

**Cross-pathogenicity of
Phytophthora infestans genotypes
and impact on their interaction with potato and tomato**

BY

HASSNA A. ALKHER

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**Department of Plant Science
University of Manitoba
Winnipeg, Manitoba**

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ABSTRACT

Late blight is one of the most devastating diseases on potato and tomato, which are important crops worldwide. The disease is caused by an oomycete, a fungus-like organism, *Phytophthora infestans*. The disease can result in a loss of an entire yield within a few days if left unchecked. Historically, late blight caused the famous Irish famine in the 1840s and continues to affect potato and tomato. The most recent pandemics of late blight that occurred in North America were caused by new *P. infestans* genotypes known as US-22, US-23 and US-24, and resulted in significant yield losses of both potato and tomato. In this study, isolates from infected potato and tomato plants collected in Canadian provinces during 2012 were identified, and some of the work was performed in collaboration with other scientists across Canada. Our results concluded a great shift of *P. infestans* populations within a single year, from US-24 and US-8 in 2011 to US-23 in 2012. Isolates of some populations showed increased insensitivity to mefenoxam as well as recombination. We confirmed an independent segregation of either *Gpi*, mating type or RG57 loci in a number of the recombinant isolates including CA12. Cross-pathogenicity of different isolates belonging to different genotypes were evaluated on susceptible and moderately resistant cultivars of potato and tomato. Isolates of genotypes US-8, US-11, US-22, US-23 and US-24 were assessed for their pathogenicity on both tomato and potato leaves as well as on tubers, at different days post inoculation (dpi). This allowed us to investigate their pathogenicity on both the original and alternative hosts of each tested isolate. Area under disease progress curve (AUDPC) was measured at 3, 5, and 7 dpi on plant leaves. We revealed higher infection caused by most US-24 isolates in all tested cultivars. Observation of more blight in Russet Burbank, the susceptible potato cultivar was recorded, followed by Sun Rise and Ultra Sweet, the susceptible and the moderately resistant

tomato cultivars, respectively. Among all tested cultivars, Kennebec, the moderately resistant potato cultivar showed less disease. Interestingly, tubers of both potato cultivars showed contrasting pathogenicity results when infected with the same isolates, with larger diseased areas on tuber slices of Kennebec, the moderately resistant cultivar and smaller ones on Russet Burbank, the susceptible cultivar. Among all of the tested genotypes, US-24 was the most aggressive on potato and tomato. However, variation was observed in the aggressiveness of US-24 isolates, where Pi19.1.11 and Pi4.3.11 were highly aggressive, whereas, Pi688, Pi166 were weakly aggressive on both hosts. Isolates of US-22 and US-23 genotypes, which were obtained from tomato, were more pathogenic to tomato. In an attempt to gather more information on virulence factors that determine *P. infestans* effects on potato and tomato, putative virulence genes (effector genes) were assessed for their role in the aggressiveness of the tested *P. infestans* isolates on potato and tomato. The expression of five RXLR putative effector genes of *P. infestans* was evaluated using qRT-PCR during the interaction of six US-24 isolates, varying in their aggressiveness with potato and tomato susceptible and moderately resistant cultivars. Gene expression was assessed 3 and 6 dpi. Among the tested candidate effector genes, DL119 was the only gene detected during the interaction between *P. infestans* and both plant species. A higher expression of DL119 was recorded in all tested isolates during their interaction with tomato cultivars compared to the potato ones. The expression of DL119 carried the same pattern in most of the treatments, with up-regulation at 3dpi then decrease at 6dpi. DL119 could play a role in pathogenicity since it showed higher expression during interaction of the isolates with tomato cultivars. The gene expression results are in line with our cross-pathogenicity result, which indicates greater aggressiveness of isolates of US-24 on tomato more than potato.

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ABBREVIATIONS

RB: Russet Burbank

K: Kennebec

SR: Sun Rise

US: Ultra Sweet

CRB: Clarified rye agar B

MS: Metalaxyl-Sensitive

MI: Metalaxyl-Intermediately Sensitive

MR: Metalaxyl-Resistant

Dpi: days post inoculation

Gpi: Glucose phosphate isomerase allozyme patterns

RFLP: Restriction Fragment Length Polymorphism fingerprinting

AUDPC: Area Under Disease Progress Curve

RXLR: effector family, which contain four amino acids; Arginine, any amino acid, Leucine, and Arginine

piEF2: *P. infestans* elongation factor

FOREWORD

The thesis was written in manuscript style as described in the Department of Plant Science thesis guideline (“A Guide to Thesis Preparation for Graduate Students in The Department of Plant Science” 2018). The thesis started with general introduction and literature review of the three manuscripts, which are the main part of the thesis. Each manuscript comprises of abstract, introduction, materials and methods, results and ending with discussion. The last part of the thesis is the general conclusion, which gives a general summary of the entire study and finding. List of references cited is included.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) are two of the most important food crops from the Solanaceae family (Friedman and McDonald 1997). Canada potato production reached 105.9 million hundredweight (4.8 million tonnes) in 2017_(Statistics Canada 2017a).

Late blight disease is a highly destructive on potato and tomato. The disease caused by the oomycete *Phytophthora infestans* (Mont) de Barry, which under favourable conditions can spread to large areas within a few days, to cause infection and yield loss. *P. infestans* infects all plant parts including tubers, stems, leaves and even fruits. Yield losses in potato and tomato are estimated in the billions of dollar annually (Haverkort et al. 2009). Disease management, including the use of resistant cultivars, crop rotation, cull removal, storage management, fungicides, scouting and forecasting techniques, is not sufficient to control late blight. The major reason for failure of these disease control strategies is the ability of the pathogen to overcome the control methods, including adaptation to fungicides, overcoming the resistance genes, and suppressing the defense mechanisms.

Variations in late blight severity and *P. infestans* resistance to fungicide coincide with an increase of genetic composition and diversity of the pathogen (Daayf and Platt 2000a; Kalischuk et al. 2012). *P. infestans* genotypes such as US-8 and US-11, which had dominated the populations of this pathogen for several years in the 1990s in North America, have been

replaced by new genotypes, with unique characteristics. This change is making it more difficult to mitigate the effects of late blight using the disease management strategies available. Therefore, monitoring and characterizing *P. infestans* populations is important for the disease management planning each year in different areas of the world.

In order to generate better solutions to manage late blight, it is also necessary to understand the interaction between the two main hosts, potato and tomato, with the new *P. infestans* strains. Previous studies had shown that during the 1990s US-8 was the most common *P. infestans* genotype on potato, whereas US-11 had been particularly troublesome on tomato (Gavino et al. 2000; Daayf and Platt 2003). Although, both genotypes can infect and cause disease on both plant species. The newer strains of *P. infestans*, mainly US-22, US-23, US-24 and US-25 can infect either one or both plant species, and with different aggressiveness levels. The aggressiveness of each genotype on its original and alternative host is poorly documented. Two of the questions that we address here are. Do the most recent genotypes cause the same disease severity levels on potato and tomato? Are they more aggressive than the old, previously dominating genotypes? The answers to these questions could guide us to provide better management plans for the protection of these important economic crops.

Like many pathogens, the oomycete *Phytophthora* spp. secretes a large number of effector proteins that modulate the host's physiological processes and facilitate colonization. The genome sequence of *P. infestans* has been published (Haas et al. 2009) and reveals the presence of great complex families of effector genes, which encode secreted proteins that are implicated in virulence (Kamoun 2006). Some of the *P. infestans* effectors have been characterized (Allen et al. 2004; Bos et al. 2006, 2009), whereas, others were only identified as secreted RxLR effector peptides. The level of effector expression may be affected by the infection stage

(biotrophic or necrotrophic), plant host, and aggressiveness of *P. infestans* isolates. Several studies concluded the manipulation by virulent isolates of different pathogens of the plant defense using effectors (Toruño et al. 2016; Wang and Wang 2018). For instance, almost 50% of *Arabidopsis* genes are reprogrammed after infection by *P. syringae* (Lewis et al. 2015). Transcriptional reprogramming of many maize genes were caused by the induction of *U. maydis* effectors (Doehlemann et al. 2008). This evidence showed that the ultimate target of the effectors, which are secreted by virulence strains, is to induce susceptibility genes and breakdown the defense mechanism of host plant. However, the effector expression level is also dependent on how resistant or susceptible the plant cultivars are to a particular pathogen as well as to different races or genotypes of the pathogens. Variation of different plant cultivars and lines responses to plant pathogen was well observed and documented in several plant-microbe interaction studies, including *Fusarium oxysporum* and cucumber (Cafri et al. 2005), *Fusarium graminearum* and grains (wheat, barley, rye) (Akinsanmi et al. 2006), *Verticillium dahliae* and potato (Alkher et al., 2009) and *P. infestans* and potato (Lebreton et al. 1999; Daayf and Platt 2002; Michalska et al. 2016).

In this project, we characterized *P. infestans* genotypes that were isolated from potato and tomato infected plants in Canada in 2012. We investigated the dramatic shift of *P. infestans* populations where the US-24 genotype was completely replaced by the US-23 genotype within one year (from 2011 to 2012). We studied potato and tomato response to the infection by different *P. infestans* isolates, using a cross-pathogenicity approach. The majority of the US-24 isolates showed an advanced level of aggressiveness on potato and tomato compared to isolates of genotypes US-8, US-11, US-23 and US-22. Isolates of genotype US-24, with different levels of aggressiveness, were selected to investigate the change in the expression of

selected putative effector genes during the pathogen-host interaction. Under the experimental design used, one gene out of five showed an expression during the interaction. We also revealed higher expression of the effector gene DL119 at 3 dpi and lesser expression at 6 dpi in most of the treatments. Based on this finding, functional analyses of this gene should be completed to confirm the role of DL119 in *P. infestans* virulence on potato and tomato.

1.2 Literature Review

1.2.1 Potato (*Solanum tuberosum* L.)

Potato (*Solanum tuberosum* L.) is considered one of the important food crop in the solanaceae family (Friedman and McDonald 1997), which also contains about 3000 species, including tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotian tabacum* L.), petunia (*Petunia exserta*), eggplant (*Solanum melongena* L.) and garden pepper (*Capsicum annuum* L.) (Bonierbale et al. 1988). Potato is also the fourth most important food crop after maize, wheat, and rice, with an annual production of more than 364 million tonnes (Statistics Canada 2017a)

About 8000 years ago, potatoes were domesticated in the highlands of South America (Hawkes 1978). The Canary Islands were the first place of recorded cultivated potato outside South America (Ríos et al. 2007), after that, the plant was brought to Europe by the Spanish explorers in 1573. Potato cultivation then expanded throughout Europe and worldwide (Romans 2005).

According to (FAOSTAT 2016), Canada is the 18th largest potato producer in the world with production 4,324 million metric tonnes. Potatoes are produced commercially in all provinces in Canada. For the most part, production is concentrated in Prince Edward Island (PEI), Manitoba (MB) and Alberta (AB). According to Statistic Canada 2017, 22.3% of total production is in PEI, followed by MB with a production of about 21%, then AB with 19.4%.

Over 55% of potatoes grown in Canada are processed, mostly into French fries. About 10-15 % is used for chips and other processed potato products.

With the high production of potatoes and the varieties available, potato is a crop that is infected by several damaging diseases such as bacterial wilt (*Pseudomonas solanacearum*), blackleg and soft rot (*Erwinia* spp.), ring rot (*Clavibacter michiganensis* ssp. *sepedonicus*), common scab (*Streptomyces scabies*), Potato Leaf roll Virus (PLRV), Potato Viruses Y and A (PVY and PVA), and mosaics (PVX, PVS, PVM, also PVY and PVA). However, the most widespread diseases are the ones caused by fungi and fungi-like organisms including powdery scab (*Spongospora subterranea*), wart (*Synchytrium endobioticum*), late blight (*Phytophthora infestans*), pink rot (*Phytophthora erythroseptica*), powdery mildew (*Erysiphe cichoracearum*), early blight (*Alternaria solani*), Fusarium dry rot and wilt (*Fusarium* spp.), and Verticillium wilt (*Verticillium albo-atrum*, *V. dahliae*) (Wale 2008). These diseases cause significant losses to potato production.

1.2.2 Tomato (*Solanum lycopersicum* L.)

The tomato, *Solanum lycopersicum* L. is a member of the Solanaceae (nightshade) family, native to South America and originally cultivated in the Andes Mountains of Peru, Bolivia and Ecuador. The crop was introduced to Europe in the 16th century. Tomato is used in different ways, fresh and processed for sauce, ketchup, and crushed diced tomato (Nonnecke 2013).

Tomato is the world's largest vegetable crop with a production estimated at 130 million tons, 88 million of which are in the fresh market and 42 million processed. Tomato largest producers are: China, EU, India, US and Turkey (eurofresh 2016). Together, they account for 70% of the world's tomato production. It is considered a perennial crop, however, in Canada it is grown as an

annual crop because of the cold weather. According to a 2017 survey, tomato yield increased about 7.4% to 78.2 tonnes per hectare (Statistics Canada 2017b). Ontario is the largest tomato producer in Canada, accounting for more than 98% of the total production of Canada.

Several diseases caused by fungi, bacteria and viruses are known to affect tomato production and quality. They include; early blight (*Alternaria solani*), late blight (*Phytophthora infestans*), gray leaf spot (*Stemphylium solani*, *S. lycopersici*), septoria leaf spot (*Septoria lycopersici*) and Verticillium wilt.

1.2.3 Late blight

Late blight is the most devastating disease on potato and tomato. The causal agent of the disease is the oomycete *Phytophthora infestans* (Mont.) de Bary, which caused the most famous epidemics in Europe in the 1840s, and led to the potato famine in Ireland (Bourke 1969; Kalischuk et al. 2012). The consequence of this famine was the death of around one million people and the displacement of another million (Rose et al. 2002; Nowicki et al. 2012). *P. infestans* is continuing to spread worldwide, causing a global threat to potato and tomato crops (Ingram and Williams 1991)

The disease impact on public and crop production is obvious. The public concern has increased in the epidemic years, especially farmers who depend on potato and tomato on their income. In unprotected potato or tomato fields, greenhouse or plastic-cover cultures, the infection by late blight can devastate the crops within 7-10 days. The economic impacts are mainly presented as yield reduction, lower quality of tuber or fruits and increased costs resulting from the use of fungicides (Nowicki et al. 2012). Depending on the disease severity and plant varieties, potato yield loss was estimated at \$6.7 billion/year (Anonymous. 1997; Chand 2009; Haas et al. 2009).

The recent late blight epidemics with new genotypes discovered since 2009 and 2010, total potato losses in the United States reached to \$3.5 billion, whereas in tomato was estimated at \$112 million. Adding to the costs above, fungicide applications needed to control late blight will increase if fungicide tolerance is an issue. According to (Gautam et al. 2013) there will be about a 25% increase in fungicide applications in 2010-2065 compared to 10 years ago.

1.2.4 Disease cycle of *Phytophthora infestans*

P. infestans is a hemibiotrophic pathogen, with a cycle of two phases. An early phase of infection, in which the pathogen requires living host cells, is followed by an extensive necrosis of host tissue resulting in colonization and sporulation (Fry and Goodwin 1997).

P. infestans is usually dispersed aerially one to several miles from the overwintering site to living potato or tomato foliage, via sporangia, which can further produce multiple zoospores per unit. Sporulation vs. germination of sporangia is a temperature-driven process, where direct germination can occur directly at temperatures above 18°C by forming invasive hyphae. Indirect sporulation occurs at temperatures below 15°C by releasing motile zoospores (Tyler 2002). The infection typically begins when a sporangiophore is formed on the infected leaves and zoosporangia or sporangia are released. Zoospores and sporangia penetrate the leaf surface either through stomata or directly through the epidermal cell wall (Lee and Rose 2010; Fawke et al. 2015). The germ tube of the zoospore is able to differentiate into an appressorium and a penetration peg forms, which aids the passage of the pathogen to the host cell wall (Mizubuti and Fry 1998). Occasionally, intracellular haustorial structures are formed. After three or four days, secondary sporulation and infection of leaves may occur to initiate once more the disease cycle (Figure 1.1).

P. infestans is a heterothallic organism with two known mating types, A1 and A2 (Brasier 1992). These mating types are physiologically differentiated by their ability to produce hormones, which are required to induce gametangia under normal conditions. When two *P. infestans* isolates are cultured together, antheridia and oogonia are formed, which may fuse to form oospores. In contrast with zoospores, oospores are large, thick-walled and dark in colour (Figure 1.1). They are distinct from the asexual spores by the long survival period on plant debris or in soil outside the living host plant (Judelson 1997). Sexual reproduction may result into increased virulence, gene transfer and genetic variation in *P. infestans*, which in turn may create new challenges for control management of late blight.

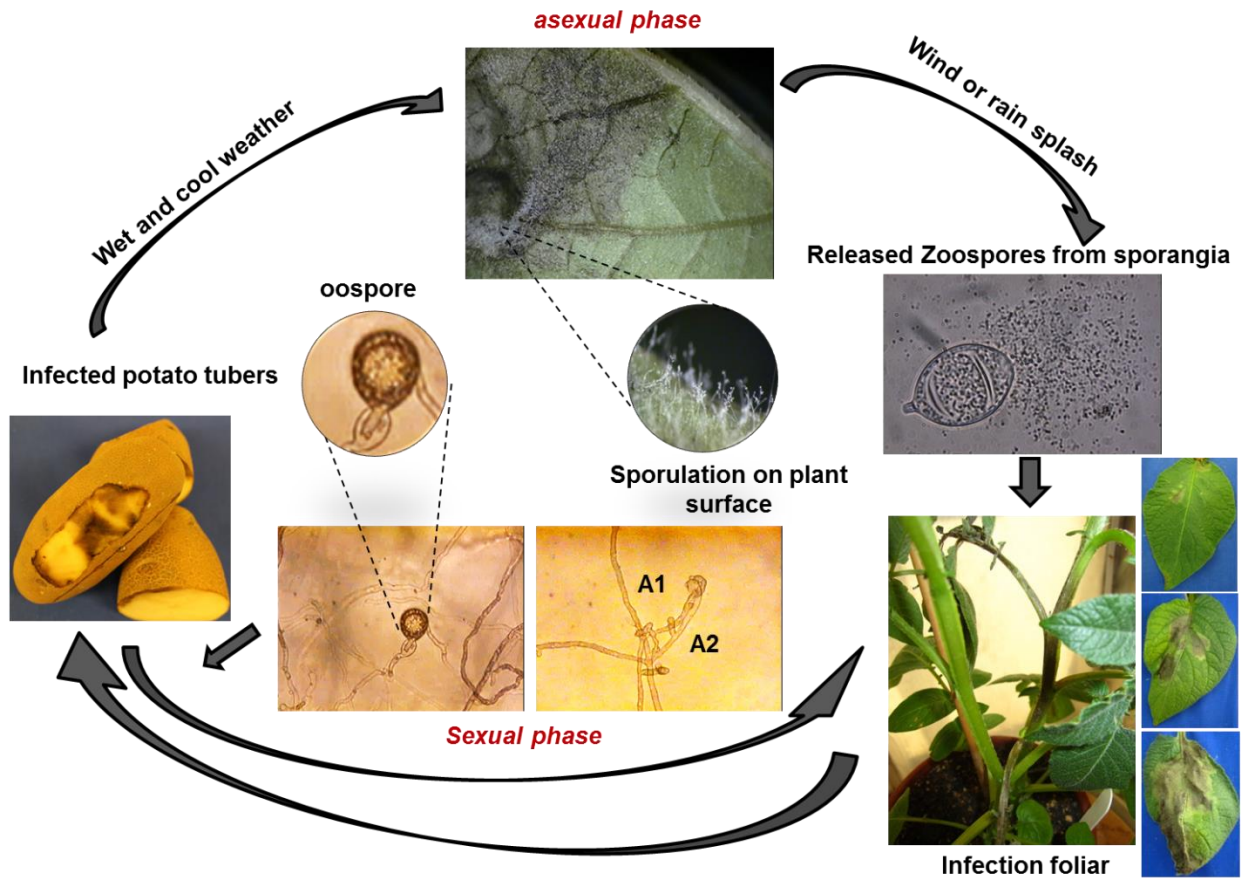


Figure 1.1 Disease cycle of late blight disease on potato and tomato.

1.2.5 Disease control strategies

P. infestans is an airborne pathogen, with its primary inoculum being the sporangia, which can disperse with winds over long distances, across fields, several states, or even intercontinentally, or through infected potato seed tubers or tomato transplants (Fry et al. 2013). The dispersal mechanisms, the frequent changes in genotypes, and the adaptation to fungicides, are factors that continuously make late blight control more challenging.

With the increasing diversity of *P. infestans* populations, no single method is sufficient for late blight management. Integrated disease management strategies include several methods. Cultural control consists of several methods that reduce the initial inoculum of late blight, including selection of well drained soils for a healthy potato crop, field scouting, removal of volunteer plants, and of the haulms when late blight severity reaches >80%, to avoid passing the infection to the tubers. When the disease reaches the tubers, cull potatoes should be frozen, crushed, fed to livestock or buried deeper than 2 feet of soil. Healthy tubers are always recommended for seeding because even the varieties with some level of tolerance sooner or later develop some blight.

Several forecasting systems are available, i.e. Blitecast and Tomcast (Krause et al. 1975; Gleason et al. 1995) and are used to adjust the intensity of scouting as well as the frequency of fungicide applications. Recommended fungicides are needed from mid-to late-season when plants are actively growing. The application of fungicides should be repeated following a schedule to eventually replace fungicides that have been washed away. The effectiveness of fungicides in controlling late blight depends on the concentration (dosage) and frequency of the applications. Applying fungicides at the low-labeled dosages more frequently is apparently more effective than

fewer applications at a higher dosage (Kirk et al. 2001). More frequent applications ensure better foliar coverage of the plants.

Several fungicides have been used to control late blight. The phenylamide fungicide metalaxyl (Ridomil) was the most effective fungicide used during the 1970s in Europe, but insensitive isolates were detected, resulting in a reduction in the effectiveness of metalaxyl against *P. infestans* (Davidse et al. 1981; Dowley and O'sullivan 1981; Haas et al. 2009; Hu et al. 2012). Mefenoxam, a formulation consisting of metalaxyl's active enantiomer was effective but not on metalaxyl-insensitive lineages such as US-6, US-7, and US-8 (Daayf and Platt 2000a; Fry et al. 2013). Mefenoxam has been the most commonly used fungicide active ingredient. It blocks RNA transcription by inhibiting RNA polymerase 1 activity (Pomerantz et al. 2014). This fungicide is still applied, but *P. infestans* isolates resistant to it can be found (Miranda et al. 2010). Successful management of late blight relies on the integration of several management strategies, including the ones cited above.

