Bacterial antagonists as a biological solution for control of potato late blight disease

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Abstract

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) De Bary, is the most devastating disease affecting the potato, accounting for over six billion United States dollars worldwide each year due to production losses and prevention measures. For many years, synthetic fungicides have been used heavily by potato producers to minimize disease severity and prevent spread. But the incidence of new, more virulent *P. infestans* genotypes – many of which now fungicide-resistant – has made it increasingly difficult and costly for producers to prevent late blight epidemics. The use of biological fungicides has thus been of considerable interest among plant pathologists in recent years for their ability to produce powerful antifungal compounds and to induce systemic resistance in host plants. This M.Sc. project, in collaboration with Agriculture and Agri-Food Canada, used detached fed-leaf bioassays to test the effectiveness of six bacterial strain whole cultures against four *P. infestans* isolates for use as potential biocontrol agents. Data analysis revealed that the level of biocontrol differed greatly among the six bacterial strains; 189 (*Pseudomonas chlororaphis*) being significantly different than the control for 28 of the 32 measurements while this was true for OY3WO11 (*Arthrobacter phenanthrenivorans*) only once. The top two candidates 189 and WAUSV36 (*Bacillus subtilis*) were then compared to the commercial synthetic fungicide Dithane^{TM} DG 75 which displayed the strongest levels of late blight control with median disease severity ratings of 0% and 3% after seven and ten days, respectively. Nonetheless, bacterial strains 189 (18% and 40%) and WAUSV36 (48% and 78%) were also significantly different from the untreated control (100% on both measurement days). Time-delay experiments were also conducted in order to determine if differences in biocontrol existed when the time between treatments and infection was increased. A significantly reduced biocontrol efficacy was detected when bacterial treatments were applied 24 hours before infection (compared to 2 and 18 hours) from measurements taken after seven days only (not ten). Integrating the use of bacterial antagonists into late blight management strategies can prove to be an effective addition to current prevention methods and help curb the heavy use of synthetic fungicides in potato production.

Résumé

Le mildiou, causé par l'oomycète *Phytophthora infestans* (Mont.) De Bary, est la maladie la plus dévastatrice affectant la pomme de terre, provoquant des coûts de plus de six milliards de dollars américains au niveau mondial à chaque année en raison de pertes de production et de mesures de prévention. Depuis plusieurs années, les fongicides synthétiques sont largement utilisés par les producteurs de pommes de terre afin de minimiser la gravité de la maladie et d'empêcher sa propagation. L'incidence de nouveaux génotypes de *P. infestans* plus virulents dont plusieurs sont maintenant résistants aux fongicides, a fait en sorte qu'il est maintenant de plus en plus difficile et coûteux pour les producteurs de prévenir des épidémies de mildiou. Depuis quelques années, l'utilisation de fongicides biologiques a soulevé l'intérêt des phytopathologistes pour leur capacité à produire de puissants composés antifongiques et à induire une résistance systémique chez les plantes hôtes. Ce projet de maîtrise, en collaboration avec Agriculture et Agroalimentaire Canada, vise à utiliser des bio-essais de feuilles détachées et nourries pour tester l'efficacité de six cultures bactériennes entières contre quatre isolats de *P. infestans* pour leur utilisation potentielle en tant qu'agents de lutte biologique. L'analyse des données a révélé que le niveau de lutte biologique différait grandement entre les six souches bactériennes; 189 (*Pseudomonas chlororaphis*) étant significativement différente du témoin pour 28 des 32 mesures ce qui était vrai qu'une seule fois pour OY3WO11 (*Arthrobacter phenanthrenivorans*). Les deux meilleurs candidats 189 et WAUSV36 (*Bacillus subtilis*) ont ensuite été comparés au fongicide synthétique commercial Dithane^{TM} DG 75 qui a présenté les niveaux les plus forts de contrôle au mildiou avec des indices médians de sévérité de la maladie de 0% et 3% après sept et dix jours respectivement. Néanmoins, les souches bactériennes 189 (18% et 40%) et WAUSV36 (48% et 78%) étaient aussi significativement différentes du témoin non traité (100% sur les deux jours mesurés). Des expériences de temporisation ont également été menées afin de déterminer si des différences dans la lutte biologique existaient lorsque le temps entre les traitements et l'infection était augmenté. Une efficacité de lutte biologique considérablement réduite a été détectée lorsque les traitements bactériens ont été appliqués 24 heures avant l'infection (par rapport à 2 et 18 heures) et ce, à partir de mesures prises après sept jours seulement (pas dix). Intégrer l'utilisation d'antagonistes bactériens dans les stratégies de

gestion de mildiou peut se révéler comme un complément efficace aux méthodes de prévention actuelles et aider à freiner l'usage intensif de fongicides synthétiques dans la production de pommes de terre.

