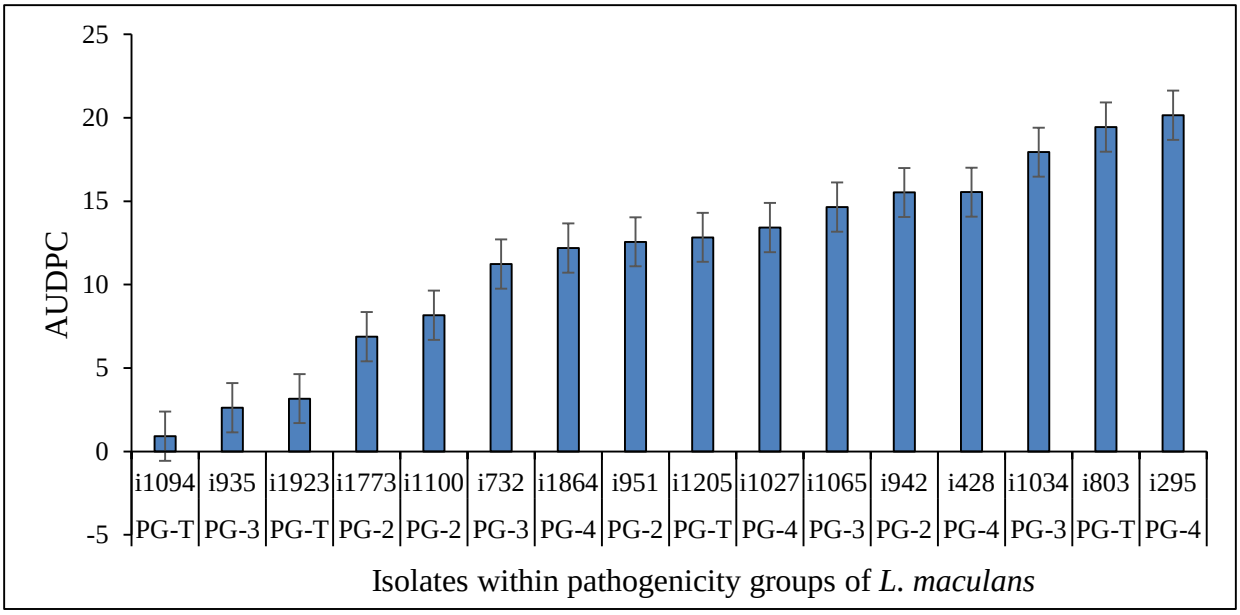

Figure A.2. Effect of temperature AUDPC values at 20°C on the isolates within pathogenicity groups of *L. maculans* aggressiveness under growth chamber conditions measure on seedlings cotyledons of canola cv. 'Westar' in the year 2013. The bars on the columns represent the standard error.