1.2.6 Reasons for late blight epidemics

1.2.6.1 *P. infestans* populations

Late blight can cause serious epidemics, as proven by the Ireland famine in 1840s. Most of the evidence of *P. infestans* migration refers to central Mexico as the initial source (Grünwald and Flier 2005). Migration out of Mexico occurred in different stages, starting with a first migration from Mexico to the United States in 1842-1843, then from the United States to Europe around 1845 (Fry and Goodwin 1997). This migration was thought to have occurred during the shipment of infected potato tubers with the genotype US-1 clonal lineage, which was also considered the

initial genotype that migrated from Mexico to the USA (Fry et al. 1993). European potato cultivars grown during the 1840s were highly susceptible to late blight. Infected potato seeds transported worldwide resulted in late blight epidemics in potato production areas. Genotypic diversity analysis of the *P. infestans* strains collected from several countries in Europe, Asia, South America, USA and Canada concluded that *P. infestans* is an organism that propagates asexually from the only A1 mating type. The populations collected were genetically quite similar, dominated by a single clonal lineage, US-1. This lineage is commonly referred to as the ‘old’ population. US-1 population comprises isolates of the A1 mating type, fungicide (metalaxyl)-sensitive, with mitochondrial DNA (mtDNA) haplotype Ib, allozymes *Gpi* 86/100, and characteristic RFLP pattern 1010101011001101000110011 with the RG57 probe (Goodwin et al. 1994a, 1994b). In the latter part of the 20th century, the global population structure of *P. infestans* began to change due to migration. This change was observed when a report of the A2 mating type was issued in Switzerland (Hohl and Iselin 1984), followed by other reports of the same mating type. This indicated that the second migration -via seed potatoes- is probably the cause of the new populations in many locations worldwide (McLeod et al. 2001). The US-1 strain that had dominated non-Mexican populations worldwide prior to 1980s was displaced by a diverse population containing both A1 (US-6, US-11) and A2 (US-7, US-8) (Spielman et al. 1991; Drenth et al. 1993; Fry and Goodwin 1997). Severe late blight on potato and tomato was observed during the epidemic years, because these new genotypes were highly aggressive and metalaxyl-resistant (Goodwin et al. 1998; Daayf and Platt 2000a; Cooke and Lees 2004; Cooke et al. 2006).

The US-8 genotype was responsible for most late blight outbreaks in potato in North America and the dominant genotype in most potato growing areas worldwide until a new outbreak of late blight hit tomato and potato in 2009 (Kalischuk et al. 2012; Nowicki et al. 2012). Reports indicated that

the significant losses were caused by the distribution of infected tomato transplants throughout home garden retail centers in the USA. Severe outbreaks in tomato fields were observed in Ontario in the same year (Fry et al. 2013).

Of the new strains, US-22 was the most widespread on tomato transplants and it was the major genotype distributed in eastern North America during 2009-2010. Compared to potatoes, US-22 was more aggressive on tomatoes, and the predominant strain in Ontario and northeastern United states (Seidl Johnson et al. 2015).

In addition to US-22, two other new genotypes, US-23 and US-24, were also detected in Western North America. The cool wet weather that occurred throughout most of the Prairie Provinces during 2009 -2010 including Alberta and Manitoba was ideal for infection and severe outbreaks of late blight (Kalischuk et al. 2012; Peters et al. 2014; Alkher et al. 2015).

Late blight caused by new genotypes is continuing to spread to other continents including Europe and Asia. In Europe, a new highly aggressive A2 lineage of *P. infestans*, known as 13-A2 (also termed 'blue13') was reported at first in the Netherlands in 2004 and it was the major threat to potato production in France (Cooke et al. 2006, 2012). Recent reports indicated that isolates belonging to this lineage spread very rapidly, causing severe infection in potato in regions beyond Europe (Chowdappa et al. 2013). During the same period (2009-2011), and for the first time in India, severe outbreaks of late blight were caused by the 13-A2 lineage on tomato, resulting in up to 100% crop losses in Karnataka state (Chowdappa et al. 2013). In contrast, *P. infestans* populations during 2009-2011 in northwestern China are distant from the European lineages, including 13-A2 (Tian et al. 2015).

The involvement of sexual reproduction in increasing the diversity of *P. infestans* populations was reported in different areas in the world. *P. infestans* reproduction mainly happens through sporangia, which can survive in soil for a short time, estimated in weeks. However, oospores (sexual spores) can survive for years in soil (Fry et al. 1993; Drenth et al. 1995). Thus, sexual reproduction would be an important factor in the epidemiology of late blight. Moreover, sexual reproduction generates new genotypes of the pathogen with unexpected traits.

Fortunately, in most parts of the world (except in Mexico), *P. infestans* populations are largely clonal with no evidence for routine sexual reproduction. Except for two recombinant populations, which occurred in the Columbia basin of the Pacific Northwest and in the northeastern part of the USA in 1993 and 2011, respectively (Gavino et al. 2000; Childers et al. 2015), the population structure usually result from asexual reproduction. However, these two reports of recombinant progeny demonstrate that sexual reproduction is possible and may happen again.

Several methods have been used to identify and characterize *P. infestans* isolates. Morphological identification is the first step usually taken to determine the blighted plant. Symptoms of late blight show as water-soaked lesion on infected leaves. Once the disease progresses, the germinated sporangia appears as white fluffy mycelium on the apical side of the leaves. Under the microscope, sporangia appear as lemon-shape structures that are carried on the sporangiophores.

Characterization of *P. infestans* relies on different methods. Phenotypic characterization is determined using fungicide sensitivity (Peters et al. 1998; Daayf and Platt 2000a) and mating type (Peters et al. 1998; Daayf and Platt 2000b) tests. However, genotypic characterization is usually based on the identification of pathogen genotypes using RG57 RFLP finger printing (Goodwin et

al. 1992) glucose-6-phosphate allozyme patterns (*Gpi*) (Goodwin 1995) and simple sequence repeat (SSR) (Cooke et al. 2006; Danies et al. 2013). The use of all the characterization methods provides better identification to each individual genotype as well as better tracking of their evolution.

Populations which occurred by few individuals can be very different compared with the populations from which they were derived (Maruyama and Fuerst 1985; Nei et al. 2006). These variations which were found between genotypes and within the same genotype are due to mutation or mitotic recombination (Goodwin et al., 1994). US-1 was named for the first genotype that brought to United States and Canada. Goodwin et al (1994) identified other genotypes that derived from the clonal linkage US-1. These genotypes were variants based on the genetic markers and they were designed as (US-1.1, US-1.2, US-1.3 and US-1.4). Later, identification of similar case was observed on US-6, where five (US-6.1- US-6.5) genotypes were found. These genotypes were identical to US-6 except for a change at a single allozyme or DNA fingerprint locus. Recently, US-23 was identical to US-23.1. The only exception was genotype US-23.1, which had gained a DNA fingerprint band in addition to those characteristic of US-23 (Alkher et al., 2015). Recombinant genotypes, which were found in Canada and named as (CA-9, CA-10, CA-11 and CA-12) showed not only changes in the allozyme (heterozygous/ homozygous) and DNA fingerprint locus but also in the mating types (Peters et al., 2014; Alkher et al., 2015)

1.2.6.2 The Plant - *P. infestans* pathosystem

The interaction between plant (i.e. potato or tomato cultivars) and *P. infestans* is usually determined by the race-specific resistant (R) proteins, which are produced by the host and the Avr

factors (effectors) produced by the pathogen. In incompatible interaction between potato plants cultivar that carries R proteins and *P. infestans* isolates with corresponding Avr proteins, the recognition of Avr proteins by the plant R proteins leads to serial physiological changes induced immediately in the plant cells. One of the first responses is the induction of reactive oxygen species (ROS) (Doke 1983), followed by the induction of hypersensitive response (HR) and production of phytoalexin. Together, they provide the plants with high production and restrict the hyphal growth of invading pathogen.

Development of cultivars with genetic resistant is the most effective and environmentally friendly approach to reduce the risk of significant yield losses caused by late blight. There is over 20 resistance genes cloned from different wild solanum species. R1-R11 was introduced from *Solanum demissum* into *Solanum tuberosum* (Black 1951; Black et al. 1953; Malcolmson and Black 1966). R1 was the first major late blight R gene that was identified and cloned (Ballvora et al. 2002). These genes have narrow spectrum because they are race-specific, and easily overcome by avr genes from *P. infestans* strains. However, research is continuing to investigate other resistance genes. Other wild solanum species such as *S. bulbocastanum*, *S. pinnatisectum*, and *S. trifidum* are promising for R genes. In *S. bulbocastanum* four different loci with broad spectrum late blight resistance have been identified, designed as *Rpi-Blb1/RB* (Helgeson et al. 1998), *Rpi-blb2* (Van Der Vossen et al. 2005), *Rpi-blb3* (Park et al. 2005a), and *Rpi-apbt* (Park et al. 2005b). Recently, other species are identified as a good source of resistance against late blight including *S. mochiquense* and *S. chacoense*. As new *P. infestans* genotypes (US-22, US-23 and US-24) are being introduced to the pathogen population, a number of resistance genes have been identified in *Solanum pimpinellifolium*, the wild relative species to tomato. Tomato resistance genes including Ph1, Ph-2, Ph-3, and Ph-5 were identified in North America and Ph-4 in Asia. These genes are

effective for some genotypes but not for others. For instance, varieties with Ph-2 and Ph-3, which are the most genes bred in tomato hybrids exhibit a significant suppression to US-23, the dominant genotype in North America (McGrath 2018).

On the other hand, *P. infestans* produces effector proteins, which are able to undergo rapid adaptation and avoid recognition by R proteins. There are different ways that effectors take to overcome R-gene mediated resistance. One of the ways is mutation of the amino acids, which lead to change in the corresponding Avr protein thus causing loss in the recognition. (Bos et al. 2006) showed that by mutating just two amino acids in AVR3a (K⁸⁰ I¹⁰³ to E⁸⁰ M¹⁰³), the pathogen is able to overcome recognition mediated by R3a.

1.2.6.3 *Phytophthora infestans* effectors

There are no potato or tomato cultivars with full resistance to late blight. The pathogen's adaptive ability has overcome all single resistance genes transferred to potato. One of the main areas of study to explore the reasons behind the success of *P. infestans* has been that of "effector", as in other oomycetes, including the genus *Phytophthora*, *Peronospora* and *Pythium* are plant fungal-like pathogens that are responsible for important crop losses (Kamoun and Smart 2005). These pathogens have an obligatory (biotrophic) life style at least in part of their life cycle (Panstruga 2003). *P. infestans* is hemibiotrophic pathogen where it divides its life cycle into two steps. The first step occurs during the early infection process and that is where the pathogen needs living cells to establish infection and colonize the tissue. In this stage, root-like structures known as haustoria form in the intercellular spaces. The second step is a switch from a biotrophic to a necrotrophic phase, where the pathogen depends on nutrients from the dead plant tissue to continue its life cycle (Kamoun and Smart 2005).

To achieve the above process and suppress plant defence mechanisms, *P. infestans* secretes an arsenal of effectors, which target plant cell compartments and alter their function. Particularly, effectors in *P. infestans* and bacteria of type III secretion system (TTSS) are defined as organized proteins into two main functional domains (Kamoun 2006). The first domain is considered as a highly conserved sequence motif at the N-terminal, which consists of a signal peptide and RXLR, (named after four amino acids; Arginine, any amino acid, Leucine, and Arginine) region. This domain is responsible for secretion and translocation of the effector into the plant cell. At the C-terminal region is the effector domain, which is responsible for the effector function (Bos et al. 2006; Kamoun 2006; Oh et al. 2009).

Several studies confirmed that the RXLR domain is required to translocate the effector inside the plant cells (Bos et al. 2006; Whisson et al. 2007; Dou et al. 2008). The virulence function is provided by the C-terminal, which is the effector domain (Schornack et al. 2009). Limited RXLR proteins were characterized due to the difficulty of predicting the C-terminal regions from the primary sequence and as there is little or no similarity of RXLR effectors with previously characterized proteins (Ryan et al. 2015).

Recent studies on oomycete effectors revealed that their functions are either to target regulatory host proteins involved in post-translational modification (PTM), or to alter PTMs associated with immunity. For instance, AVR3 of *P. infestans* targets the ubiquitin E3 ligase, which has important roles in cell death and perception of range of pathogen elicitors (Gilroy et al. 2011; Boevink et al. 2016). The reduction of salicylic acid- triggered immunity was mediated by HaRxL44 effector in *Hyaloperonospora arabidopsidis*, through a subunit of the mediator complex (Caillaud et al. 2013). Boevink et al. (2016) revealed the role of *P. infestans* core effector (Pi04314)

in enhancing susceptibility and promoting disease by co-opting host substrates (protein phosphatase 1 catalytic isoforms PP1c).

Research is continuing on the role of oomycete effectors in manipulating plant physiology for the promotion of disease (Haas et al. 2009; Rovenich et al. 2014; Sugawara et al. 2018; Uhse and Djamei 2018).

1.2 Objectives

My hypothesis is that *P. infestans* isolates belong to different genotypes that vary in their aggressiveness on potato and tomato based on the number of cycles they spent on their host of origin. The other hypothesis is that the variation among these isolates is at least partly related to the effectors they secrete in the plant cell, which result in the suppression of defense responses. These hypotheses will be tested by I) characterizing the new *P. infestans* genotypes that appeared in Canada in a recent survey, using different methods; II) determining the cross-pathogenicity of *P. infestans* isolates on potato and tomato by inoculating susceptible and moderately resistant cultivars of both plant species, and by monitoring the disease progress over time; III) studying the expression of selected effector genes during the *P. infestans*- potato/ tomato interactions by testing the expression of these genes during the interaction between susceptible/moderately resistant cultivars with weakly and highly aggressive isolates of *P. infestans*.

The research accomplished in this thesis is presented in several chapters:

Chapter 2: characterization of *Phytophthora infestans* populations in Canada during 2012 (H. Alkher, M. R. Islam, C. Wijekoon, M. Kalischuk, L. M. Kawchuk, R. D. Peters, K. I. Almughrabi, K. L. Conn, K. F. Dobinson, D. Waterer, and F. Daayf. 2015. Canadian Journal of Phytopathology. Vol. 37, Iss. 3, P. 305-314)

Chapter 3: Cross- pathogenicity of *Phytophthora infestans* genotypes on potato and tomato

Chapter 4: Expression of putative RXLR effectors, in particular the DL119 (PITG-12773-2) gene during the interaction of weakly and highly aggressive *P. infestans* isolates with potato and tomato



Characterization of *Phytophthora infestans* populations in Canada during 2012

H. Alkher, M. R. Islam, C. Wijekoon, M. Kalischuk, L. M. Kawchuk, R. D. Peters, K. I. Al-Mughrabi, K. L. Conn, K. F. Dobinson, D. Waterer & F. Daayf

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CHAPTER 2: CHARACTERIZATION OF *PHYTOPHTHORA INFESTANS* POPULATIONS IN CANADA DURING 2012

Alkher, H. ; Islam, M. R. ; Wijekoon, C. ; Kalischuk, M. ; Kawchuk, L. M. ; Peters, R. D. ; Al-Mughrabi, K. I. ; Conn, K. L. ; Dobinson, K. F. ; Waterer, D. ; Daayf, F.

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Canadian Journal of Plant Pathology, 23 May 2015

The first Author carried out experiments to characterize *P. infestans* isolates that were collected from Manitoba in 2012 by using mating type, *GPI*, fungicide sensitivity and RG57 RFLP fingerprinting techniques. She also compiled and interpreted the results gathered from other provinces.

2.1 Abstract

The late blight pathogen (*Phytophthora infestans*) continues to cause major losses on potato and tomato in Canada and worldwide. An increased diversity of *P. infestans* and dramatic shifts in pathogen populations have occurred in Canada in recent years. In 2011, a survey identified different genotypes of *P. infestans* in Canada, including the new US-22, US-23 and US-24 genotypes, which were dominant in various Canadian provinces. In 2012, analysis of samples collected from infected potato and tomato plants from different regions in Canada indicated a rapid change in *P. infestans* populations in most provinces within a single year. For example, in Prince Edward Island, the US-8 genotype that dominated the *P. infestans* landscape for many years was displaced by the US-23 genotype, a phenomenon similar to that which occurred in western Canada in prior years. In British Columbia, however, US-11 and the new CA-12 were the dominant genotypes while in Ontario the tested isolates were US-22. Evidence for recombination was found, and increasing insensitivity to mefenoxam was apparent among isolates of some populations.

Independent segregation of either *Gpi*, mating type or RG57 loci occurred in a number of the recombinant isolates, resulting in increased diversity of *P. infestans* populations. The unexpected change in composition of *P. infestans* genotypes supports the need for continued monitoring of this pathogen.

2.2 Introduction

Late blight is one of the most damaging diseases of potato (*Solanum tuberosum* L.) and tomato (*S. esculentum* L.) (Fry and Goodwin 1997; Chowdappa et al. 2013) and is caused by *Phytophthora infestans* (Mont.) de Bary. This pathogen is the most widely studied oomycete, with 1230 papers published in the last 10 years, and is one of the top 10 oomycete pathogens studied using molecular techniques (Kamoun et al. 2015). This disease decimated potato crops in Ireland (Woodham Smith 1962), leading to the starvation of a million people in the mid-1840s. Even 170 years later, the disease continues to cause significant losses in potato production worldwide of up to \$6.7 billion annually (Vleeshouwers et al. 2011).

Phytophthora infestans is an oomycete that has the ability to reproduce both sexually and asexually. In the sexual phase, the hyphae of the opposite mating types interact with each other, inducing the formation of oogonia and antheridia. The successful association of these structures leads to the formation of oospores, which can tolerate harsh conditions and subsist in the soil between growing seasons (Fry and Goodwin 1997; Andersson et al. 1998). The pathogen can spread asexually by sporangia, which are dispersed by rain splash or wind, and generally survives in potato tubers between crop seasons (Aylor 2003).

After the US-1 clonal lineage had long dominated *P. infestans* populations in North America, severe damage by this pathogen was reported in potato fields after a new genotype, US-6, was

detected in the western United States (Goodwin et al. 1994a, 1996; Goodwin 1995; Chycoski and Punja 1996; Peters et al. 1998). Increased losses of potato in both Canada and the United States followed, with the appearance of other new genotypes in the early 1990s, namely US-7 and US-8 (Goodwin et al. 1995; Fry and Goodwin 1997). Eventually, US-8 became the dominant genotype in most Canadian provinces (Peters et al. 1998) except for British Columbia, where the US-11 genotype dominated until 1996. Although changes in several *P. infestans* characteristics ensued in subsequent years (Daayf and Platt 1999, 2000a), US-8 and US-11 remained the predominant genotypes in Canada. More recently, a severe late blight pandemic occurred in 2009 when infected tomato transplants were shipped to garden centres in large retail stores throughout the northeast United States (Fry et al. 2013), and the disease spread quickly throughout many states and into Canada. Late blight led to losses of millions of dollars for growers in the United States and some tomato growers totally abandoned production (Hu et al. 2012).

Surveys conducted in 2010 on the *P. infestans* population in the Canadian provinces indicated that a new genotype, US-23, was dominant in western Canada (BC, AB, SK and MB). This genotype was isolated from both potato and tomato, but was more severe on tomato (Hwang et al. 2014). US-24, another new genotype, which infects mainly potato, was detected in Manitoba for the first time in 2010. In 2011, US-23 and US-24 became the most predominant *P. infestans* genotypes across much of Canada, except in Ontario, where all isolates originated from tomato plants and belonged to five different genotypes (CA-9, CA-10, CA-11, US-11 and US-22) and Prince Edward Island where the US-8 genotype still predominated (Peters et al. 2014). Based on common markers used to characterize *P. infestans* genotypes, including allozymes of glucose-phosphate isomerase (*Gpi*), mating type, RG57 fingerprinting and sensitivity to metalaxyl-m, US-1, US-6, US-11, US-23 and US-24 are of the A1 mating type, whereas US-7, US-8 and US-22 are A2. Moreover,

isolates characterized as US-1, US-22 and US-23 are generally sensitive to metalaxyl-m, whereas US-6, US-7, US-8 and US-11 are commonly insensitive to metalaxyl-m, although an increase in insensitivity to this fungicide has been reported (Seidl and Gevens 2013). US-24 often has intermediate insensitivity to metalaxyl-m (Kalischuk et al. 2012; Peters et al. 2014).

An increased diversity of *P. infestans*, reflected by changes in genotypes as described by the markers employed in the current study, indicates a dramatic shift in populations of this pathogen. Factors contributing to this shift may include sexual recombination, climate change, mutations, and migration of genotypes via infected plants and/or potato tubers (Goodwin et al. 1995; Chycoski & Punja 1996).

Since *P. infestans* remains a major pathogen with an increasing ability to adapt to changing circumstances, monitoring its diversity is important in order to better predict future population changes, to help detect the presence of sexual reproduction, and understand the pathogen's response to current management strategies and environmental changes. Ultimately, monitoring the population structure of *P. infestans* will provide vital information towards better management of late blight. The goals of this study were: (i) to characterize the genetic diversity of *P. infestans* isolates collected in Canada in 2012, thereby identifying fluctuations in genotypes, and (ii) to assess the effect of these changes on pathogen behaviour, such as response to fungicides in comparison with strains from previous years.

2.3 Materials and methods

2.3.1 Collection and purification of isolates

One hundred and nineteen diseased tomato and potato samples from commercial fields and greenhouses (Table 1.1) were received from Prince Edward Island (PEI), New Brunswick (NB), Quebec (QC), Ontario (ON), Manitoba (MB), Saskatchewan (SK), Alberta (AB) and British Columbia (BC), Canada in 2012. Segments of infected tomato leaves and fruits, and potato leaves, stems, and tubers, were transferred to Petri dishes and placed in a plastic container, and kept in a moist chamber in the dark at 18°C for 24 h. Potato tuber and tomato fruit tissues were surface-sterilized with 10% bleach before sectioning. The samples were examined for the presence of *P. infestans* sporangia using a dissecting microscope. A small agar fragment on the tip of a needle was used to collect sporangia from the incubated plant materials, which were then transferred onto clarified rye agar B (CRB) containing 2% sucrose and incubated at room temperature for 24 h. Single germinated sporangia were transferred separately onto new CRB.