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Contribution of authors

Nicholas Foran, a Master's student in the Department of Plant Science, McGill University was the primary investigator and writer for this paper. He is responsible for the majority of experimental manipulations and interpretations presented here.

Dr. Susan Boyetchko and Dr. Patrice Audy, research scientists at Agriculture and Agri-Food Canada, along with all their research technicians, are responsible for the experimental design and technical know-how of the project. Their teams are also responsible for a considerable amount of experimental manipulations performed before and during the student's involvement in the project.

Dr. Valérie Gravel, professor in the Department of Plant Science, McGill University is responsible for assisting Nicholas with the statistical analysis of all experimental data and for providing editorial aid during the redaction of this paper.

Chapter 1 : Introduction

1.1 Problem definition

Potato late blight is well known as being the most costly potato disease worldwide (Goss *et al.*, 2014). Accounting for well over six billion United States dollars worldwide each year and problematic wherever potatoes are grown, its significance for potato producers definitely cannot be overlooked (Al-Mughrabi, 2012; Johnson *et al.*, 2015). Late blight is caused by the plant pathogen *Phytophthora infestans* (Mont.) De Bary and is responsible for such epidemics as the Irish potato famine in 1845 (Ristaino, 2002). Today, late blight remains problematic due to the ubiquitous presence of both mating types, its ability to produce long-living extreme weather-tolerant oospores through sexual reproduction, by quickly spreading through international trade routes and from the mutation of many *P. infestans* isolates towards fungicide-resistant genotypes (Mizubuti *et al.*, 2007; Peters *et al.*, 2014). The disease triangle for potato late blight can be characterized as a function of the pathogen *P. infestans*, the environment (ideal temperature and humidity conditions) and the potato plant host (Al-Mughrabi, 2012). When it comes to the environment, disease development and consequential crop losses depend in part on the aerial transport of pathogenic sporangia that are well adapted for aerial dispersal; sporangia being able to travel up to distances of 40-60 km (Fall *et al.*, 2014). Because potato late blight is capable of causing huge economic damages at a frightening speed, potato producers must rely on nearly weekly synthetic fungicide applications on plant surfaces before incoming sporangia arrive (Hadwiger *et al.*, 2015). However, the heavy use of these synthetic pesticides has raised serious concerns concerning human health effects and damages to the environment (Garron *et al.*, 2009; Macphail Ecological Woods Forestry Project, 2011). In addition, the use of synthetic fungicides as the gold-standard treatment against late blight has favoured the development of fungicide-resistant *P. infestans* genotypes (Saville *et al.*, 2015). In Canada, the abundant use of metalaxyl, the only truly systemic fungicide available to prevent late blight epidemics, has resulted in the loss of its effectiveness through said fungicide resistance (Agriculture and Agri-Food Canada, 2013). Organic fungicides such as copper-based treatments can be used as alternatives yet they are generally not as effective as their synthetic equivalents and can introduce toxic reactive

oxygen species (ROS) into the rhizosphere (Glover *et al.*, 2011; Ferreira *et al.*, 2014). The development of disease resistant potato cultivars has also been explored as a means of preventing potato late blight (Zakharchenko *et al.*, 2011). However producing a new cultivar through genetic breeding can take a very long time and speeding up this process through genetic engineering can often result in many unpredictable new traits (Zakharchenko *et al.*, 2011). Although crop production practices are essential to any late blight management strategy, the aggressiveness of newer *P. infestans* genotypes has made it impossible to rely on these measures alone – especially if tomatoes are also grown nearby as the disease can spread from one plant to the other (Nowicki *et al.*, 2012). One of the earliest published reports on the use of biological control agents against *P. infestans* is by Jindal *et al.* (1988) where they applied *Penicillium aurantiogriseum* and *Stachybotrys atra* suspensions to whole potato plants. Their results were positive; late blight intensity being reduced by 93% and 84% respectively (Jindal *et al.*, 1988). The use of bacteria as biocontrol agents for the treatment of potato late blight has since increased in interest during recent years, with many studies reporting positive results (Bengtsson *et al.*, 2015). In addition to providing effective alternatives to environmentally-taxing synthetic fungicides without the need for developing potentially less marketable potato varieties, many biofungicides have the potential to provide additional agricultural benefits such as secreting plant growth regulating hormones, fixing atmospheric nitrogen and improving phosphorus nutrition (Zakharchenko *et al.*, 2011). Unlike synthetic fungicides, many microorganisms may also have the potential to increase in antagonistic activity over time by successfully colonizing plant surfaces (Wharton *et al.*, 2012).