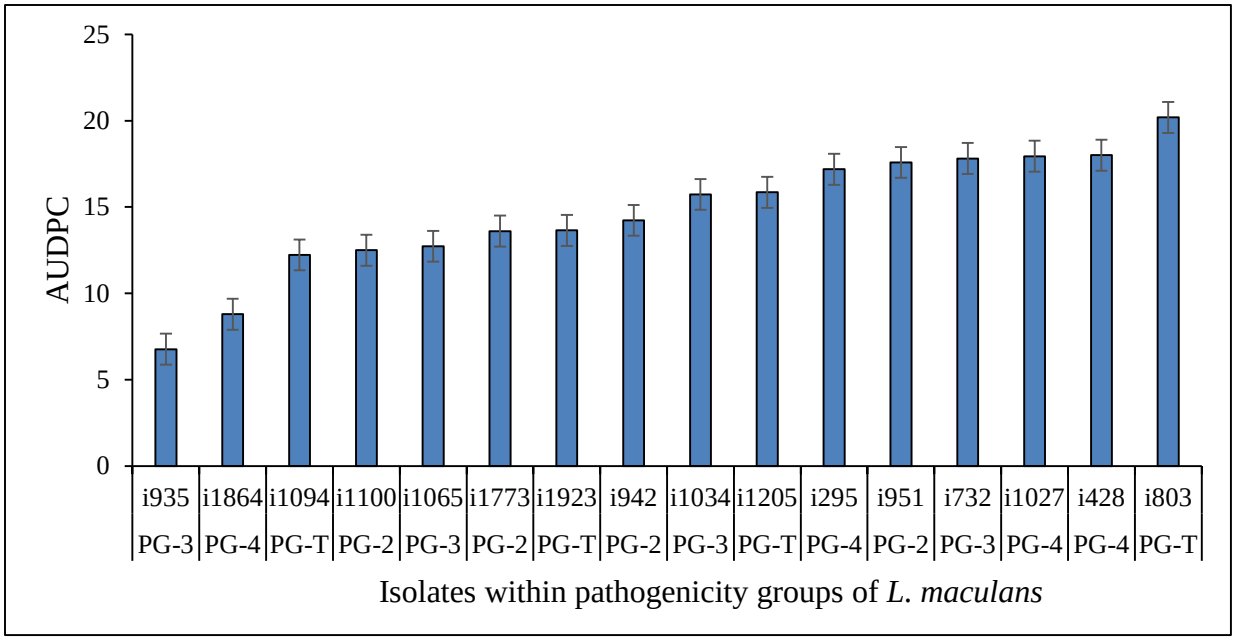

Figure A.3. Effect of temperature AUDPC values at 25°C on the isolates within pathogenicity groups of *L. maculans* aggressiveness under growth chamber conditions measure on seedlings cotyledons of canola cv. 'Westar' in the year 2013. The bars on the columns represent the standard error.

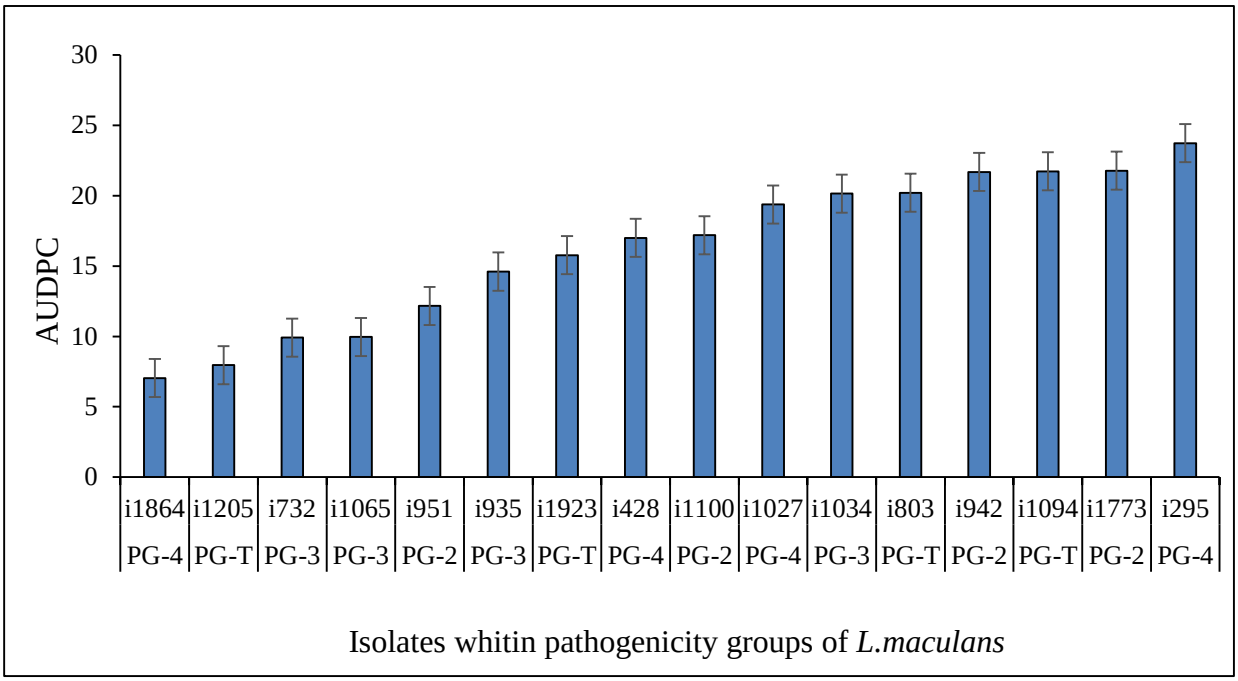

Figure A.4. Effect of temperature AUDPC values at 30°C on the isolates within pathogenicity groups of *L. maculans* aggressiveness under growth chamber conditions measure on seedlings cotyledons of canola cv. 'Westar' in the year 2013. The bars on the columns represent the standard error.