2.3.2 Glucose phosphate isomerase allozyme patterns (Gpi)

Some genotypes of *P. infestans* can be distinguished on the basis of glucose phosphate isomerase (*Gpi*) allozyme profiles determined using cellulose-acetate electrophoresis (Oudemans and Coffey 1991; Goodwin et al. 1996). Isolates were grown on V8 medium for two weeks and then two wefts of mycelium were placed in a 1.5 mL Eppendorf microcentrifuge tube and homogenized in 100 µL of sterile water using sterile micro-pestles. After vortexing, samples were centrifuged for 5 min at 12 000 g to remove cell debris. A 10-µL aliquot from each sample supernatant was transferred into one of the wells of the sample applicator (super Z-12 Applicator kit, Helena laboratories,

Beaumont, TX), which was used to load the gels (Goodwin et al. 1995). The running dye was spotted on each side of the gel to monitor the electrophoresis run at 175–200 v for 15–20 min. The gels were stained with agar overlays and the reactions stopped after the banding patterns were clear (Goodwin et al. 1995). The bands were then compared with the reference isolates (Figure 2.1).

2.3.3 Mating types

Isolates were grown on clarified rye agar B medium (CRB) for 2–3 weeks. Using new CRB plates, mycelial discs of each isolate, 5-mm in diameter, were paired separately with those of known A1 and A2 isolates. The tester disc and the unknown isolate disc were placed on opposite sides of each Petri dish. Plates were incubated for approximately 2 weeks in the dark, and examined microscopically for the presence of gametangia and mature oospores at the interface between the growing colonies. Isolates that formed oospores with the A1 and not the A2 tester were considered the A2 mating type, and isolates that formed oospores with the A2, and not with the A1 tester, were considered the A1 mating type. The test was repeated twice with three replicates for each isolate.

2.3.4 Metalaxyl-m sensitivity

In vitro testing for sensitivity to metalaxyl-m was done with a protocol modified from Peters et al. (1998) and Daayf et al. (2000). Metalaxyl-m (Metalaxyl-m 90% w/w; technical grade; Syngenta Canada, Guelph, ON) was prepared as a 100 mg mL⁻¹ stock solution in pure dimethyl sulfoxide (DMSO) and added to molten clarified pea agar after autoclaving to achieve a concentration of 100 µg mL⁻¹. Agar plugs (5 mm in diameter) taken from the margin of 2-week-old cultures of *P. infestans* were transferred to Petri plates (60 × 15 mm) containing clarified pea medium amended

with 0 or 100 μg metalaxyl-m mL^{-1} . Growth was measured after incubation for 7 and 14 days in the dark at 15°C using digital calipers. Two measurements, perpendicular to one another, were taken from each of two replicate plates. Means were calculated and the diameter of the inoculation plug (5 mm) was subtracted from each mean. Three categories of fungicide sensitivity, expressed as mean growth (colony diameter) in the presence of 100 μg metalaxyl-m mL^{-1} as a percentage of mean growth in the absence of metalaxyl-m, were recognized: metalaxyl-sensitive (MS) $\leq 10\%$ growth, metalaxyl-intermediately sensitive

Table 2.1 *Phytophthora infestans* isolates collected in 2012 as characterized with phenotypic and genotypic markers.

<i>Province</i>	<i>No. of isolates</i>	<i>Gpi Allozym</i> ^a	<i>Mating type</i>	<i>Mefenoxam</i> ^b	<i>RG57 RFLP</i> ^c	<i>genotype</i>	<i>Host</i> ^f	<i>Plant tissue</i> ^e
<i>British Columbia</i>	4	100/100/122	A1	R	1,4,5,10,13,14,17,20,21,24,25	CA12	P	L
	4	100/100/111	A1	I	1,3,5,6,7,10,13,14,16,18,20,21,24,25	US11	p	L
<i>Alberta</i>	25	100/100	A1	4 I/ 21 S	1,2,5,6,10,13,14,17,20,21,24,24a,25	US23	P	L
<i>Saskatchewan</i>	2	100/100	A1	S	1,2,5,6,10,13,14,20,21,24,24a,25	US23.1	P	F
		100/100	A1	S	1,2,5,6,10,13,14,17,20,21,24,24a,25	US23	p	T
<i>Manitoba</i>	9	100/100	A1	4 S/ 6 I/ 1nd*	1,2,5,6,10,13,14,20,21,24,24a,25	US23	T	L
	1	100/100	A1		1,2,5,6,10,13,14,17,20,21,24,24a,25	US23.1	P	F
	1	100/100/111	A1		1,3,5,7,10,13,14,16,20,21,23,24,25	US24		L
<i>Ontario</i>	8	100/122	A2	3 I/ 5 S	1,5,13,14,16,20,21,24,25	US22	T	L
<i>Quebec</i>	1	100/100	A1	I	1,2,5,6,10,13,14,17,20,21,24,24a,25	US23	p	L
<i>New Brunswick</i>	3	100/100	A1	2 I/ 1 R	1,2,5,6,10,13,14,17,20,21,24,24a,25	US23	p	L
<i>Prince Edward Island</i>	1	100/111/122	A1	S	1,5,10,13,14,16,20,21,23,24,25	US-8	P	L
	1	100/111/122	A1	I	1,2,5,6,10,13,14,17,20,21,24,24a,25	US23	P	L
	59	100/100		58 I/ 1 R	1,2,5,6,10,13,14,17,20,21,24,24a,25	US23	p	L S

a: Glucose-6-phosphate isomerase. 100/100/122 (CA12), 100/100/111 (US11), 100/100 (US23), 100/122 (US-22), 100/111/122 (US-8). 100, 111 and 122 are the locations and the names of homodimer bands of the allozymes. Alleles were originally named on the basis of their migration distance relative to that of 100 allele.

b: Mefenoxam rate; S = sensitive, I = intermediate, R = resistant.

c: Restriction fragment length polymorphism using RG57 probe. From left to right, the numbers from 1-25 indicate to the identified bands with their presence or absence for each genotypes.

f: P = potato, T = tomato

e: L = leaf, F = fruit, S = stem.

*nd = no data

(MI) = 10–60% growth, and metalaxyl-resistant (MR) \geq 60% growth. The experiment was repeated for each isolate and the data pooled for reporting.

2.3.5 RG57 Restriction fragment length polymorphism fingerprinting

RFLP fingerprinting using the RG57 probe was performed according to (Goodwin et al. 1992). After growing in pea broth for 14 days at 18°C, mycelia were vacuum-filtered using Whatman filter paper no. 1, lyophilized overnight, and ground in liquid nitrogen. Total DNA extraction followed the protocol of W.E. Fry (personal communication). Twenty μ g of genomic DNA was incubated overnight with 70 U EcoRI then loaded onto a 0.8% agarose gel and run at 50 V for 8 hours. The DNA was transferred by capillary overnight onto Hybond N+ membrane. A 250 ng RG57 probe was prepared by digesting the plasmid with EcoRI followed by a gel extraction. The RFLP banding was visualized with an AlkPhos labelled probe (GE Healthcare/Amersham).

2.3.6 Microsatellite analyses

Microsatellite analysis was conducted following Lees et al. (2006) and Danies et al. (2013) in order to differentiate clonal lineages. Isolates of *P. infestans* collected in 2012 were tested with eight polymorphic markers (Pi4b, PiG11, D13, Pi63, Pi89, Pi16, Pi56 and Pio4). PCR amplification was conducted with standard conditions: 95°C for 10 min; followed by 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 60 s; and a final extension at 72°C for 20 min. PCR amplicons were compared with pure isolates of five known genotypes: US-8, US-11, US-22, US-23 and US-24 (Table 2.3).

2.4 Results

One hundred and nineteen *P. infestans* isolates were successfully isolated from tissue samples originating from Canadian provinces including PEI, NB, QC, ON, MB, SK, AB and BC (Table 2.1). The majority of *P. infestans* isolates were taken from infected potato leaves, stems or tubers, except for the eight isolates from ON, one from MB, and one from SK, which were obtained from tomato tissues. Glucose-6-phosphate isomerase (*Gpi*) and DNA restriction fragment length polymorphism (RFLP) analyses identifies four *Gpi* allozyme profiles in the *P. infestans* isolates collected from 2012, including *Gpi* 100/111/122 for CA-12, CA-13 and CA-14; *Gpi* 100/100/111 for US-11 and US-24; *Gpi* 100/100 for US-23 and US-23.1; and *Gpi* 100/122 for US-22 (Table 2.1). Figure 2.1 shows the pre-dominance of the 100/100 *Gpi* profile in Canada, with 84% of the isolates tested.

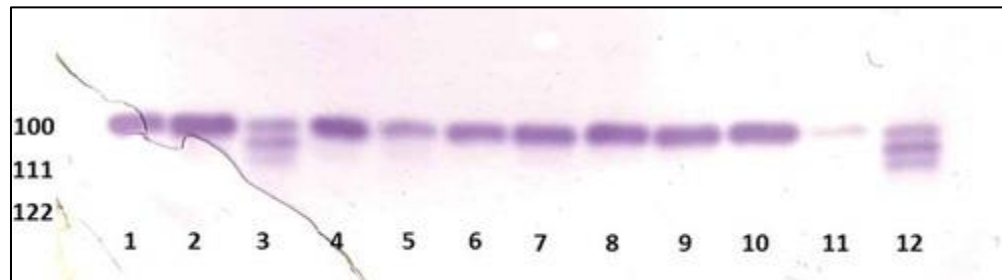


Figure 2.4 The allozyme banding patterns of *Phytophthora infestans* isolates in 2012.

Lane 1: Pi 1-4-12, **lane 2:** Pi 2-3-12, **lane 3:** Pi 3-2-12, **lane 4:** Pi 4-3-12, **lane 5:** Pi 5-2-12, **lane 6:** Pi 6-1-12, **lane 7:** Pi 7-1-12, **lane 8:** Pi 8-1-12, **lane 9:** Pi 9-1-12, **lane 10:** Pi 11-1-12, **lane 11:** Pi 12-4-12, **lane 12:** Pi 4-3-11.

RFLP profiles of the 2012 isolates indicated that the majority belonged to the US-23 genotype (Table 2.1, Figure. 2.2). This genotype was recovered from tomato fruit tissues and from all parts

of potato plants and was dominant in AB, SK, MB, QC, NB and PEI. US-11 was only found in four samples, all from BC (Table 2.1). US-22 was only found on tomato in ON, where it was dominant. The CA-12 genotype was found among the BC isolates. This genotype has an identical *Gpi* profile to US-8, but a different RFLP profile (1, 4, 5, 10, 13, 14, 17, 20, 21, 24, 25) with two additional bands: 4 and 17, and two missing ones: 16 and 23. Two isolates from PEI were designated as CA-13 and CA-14. Both had the 100/111/122 *Gpi* banding pattern, characteristic of US-8. However, both had the A1 mating type and CA-13 had the US-8 RFLP profile, while CA-14 had the US-23 RFLP profile. Only one US-24 isolate was found in 2012 in MB. Two isolates, one from MB, and one from SK, had the US-23.1 genotype, which is identical to US-23 in all markers, except that it has an additional RFLP band 17 (Table 2.1). Both of these isolates were from tomato.

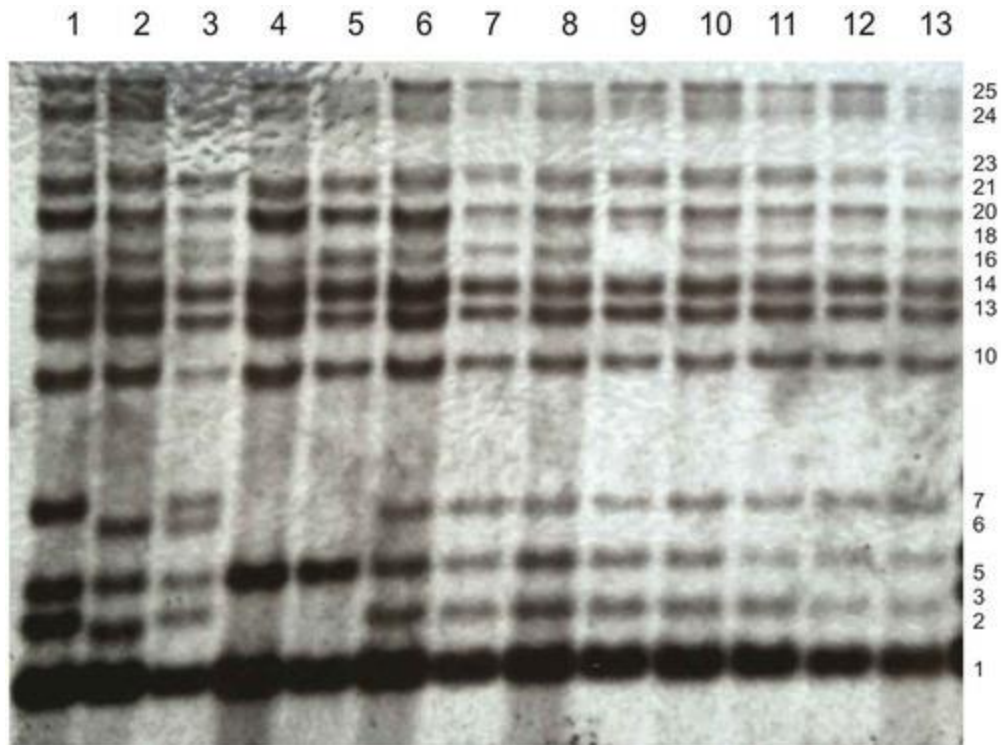


Figure 2.2 Restriction fragment length polymorphism (RFLP) analysis of the *Phytophthora infestans* isolates from 2012 with the moderately repetitive RG57 loci and EcoRI.

Genotype designations are L1 = US-24, L2 = US-23, L3 = US-11, L4 = US-8, L5 = CA-12, L6 = CA-14, L7, L8, L10, L11, L12 and L13 = US-23. The genotype US-23.1 (L9) resembles US-23 except DNA fragment 17 was missing. Genotype CA-12 was distinguished by the unique DNA 1, 5, 10, 13, 14, 17, 20, 21, 24, 25 RG57 RFLP pattern. Genotypes CA-13 and CA-14 have RFLP patterns identical to US-8 and US-23, respectively.

2.4.1 Mating type analysis

Isolates from all provinces, except ON, were of the A1 mating type. Of these, BC isolates were US-11 and the remainder were US-23, US-24, and recombinants US-23.1, CA-12, CA-13 and CA-14. The only A2 mating type isolates in all the collected samples were from ON (US-22 genotype). The CA-12 isolates had the *Gpi* 100/111/122 profile, just like the US-8/A2 isolates. However, they were of the A1 mating type. Another unique isolate, designated CA-13, was found in PEI, with *Gpi* 100/111/122 and RFLP profile 1, 5, 10, 13, 14, 16, 20, 21, 23, 24, 25, although it was of the A1 mating type, which segregates independently from other genotype markers.

2.4.2 Changes in genotypes of Canadian *P. infestans* isolates from 2011 to 2012

Genotype frequencies in the Canadian *P. infestans* populations from 2011–2012 are summarized in (Table 2.2). The percentage of each genotype in Canada indicates that a rapid change in frequency occurred among genotypes of *P. infestans* from potato and tomato. An increase in recovery, from 11.6% to 82.4%, of the US-23 genotype was observed in potato during this period. The recovery of US-11 isolates decreased in both potato and tomato, from 7.2% to 3.4% and from 0.7% to nil, respectively. US-22 was a dominant genotype in Ontario, found only on tomato and only in this province. It did not show a significant change in frequency from 2011 to 2012 (Table 2.2) with a slight decrease from 7.2% to 6.7%. No US-8, CA-9, CA-10 or CA-11 genotypes were found among the samples collected in 2012. On the other hand, new genotypes with recombinant profiles appeared, including US-23.1, CA-12, CA-13 and CA-14 with 1.7%, 3.4%, 0.8% and 0.8% frequency, respectively. Recombinant isolates CA-12, CA13 and CA-14 were all isolated from potato, whereas the two recombinant US-23.1 isolates were both isolated from tomato.

Table 2.2 Changes in genotypes of potato and tomato *P. infestans* isolates across Canada from 2011 to 2012.

<i>Host</i>	<i>Genotype</i>	<i>2011 (Peters et al. 2014)</i>	<i>2012 (This study)</i>
<i>Potato</i>	US-8	37%	-
	CA-13	-	0.8%
	CA-14	-	0.8%
	US-11	7.2%	3.4%
	US-23	11.6%	82.4%
	US-23.1	-	-
	US-24	29.7%	0.8%
	CA-12	-	3.4%
<i>Tomato</i>	US-11	0.7%	-
	US-22	7.2%	6.7%
	US-23	2.2%	-
	US-23.1	-	1.7%
	CA-9	0.7%	-
	CA-10	3%	-
	CA-11	0.7%	-
<i>Total</i>		100%	100%

2.4.3 Metalaxyl-m sensitivity analysis

Of all the collected isolates, 33 were sensitive to metalaxyl-m, 79 had intermediate resistance, and only six were resistant (sensitivity to metalaxyl-m was not determined for one isolate). All of the US-23 isolates collected from PEI showed intermediate insensitivity to metalaxyl-m except for one that was resistant (Table 2.1). Both US-23 and US-22 genotypes in other provinces ranged from sensitive to intermediate resistant, except for one isolate from NB that was resistant to metalaxyl-m. US-11 isolates were found only in BC and had intermediate resistance to metalaxyl-m. Interestingly, all of the four recombinant isolates of the CA-12 genotype were resistant to metalaxyl-m. US-11 and CA-12 completely displaced the US-23 genotype in BC. Another unique finding was that the CA-13 isolate from PEI was sensitive to metalaxyl-m. This isolate was initially determined to be US-8 based on the *Gpi* allozyme assay and RFLP profile, but it was of the A1 mating type. Among all PEI isolates, CA-13 was the only isolate that showed sensitivity to metalaxyl-m.

2.4.4 Microsatellite analyses

Eight microsatellite markers were considered in this study. The microsatellite PCR amplicons were compared with the size standards and the allelic compositions were scored accordingly. Some of the microsatellite markers such as Pi4B, PiG11, D13, Pio4 and Pi16 produced multiple fragment patterns among the different genotypes, while pi56, Pi89 and Pi63 produced only two different fragment patterns, respectively. Alleles at eight selected microsatellite loci successfully differentiated the most common five clonal lineages (US-8, US-11, US-22, US-23 and US-24 (Table 2.3). The majority of the tested isolates were identified as US-8 or US-23 based on their fragment identities. Genotype CA-14 shares the US-23 fragment pattern. However, genotype CA-

13 is only different from US-8 at the Pi16 locus. Interestingly, US-23.1 differed from the typical US-23 at markers Pi4B and Pio4, whereas CA-12 varied from US-8 at markers Pi4B and Pi16 forming unique fragments compared with other isolates (Table 2.3).

Table 2.3 Microsatellite analyses of *Phytophthora infestans* isolates collected in 2012.

<i>Microsatellite markers</i>	<i>Isolates</i>								
	<i>US-8</i>	<i>US-11</i>	<i>US-22</i>	<i>US-23</i>	<i>US-24</i>	<i>US-23.1</i>	<i>CA-12</i>	<i>CA-13</i>	<i>CA-14</i>
<i>Pi4B</i>	213	213	213	213	217	196	213	213	213
	226	213	213	217	226	200	220	226	217
<i>PiG11</i>	155	131	131	140	155	140	155	155	140
	155	155	155	155	155	155	155	155	155
<i>D13</i>	106	106	106	134	106	134	106	106	134
	110	106	106	134	110	134	110	110	134
<i>Pio4</i>	166	166	166	170	166	170	170	166	170
	170	170	170	170	170	177	170	170	170
<i>Pi16</i>	173	177	177	177	173	173	170	160	177
	177	177	177	177	177	177	177	166	177
<i>Pi56</i>	250	230	230	230	230	230	250	250	230
	255	250	250	250	250	250	255	255	250
		255	255	255	255	255			255
<i>Pi89</i>	179	177	177	179	177	179	179	179	179
	179	179	179	179	179	179	179	179	179
<i>Pi63</i>	279	279	279	270	279	270	279	279	270
	279	279	279	279	279	279	279	279	279

The data presented in the table for each microsatellite marker under each genotype show the band size (bp) of amplicons.

2.5 Discussion

Late blight caused by *P. infestans* continues to be the most devastating disease of potato and tomato worldwide by destroying the foliage, fruit and tubers to dramatically impact yield. In recent years, disease severity and pathogen diversity rapidly increased with the occurrence of new genotypes in North America and elsewhere (Gisi et al. 2011; Hu et al. 2012; Kalischuk et al. 2012; Chowdappa et al. 2013; Fry et al. 2013). Such rapid changes in *P. infestans* populations suggest that sexually reproducing populations of *P. infestans* may now be established in Canada.

Severe outbreaks of *P. infestans* on tomato and potato are of concern with the rapid displacement of common genotypes by new ones that are segregating independently for different biochemical and molecular markers (Peters et al. 2014). In 2012, 119 isolates were collected from various Canadian provinces, and surprisingly, US-24, which dominated some Canadian regions in 2011, was displaced by the US-23 genotype. Even the US-24 and US-11 genotypes found in AB in 2011 were displaced by US-23 in 2012. The tested *P. infestans* isolates from MB were all US-23 except for one US-24 and one US-23.1 isolate. Importation of infected tomato plants and potato seed likely contributed to such rapid changes in genotypes, also witnessed in PEI, where the previously dominant genotype US-8 was displaced by US-23. However, factors that contribute to the displacement of common genotypes by new ones did not appear to affect the ON *P. infestans* population, based on the tomato isolates tested. The US-22 genotype continued to be predominant in the province, suggesting that environmental conditions and host specificity may be facilitating the establishment of this particular genotype in this region.

Genetic changes have always been linked to the clonal propagation of *P. infestans* genotypes and the movement of this pathogen (Sujkowski et al. 1994; Abu-El Samen et al. 2003). Genetic

diversity can be affected by mutation and mitotic recombination. Several studies showed that asexual progenies of *P. infestans* differ from their parents in growth rate, colony morphology, as well as pathogenicity (Caten and Jinks 1968; Abu-El Samen et al. 2003).

Chamnanpant et al. (2001) reported that mitotic gene conversion occurs at unusually high frequencies in *P. sojae*, supporting the potential for rapid variation. Populations with large genome sizes such as *P. infestans* seem to have higher gene diversity generated through mutation and random genetic drift (Hartl and Clark 1997). In the present study, recombination appears to have occurred in the BC and PEI isolates (Table 2.1), supported by the presence of both mating types (A1, A2) in BC over many years (Chycoski and Punja 1996), which may have caused generation of new genotypes. The genotype CA-12 had *Gpi* 100/111/122, which is identical to that of the US-8 genotype, but with an A1 mating type and RFLP profile 1, 4, 5, 10, 13, 14, 17, 20, 21, 24, 25. Furthermore, results of the metalaxyl-m sensitivity analysis showed that this genotype was resistant, as previously reported for US-8 isolates. This suggests that CA-12 is probably a result of recombination of either US-11 or US-23 with US-8. The presence of both mating types in the same area increases the chances of sexual reproduction that produces oospores, able to survive without the presence of a host (Turkensteen et al. 2000) Evidence of such recombination was reported in Ontario in 2011 (Peters et al. 2014), where both A1 and A2 mating types were present in close proximity, and where novel CA-9, CA-10 and CA-11 genotypes were recovered.

Other potential recombinant isolates included two from PEI in 2012. One of the isolates, designated CA-13, had *Gpi* 100/111/122 and an RFLP profile characteristic of US-8, but it was of A1 mating type. The other isolate was designated CA-14, with an RFLP profile characteristic of US-23, A1 mating type, and the *Gpi* 100/111/122 that is usually associated with the US-8 genotype. These results show independent segregation at mating type and *Gpi* loci. Moreover,

polymorphism was detected in two isolates from Manitoba and Saskatchewan designated as US-23.1. Independent segregation at *Gpi*, RFLP or mating type loci strongly suggests that all of these standard characterization tests should be performed continuously. With the consistent appearance of independent segregation in these markers, one marker is not sufficient to predict the characteristics of any isolate.

Previous studies indicated that the US-23 genotype was sensitive to metalaxyl-m, whereas both US-8 and US-11 genotypes were metalaxyl-m-insensitive (Peters et al. 2014). Our results showed a shift in the response of the US-23 genotype to this fungicide, with many isolates showing intermediate resistance, and some having resistance to metalaxyl-m. This is consistent with recent findings in Wisconsin, USA, wherein populations of the US-23 genotype were predominantly intermediate in sensitivity to mefenoxam (Seidl and Gevens 2013). Therefore, monitoring fungicide responses among *P. infestans* genotypes is imperative to plan for best management of late blight in current and subsequent seasons. Monitoring movement and evolution of the *P. infestans* populations will be helpful in making management decisions and developing strategies to delay or prevent late blight.

In conclusion, analysis of Canadian *P. infestans* isolates in 2012 showed rapid changes in the regional distribution of genotypes, with most displaced partially or completely by US-23 within a single year, with the exception of that in BC. This resembles the displacement of a number of genotypes by US-8 in most of Canada in the 1990s. Although there was no occurrence of US-23 in BC in 2012, the new genotype CA-12 was described and was likely the result of recombination between US-8 A2 isolates and US-23 A1 isolates, which were present in 2011, although not both in BC. In 2011, A1 and A2 mating types were found together in the same province in ON, QC and PEI (Peters et al. 2014). The presence of both mating types in close proximity increases the chances

for generation of recombinant isolates and new genotypes. As previously reported, some new genotypes are exhibiting independent segregation at different loci (Peters et al. 2014). This suggests that relying on the results of only some markers for accurate estimation of population characteristics is insufficient. Limiting the movement of infected potato seed and transplant hosts of *P. infestans* from one region to another is critical to mitigate epidemic risks and to reduce the chances of sexual reproduction.

CHAPTER 3: CROSS-PATHOGENICITY OF *PHYTOPHTHORA INFESTANS* ON POTATO AND TOMATO

3.1 Abstract:

Late blight is a disease that has devastating effects on very important economic crops globally. It is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary. The genetic composition of *P. infestans* populations in Canada has changed dramatically over the last 5-10 years, with the appearance of new genotypes that differ from the old pathogen populations in terms of genetic profiles and several traits such as fungicide sensitivity. The majority of damages and yield losses caused by late blight occur on potato and tomato crops. Cross infection of *P. infestans* isolates on potato and tomato has been studied using the old genotypes US-1, US-8 and US-11. These genotypes have been almost completely displaced by other genotypes. Determining the effects of the new genotypes originating from either potato or tomato on their original and alternative hosts is needed and would be beneficial for future control strategies. In this study, two potato (Russet Burbank, Kennebec) and two tomato (Sun rise, Ultra sweet) varieties, representing susceptible and resistant lines, were inoculated with isolates belonging to genotypes US-24 isolated from potato, and US-22 and US-23 from tomato. One isolate from each of US-8 and US-11 was included as extra controls. The infection was assessed at 3, 5, 7 days after inoculation on leaves and at 5 days post inoculation on potato tubers. Comparison of the area under disease progress curves (AUDPC) revealed higher infection caused by most US-24 isolates in all tested cultivars of potato and tomato. More disease was recorded in Russet Burbank, Sun Rise and Ultra sweet cultivars compared to Kennebec. The US-22 and US-23 isolates, which were obtained from tomato, are more virulent to tomato. These isolates caused significantly higher infection on tomato varieties compared to the potato cultivars. The lesion area of inoculated tubers of moderately resistant potato cultivar

(Kennebec) was larger than that of the susceptible cultivar (Russet Burbank), which contrasted with the foliar infection results. This study provided essential information about the performance of different *P. infestans* genotypes during their interaction with potato and tomato, which may be helpful in planning for better control strategies that involve fungicide application, as well as for potato operations.

3.2 Introduction

Late blight is one of the most threatening plant diseases. It is caused by the oomycete *Phytophthora infestans* (Mont) de Bary. It is the most devastating disease on the economically important crops, potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) (Benson 1997). Under optimal conditions, the disease can destroy an entire field within 2-3 days (Vleeshouwers 2011). Sporangia are the main source of inoculum (Fry 2008). They disperse via wind or rain-splash, and then settle on the host's surface. Under favourable conditions, the sporangia start germinating at temperatures above 12°C (Sabaratnam 2018). Once the plant is colonized, symptoms appear on the leaves as water-soaked areas with an increase in size with more sporangia sporulation. These symptoms are more obvious on the apical side of the leaves, as fluffy, white sporangial masses (Nowicki et al. 2012). Under wet conditions and optimal temperature, epidemics can be rapid due to successful inoculum production. Such epidemics result from many sequential cycles of infection.

The hosts of *P. infestans* are mainly plants from the Solanaceae family, including potato and tomato. However, late blight was also reported on non-Solanaceae plants such as maple (*Acer*

sp.), morning glory (*Ipomoea hederacea*) and fleabane (*Erigeron multicaulis*) (Erwin and Ribeiro 1996). Within the Solanaceae family, there are some wild, ornamental, and vegetative plants that are hosts to *P. infestans*, including wild nightshade, bitter nightshade, petunia, ground cherry, eggplant and tomatillo (Peterson 1947; Beckett et al. 2005).

The pathogenicity of *P. infestans* isolates representing different genotypes has been previously studied on potato, tomato and other Solanaceae (Shattock 2002; Fontem et al. 2004; Sedláková et al. 2011) plants. However, most reports provided information on the infection of such genotypes on their hosts of origin. Multiple hosts of a pathogen such as *P. infestans* allow for the disease to be spread more widely and result in increasing disease incidence. Severe infections and high disease incidences are determined by different factors, including the *P. infestans* genotypes present in the growing area, the plant species and its susceptibility to these genotypes, environmental conditions, and the host of origin of the isolates.

Cross-infection has been documented on *P. infestans* (Daayf and Platt, 2003) as well as on other pathogens such as *Verticillium dahliae*, *Fusarium oxysporum*, *Sclerotinia* species and *Phytophthora capsici*. Most reports indicate that isolates of this pathogen are more aggressive on their hosts of origin. Daayf and Platt (2003) studied the differential pathogenicity of US-11 and US-8 genotypes of *P. infestans* on several potato and tomato varieties. They showed that US-8 isolates were highly aggressive on both hosts. However, less damage was observed on the foliar part of potato infected by US-11, which was more dominant on tomato than on potato. Similar results were found with *Verticillium* species, which vary in pathogenicity on different hosts, but commonly cause more infection on the host from which they were collected (Bhat and Subbarao

1999; Fontem et al. 2004; Alkher et al. 2009). The continuous changes in the population structure of *P. infestans* means that the nature of the interaction between the pathogen genotypes and the hosts is also continuously changing. As a result, severe epidemics may occur and directly impact such economic crops as potato and tomato. It is unknown how new genotypes collected from tomato will impact potato and *vice-versa*. This fact reveals the importance of studying the cross-pathogenicity of *P. infestans* isolates on its hosts for to better understand of the epidemics of late blight disease and how to predict such epidemics. Therefore, with the appearance of new genotypes, it is very important to know how they behave on both their host of origin as well as on alternative hosts. Such results will be helpful in predicting the impact of this disease and in improving disease control strategies.

The objectives of this study were to evaluate the aggressiveness of genotypes of *P. infestans* found in Canada and newer ones collected between 2009-2012, on their original and alternative hosts, namely potato and tomato. In addition, the aggressiveness of newly-introduced genotypes will be assessed on potato tubers to complete the picture of the potential impact of new pathogen populations on potato production.

3.3 Materials and methods

3.3.1 Whole plant inoculation

3.3.1.1 Plant material

The potato cultivars, Russet Burbank (RB, susceptible) and Kennebec (K, moderately resistant), tomato cultivars, Sun Rise (SR, susceptible) and Ultra sweet (US, moderately resistant)

were planted in clay pots filled with mixture of soil- sand- peat and perlite (4:4:4:1) and kept in a growth room at 20 ± 2 °C and 16 h photoperiod.

3.3.1.2 *Phytophthora infestans* isolates and inoculum preparation

The *P. infestans* isolates were used in this experiment were illustrated in Table 3.1. The isolates were grown on rye agar medium, which contains 60g of rye grain boiled for 1h. The filtered supernatant was mixed with 15g of Agar, 20g of sucrose and 0.05g of b-sitosterol. The volume was adjusted to 1L with distilled water. The isolates were kept in rye medium under room temperature for 5-7 days. For inoculum suspension preparation, a piece of growing mycelium on the rye media was transferred into clarified V8 medium prepared following the method described by Fry protocol . Briefly, 150 ml of V8 juice was mixed with 1.5g of calcium carbonate CaCO_3 . The mixture was stirred until CaCO_3 is dissolved. To clarify the media, the mixture was spun at high speed in the centrifuge. Fifteen g of agar was added to the supernatant and adjusted with water to 1L. Plates were kept in room temperature about 5 to 7 days - depending on the growth speed- until the growth reaches 75% of the plate surface.

Inoculum suspension was prepared for each isolate by “smashing” the mycelium with sterile water using sterilized glass stick. The free mycelium plates were kept overnight to allow the sporangia to germinate and maintain the desirable amount of inoculum. The germinated sporangia were harvested in the following day using Sato’s solution, which consist of 40mL 0.25M HEPES (5.95g/100ml), 20mL 5mM MgSO_4 (0.62g/500ml), 40mL 10mM CaCl_2 (0.55g/500ml) in 1L ultrapure 18Mohm (Sato 1994). Six-week growing plants were inoculated with sporangial suspension of 10^4 sporangia/mL, and with water for non- inoculated control plants following the method described by (Wang et al. 2004). The second, third and fourth leaves from the top of each

plant were inoculated by placing 100 µl of isolate suspension distributed into 10 µl droplets on the leaf surface. Inoculated plants were kept in misting chambers with 100% humidity for 48 h (Wang et al. 2004), with 3 replicates per treatment, and the experiment was repeated two times.

3.3.2 Tuber inoculation

Two-millimetre slices of surface-sterilized Kennebec and Russet Burbank potato tubers were used to assess the aggressiveness of different *P. infestans* isolates. One hundred microliter of 10^4 sporangia/ml suspension was placed in the center of each potato slice, which was incubated in misted Petri dishes for 5 days in the dark. Control slices were inoculated using sterilized water. There were six replicates per treatment, and the experiment was repeated two times.

3.3.3 Disease assessment

Based on the disease progress, the assessment was designed to be at 3, 5, 7 days post-inoculation (dpi). Lesions of each inoculated leaf were measured individually using the Assess 2.0 software (Lamari 2008). Measurement of percent infection was determined by calculating the percentage of the lesion area of each leaf over the total area of the leaf. A total number of 12 leaves for each treatment, at different time points over time, were used to calculate the Area Under Disease Progress Curve (AUDPC), which allows estimation of the overall accumulated percent infection for each treatment.

Percent infection on potato tuber slices was estimated using the same software. Infection on six slices of each treatment was assessed 5dpi.

3.3.4 Statistical analysis

Statistical analyses were performed using PROC MIXED with Statistical Analysis Software (SAS) (SAS Institute, Cary, NC, USA; 9.1 for Windows). PROC UNIVARIATE was used to check the normality of the data. The outliers (unfit values) were removed based on residuals comparison to critical values for studentized residuals (Lund 1975). Normality of the data was also confirmed by checking Shapiro–Wilk test. Mean values were separated using least squared means and letters assigned by the macro PDMIX800.sas (Saxton 1998) with $\alpha = 0.05$.

Table 3.1 *P. infestans* isolates sampled from potato/ tomato used in this study.

<i>Isolate No</i>	<i>Genotype</i>	<i>Mating type</i>	<i>Host of origin</i>	<i>Year of isolation</i>	<i>Location/Province of origin</i>
<i>D1-9-01d</i>	US-8	A2	potato	2001	MB
<i>Pi2-1-09</i>	US-11	A1	Potato	2009	MB
<i>661</i>	US-24	A1	Potato	2010	MB
<i>688</i>	US-24	A1	Potato	2010	MB
<i>3.3.11</i>	US-24	A1	Potato	2011	MB
<i>4.3.11</i>	US-24	A1	Potato	2011	MB
<i>5.1.11</i>	US-24	A1	Potato	2011	MB
<i>10.1.11</i>	US-24	A1	Potato	2011	MB
<i>19.1.11</i>	US-24	A1	Potato	2011	MB
<i>17.1.11</i>	US-24	A1	Potato	2011	MB
<i>164man</i>	US-23	A1	Tomato	2011	MB
<i>6.1.11</i>	US-23	A1	Tomato	2011	MB
<i>067ON</i>	US-22	A2	Tomato	2011	ON

3.4 Results

Potato and tomato cultivars were inoculated with US-24 isolates obtained from potato infected tissues (611, 688, 3.3.11, 4.3.11, 5.1.11, 10.1.11, 19. 1.11 and 17.1.11), US-23 isolates obtained from tomato (164 man and 6.1.11), and one US-22 isolate from tomato (067ON). Two old genotype isolates obtained from potato were also included; US-11 (Pi2-1-09) and US-8 (D1-9-01d). Symptoms of late blight were visible for some isolates starting at 3dpi as small lesions. For others, the symptoms were either late or not visible in some tested cultivars.

3.4.1 Whole plant inoculation

3.4.1.1 Symptoms on potato

At three days after inoculation, symptoms were observed on inoculated leaves of the susceptible Russet Burbank cultivar. The highest value of AUDPC was obtained on both potato cultivars inoculated with three *P. infestans* isolates (17.1.11, 19.1.11 and 4.3.11) belonging to the US-24 genotype. All three isolates caused higher infection in Russet Burbank (susceptible, S) than Kennebec (moderately resistant, MR). These isolates were considered as highly aggressive. Pi 3.3.11 is another US-24 isolate that caused moderate infection on both potato cultivars. Very late symptoms and small lesions were observed on inoculated potato cultivars with isolate D1-9-01d (US-8 genotype). The US-24 isolates 661 and 688 caused very low to no infection, thus classified as weakly aggressive isolates. Tomato isolates that belong to the US-22 and US-23 genotypes as well as the tested US-11 isolate were weakly aggressive on potato cultivars, based on AUDPC values (Figure 3.1).

3.4.1.2 Symptoms on tomato

Overall, most of the isolates showed earlier symptoms on tomato cultivars compared to potatoes, and these symptoms appeared earlier in Sun Rise (susceptible, S) than in Ultra sweet (moderately resistant, MR). Starting at 2 dpi, late blight disease showed as dark spots on the susceptible plant leaves, at the area where the inoculum was placed. By 5 dpi, lesions enlarged to reach -for some isolates- more than 50% of the total leaf area. This severe infection was caused by the same US-24 isolates that were highly, moderately, or weakly aggressive on potatoes, including Pi 3.3.11, Pi 5.1.11 and Pi 10.1.11. The cultivar Ultra Sweet showed tolerance to the disease caused by all of the isolates more than the susceptible cultivars of both plant species.

Tomato isolates from the US-22 and US-23 genotypes, and the US-11 isolate, caused more symptoms with higher AUDPC values on the susceptible tomato cultivar compared with the moderately resistant cultivar, and to potato. Isolate Pi 6.1.11 was the only isolate sampled from tomato in 2011 in Manitoba. This isolate belongs to the US-23 genotype. Its virulence was obvious on the susceptible tomato cultivar sunrise, with the AUDPC value approximately fifteen times higher than the value calculated for the Ultra Sweet cultivar (Figure 3.2). Isolates D1-9-01d (US-8) and 067ON (US-22) caused higher infection only on the susceptible tomato cultivar.

3.4.2. Tuber inoculation

Kennebec and Russet Burbank inoculated tuber slices were evaluated for late blight infection at 5 dpi. There were obvious differences in the aggressiveness of *P. infestans* isolates on potato tubers, compared with the controls (Figure 3.3). Isolates from the US-24 genotype caused higher infection on Kennebec tubers than Russet Burbank, regardless of their level of aggressiveness. On the other

hand, isolates of genotypes US-8, US-11, US-22 and US-23 (tomato isolates) caused larger infection lesions on Russet Burbank compared to Kennebec (Figure 3.4).

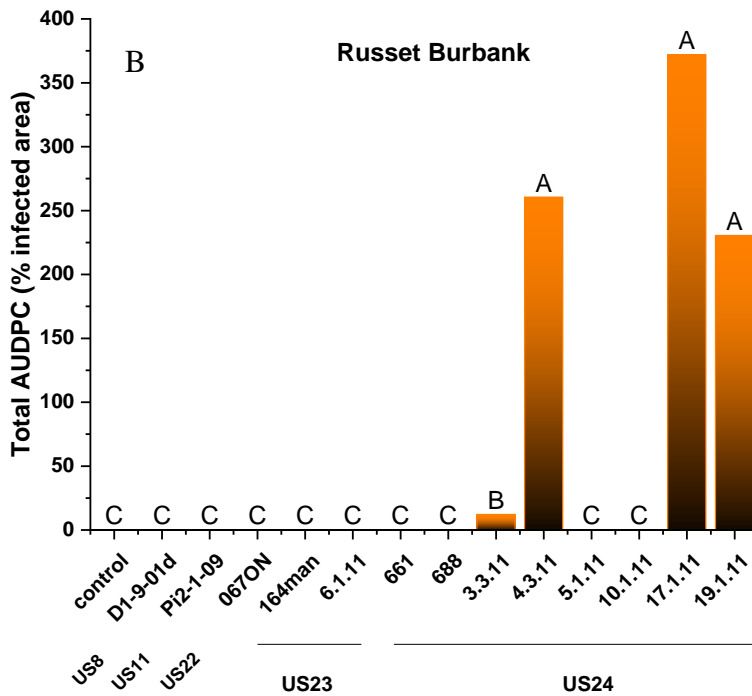
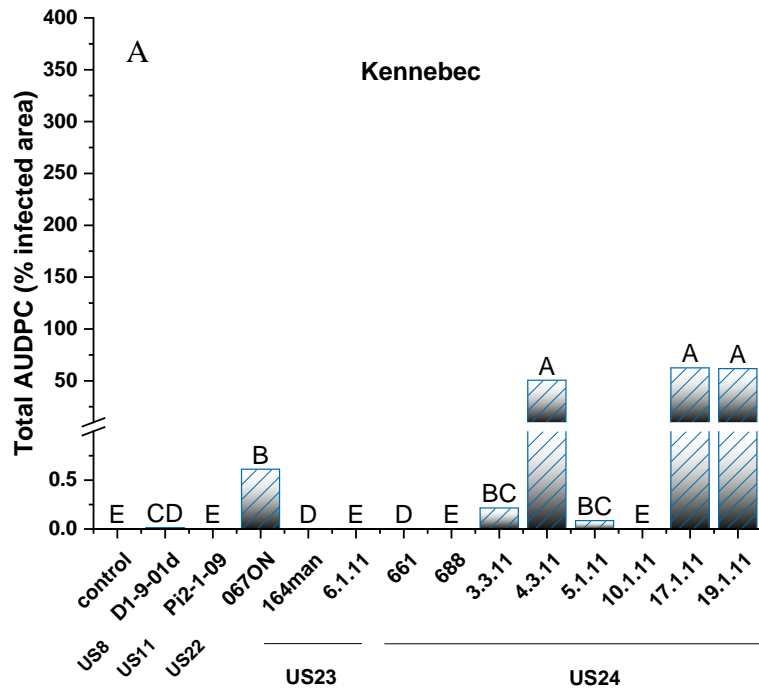


Figure 3.1 Total Area Under Disease Progress Curve (AUDPC) of percent of infection on potato cultivars. Kennebec (A) and Russet Burbank (B) inoculated with *P. infestans* strains from different genotypes. Strains from left to right are; D1-9-01d (US-8), Pi2-1-09 (US-11), 661 to 17.1.11 (US-24), 164man and 6.1.11 (US-23), 067ON (US-22). Isolates of US-24 (Pi4.3.11,

Pi17.1.11 and Pi19.1.11) showed significant high AUDPC on both potato cultivars. in Russet burbank, except the isolates (Pi3.3.11, Pi4.3.11, Pi17.1.11 and Pi19.1.11) no significant differences between the isolates of different genotypes and the control (inoculated plants with water). For isolates details refer to table 3.1

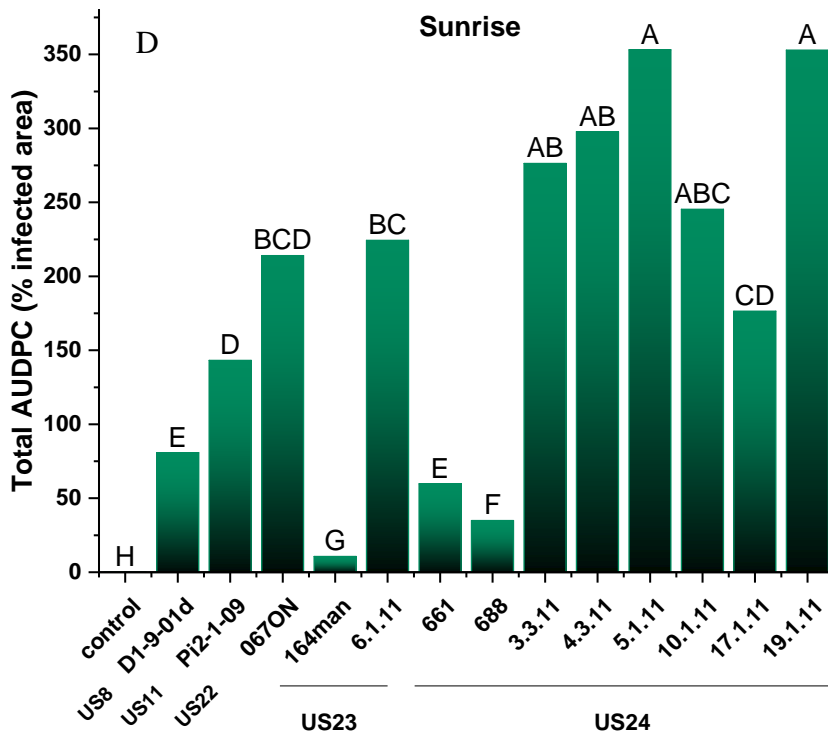
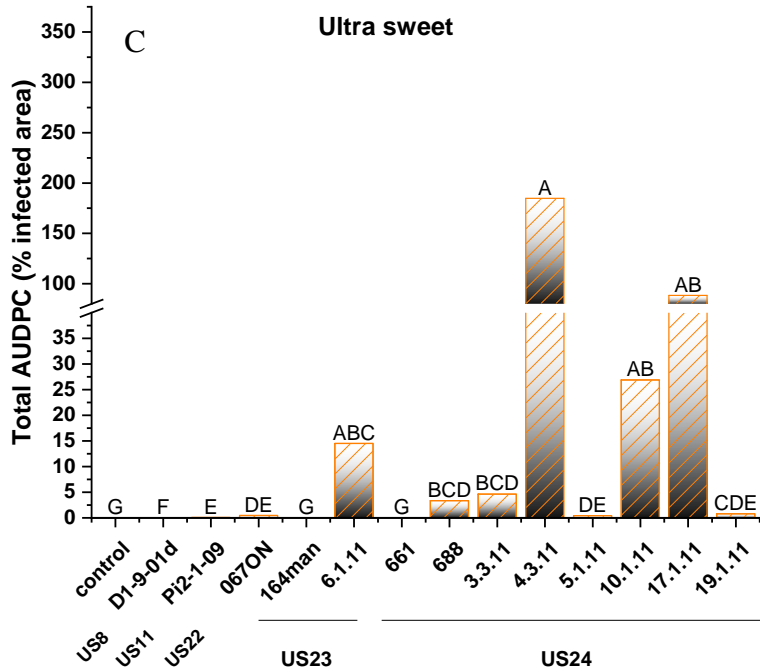


Figure 3.2 Total Area Under Disease Progress Curve (AUDPC) of percent of infection on tomato cultivars; Ultra sweet (C) and Sun Rise (D) inoculated with *P. infestans* strains from

different genotypes. Strains from left to right are; D1-9-01d (US-8), Pi2-1-09 (US-11), 661 to 17.1.11 (US-24), 164man and 6.1.11 (US-23), 067ON (US-22).