APPENDIX B. AUDPC MEANS VALUES FOR PGS, ISOLATES AND COLONY AGE INTERACTION

Table B.1 Means for area under the disease progress curve (AUDPC) values for the interaction between pathogenicity groups, isolates and colony age of *L. maculans* on canola cv. Westar under a greenhouse environment.

		Colony age (days)[3]				
PG's[1]	Isolate[2]	7	10	14	21	Isolate means[4]
		----------AUDPC----------				
PG-2	BL 1017	29.23	26.90	32.14	30.04	29.58
PG-2	BL 1060	42.33	37.67	37.31	24.08	35.35
PG-2	BL 1071	23.36	26.72	37.14	18.43	26.41
PG-2	BL 1075	28.86	29.27	36.59	12.87	26.90
PG-2	BL 1083	41.90	39.90	33.19	34.70	37.42
PG-2	BL 1084	20.22	30.69	29.85	23.58	26.09
PG-2	BL 1091	34.22	33.67	33.57	29.43	32.72
PG-2	BL 1100	32.96	53.45	43.04	34.21	40.91
PG-2	BL 1154	30.09	39.67	26.79	29.29	31.46
PG-2	BL 1219	24.40	24.19	28.39	13.45	22.60
PG-2	BL 1253	36.72	36.09	38.51	21.92	33.31
PG-2	BL 1773	39.92	49.84	51.55	31.34	43.16
PG-2	BL 1791	33.71	34.17	37.40	26.82	33.02
PG-2	BL 483	26.93	28.47	34.28	20.10	27.45
PG-2	BL 927	24.31	38.01	30.61	21.41	28.59
PG-2	BL 942	42.39	48.89	36.77	34.69	40.68
PG-2	BL 951	38.83	41.40	49.81	26.76	39.20
PG-2	BL 991	38.98	22.74	31.28	21.98	28.74
PG-3	BL 1034	44.05	40.57	56.50	46.54	46.92
PG-3	BL 1062	42.83	37.89	33.76	29.42	35.97
PG-3	BL 1065	43.15	37.20	46.45	49.38	44.04
PG-3	BL 1097	50.46	35.82	38.25	32.33	39.21
PG-3	BL 1104	38.35	37.62	34.15	43.36	38.37
PG-3	BL 1106	45.89	38.63	39.98	32.40	39.23
PG-3	BL 1790	42.98	39.55	41.12	33.50	39.29
PG-3	BL 1814	45.84	43.32	45.41	31.44	41.50
PG-3	BL 1855	37.25	35.09	43.86	52.79	42.25
PG-3	BL 1884	33.03	38.79	28.29	23.33	30.86
PG-3	BL 526	32.68	45.28	26.49	28.79	33.31
PG-3	BL 732	48.89	38.27	40.95	45.59	43.42

Table B.1 Means for area under the disease progress curve (AUDPC) values for the interaction between pathogenicity groups, isolates and colony age of *L. maculans* on canola cv. Westar under a greenhouse environment (Continued).