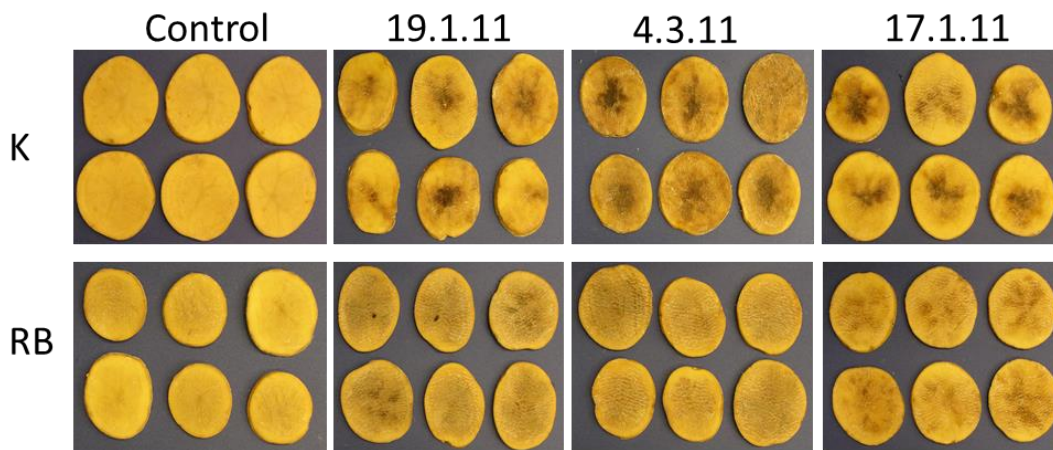


Figure 3.3 late blight in infected tuber slices at 5 dpi

Healthy (uninoculated control) tuber control and typical granular brown rot of internal tissue of Kennebec (K) and Russet Burbank (RB) infected tuber slices with different *P. infestans* isolates. 19.1.11, 4.3.11, and 17.1.11

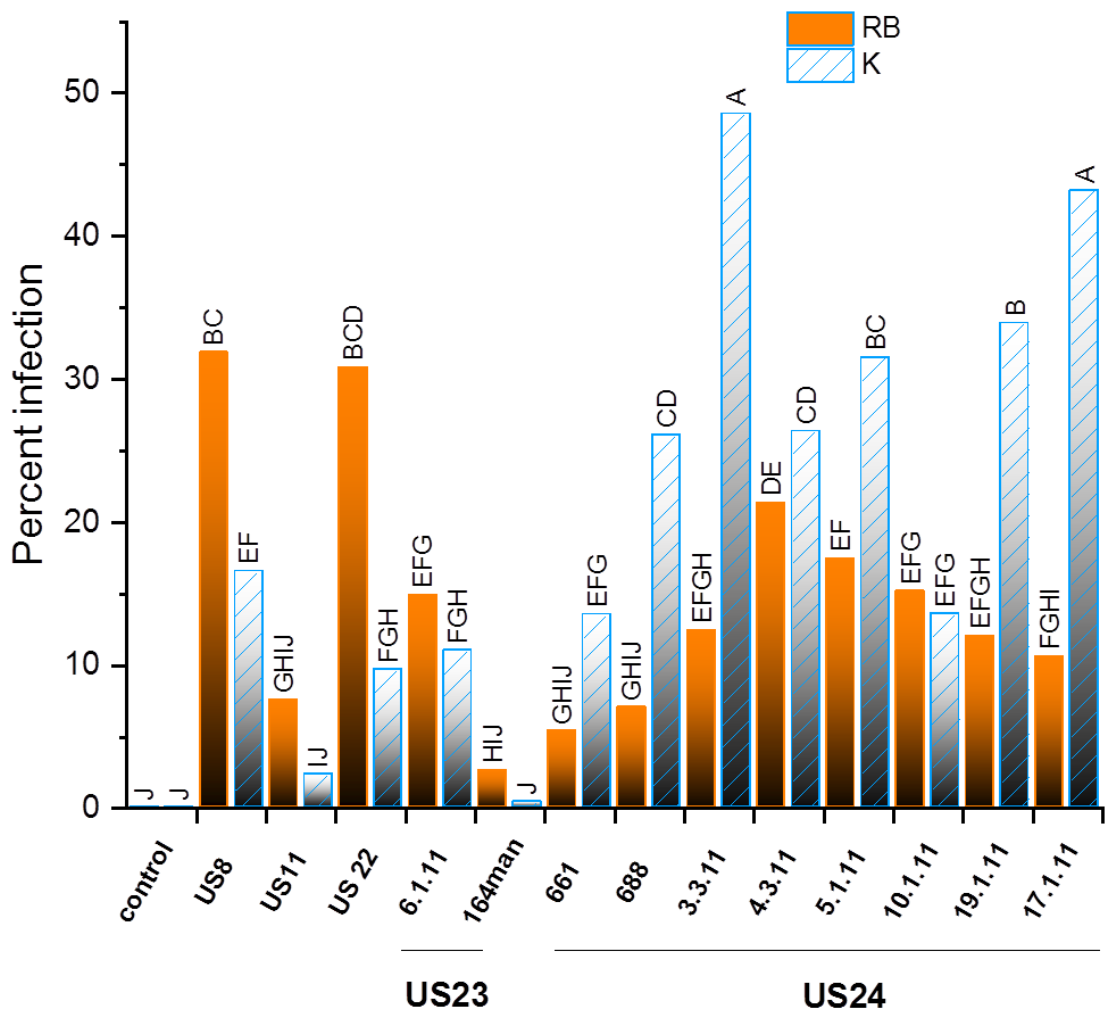


Figure 3.4 Assessment of percent infection of late blight on Kennebec and Russet Burbank potato tuber slices at the fifth day post-inoculation. Control (healthy tubers), Strains from left to right are; D1-9-01d (US-8), Pi2-1-09 (US-11), 661 to 17.1.11 (US-24), 164man and 6.1.11 (US-23), 067ON (US-22).

3.5 Discussion

Canadian populations of *P. infestans* changed rapidly during two periods of late blight epidemics. The first change was during the 1990s, where pathogen populations were composed of the single clonal lineage US-1 genotype (Goodwin et al. 1994a). By 1996, the more aggressive genotype US-8 predominated across most of the Canadian provinces (Peters et al. 1998). The second change was during the severe epidemics that occurred in 2009 due to strains which were genetically distinct from the previous *P. infestans* genotypes. Generally, in 2011, US-23 and US-24 were the most widely distributed genotypes among Canadian provinces, except Ontario, which was dominated by the US-22 genotype. Most US-24 genotype isolates were obtained from potato infected plants (Peters et al. 2014). On the other hand, US-23 was obtained from both potato and tomato (Peters et al. 2014). US-22 was an exceptional genotype, it was only isolated from infected tomato plants and its distribution was strictly in tomato growing areas (Peters et al. 2014). In 2012, US-23 has been the most dominant genotype in Canadian provinces (except Ontario) (Alkher et al. 2015). However, from 2013 to 2016, US-23 became the dominant genotype in all Canadian provinces. These dramatic population shifts have been accompanied by increased disease management problems due to the increase in aggressiveness and fungicide insensitivity of many of the new genotypes or some isolates of the same genotype.

In this study, isolates from genotypes US-8, US-11, US-22, US-23 and US-24 were tested for their pathogenicity on two potato and two tomato cultivars. The infection percentage was calculated in serial time starting at 3, 5 then 7 days post-inoculation. Cumulative areas under disease progress curve (AUDPC) were used to present the results of the pathogenicity of the tested isolates. In general, isolates belonging to the US-24 genotype were the most pathogenic ones in all tested cultivars. The significant disease infection was observed on Russet Burbank, Sun Rise and

Ultra Sweet with high AUDPC. Kennebec cultivar showed the lowest disease infection based on the AUDPC compared to other tested cultivars. The variation of aggressiveness was observed among the tested genotypes and between isolates from the same genotype. Determination of the cultivar response to late blight is helpful not only to define the resistance or the susceptibility of the cultivars but also it is meaningful for breeding and fungicide application programs (Razukas et al. 2008).

Several studies have reported differences in aggressiveness among isolates belonging to the same pathotype or sharing similar genotypes (Mundt 2002; Milus et al. 2006). For instance, isolates of the same pathotype of *Cochliobolus carbonum* showed significant differences and variation for infection efficiency, lesion length and sporulation capacity (Hamid et al. 1982; Pariaud et al. 2009). Variations in aggressiveness (lesion expansion rate, latent period, sporulation and infection efficiency) were also documented among 17 *P. infestans* isolates in a Northern Ireland population. These isolates share an identical multilocus genotype, including allozyme profiles and mtDNA haplotypes, belong to the same mating type and share the ability to overcome the specific R1 resistance gene, and have the same response to fungicides (Carlisle et al. 2002). These findings support our results, where we found the isolates PI688, 661 and 164man to be less aggressive even if they belong to the US-24genotype. This suggests that mutations leading to increased aggressiveness have rapidly accumulated within the phylogenetic lineages.

A significant variation was found between the isolates from old genotypes such as US-8, US-11 (D1-9-01d, Pi2-1-09), and those from newer genotypes. Isolates from old genotypes exhibited very weak aggressiveness on potato cultivars and on the moderately resistant tomato cultivar. However, the infection on the susceptible tomato cultivar sunrise was clearly visible,

especially in plants inoculated with US-8. Results of evaluating the pathogenicity of isolates from old genotypes (US-8, US-11), and the ones from newer genotypes (US-22, US-23 and US-24) conflict with previous research finding, where worldwide problem and notoriety on potato fields was created by the lineage US-8 (A2 mating type) (Kato et al. 1997; Lambert and Currier 1997). Significant severe infection, short latent period, and lesion area had been observed on potato cultivars when plants were inoculated with US-8 and US-7 compared to the ones inoculated with US-1 (Kato et al. 1997). At that time, US-8 and US-7 were new genotypes introduced to North America and replacement of US-1 by US-8 had occurred. There has been a similar displacement in North America more recently, where US-8, which is now considered as an old genotype was replaced by new genotypes US-24 or US-23 in 2011 and 2012 (Alkher et al., 2015). Thus, it may seem possible that differential pathogenic fitness has contributed to the predominance of the new clonal lineages.

Potato plants differentiate the level of aggressiveness based on the response of different plant parts to the pathogen (Peters et al. 1999). In this study, Russet Burbank potato cultivar exhibited a significantly low response to the disease infection on leaf surface caused especially by most US-24 isolates, compared to Kennebec. However, the opposite response was observed on Russet Burbank tuber slices inoculated with the same isolates. On the other hand, the foliage of Kennebec tolerated the blight more, compared to its tuber. This result is an agreement with (Bonde et al. 1940; Gallegly 1968), who reported a positive correlation between the resistance of potato foliage to infection by *P. infestans* and the susceptibility of the tuber. However, many exceptions to this rule (foliar resistance- tuber resistance) were also documented (Toxopeus 1958; Lapwood 1961; Eide and Lauer 1967; Stewart et al. 1983).

Susceptibility of some economically important crops to different diseases has been studied in detached leaf assays and field trials. (Platt and McRae 1990) determined the susceptibility of Russet Burbank inoculated with A1 isolates of *P. infestans*. This finding is supporting our result, where it confirmed that Russet Burbank was more susceptible to the isolates compared to Kennebec and tomato cultivars. (Legard et al. 1995) examined the susceptibility of different tomato cultivars to late blight. Both detached leaf and field experiments indicated that Sunrise is more susceptible than Pik Rite. This information supports our results and suggests ranking Russet Burbank and Sun Rise as susceptible cultivars. Our finding provides information essential for cultivar selection and the planning of effective disease control strategies. By using tolerant cultivars, it becomes possible to reduce the inoculum overtime, which means reducing the genotype population in the productive areas.

Aggressiveness of isolates originating from potato on other hosts has been reported in different pathosystems. Cross-pathogenicity of *Verticillium dahliae* between potato and sunflower using isolates originating from potato and sunflower, showed that potato isolates were more aggressive on both hosts (Alkher et al. 2009). Similar results obtained when the same *V. dahliae* potato and sunflower isolates infect olive varieties. More wilting was observed on olive trees inoculated by potato isolates than the sunflower ones (Gharbi et al. 2015). It is not clear why potato isolates of different pathogens tend to cause more disease and exhibit more aggressiveness on other hosts compared with the isolates that originated from those plants. This suggests that there might be an enhancement by potato plant substances or elements to increase the fitness of these pathogens.

Increasing the aggressiveness of *P. infestans* isolates with an increase of fungicide resistance has been reported (Davidse et al. 1981; Deahl et al. 1993; Gisi and Cohen 1996; Vega-

Sánchez et al. 2000; McLeod et al. 2001). For instance, US-8 and US-11 isolates were the most aggressive isolates on potato and tomato plants. Both genotypes showed some level of resistance to metalaxyl (Hu et al. 2012). Mefenoxam, the active R-isomer of metalaxyl, has been used lately to control late blight disease. Saville et al. (2015) concluded that only US-8 and US-11 isolates are insensitive to mefenoxam while all other genotypes including US-20, US-21, US-22, US-23, and US-24 were sensitive. This indicated that fungicide resistance does not necessarily associate with the level of aggressiveness of *P. infestans* isolates.

It had been shown by different studies that pathogen populations isolated from a given cultivar in the field are more aggressive on this specific cultivar than others (Ahmed et al. 1995; Bhat and Subbarao 1999; Resende et al. 2006; Alkher et al. 2009). Our results concur with the above studies. We observed that *P. infestans* strains isolated from potato and that belong to the same genotype (US-24) are more aggressive on potato than the isolates obtained from tomato (US-22 and US-23). Similar results were observed on tomato plants infected by potato isolates. Our result with *P. infestans* tomato isolates strengthen the evidence, where they caused more infection on tomato cultivars and less to none on Kennebec and Russet Burbank potato cultivars, respectively. Although the results are in agreement with the theory of higher aggressiveness on the host of origin, we cannot confirm it, as other research showed contrasting results, where they either had mixed results or found no evidence for quantitative adaptation to the host of origin (Jeffrey et al. 1962; Zhan et al. 2002; Daayf and Platt 2003).

In fact, the “host of origin” phrase should be used with caution since it is practically impossible to know how many generation a particular isolate or genotype spent in that host. Moreover, large numbers of isolates should always be included in this type of study to ensure they present the original population properly. Another point we should mention is that plant growth

stages should be comparable among the comparative studies, since a differential effect between seedling and adult plants was also reported (Milus and Line 1980; Zhan et al. 2002).

Goodwin et al. (1992) showed that *P. infestans* potato and tomato isolates are genetically distinct. In addition, the distribution of *P. infestans* genotypes in Canada during 2010-2013 showed that US-22 is a dominant genotype in Ontario (Alkher et al., 2015), which is the largest tomato producer in Canada, and this particular genotype was isolated only from tomato. This suggests that specificity of this genotype to tomato occurred. However, the lesser developed tomato industry in provinces other than Ontario does not allow validation of this theory.

Based on this and previous studies, we can conclude that there are several factors that can have an effect on the aggressiveness and the variation of *P. infestans* including mutation, fungicide sensitivity, host of origin, different plant response, variation among genotypes and variation within isolates of the same genotype. Such information on each individual factor is essential and will be added to each other to understand more about the pathosystem and to improve the epidemiological strategies of late blight disease on potato and tomato.

**CHAPTER 4: DIFFERENTIAL EXPRESSION OF PUTATIVE
PHYTOPHTHORA INFESTANS EFFECTOR GENE DL119 DURING THE
INTERACTION WITH POTATO AND TOMATO CULTIVARS**

4.1 Abstract

Phytophthora infestans delivers an arsenal of effectors to manipulate plant processes. Knowing the expression level and phase (biotrophic or necrotrophic) of effector expression is a critical starting point to determine effector genes that are relevant to the virulence of *P. infestans*. It also provides better understanding to how these effectors could reflect the behaviour of this pathogen during its interaction with different hosts. In this study, five putative effector genes (DL32, DL119, DL95, DL142 and DL31) were tested for their expression during the *P. infestans*-host interaction using qRT-PCR. The interaction included *P. infestans* from the US-24 genotype, with three highly aggressive isolates (Pi19.1.11, Pi17.1.11, Pi4.3.11), one moderately aggressive isolate (Pi3.2.12), and two weakly aggressive isolates (Pi10.1.11 and Pi688) that were inoculated on potato (Kennebec, Russet Burbank) and tomato (Ultra Sweet, Sun Rise) cultivars. Gene expression was evaluated at 3 (biotrophic) and 6 (necrotrophic) days post-inoculation. Among the five effector genes tested, only DL119 was expressed in all the treatments at both time points. The expression of DL119 was significantly higher in isolates infecting tomato than potato cultivars, regardless of their level of aggressiveness. We also observed a similar expression trend among time points in most interactions. The expression peaked at 3dpi, which represent the biotrophic phase then regressed at 6 dpi, the necrotrophic phase. No expression of DL119 was detected in Russet Burbank inoculated with highly aggressive isolates. The weakly aggressive isolate Pi688 showed an expression of this gene only on tomato cultivars. The results in this study linked the importance

of DL119 in the performance of *P. infestans* isolates and its effect on the interaction with potato and tomato cultivars, thereby adding to the evidence of the potential role of DL119 as an effector gene in the interaction of *P. infestans* with its hosts.

4.2 Introduction

Phytophthora infestans (Mont.) de Bary is one of the most pathogenic oomycetes that has devastating effects on crops worldwide. It is the causal agent of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) late blight, which has been a well-known disease since the 1840s, when it caused the Irish potato famine (Austin Bourke 1964; Fry 2008). The disease causes significant losses in potato and tomato productions (Fry 2008; Haverkort et al. 2008; Nowicki et al. 2012). The epidemics of late blight occur either clonally or sexually (Fry 2008). Sporangia, produced by asexual reproduction, are the major inoculum source of epidemics and distribution of this pathogen. It is more common in South and North America, Africa, Asia, and parts of Europe. The sexual reproduction was initially limited to some areas of Mexico (Runno-Paurson et al. 2009, 2016; Yuen and Andersson 2013). This limitation of sexual reproduction no longer applies only to the Toluca Valley in Mexico because evidence of recombination has been reported in other parts of the world, including the United States and Canada (Kalischuk et al. 2012; Danies et al. 2014; Peters et al. 2014).

The persistent diversity of *P. infestans* population and the consistent damages they cause are indicative of the adaptive ability of the pathogen and its capacity to overcome the many barriers and defence mechanisms of the host plant. Over the last 15 years, much progress has been made in the field of plant-microbe interactions including the discovery of genes involved in the pathogen's virulence. In the oomycetes, virulence genes encode small molecules and proteins known as effectors, which are capable of modifying host cell structures or functions (Anderson et al. 2015). *P. infestans* secretes the largest number of effectors, reaching up to 563 genes, compared to other *Phytophthora* species. Effector proteins are either apoplastic or cytoplasmic. Apoplastic effectors are secreted into the extracellular space of the host plant. The role of the apoplastic

effectors is to interfere with the plant defense proteins located in the extracellular space. Glucanases, serine and cysteine proteases are considered as apoplastic proteins that act as inhibitors of plant hydrolases (Rose et al. 2002; Tian et al. 2006; Damasceno et al. 2008). Cytoplasmic effectors account for the majority of proteins secreted into plant cells. They are characterized by a signal peptide that is a N-terminal region responsible for secreting and translocating the effectors inside the plant cells. The C-terminal region is involved in the protein function and activity (Kamoun 2006; Morgan and Kamoun 2007). The unique feature of cytoplasmic effectors is a conserved motif in their N-terminal region. This motif is defined as RXLR, which is the amino acid sequence of Arg-(X, any residue)-Leu-Arg. It is reported that the RXLR motif has a role in translocating the effectors inside host cells but is not involved in the function of the effectors (Whisson et al. 2007; Dou et al. 2008). Some RXLR effector genes of *P. infestans*, with their avirulence function, were predicted, including Avr3a, Avr4, AVRblb1/ipi0 (Haas et al. 2009), Pi04089 and Pi0431/RD24 (Boevink et al. 2016).

Like other hemibiotrophic pathogens, *P. infestans* exhibits distinct phases of its life cycle: an early asymptomatic biotrophic phase and a later necrotrophic stage. In the early stages of infection, including spore germination, appressoria and haustoria formation, the pathogen proliferates asymptotically in the host by suppressing programmed cell death (PCD) or preventing host defense response. However, in later stages of infection, the pathogen undergoes a physiological transition from biotrophic growth to a distractive necrotrophic phase (Lee and Rose 2010). During *P. infestans*-host interaction, differential expression of some effector genes of the pathogen was observed during biotrophic and necrotrophic phases. Increase in expression of some effector genes was recorded during appressoria formation stage (Vetukuri et al. 2017). However, the expression of other genes was boosted later in the biotrophic phase, such as Pi14054, which

expressed at 36 h after infection (Hass et al. 2009; Vetukuri et al. 2017). Other effectors including NPP1, which acts as a toxin, expresses during the necrotrophic phase (Qutob et al. 2002; Bos et al. 2006), indicating that effector expression can be stage-specific during *P. infestans* interaction with its host plants.

In a previous study (chapter 3, unpublished data), we showed that among all isolates collected, those belonging to the US-24 genotype were the most aggressive on potato and tomato, compared to those belonging to other tested genotypes. Severe infection on tomato susceptible cultivars was caused by most isolates, particularly US-24. This variation in aggressiveness among the isolates in different plant species as well as on cultivars within each species could be related to different factors, including pathogen virulence genes and plant susceptibility/ tolerance factors.

Following our lab's efforts to characterize the factors involved in the different levels of strains' aggressiveness, we tested five transcript-derived fragments (TDFs) of *P. infestans* revealed by (Henriquez and Daayf 2010) for their expression in highly and weakly aggressive isolates. These fragments had been shown to express only in the interaction of highly aggressive isolate with a susceptible potato cultivar, as opposed to other treatments involving a weakly aggressive isolate and a tolerant potato line. It was then suggested that these fragments may act as potential pathogenicity factors of *P. infestans*.

Our hypotheses are that, i) The expression of TDFs in highly aggressive isolates of US-24 is greater than in the weakly aggressive isolate during their interaction with susceptible and moderately resistant cultivars of potato and tomato. ii) The expressions vary in the different phases of *P. infestans* (biotroph/necrotroph) infection.

4.3 Materials and methods

4.3.1 *P. infestans* genes selection and primers design

P. infestans genes were chosen based on the results of (Henriquez and Daayf 2010) (Table 4.1). Seven transcript derived fragments were only present in the compatible interaction of *P. infestans* and susceptible potato cultivar. The National Center for Biotechnology Information (NCBI) database was researched to determine any similarity of these fragments to other pathogenesis genes. Blast results of the seven fragments showed similarity to several genes that contain the RXLR motif of *P. infestans* T30-4 strain genome (Table 4.1). Some of these genes were tested for their expression on selected isolates of *P. infestans*

Primers were designed for each gene using GenScript Real-time PCR (TaqMan) Primer Design. The size ranged from 150-200 bp with an annealing temperature of 58-60° C. In addition, primers specific for elongation factor gene piEF2 as housekeeping gene of *P. infestans* (Belhaj et al. 2017) (Table 4.2) were used in this study.

4.3.2 Plant growth, *P. infestans* isolates selection and inoculation process

The initial experiment was conducted using only one highly aggressive isolate (Pi19.1.11), and one weakly aggressive isolate (Pi10.1.11). Both isolates are of the A1 mating type and belong to the US-24 genotype. The purpose of this experiment was to elucidate the stability of the aggressiveness levels of these isolates by measuring the percent of infection and to design the time points for gene expression experiment.

Potato cultivars (Kennebec, Russet Burbank), and tomato hybrids (Sun Rise and Ultra Sweet) were grown in 15 cm clay pots filled with a mix of soil- sand- peat and perlite (4:4:4:1) and kept in a growth room for 6 weeks at 20±2 °C and 16 h photoperiod.

P. infestans isolates Pi19.1.11 and Pi 10.1.11 were re-incubated on clarified V8 medium after they have been in rye agar media. The growth of each colony takes about 5-7 days to fill 75% of the plate. Mycelium was “smashed” from the media surface using distilled water. Propagated sporangia were harvested using Sato’s solution (Sato 1994) and then used to prepare the pathogen suspension. Final concentration of the pathogen suspension was 2 X10⁴ sporangia/ml. The inoculation process was done by applying 100µl of the suspension (~ 10 µ/ drop) on the 2nd, 3rd, and 4th top plant leaves. The inoculated plants were then incubated in the misting chambers with 100% humidity for 2 days then moved out in the growth room for disease assessment. Samples in each time point (1, 2, 3, and 6 days post-inoculation) were detached and kept at -80 °C for RNA extraction.

4.3.3 Disease assessment and tissue sampling

The symptomatic leaves of late blight were collected at 1, 2, 3, and 6 dpi and then photographed along with non-infected plant leaves (control). Percent of infection of inoculated plants with Pi19.1.11 and Pi10.1.11 were assessed using Assess2.1 software (Lamari 2008).

4.3.4 RNA extraction and cDNA synthesis

Total RNA was extracted from all treatments (non-inoculated plant control/ pathogen mycelium/ Kennebec + Pi10.1.11/ Kennebec + Pi19.1.11- Russet Burbank + Pi10.1.11/ Russet Burbank + Pi19.1.11- Sun Rise + Pi10.1.11/ Sun Rise + Pi19.1.11- Ultra Sweet+ Pi10.1.11/ Ultra Sweet + Pi19.1.11), using a RNeasy Plant Mini kit from QIAGEN (cat. Nos. 74903 and 74904). 100 mg of

plant material at 1, 2, 3, and 6 dpi was required for RNA extraction based on the kit protocol. The quantity and the quality of total RNA were analyzed using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). The DNase-treated RNA was reverse transcribed using a Clontech kit (SMARTTM MMV Reverse Transcriptase).

4.3.5 Quantitative Real-Time PCR

Quantitative Real-Time RT-PCR was achieved as described in the protocol of PowerUp SYBER Green Master Mix (ThermoFisher Scientific, USA) using a CFX96 Thermal Cycler (BioRad, Hercules, CA, USA). Each reaction of qPCR was carried out in 10 μ l that include 2 μ l of cDNA, 5 μ l Super mix (ThermoFisher Scientific, USA), 1 μ l of each primer (Table Figure 4.2), and 1 μ l of nuclease-free water. The following qPCR cycling program was used for all sets of primers: the thermocycler program included activation 50 °C (2 min), followed by polymerase activation 95 °C (2 min), 40 cycle of denaturation 95 °C (15 sec) and annealing 60 °C for 1 min. The data were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Based on the initial experiment of disease assessment and gene expression obtained with Pi10.1.11 and Pi19.1.11, other highly and weakly aggressive *P. infestans* isolates were included in this study to test the expression of five putative effector genes. Time points after infection were also minimized to only 3 and 6 dpi because of the lack of amplification at 1 and 2 dpi in the initial experiment. The RNA extraction and qPCR quantification procedures were followed as described for the initial experiment. Treatments of gene expression experiment with six *P. infestans* isolates are summarized in Table 4.3.

4.3.6 Statistical analysis

Statistical analyses were performed using PROC MIXED with Statistical Analysis Software (SAS) (SAS Institute, Cary, NC, USA; release 9.1 for Windows). PROC UNIVARIATE was used to check the normality of the data. The outliers (unfit values) were removed based on residuals comparison to critical values for studentized residuals (Lund, 1975). Normality of the data was also confirmed by checking Shapiro–Wilk test. Gene expression data were normalized by log10 transformation. Mean values were separated using least squared means and letters assigned by the macro PDMIX800.sas (Saxton, 1998) with $\alpha = 0.05$.

4.4 Results

4.4.1 Disease assessment

Disease assessment was only carried out for the initial experiment for plants inoculated with Pi10.1.11, and Pi19.1.11.

Control and inoculated potato and tomato plants using isolates Pi10.1.11 and Pi19.1.11 were assessed for disease development at 1, 2, 3 and 6 dpi using the Assess2.1 software. No visible symptoms appeared 1 and 2 dpi either in potato or in tomato inoculated plants. At 3 dpi, the symptoms started showing, but with no significant differences among all cultivars. Significant differences started showing among the treatments at 6 dpi. Pi19.1.11 caused a higher infection on both potato cultivars, with more in Russet Burbank than in Kennebec. No significant differences were seen on the cultivars infected with the weakly aggressive isolate Pi10.1.11. However, percent

infection was significantly different among treatments where plants were inoculated with the different isolates (Figure 4.1, A).

Consistent results were recorded on the tomato hybrids inoculated with either the highly or the weakly aggressive isolate. Pi19.1.11 caused more disease on the susceptible tomato cultivar, Sun Rise, which showed two times lesion size than on the potato susceptible cultivar Russet Burbank. The same isolate caused less infection on the moderately resistant cultivar, Ultra Sweet, which showed an equal infection as Sun Rise inoculated with the weakly aggressive isolate (Figure 4.1, B).

4.4.2 Reverse transcriptase and qRT-PCR analysis

The expression of some genes at different time points was assessed on an agarose gel. Among five tested genes, DL119-ssor (PITG_12737.2) was the only gene expressed in all treatments. However, clear bands were detected only at 3, 6 dpi. Quantitative real time PCR showed similar results, where no amplifications were detected for DL142, DL 20, DL31, DL32 genes. Due to the low biomass of *P. infestans* isolates in infected tissue at early stages, only 3 and 6 dpi were kept for analysis. These initial results indicated the activity of DL119 compared with other genes during *P. infestans* and host interaction.

Confirmation of the involvement of DL119 in pathogenicity required the inclusion of more isolates, which varied in their aggressiveness on potato and tomato. As a result, another four isolates (Pi17.1.11, Pi 3.4.11, Pi 3.2.12, and Pi688) were tested to detect the expression of the effector gene during the interaction with host plants. Due to the variation of the expression of DL119 in the different isolates during their interaction with either potato or tomato at different time points, fold changes of the gene were analyzed for each isolate-host interaction individually.

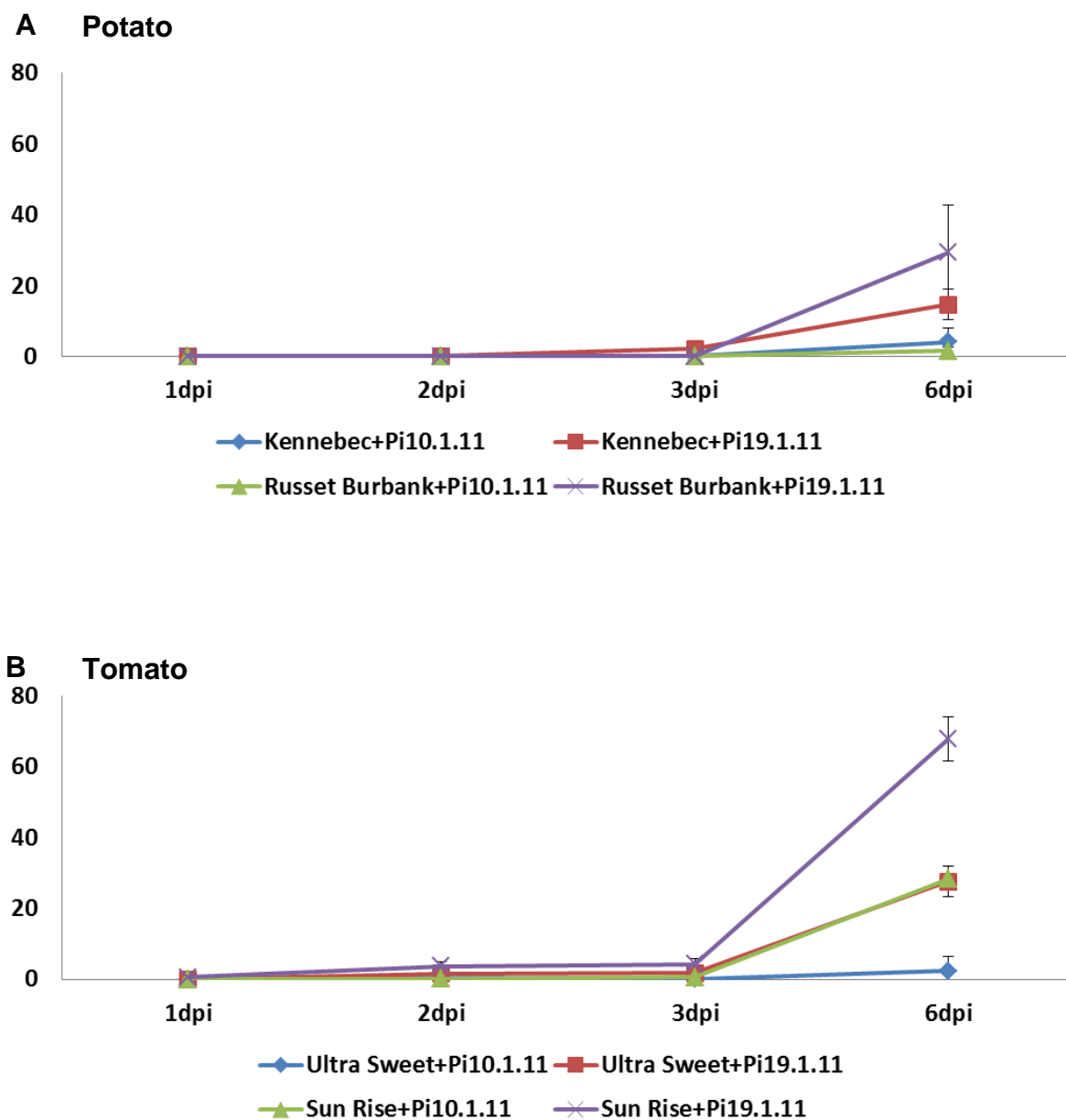


Figure 4.1 Percent infection (foliar lesions) of inoculated potato and tomato cultivars with a weakly aggressive isolate Pi10.1.11 and a highly aggressive isolate Pi19.1.11 in different time points (1dpi, 2 dpi, 3dpi and 6 dpi). A) Russet Burbank potato susceptible cultivar and Kennebec moderately resistant cultivar with weakly aggressive isolate Pi10.1.11 and highly aggressive isolate Pi19.1.11 and B) Sun Rise tomato susceptible cultivar and Ultra Sweet moderately resistant cultivar with Pi10.1.11 and Pi19.1.11.

4.4.3 Expression of DL119 in *P. infestans*-potato and tomato interactions

Regardless of the time points (3 and 6 dpi), the expression of DL119 was significantly different among all potato and tomato cultivars at ($P < 0.05$). Isolates of Pi19.1.11, Pi17.1.11 and Pi4.3.11 are highly aggressive on potato and tomato. They caused severe symptoms on both plant species, while Pi10.1.11 and Pi688 are weakly aggressive isolates on potato but they caused significant infection on tomato compared to potato. Another isolate (Pi3.2.12), which is mildly aggressive on both plant species, was also included. The isolate Pi19.1.11 had the lowest level of expression of DL119 during the interactions compared to other isolates. In potato, the expression was only detected on Kennebec (K), the moderately resistant potato cultivar at 6 dpi, however, the gene expression was detected at both time points in the tomato cultivars. In the early stage, the expression started high during the interaction of Pi19.1.11 with tomato cultivars, slightly higher in the susceptible than the moderately resistant one. After 3 dpi, the expression was reduced to the half-fold at 6dpi as it was in the early stage. Significant reduction was detected in the susceptible tomato cultivar Sun Rise (SR) (Figure 4.2, A). We had observed similar expression trends in all other isolates except the weakly aggressive isolate Pi10.1.11 during its interaction with the moderately resistant potato cultivar. No expression of DL119 was detected in the isolate Pi17.1.11 in the interaction with Russet Burbank (RB), the susceptible potato cultivar. However, during the interaction with Kennebec, the expression was higher at 3dpi, and then it decreased slightly at 6dpi. During the interaction of Pi17.1.11 with the susceptible tomato cultivar, the gene showed significant high induction at 3dpi compared to 6dpi as well as its expression in the moderately resistant tomato cultivar Ultra Sweet (US) at both time points (Figure 4.2, B). Similar induction of the gene was observed during the interaction of the isolates Pi4.3.11 with potato cultivars, where the expression was detected only with Kennebec in both time points. Interestingly, among the

tested isolates, Pi4.3.11 was the only isolate that had higher expression in the moderately resistant tomato line than in the susceptible line at 3 and 6 dpi (Figure 4.2, C). Only at later stage of infection (6dpi), the isolate Pi3.2.12 induced the gene in potato. However, with the tomato lines the isolate expressed the gene at both time points, higher at 3dpi than at 6dpi (Figure 4.2, D). Remarkably, Pi10.1.11 and Pi688, which are weakly aggressive isolates, had higher expression of DL119 compared to the highly aggressive isolates, Pi19.1.11 and Pi3.2.12. During the interaction of Pi10.1.11 with potato and tomato susceptible cultivars, the expression was higher at 3dpi than at 6dpi. On the other hand, increase in the expression toward 6dpi was observed during the isolate interaction with the moderately resistant cultivars of both plant species (Figure 4.2, E). No expression of DL119 was detected in the weakly aggressive isolate Pi688 during the interaction with potato cultivars, however, significant induction was observed in Pi688 in the interaction with Sun Rise (SR). This expression was two-folds higher at 3dpi compared to the expression at 6dpi (Figure 4.2, F).

Table 4.1 RxLR effectors of *P. infestans* identified based on the blast similarity with transcript derived fragments (TDFs) from Henriquez and Daayf (2010).

Differentially expressed TDFs (ssor) as reported by Henriquez and Daayf, 2010		TDFs Show similarity with the RxLR effectors of <i>P. infestans</i> T30-4 genome database	
<i>Accession number</i> ^a	<i>TDFs</i> ^b	<i>Locus number</i> ^c	<i>Gene name</i>
GH456578.1	DL32-ssor	PITG_13959.2	Secreted RxLR effector peptide
GH456575.1	DL119-ssor	PITG_12737.2	Secreted RxLR effector peptide
		PITG_04050.1	Secreted RxLR effector peptide
GH456580.1	DL95-ssor	PITG_12761.2	Secreted RxLR effector peptide
GH456571	DL12-ssor	None	None
GH456572	DL142-ssor	PITG_09773.1	Secreted RxLR effector peptide
		PITG_09689.1	Secreted RxLR effector peptide,
		PITG_13072.2	Secreted RxLR effector peptide
		PITG_07435.1	Secreted RxLR effector peptide
GH456588	DL31-ssor	PITG_04350.1	Secreted RxLR effector peptide
		PITG_21984.2	Secreted RxLR effector peptide
		PITG_22871.1	Secreted RxLR effector peptide
		PITG_07954.1	Secreted RxLR effector peptide
		PITG_06076.1	Secreted RxLR effector peptide
GH456591	DL20-ssor	PITG_14062.2	RxLR effector family peptide
		PITG_21190.2	Secreted RxLR effector peptide

a: accession number of the genes as reported in NCBI database

b: TDFs (ssor): potential pathogenicity factor

c: Locus number and name of similar genes to TDF (ssor) according to the blast of NCBI database

Table 4.2 Designed primers for RXLR genes.

Primer name	TDFs ^a	Accession # ^b	Sequence 5'-3'
PITG_09773	DL142	GH456572	FP/ CTTCGCTGTGATCGCTGCAG
			RP/ AGTGTTCTTTCGTGAGACCCC
PITG_12737	DL119	GH456575.1	FP/ GCTTTGGAGCTGGCTACGCT
			RP/ TCCCCCACCTTCTTGA ACTTGT
PITG_13959	DL32	GH456578.1	FP/ AGTGAGTTGAACGTGGATGTTTAC
			RP/ GAACTCCTTATCGCGGTAAGATGTA
PITG_21190	DL20	GH456591	FP/ ATCGATGCAGAACAGCGGGTC
			RP/ GCGTTGTCTTGACGTAGTCATCAG
PITG_21984	DL31	GH456588	FP/ GACAACGACAACGATGAAGAGAGG
			RP/ GCGTTAGAACATTTTCCGCGCCA
piEF2 ^c			FP/ CACCGTGGTATGGGCCAGAT
			RP/ ATCAGAGCAGGCTCCGACAC

a: transcript derived fragments as indicated by Henriquez and Daayf 2010

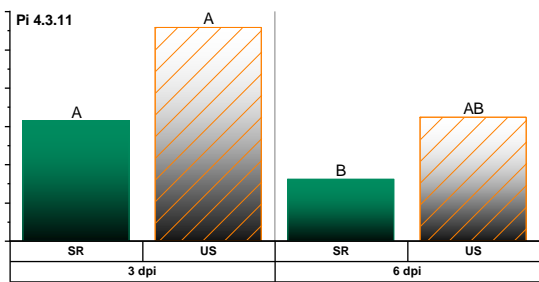
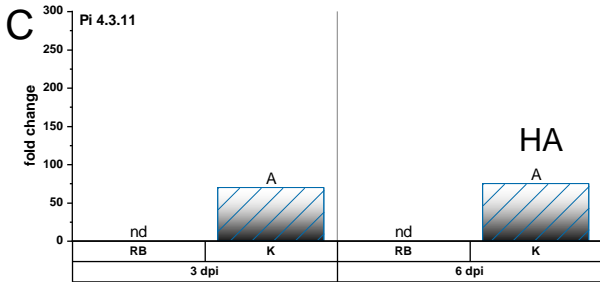
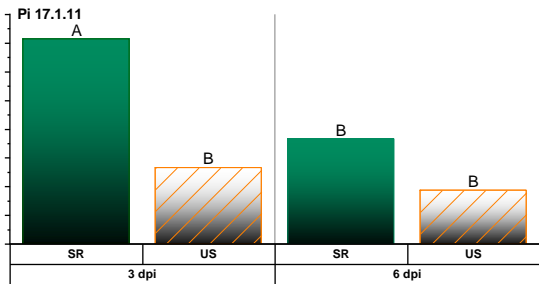
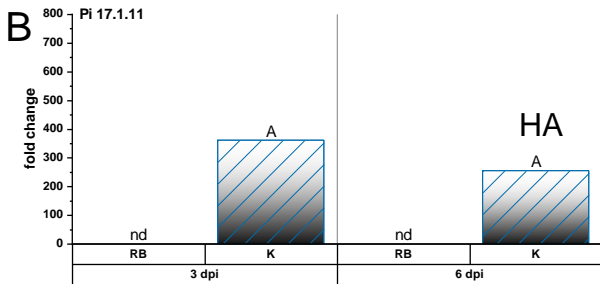
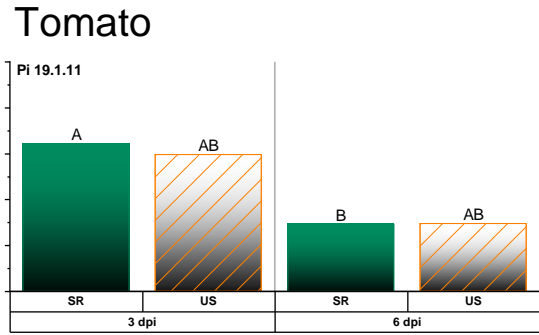
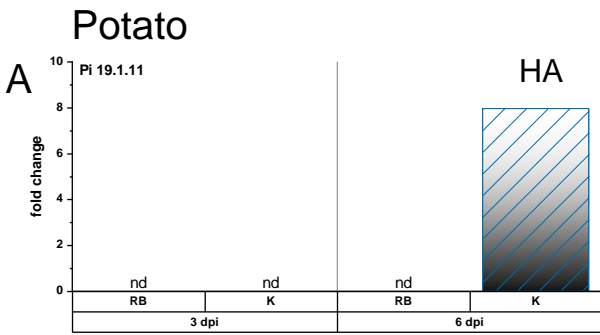
b: accession numbers as referred in NCBI database

c: *P. infestans* elongation factor

Table 4.3 Treatments for gene expression of DL119.