		Colony age (days)[3]				
PG's[1]	Isolate[2]	7	10	14	21	Isolate means[4]
		----------AUDPC----------				
PG-3	BL 935	48.75	38.00	43.91	37.59	42.06
PG-4	BL 1027	61.67	64.71	46.75	53.54	56.67
PG-4	BL 1161	36.69	36.17	21.71	26.10	30.17
PG-4	BL 1803	24.63	40.00	56.38	65.25	46.56
PG-4	BL 1864	58.02	40.23	60.10	57.21	53.89
PG-4	BL 1868	58.46	49.58	31.50	24.50	41.01
PG-4	BL 1893	32.21	41.88	39.69	40.29	38.52
PG-4	BL 1935	12.96	12.46	28.58	18.33	18.08
PG-4	BL 295	52.44	46.67	56.50	36.92	48.13
PG-4	BL 303	47.52	33.69	36.23	24.46	35.47
PG-4	BL 428	59.21	35.42	41.50	52.98	47.28
PG-4	BL 44	59.24	40.46	43.26	26.93	42.47
PG-4	BL 532	38.35	43.00	45.10	44.58	42.76
PG-4	BL 6	20.13	13.33	27.79	17.96	19.80
PG-4	BL 64	39.29	37.71	30.92	20.04	31.99
PG-4	BL 674	26.35	42.16	54.50	41.57	41.15
PG-4	BL 85	50.33	36.13	39.58	56.92	45.74
PG-T	BL 1094	12.54	23.92	31.41	29.59	24.36
PG-T	BL 1193	6.34	12.27	11.47	6.33	9.10
PG-T	BL 1205	19.30	32.71	26.00	26.73	26.18
PG-T	BL 1217	13.73	20.00	13.13	14.88	15.43
PG-T	BL 1240	3.58	3.25	10.69	6.29	5.95
PG-T	BL 1242	8.80	12.15	4.04	4.75	7.43
PG-T	BL 1797	13.39	17.99	11.71	8.42	12.88
PG-T	BL 1808	4.46	5.00	5.08	5.35	4.97
PG-T	BL 1820	19.09	28.16	26.85	26.33	25.11
PG-T	BL 1886	8.29	10.70	8.91	5.83	8.43
PG-T	BL 1895	8.63	5.71	8.63	5.71	7.17
PG-T	BL 19	11.00	5.33	7.43	8.17	7.98
PG-T	BL 1905	12.25	11.89	9.04	7.42	10.15
PG-T	BL 1912	2.71	4.67	5.15	12.52	6.26
PG-T	BL 1923	30.89	36.43	26.63	18.59	28.13
PG-T	BL 790	22.24	15.72	25.75	13.11	19.21

Table B.1 Means for area under the disease progress curve (AUDPC) values for the interaction between pathogenicity groups, isolates and colony age of *L. maculans* on canola cv. Westar under a greenhouse environment (Continued).

		Colony age (days)[3]				
PG's[1]	Isolate[2]	7	10	14	21	Isolate means[4]
		----------AUDPC----------				
PG-T	BL 803	31.68	37.59	18.79	20.71	27.19
PG-T	BL 952	7.42	4.45	7.82	8.06	6.94
AUDPC means[5]		31.66	31.57	32.12	27.42	30.69
LSD (0.05)† 2.88						
LSD (0.05)†† 8.66						
LSD (0.05)††† 11.76						

[1]PG's=Pathogenicity groups of *L. maculans.*

[2]Isolates of *L. maculans.*

[3]Spore age of *L. maculans* isolates at 7, 10, 14, and 21 days of being culture on V8 media.

[4]Isolates means of AUDPC within isolates.

[5]AUDPC means among isolates for each spore age.

† Compare means within isolates.

†† Compare means among isolates.

†††Compare means across isolates by spore age.

APPENDIX C. MEANS VALUES FOR COLONY DIAMETER AND SPORE PRODCUTION AT DIFFERENT TEMEPRATURES

Table C.1. Mean values for colony diameters of *L. maculans* isolates belonging to different pathogenicity groups after 12 days of incubation on artificial culture media.

		Culture media[3]		
PG's[1]	Isolate[2]	V8	PDA	Isolates means[4]
		----------Colony diameter (mm)----------		
PG-2	BL 483	29	35	32
PG-2	BL 927	45	38	42
PG-2	BL 942	56	37	47
PG-2	BL951	47	35	41
PG-2	BL 991	59	34	46
PG-2	BL 1017	40	32	36
PG-2	BL 1060	57	37	47
PG-2	BL 1071	55	40	48
PG-2	BL 1075	45	37	41
PG-2	BL 1083	53	28	41
PG-2	BL 1084	44	36	40
PG-2	BL 1091	59	44	52
PG-2	BL 1100	58	34	46
PG-2	BL 1154	59	38	48
PG-2	BL 1219	50	39	44
PG-2	BL 1253	55	38	46
PG-2	BL 1773	54	32	43
PG-2	BL 1791	51	42	47
PG-3	BL 526	62	54	58
PG-3	BL 732	46	42	44
PG-3	BL 935	48	42	45
PG-3	BL 1034	59	46	53
PG-3	BL 1062	51	45	48
PG-3	BL 1065	53	36	44
PG-3	BL 1097	51	38	44
PG-3	BL 1104	53	32	42
PG-3	BL 1106	61	44	52
PG-3	BL 1790	52	37	45
PG-3	BL 1814	46	45	45
PG-3	BL 1855	57	50	53
PG-3	BL 1884	52	44	48

Table C.1. Mean values for colony diameters of *L. maculans* isolates belonging to different pathogenicity groups after 12 days of incubation on artificial culture media (continued).