<i>Host</i>	<i>cultivar</i>	<i>Isolates*</i>	<i>Time point (dpi)</i>
<i>Potato</i>	Russet Burbank (RB)	Pi19.1.11	3 and 6
		Pi17.1.11	
		Pi4.3.11	
		Pi3.2.12	
		Pi10.1.11	
		Pi688	
	Kennebec (K)	Pi19.1.11	
		Pi17.1.11	
		Pi4.3.11	
		Pi3.2.12	
		Pi10.1.11	
		Pi688	
<i>Tomato</i>	Sun Rise (SR)	Pi19.1.11	
		Pi17.1.11	
		Pi4.3.11	
		Pi3.2.12	
		Pi10.1.11	
		Pi688	
<i>Tomato</i>	Ultra Sweet	Pi19.1.11	
		Pi17.1.11	
		Pi4.3.11	
		Pi3.2.12	
		Pi10.1.11	
		Pi688	

*All isolates were used are US-24, and A1 mating type. Pi10.1.11 (weakly aggressive) and Pi19.1.11 (Highly aggressive) were used for the initial experiment as well as the gene expression experiment. Pi17.1.11, Pi4.3.11 (highly aggressive), Pi3.2.12 (moderately aggressive), Pi688 (weakly aggressive) isolates were included in the gene expression study.



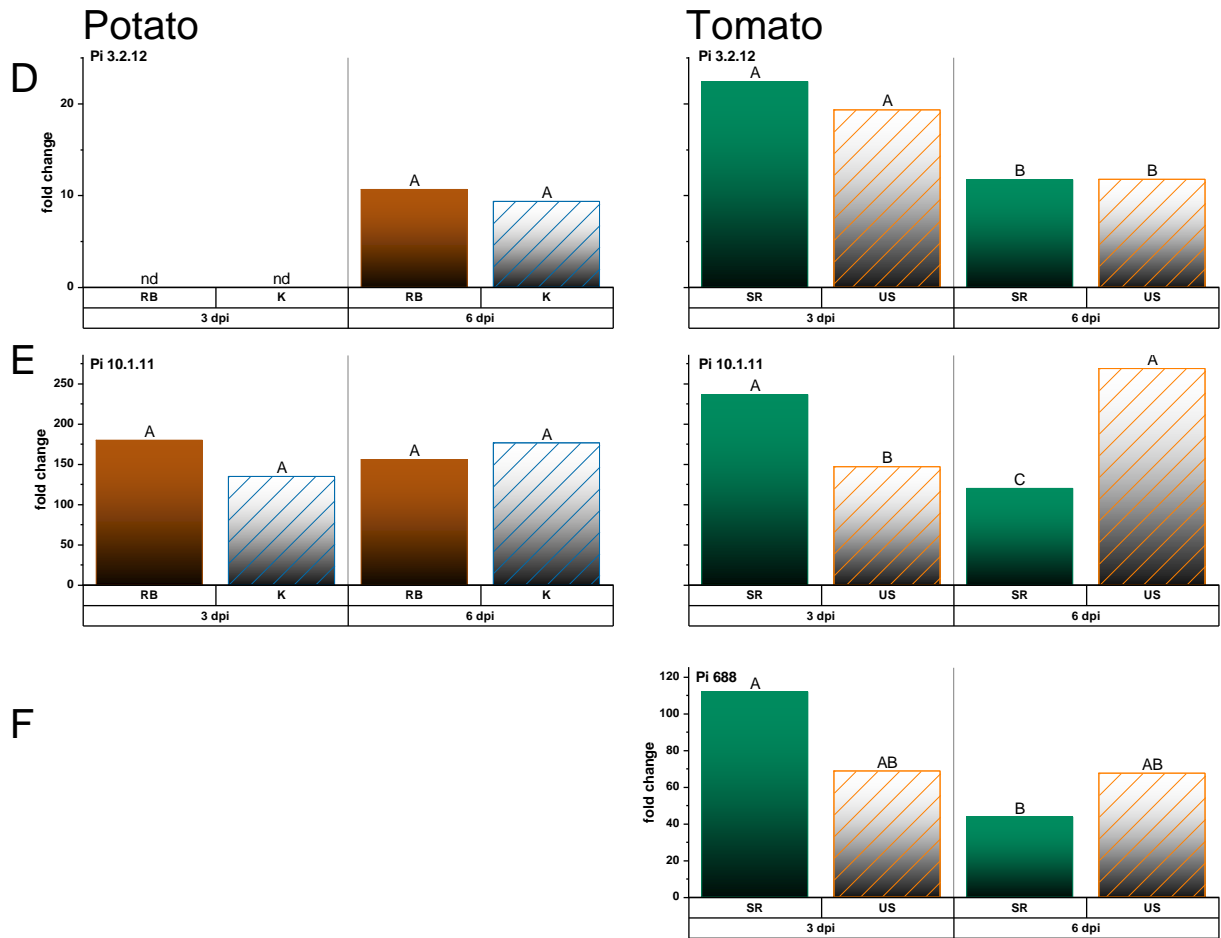


Figure 4.2 The fold change of expressed DL119 effector gene in *P. infestans* isolates at 3 and 6 day post inoculation in potato and tomato. A, B, C: represent the highly aggressive (HA) isolates, D: moderately aggressive isolate, E, F: weakly aggressive isolates with potato cultivars; K: Kennebec, RB: Russet Burbank and tomato cultivars; SR: Sun Rise, US: Ultra Sweet. Potato treatments are represented on the left, whereas tomato treatments are on the right of the figure. DL119 expression was not detected in the highly aggressive (HA) isolates during the interaction with Russet burbank (RB) at both time points. The expression of the gene was significantly reduced from 3dpi to 6dpi in all isolates during thier interaction with tomato susceptible cultivar, Sun Rise (SR). No expression of DL119 in potato cultivars-Pi688 interaction (F, left).

4.5 Discussion

We used quadratic host-*P.infestans* systems to assess the expression of putative *P. infestans* effector genes during the *P. infestans*- potato and *P. infestans*- tomato interactions. The quadratic system included Russet Burbank (RB) and Sun Rise (SR), susceptible lines of potato and tomato, respectively. It also included Kennebec (K) and Ultra Sweet (US), moderately resistant lines of potato and tomato, respectively, during their interaction with weakly and highly aggressive isolates of *P. infestans*. We used the information in a previous study published by Henriquez and Daayf (2010) to test our hypothesis. In their study, they investigated several expressed transcript-derived fragments (TDFs), which were found to be differentially expressed during the interaction between potato and *P. infestans* isolates. Among these fragments, seven were associated with *P. infestans* pathogenicity because they better or only in a highly aggressive isolate as opposed to a weakly aggressive one. No further investigation was done in terms of characterizing these fragments or studying their role in pathogenicity. Our hypothesis of this study was that these unknown fragments could express differently during *P. infestans* interaction with potato and tomato because the isolates varied in their aggressiveness on both hosts. Moreover, *P. infestans* is a hemibiotrophic pathogen that involves biotrophic and necrotrophic phases during its life cycle. Based on this fact, we expected differential levels of gene expression dependent on different phases.

The fragments were designated as DL32, DL119, DL95, DL12, DL142, DL20 and DL31 (Henriquez and Daayf 2010). We determined the similarity of these fragments to other genes using NCBI database. Interestingly, the blast results showed that all the TDFs are sharing similarity with secreted RxLR effector peptides of the *P. infestans* strain T30-4 in the genome database, except DL12. This supported the suggestion that these fragments may be involved in the pathogenicity of aggressive *P. infestans* isolates.

Gene expression was assessed at 1, 2, 3, and 6 dpi to include both biotrophic and necrotrophic phases of the interaction. The potato and tomato plants inoculated with either isolate did not exhibit visible symptoms in the early stages (1 and 2 dpi), neither in the susceptible nor in the moderately resistant lines. Visible late blight symptoms started to show at 3 and 6 dpi. The highly aggressive isolate Pi19.1.11 caused a more severe infection on Russet Burbank and Sun Rise (susceptible lines) than on Kennebec and Ultra Sweet (moderately resistant). On the other hand, the weakly aggressive isolate Pi10.1.11 produced fewer symptoms on moderately resistant than on susceptible lines of both plant species. This is in line with Pi19.1.11 being more aggressive than Pi10.1.11. This is interesting because our previous studies had shown contrasting results between potato and tomato in terms of disease response to *P. infestans* genotypes (chapter 3, not published data). However, it is probable that such results may change with individual genotypes or possibly even within genotypes if another factor of diversity, i.e. physiological races, is involved.

RNA concentration of samples from each treatment was high enough to synthesize cDNA for gene expression, however, the results of real-time PCR showed no amplification of the tested genes at 1 and 2 dpi regardless of the treatment. The conflict of high RNA concentration and lack of amplification at early stages of infection could be explained by the fact that the RNA is essentially pooled RNA, which contains RNA from both plant tissue and the pathogen. The fact that the ratio of RNA from plant tissue is usually greater than the RNA from *P. infestans* makes the amplification of the pathogen's material not easily detectable and resulting in no measurable expression of the genes at early stages. On the other hand, at 3 dpi and 6 dpi, expression of DL119 was detected during the interaction of potato and tomato cultivars with both the highly and the weakly aggressive isolates, with a higher concentration in plants inoculated with the weakly than

the ones inoculated with the highly aggressive isolate. This is opposite to our initial expectation that DL119 would express more in the highly aggressive isolate. The only explanations could be that the highly aggressive isolate expressed more DL119 earlier than the tested time. Another possibility to explain the low expression of DL119 in the highly aggressive isolates is that the amount of expressed gene (e.i DL119) is dependent on the level of aggressiveness of the isolate, the more aggressive the less amount of gene expressed. Other consideration is that the results obtained by Henriquez and Daayf (2010) used isolates from the US-8 and US-11 genotypes, whereas here we used US-24, and US-23 isolates. DL119 was the only gene among the selected genes that showed detectable expression. It is not clear if the other genes did not express or their expression occurred outside the tested time window. Effector genes of different oomycetes were reported with diverse temporal expression (Schornack et al. 2009). RxLR effectors had been shown to be induced in early stages of potato infection by *P. infestans*, i.e., during germination cyst stage (Whisson et al. 2007). Moreover, other effectors such as Avr3a, Avr4 showed up-regulation of their transcription during early stages of infection at 2-3 dpi (Haas et al. 2009). The temporal expression of DL119 at 3 and 6 dpi may indicate its involvement in processes that are induced in the biotrophic phase of the infection or have enzymatic activities such as apoplastic enzymatic inhibition (Bos et al. 2006).

P. infestans is a hemibiotrophic pathogen, requiring living cells to continue its life cycle. The biotrophic phase was estimated between the germination up to 3 day after infection (Vargas et al. 2012). At this stage, the pathogen colonizes the plant tissue and starts the necrotrophic phase where it benefits from the utilized plant nutrition. This transition from biotrophic to necrotrophic phase may be associated with the recorded pathogenicity (Figure 4.2, A & B) and qRT-PCR results. Pathogenicity assessment of the highly and weakly-aggressive isolates showed that symptoms

started to be observed at 3 dpi and increased with time. At 6 dpi, necrotic areas were noticeable particularly on infected susceptible cultivars of potato and tomato. QRT-PCR for gene expression showed parallel results, where high expression of DL119 was measured 3dpi (biotrophic phase), then the expression was reduced at late stage of infection, 6dpi (necrotrophic phase) when the necrotic area enlarged and most living plant tissue was utilized. These observations could be linked to the possible role of DL119 on pathogenicity of *P. infestans* particularly on the tomato susceptible cultivar compared to other tested cultivars (Figure 4.2).

The overall expression of DL119 during the interaction between *P. infestans* isolates and potato cultivars was relatively low. Interestingly, the expression was detected only in infected Kennebec during interaction with highly aggressive isolates (Pi19.1.11, Pi17.1.11 and Pi4.3.11) compared to the expression of the gene on Russet Burbank. The detection of the effector gene in Kennebec could be associated with interference with the partial resistance factors of the potato cultivars and the survival of the pathogen within plant tissue. We observed that the amount of living plant tissue in the extracted samples is important during qRT-PCR analysis. Kennebec inoculated with *P. infestans* isolates showed less necrotic lesions compared to Russet Burbank. The highly aggressive isolates of *P. infestans* (Pi19.1.11, Pi17.1.11 and Pi4.3.11) exhibited severe infection and large necrotic lesions on Russet Burbank. However, the infection and the lesion size were reduced gradually with the level of aggressiveness of the mildly aggressive isolate Pi3.2.12, to the weakly aggressive isolates Pi10.1.11 and Pi688. The fact that *P. infestans* requires living cells to continue the life cycle and colonizes the plant host suggests the importance of including the living plant tissue during the extraction of RNA to detect gene expression. There was no significant difference on the expression of DL119 in infected potato cultivars inoculated with the weakly aggressive isolate Pi10.1.11 in either time points, which may indicate the failure of the isolate to infect potato.

This scenario differs from what we had observed on tomato cultivars inoculated with Pi10.1.11. It exhibited significant gene expression on the susceptible and moderately resistant cultivars at both 3 and 6 dpi. This confirms that the differential level of expression depends on different plant hosts and the susceptibility of tomato to late blight.

Chaudhari et al. (2014) and Upson et al. (2018) discussed how a particular effector is able to behave differently, where it may act as a virulence factor on a specific host and an avirulence factor on another one. This phenomenon was also observed in plants that belong to the same species. The finding could support our result of the differential expression of DL119 between potato and tomato. We had observed a change in expression of this gene in different *P. infestans* isolates that possess different levels of aggressiveness. It is not surprising to obtain such differences since we examined the expression in a quadratic system. Factors involved in such an experiment include plant hosts and susceptibility of the cultivars within each host, aggressiveness of the isolates, also different times after infection, considered as causes of differential expression of effector gene to enhance the pathogen to initiate the disease or to process colonization of host cells. Halterman et al. (2010) investigated the extreme variability of the locus of *ipiO* in different *P. infestans* isolates. This gene encodes effector proteins. This variability was found not only in the presence or the absence of specific alleles but also in the copy number, resulting in different recognition and response to resistant plant defense genes. Similar situations could possibly occur in other virulence genes of *P. infestans*. This could be another explanation for the variation of DL119 expression in the tested *P. infestans* isolates.

We demonstrated in cross-pathogenicity experiment the ability of potato to tolerate *P. infestans* isolates more than tomato. We also showed particularly the increased virulence of US-24 genotype on tomato compared to potato. Here we associate the aggressiveness of tested isolates with the

expression of DL119 and its role in pathogenicity from the plant response perspective. Our results showed clearly high expression of DL119 induced by *P. infestans* tested isolates during the interaction with tomato cultivars at both time points (3 and 6 dpi) regardless of the isolates' aggressiveness. This observation clearly suggested a correlation between aggressiveness of isolates and expression of DL119 as well as its relationship to the interaction with different defense responses of the two host plant species. Alongside the study of DL119 roles in *P. infestans* pathogenicity, it is important to combine this research with greater understanding of potato and tomato defense mechanisms.

In this study, we examined isolates that belong to the US-24 genotype, which seem to infect potato more than tomato. US-22 and US-23 on the other hand, are the major threat to tomato crops. Inoculation of US-24 experimentally showed the failure of tomato cultivars to exhibit defensive mechanism to suppress uncommon genotype infection. Recently, resistance genes against late blight were reported on some tomato cultivars. Ph-1 to Ph-5 are tomato resistance genes, which are discovered from wild tomato *Solanum pimpinellifolium* (Chunwongse et al. 2002; Merk et al. 2012). Ph-2 and Ph-3, in combination, showed efficient effects to suppress infection caused by US-22 and US-23 genotypes but ineffective against US-24 (McGrath 2018). This means that even if Sun Rise and Ultra sweet tomato hybrids were carrying one of the resistance genes, they would develop infection if infected by US-24.

In conclusion, the road to clearly understand the roles/functions of the selected putative pathogenicity genes is still long. However, the differential expression of DL119 may mean that this gene's involvement is restricted to a limited infection stage, i.e., biotrophic phase. The role of DL119 may be different between potato and tomato, as evidenced by the different expression patterns in the two species. Such patterns go along with the findings of the cross-pathogenicity

tests, where most isolates were more aggressive on the susceptible tomato hybrid. The lack of expression of DL119 in the weakly aggressive isolate (pi688) during the interaction with potato cultivars supports the involvement of this gene in pathogenicity since this particular isolate did not cause any infection on potato compared to tomato. However, this is not enough to conclude that DL119 may count as one of the essential effector genes for the pathogen to invade the plant host. Future studies should focus on the localization and functional characterization of DL119 to understand the host target. This could be achieved by knocking down the gene and test it on host plants in order to determine the mode of action of the effector toward infection. The finding of such future study will help develop more effective control against different genotypes and it will be very helpful in different growing areas dependent on which crops are growing. Understanding the *P. infestans* and host interaction from the effectors' perspective will help improve breeding programs to develop the best cultivars of potato and tomato against late blight disease.

CHAPTER 5: CONCLUSION

Potato and tomato are valuable crops and two of the major food crops worldwide. However, both crops are infected by an important plant pathogen, *P. infestans*. Late blight is a destructive disease in potato and tomato production areas. Historically, it caused one of the most devastating famines, which is well known as the Irish potato famine and that occurred in the 1840s. The disease is continuing to affect populations worldwide both economically and socially. Together, losses of crops and fungicide application total cost were estimated to \$ 3 - 5 billion dollar annually in Canada (Agriculture and Agri-Food Canada 2015).

Late blight is caused by the oomycete *Phytophthora infestans*, which is a hemibiotrophic pathogen that requires living cells at early infection stages to continue its life cycle (biotrophic phase). Later, the pathogen switches to rely on dead tissue to maintain the needs of nutrients and complete the cycle (necrotrophic phase). The pathogen requires a misty cool environment in order to cause infection. The major and common inoculum is the asexual spores (sporangia or/ and zoospores), however, when the two mating types, A1 and A2 of the pathogen are present at close proximity, sexual spores may form (oospores).

Late blight is a devastating disease on two major important crops, and affects the industry by reducing the production significantly. One of the reasons behind these devastating effects is the rapid genetic change in *P. infestans* populations worldwide (Kalischuk et al. 2012). Continuous evolution of different genotypes of *P. infestans* is enhancing the chances of more epidemics to occur. Such dynamic diversity makes the disease management continuously more challenging. Thus, continuing to track the distribution and characterizing *P. infestans* genotypes is important to

try to predict the subsequent population structure which could in turn influence rapid epidemics in potato and tomato production regions.

In this study, we characterized the new *P. infestans* genotypes in Canadian provinces during 2012. The collected isolates were obtained from our collaborators from different infected tomato and potato regions. Using mating types, allozyme patterns, fungicide sensitivity, RG57 RFLP and mtDNA analysis, we observed an increase in the diversity of *P. infestans* populations in a single year. The diversity resulted from the dramatic shifts of the genotypes and the appearance of new recombinant genotypes in some regions. For instance, *P. infestans* populations were dominated by US-24 in 2011 in Manitoba. In a single year, an extreme shift from US-24 to US-23 occurred. Moreover, a similar shift was observed in Prince Edward Island, where US-8, the dominant genotype for many years was completely replaced by US-23. Considering recombinant genotypes such as CA-12 in 2012 and CA-9, 10 and 11 in 2011 in increasing the diversity of *P. infestans* is critical. The results of the characterization showed clearly the independent segregation in either RG57-RFLP, mating type or in the allozyme pattern (*Gpi*). This change in the genetic profile of *P. infestans* isolates increases the diversity of the pathogen populations. Monitoring *P. infestans* genotypes every year is very important because unexpected epidemics by different genotypes could be possible.

The appearance of new genotypes such as US-22, US-23 and US-24 in North America was an incentive for us to study the behavior of isolates belonging to these genotypes on potato and tomato. The cross-pathogenicity study was designed in a quadratic potato-*P. infestans*, tomato-*P. infestans* system. The results indicated that the US-24 genotype was more aggressive compared to US-22 and US-23 on all cultivars used in this study. Sun Rise, tomato susceptible cultivar displayed severe symptoms induced by US-24. Isolates belonging to US-22 and US-23 exhibited

large necrotic lesions on Sun Rise and less to none on Ultra Sweet, tomato moderately resistant hybrid and Kennebec and Russet Burbank, moderately resistant and susceptible potato cultivars, respectively. The results showed that US-24 isolates are highly aggressive on both potato and tomato, but more isolates should be tested to extrapolate to the whole genotype.

Variations in the aggressiveness of *P. infestans* genotypes were reported previously in several studies (Tellier and Brown 2007; Montarry et al. 2008). US-1, which was the initial *P. infestans* genotype, was less aggressive on potato compared to US-7 and US-8 genotypes that caused severe infection on potato. However, US-11 was prevalent on tomato. Recently, researchers are putting great efforts to understand the plant-microbe interaction from different angles and highlighting the involvement of pathogen's effectors (virulence factors) in suppressing the defense mechanisms in their respective plant hosts. In addition, emphasize the effector ability to alter cell mechanisms to manipulate hosts' environments in a way that benefits the pathogen's growth. From this perspective, we hypothesized that one of the major reasons for the aggressiveness of some isolates used in our studies is the activation of effector genes during the interaction between the isolates and potato and tomato cultivars. Previous studies demonstrated the diverse patterns of temporal expression of RXLR effectors during their interaction with plant hosts (Bos et al. 2006; Whisson et al. 2007; Haas et al. 2009; Vetukuri et al. 2017). Their expression varied from pre-infection stages and early stages of the infection to the late expression during the necrotrophic phase. This information guided our experiment design, where we used different isolates varying in their level of aggressiveness on potato and tomato cultivars. The expression of the selected effector genes was analyzed at 3 and 6 dpi. Our selective effector genes were chosen from previous findings of transcript-derived fragments expressed during the compatible interaction between a highly aggressive isolate of US-8 and potato susceptible cultivar, Russet Burbank (Henriquez and Daayf,

2010). Since the expression was detected during the compatible interaction between the pathogen and the host, it might be involved in the aggressiveness of the US-8 isolate. This finding is important to extend our current study and assess the expression of the fragments in weakly and highly aggressive isolates of the new genotypes during their interaction with potato and tomato cultivars. Unfortunately, only isolates from US-24 were used in the gene expression study. The difficulty of including US-23 isolates was due to the failure of maintaining the isolates on the artificial media. Another difficulty that limited our study to examine the expression only at 3 and 6 dpi is that we could not obtain the optimum biomass of the pathogen at early stages (1 and 2 dpi), where the colonization was very low during both time points. We observed the activation of one effector gene (DL119) among the tested genes (DL32, DL119, DL95, DL142 and DL31) in six selected isolates. The isolates ranged from weakly aggressive (Pi10.1.11, Pi688), moderately aggressive (Pi3.2.12) to highly aggressive (Pi19.1.11, Pi17.1.11 and Pi4.3.11). Our results showed significant higher expression of DL119 on tomato cultivars (Sun Rise and Ultra Sweet) compared to potato cultivars (Russet Burbank and Kennebec). The expression trend was similar on all the treatments, where it started higher at 3 dpi and then decreased to half-fold at 6 dpi. The results suggest that DL119 may affect the pathogenicity of *P. infestans* as its expression was determined in all isolates at both times on tomato lines. The result is in line with our cross- pathogenicity study, where most isolates were more aggressive on the susceptible tomato cultivar. The lack of expression of DL119 in the weakly aggressive isolate (pi688) during the interaction with potato cultivars supports the finding since this particular isolate did not cause any infection on potato compared to tomato. DL119 may not count as one of the essential effector genes that are important for the pathogen to invade the plant host, but it may be involved in association with other genes to suppress plant defences or alter the cell metabolism.

Late blight is a complex plant disease that affects important crops worldwide. The complexity is due to the rapid evolution of pathogen genotypes, which in turn, evolve new invading strategies that help cause epidemics. Clonal lineages and recombinant genotypes have been discovered in North America and worldwide with insensitivity to fungicides and different genetic profiles. Moreover, adding to the above reasons of complexity, *P. infestans* secretes an arsenal of effector proteins that manipulate plant defense mechanisms and promote cell invasion. Continuing to monitor the distribution and evolution of *P. infestans*, along with molecular research on virulence factors (effectors) constitute a good strategy to improve disease control techniques and develop cultivars with more durable resistance against late blight disease.

Future work should focus on dissecting the function and role of DL119 in pathogenicity of *P. infestans* genotypes. Searching for possible involvement of susceptibility factors from the plant host that enhance susceptibility and promote pathogen growth along with effectors such as DL119 is another target to understand the differential interactions in this pathosystem.

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APPENDICES

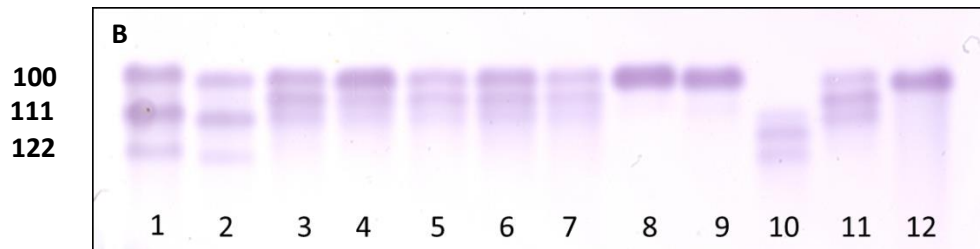
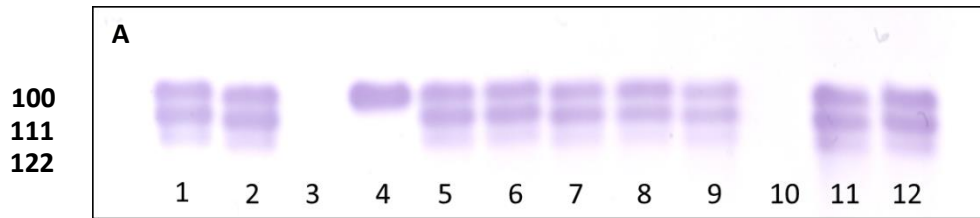
Appendix 1 (Chapter 2):

Summary of the results of characterization markers of *P. infestans* isolates collected in 2011, 2012, and 2013 in Manitoba. Mefenoxam rating on 100 ppm; MS= mefenoxam sensitive, MMS= mefenoxam moderately sensitive, MMR= mefenoxam moderately resistant, MHR= mefenoxam highly resistant. ND= No data.

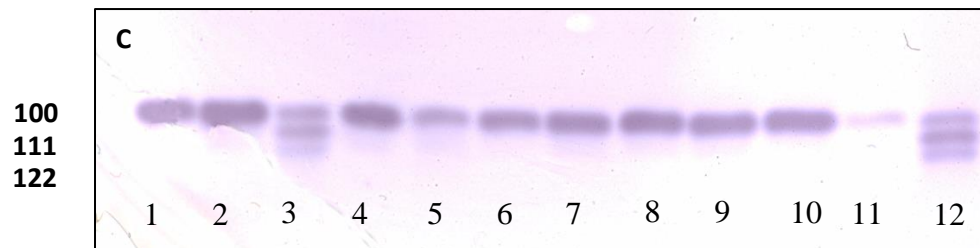
2011				
<i>isolates</i>	<i>Mating type</i>	<i>GPI</i>	<i>Mefenoxam rating on 100 ppm</i>	<i>RG57 RFLP</i>
<i>6-1-11</i>	A1	100/100	MMR	US23
<i>15-1-11</i>	A1	100/100/111	MMR	US24
<i>Pi 18-1-11</i>	A1	100/100/111	MMR	US24
<i>Pi 19-1-11</i>	A1	100/100/111	MMR	US24
<i>8-1-11</i>	A1	100/100/111	MMR	US24
<i>9-1-11</i>	A1	100/100/111	MMR	US24
<i>10-1-11</i>	A1	100/100/111	MMR	US24
<i>11-1-11</i>	A1	100/100/111	MMR	US24
<i>12-1-11</i>	A1	100/100/111	MMR	US24
<i>Pi 17-1-11</i>	A1	100/100/111	MMR	US24
<i>7-1-11</i>	A1	100/100/111	MMR	US24
<i>2-1-11</i>	A1	100/100/111	MMR	US24
<i>3-1-11</i>	A1	100/100/111	MMR	US24
<i>4-1-11</i>	A1	100/100/111	MMR	US24
<i>13-1-11</i>	A1	100/100/111	MMR	US24
<i>14-1-11</i>	A1	100/100/111	MMR	US24
<i>Pi-16-1-11</i>	A1	100/100/111	MMR	US24
<i>5-1-11</i>	A1	100/100/111	MMR	US24
2012				
<i>Pi 8-1-12</i>	A1	100/100	MMR	US23
<i>Pi 9-1-12</i>	A1	100/100	MS	US23
<i>Pi 11-1-12</i>	A1	100/100	MS	US23
<i>Pi 12-4-12</i>	A1	100/100	MMR	US23
<i>Pi 7-1-12</i>	A1	100/100	MMR	US23
<i>Pi 1-4-12</i>	A1	100/100	MS	US23
<i>Pi 2-3-12</i>	A1	100/100	MS	US23
<i>Pi 3-2-12</i>	A1	100/100/111	MMR	US24
<i>Pi 4-3-12</i>	A1	100/100	MMR	US23
<i>Pi 5-2-12</i>	A1	100/100	MMR	US23
<i>Pi 6-1-12</i>	A1	100/100	MMR	US23
2013				
<i>Pi2-3-13</i>	A1	100/100	MMR	US23
<i>Pi3-x-13</i>	ND	100/100	MMR	US23

<i>Pi4-x-13</i>	ND	100/100	MHR	US23
<i>Pi5-x-13</i>	ND	100/100	MMR	US23
<i>Pi6-x-13</i>	ND	100/100	MMR	US23

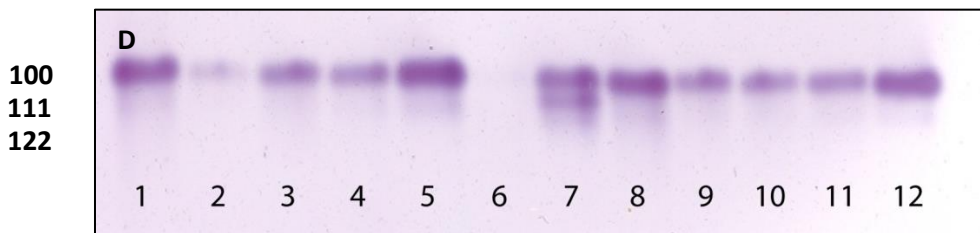
2011



2012



2013



Allozyme banding patterns of *P. infestans* isolates from Manitoba. A) Lane (L) 1: US-11, L 2: 688, L 3: US-8, L 4: 303, L5: 4-1-11, L6: 4-3-11, L7: 5-1-11, L8: 5-4-11, L9: 7-1-11, L10: 8-1-11, L11: 9-1-11, L12: 10-1-11. B) L1: US-8, L2: US-8, L3: US-11, L4: 8-1-11, L5: pi 19-1-11, L6: 688, L7: pi17-1-11, L8: pi6-1-11, L9: 164man, L10: 067ONt, L11: 067ON, L12: *P. necotiana*. C) L1: Pi.1-4-12, L2: Pi.2-3-12, L3: Pi.3-2-12, L4: Pi.4-3-12, L5: pi Pi.5-2-12, L6: Pi.6-1-12, L7: Pi.7-1-12, L8: Pi.8-1-12, L9: Pi.9-1-12, L10: Pi.11-1-12, L11: Pi.12-4-12, L12: Pi.4-3-11. D) L1: Pi2-3-13, L2: Pi 3-x-13, L3: Pi 4-x-13, L4: Pi 5-x-13, L5: Pi 6-x-13, L6: Pi11-2-12, L7:Pi 10-1-11, L8: Pi2-3-13, L9: Pi 3-x-13, L10: Pi 4-x-13, L11: Pi 5-x-13, L12: Pi 6-x-13.

Appendix 2 (Chapter 4):

EST#: DL119

Sequence

TCCGCTAGTGCAGCTGCCTTGCGTGACGATCGTCATGTTTCATGAACAATACGATCGT
TATCGGCAACGTCGCCAAAAGCTCAAAGCAGCGGTGGAATCTGCGGGAATGCGGGT
TGATGATTCTGCGGCTGGGCTTTATCTGTGGACAACAAACGGTACACCCTGCAGAGA
ACTCACGCACTGGTTTGCCGAGCGGGGTATTCTTGTCGCTCCAGCT

EST#: DL142

Sequence

GCGGTAAAGCTTTACCAGGCAGGCGAAGTCCTTGCCGACTACAAGCTTGATCGAAC
AATTATCGAAGCCAACATCTTTACGATGACTTCCCACTTCTAGCTTGGG

EST#: DL32

Sequence

GTAACGCAGGCGTCCTAAGATGAGCTCAACGAGAACAGAAATCTCGTGTGGAACAG
AAGGGTAAACTAACCTGTCTCACGACGGCCTAAACCCAGCTCACGTTCCCTATTGG
TGAT

EST#: DL31

Sequence

CTATTCTTGGTCTACCCAGTCGTGCGCCGATTCTTCGGCCTTTTTGCTGTCGCCAGAT
TGGTGTTGCTGAGCAGCTTCCAACAGGCTTGTGTGAACTTCCAATTGCTCGTCGGCG
GCATTCTTTAGAGCCTTTTTTGACCCTCCAGAGGACCCGCAGCCAGTGATTGCAAGT
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CTG

EST#: DL20

Sequence

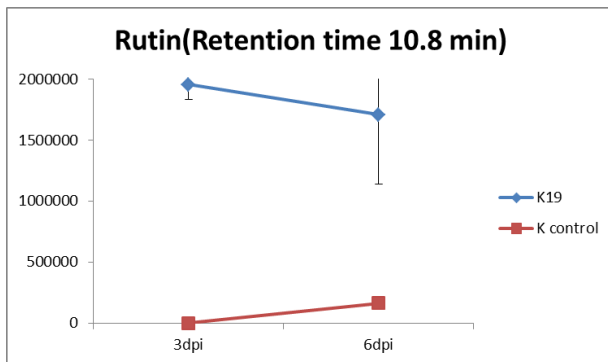
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Appendix 3 (Chapter 4):

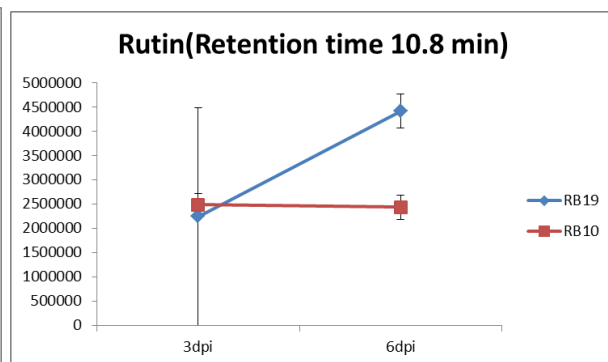
Area of peaks of secondary metabolites of potato and tomato plants inoculated with *P. infestans* as detected by high performance liquid chromatography (HPLC).

Rutin:

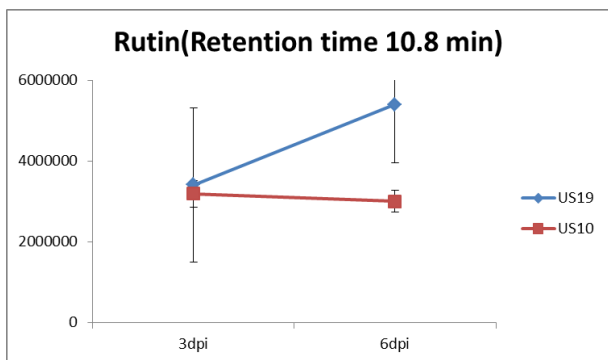
Kennebec



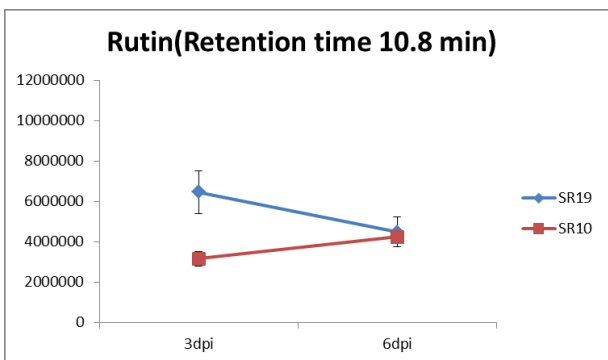
Russet Burbank



Ultra

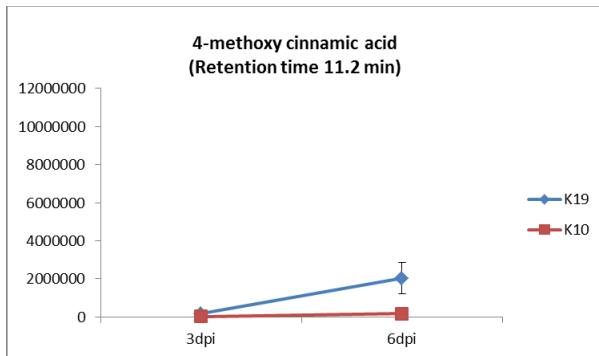


Sun Rise

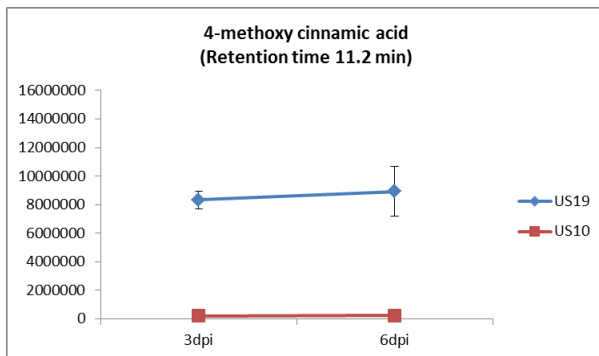


4-methoxy cinnamic acid

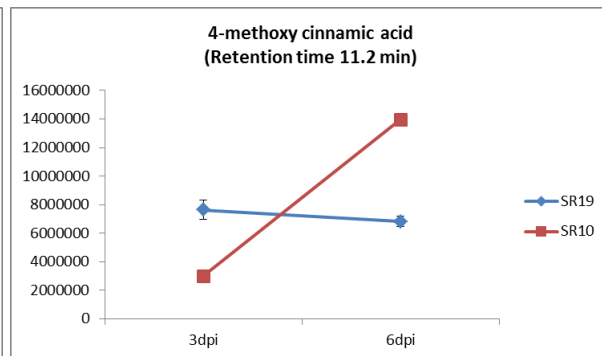
Kennebec



Ultra

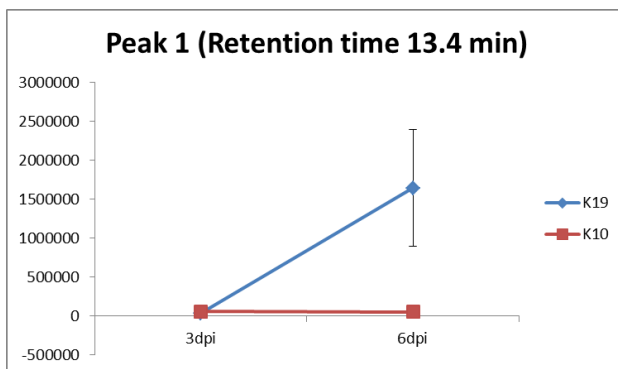


Sun Rise

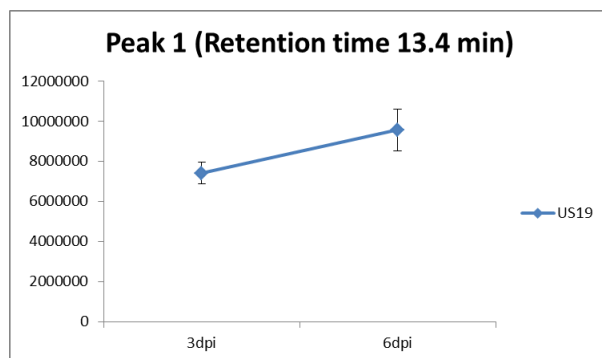


Peak 1:

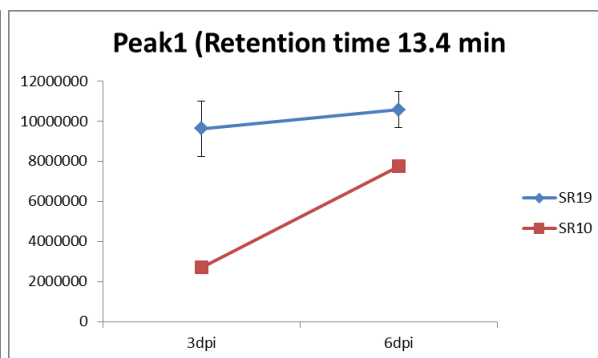
Kennebec



Ultra

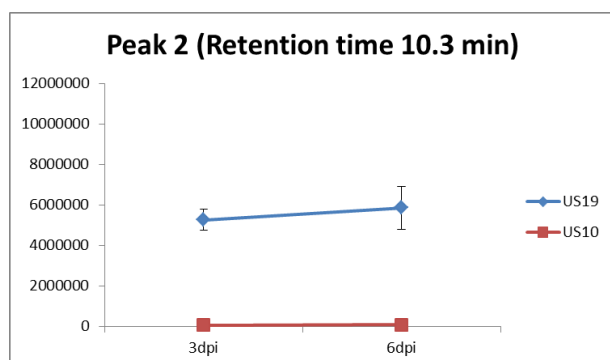


Sun Rise

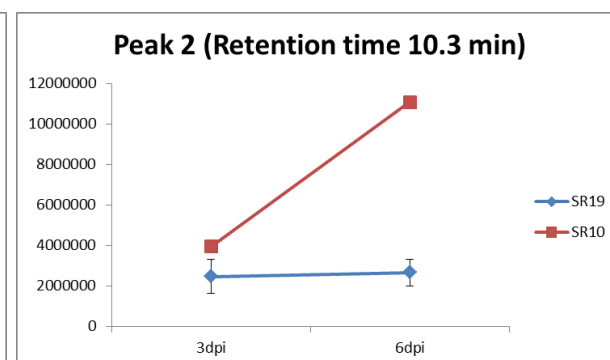


Peak 2:

Ultra



Sun Rise



K19: Kennebec potato cultivar inoculated with highly aggressive isolate Pi 19.1.11, **K10:** Kennebec inoculated with weakly aggressive isolate Pi10.1.11, **RB19:** Russet Burbank inoculated with Pi19.1.11, **RB10:** Russet Burbank inoculated with Pi10.1.11, **US19:** Ultra sweet tomato hybrid inoculated with Pi19.1.11, **US10:** Ultra sweet inoculated with Pi10.1.11, **SR19:** Sun Rise tomato hybrid inoculated with Pi19.1.11, **SR10:** Sun Rise inoculated with Pi10.1.11.