PG's[1]	Isolate[2]	Culture media[3] V8	PDA	Isolates means[4]
		----------Colony diameter (mm)----------		
PG-4	BL 6	52	48	50
PG-4	BL 44	57	44	51
PG-4	BL 64	60	49	55
PG-4	BL 85	52	40	46
PG-4	BL 295	60	46	53
PG-4	BL 303	65	52	58
PG-4	BL 428	61	50	55
PG-4	BL 532	59	47	53
PG-4	BL 674	56	51	53
PG-4	BL 1027	60	49	54
PG-4	BL 1161	58	50	54
PG-4	BL 1803	50	44	47
PG-4	BL 1864	73	51	62
PG-4	BL 1868	60	51	56
PG-4	BL 1893	67	50	59
PG-4	BL 1935	62	41	52
PG-T	BL 19	54	46	50
PG-T	BL 790	47	41	44
PG-T	BL 803	54	51	53
PG-T	BL 952	50	45	47
PG-T	BL 1094	55	45	50
PG-T	BL 1193	54	49	52
PG-T	BL 1205	52	43	47
PG-T	BL 1217	49	47	48
PG-T	BL 1240	58	49	54
PG-T	BL 1242	58	50	54
PG-T	BL 1797	55	46	51
PG-T	BL 1808	58	49	54
PG-T	BL 1820	51	42	47
PG-T	BL 1886	53	44	48
PG-T	BL 1895	52	46	49
PG-T	BL 1905	50	45	48
PG-T	BL 1912	58	49	53
PG-T	BL 1923	54	48	51
Means[5]		55	44	49
LSD (0.05)† 1.67				

Table C.1. Mean values for colony diameters of *L. maculans* isolates belonging to different pathogenicity groups after 12 days of incubation on artificial culture media (continued).
LSD (0.05)†† 4.57
LSD (0.05)†††5.04

[1]PG's=Pathogenicity groups of *L. maculans.*

[2]Isolates of *L. maculans.*

[3]Culture media, V8 = V8; PDA= potato dextrose agar. Values are means of four readings.

[4]Isolates means of AUDPC within isolates.

[5]AUDPC means among isolates for each culture media.
† Compare means within isolates of the same PG.
†† Compare means among isolates of all PG.
†††Compare means of isolates across culture media.

Table C.2. Means values for spore production interaction between pathogenicity groups, isolates and artificial culture medium of *L. maculans* on laboratory conditions in Fargo, ND 2013 to 2014.

		Incubation media[3]		
PG's[1]	Isolate[2]	V8	PDA	Isolates means[4]
		--Spore production(10^5ml^{-1})--		
PG-2	BL 483	150	28	89
PG-2	BL 927	96	31	63
PG-2	BL 942	166	35	100
PG-2	BL951	148	33	90
PG-2	BL 991	410	55	233
PG-2	BL 1017	149	58	103
PG-2	BL 1060	242	33	137
PG-2	BL 1071	167	22	95
PG-2	BL 1075	186	52	119
PG-2	BL 1083	309	22	166
PG-2	BL 1084	152	38	95
PG-2	BL 1091	231	69	150
PG-2	BL 1100	332	40	186
PG-2	BL 1154	235	53	144
PG-2	BL 1219	227	40	133
PG-2	BL 1253	216	55	136
PG-2	BL 1773	213	72	143
PG-2	BL 1791	367	29	198
PG-3	BL 526	365	92	229

Table C.2. Means values for spore production interaction between pathogenicity groups, isolates and artificial culture medium of *L. maculans* on laboratory conditions in Fargo, ND 2013 to 2014 (continued).

PG's[1]	Isolate[2]	Incubation media[3] V8	PDA	Isolates means[4]
		--Spore production(10^5ml^{-1})--		
PG-3	BL 732	239	78	158
PG-3	BL 935	121	78	100
PG-3	BL 1034	209	70	139
PG-3	BL 1062	188	81	135
PG-3	BL 1065	294	44	169
PG-3	BL 1097	136	45	90
PG-3	BL 1104	244	53	149
PG-3	BL 1106	181	65	123
PG-3	BL 1790	150	75	113
PG-3	BL 1814	141	77	109
PG-3	BL 1855	230	88	159
PG-3	BL 1884	211	89	150
PG-4	BL 6	65	42	53
PG-4	BL 44	421	142	282
PG-4	BL 64	427	133	280
PG-4	BL 85	203	107	155
PG-4	BL 295	392	119	255
PG-4	BL 303	65	52	59
PG-4	BL 428	573	161	367
PG-4	BL 532	102	47	75
PG-4	BL 674	281	105	193
PG-4	BL 1027	283	101	192
PG-4	BL 1161	68	54	61
PG-4	BL 1803	334	105	220
PG-4	BL 1864	494	155	324
PG-4	BL 1868	529	148	339
PG-4	BL 1893	258	112	185
PG-4	BL 1935	481	132	306
PG-T	BL 19	157	23	90
PG-T	BL 790	332	38	185
PG-T	BL 803	114	18	66
PG-T	BL 952	383	31	207
PG-T	BL 1094	236	38	137

Table C.2. Means values for spore production interaction between pathogenicity groups, isolates and artificial culture medium of *L. maculans* on laboratory conditions in Fargo, ND 2013 to 2014 (continued).

PG's[1]	Isolate[2]	Incubation media[3] V8	PDA	Isolates means[4]
		--Spore production(10^5ml^{-1})--		
PG-T	BL 1193	332	42	187
PG-T	BL 1205	325	29	177
PG-T	BL 1217	297	32	165
PG-T	BL 1240	507	45	276
PG-T	BL 1242	402	45	224
PG-T	BL 1797	372	43	207
PG-T	BL 1808	543	45	294
PG-T	BL 1820	232	23	128
PG-T	BL 1886	340	37	189
PG-T	BL 1895	167	39	103
PG-T	BL 1905	437	56	246
PG-T	BL 1912	544	64	304
PG-T	BL 1923	529	42	286
Spore means[4]		280	64	172
LSD (0.05)† 17.81				
LSD (0.05)†† 49.93				
LSD (0.05)††† 79.71				

[1]PG's=Pathogenicity groups of *L. maculans.*

[2]Isolates of *L. maculans.*

[3]Culture media, V8 = V8; PDA= potato dextrose agar. Values are means of four readings.

[4]Isolates means of AUDPC within isolates.

[5]AUDPC means among isolates for each culture media.

† Compare means within isolates.

†† Compare means among isolates.

†††Compare means across isolates by culture media

Table D.1. Summary of traits evaluated on *L. maculans*.

		Means values of traits evaluted on L.maculans isolates																			
PGs[1]	Isolates[2]	Aggressive[3]				Aggressive temp[4]				Colony5		Spore[6]		Colony temp[7]				Spore temp[8]			
		7	10	14	21	15	20	25	30	PDA	V8	PDA	V8	15	20	25	30	15	20	25	30
		--------days--------				---------^{0}C---------								--------^{0}C---------				----------^{0}C----------			
PG-2	BL 1100	33	53	43	34	7	8	13	18	34	58	40	332	14	38	41	43	51	76	94	106
	BL 951	39	41	50	27	5	13	18	12	35	47	33	148	9	32	42	43	44	59	78	92
PG-3	BL 1034	44	41	57	47	6	18	16	20	46	59	70	209	16	45	47	49	56	79	92	103
	BL 1065	43	37	46	49	7	15	13	10	36	56	44	294	11	43	46	48	55	74	92	105
PG-4	BL 295	52	47	57	37	11	20	17	24	46	60	119	392	13	41	49	51	55	76	98	110
	BL 428	59	35	42	53	11	16	18	17	50	61	161	573	18	42	48	52	59	79	101	114
PG-T	BL 1923	31	36	27	19	3	13	16	8	43	52	42	529	14	34	37	41	50	78	87	100
	BL 1205	32	38	19	21	2	3	14	16	48	54	29	325	10	28	38	40	48	68	82	93

[1] PGs= Pathogenicity groups of L. maculans.

[2] Isolates of L. maculans.

[3] Aggressiveness of L. maculans isolates on seedlings at 7, 10,14, and 21 days old colonies.

[4] Agressiveness of L. maculans isolates on seedlings at different temperatures 15, 20, 25, and 30°C.

[5] Isolates colony growth on different culture media.

[6] Isolates spore production on different culture media

[7] Isolates colony growth at different temperature different temperatures 15, 20, 25, and 30°C.

[8] Isolates spore production at different temperature different temperatures 15, 20, 25, and 30°C.