1.2 Rationale for research

Several biopesticide products (of which biofungicides are one type) are already registered for the treatment of late blight in Canada or have pending registrations (Glover *et al.*, 2011; Reuters, 2012). However these products have elicited mixed results and as of yet have not demonstrated sufficient and consistent levels of late blight suppression in order to significantly curb the heavy use of synthetic and copper-based fungicides (Glover *et al.*, 2011). Continued biopesticide research is therefore needed. This study investigated the biocontrol abilities of six bacterial strains for the treatment of potato late blight disease on detached fed-leaves. These

results, in conjunction with those obtained from various Agriculture and Agri-Food Canada (AAFC) research teams across Canada, will help AAFC develop a commercial biopesticide product for use against potato late blight.

1.3 Hypotheses

The objectives of this study were supported by the hypotheses:

I. *Phytophthora infestans* isolates demonstrate varying degrees of pathogenicity and are capable of inducing destructive levels of disease severity among detached fed-potato leaves.

II. Select soil-borne bacterial strains can be effective agents in protecting potato plants against late blight in detached fed-leaf bioassay experiments.

III. Whole cultures of bacterial strains can be of similar or greater effectiveness as a commercial synthetic fungicide used by the potato industry today.

IV. Whole cultures of bacterial strains will remain effective biocontrol agents when the delay between treatment and infection is increased up to 24 hours.

1.4 Objectives

The objectives were to:

I. Identify four *P. infestans* isolates which have strong pathogenicity and are a reasonably good representation of the current Canadian genotypes found in potato production areas.

II. Identify two bacterial strains which are effective biocontrol agents against four aggressive isolates of *P. infestans*.

III. Determine if these two bacterial strains can be of similar or greater effectiveness as a commercial synthetic fungicide counterpart.

IV. Determine if there are any differences in effectiveness for these two bacterial strains when the treatment-to-infection delay time changes from 2 hours to 18 hours to 24 hours.

Chapter 2 : Literature review

2.1 Potatoes

Solanum tuberosum L., commonly known as the potato plant, is a perennial herb used as an annual crop for primarily agricultural purposes and is a member of the Solanaceae (nightshade) family (Struik, 2007; Agriculture and Agri-Food Canada, 2013). Tuber-bearing potatoes are thought to have originated in the Andes Mountains of Peru and Bolivia, where current wild type species are found to be quite phylogenetically primitive (Agriculture and Agri-Food Canada, 2013). Although authors dispute the number of series under which to classify them, more than 200 potato species have been described. The series *Tuberosa* - a large and variable group without clear morphological distinctions - includes the cultivated potato which is quite unusual as a crop plant due to its particularly large secondary genepool consisting of related tuber-bearing wild types (Van den Berg and Jacobs, 2007). In terms of worldwide production, the potato ranks fourth after rice, wheat and corn and is the most important vegetable crop in Canada, accounting for 30% of all vegetable receipts and 15% of all horticultural receipts in 2013 (Agriculture and Agri-Food Canada, 2013; Agriculture and Agri-Food Canada, 2015). However, yields are not homogeneous across the globe. In North America where cooler climates, higher rainfall averages, mechanization, superior production systems and higher inputs are in place (promoting better soil structure and discouraging disease), yields are approximately double those of Latin America, Asia, and some parts of Europe (Donnelly and Kubow, 2011). Lacking many of the favourable conditions listed above, Africa remains the continent with the lowest yields (Donnelly and Kubow, 2011). Despite this, potato growing areas have recently doubled in many developing countries of Asia, Latin America and Africa – with China and India now becoming the first and third largest potato producers in the world (Russia being the second) (Donnelly and Kubow, 2011).

The potato has been a food source for humans for over 10,000 years. Chuño is an aboriginal potato preparation still practiced in the Andes Mountains which includes such traditional practices as the removal of tuber skins via trampling. Both chuño and papa seca

(another dry potato product) are still used in traditional stews and soups today (Donnelly and Kubow, 2011). From their Central American origins, potatoes were subsequently introduced into Europe during the sixteenth century. The first Canadian shipment of potatoes occurred in 1623; when the tubers were unloaded in Annapolis Royal, Nova Scotia (Agriculture and Agri-Food Canada, 2013). Potato plants are composed of several different gross structures, both above and below-ground. Fig. 2-1 demonstrates the major morphologies of the potato plant:

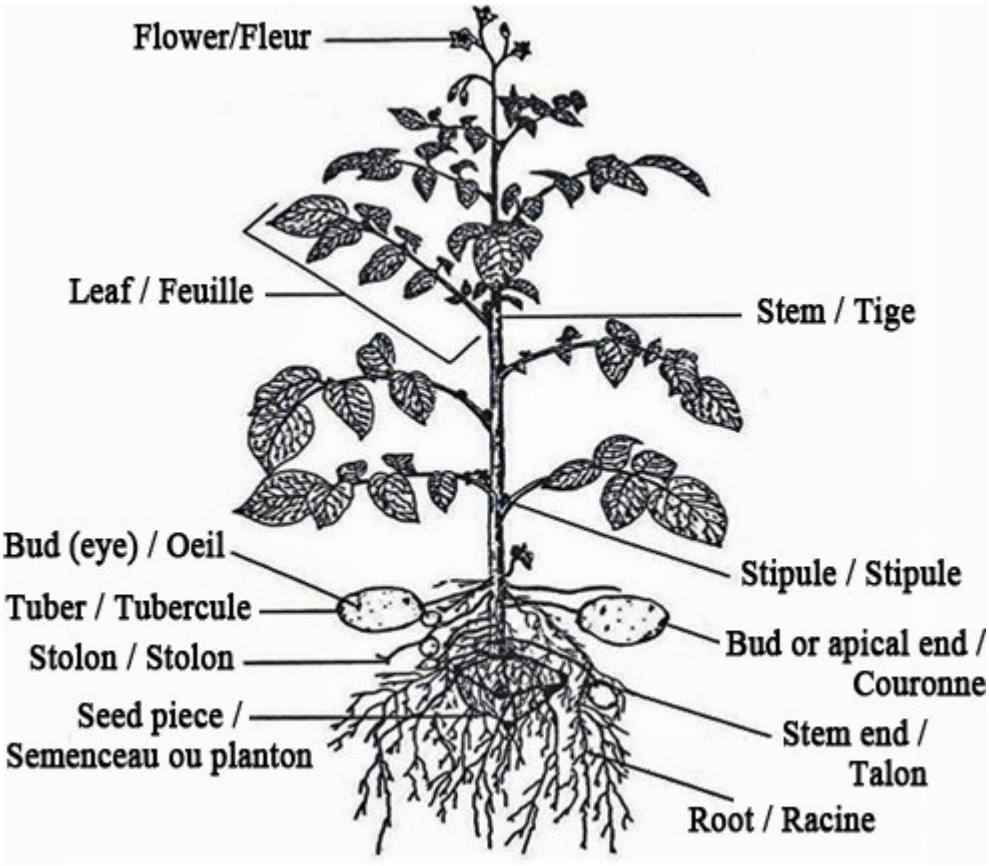

Figure 2-1. Potato plant.
This diagram adapted from the Canadian Food Inspection Agency (2013) illustrates how the plant comprises of a moderate amount of leaves, with the roots being demonstrated at the bottom of the image. From top to bottom: the flowers, leaves, stem, stipules, tubers (with eyes and apical ends), stolons, the seed piece, stem ends and the roots. New tubers can be seen growing at the ends of some of these stolons (Canadian Food Inspection Agency, 2013).

The foliage (leaves and stems) which makes up the bulk of the plant above-ground is particularly vulnerable to harsh environmental conditions and pests (Canadian Food Inspection

Agency, 2013). The potato leaf is typically composed of between two and four primary leaflets situated on both sides of the mid-rib with a terminal leaflet on the end. Smaller leaflets, called secondary leaflets, adorn the spaces between the primary leaflets. These are located irregularly along the mid-rib. Smaller tertiary leaflets can also be found around the mid-rib as well. These different components are what make up the compound potato leaf (Canadian Food Inspection Agency, 2013). The arrangement of primary, secondary and tertiary leaflets can depend on the variety of potato being studied. Leaflets can be light green, darker green or grey-green in colour. Leaves can either have an open (where leaflets are quite separate with evident spaces between primary leaflets) or closed (where leaflets are overlapping with very limited space between them) leaf structure (Canadian Food Inspection Agency, 2013). Several stems can actually be formed from one seed tuber because several of their buds can produce viable shoots. What thus typically happens is the growth of multiple stems originating from the same seed. These stems, especially in the later stages of development, can compete for light and nutrients and eventually become independent units (Struik, 2007). Tubers are the swollen part of rhizomes or stolons that are typically developed underground for nutrient storage by the plant. They contain elevated levels of starch and storage proteins. Depending on potato variety and environmental conditions, tubers can vary in shape (most commonly forming cylindrical, elliptical, oblong, oval or round shapes) and size (Struik, 2007).

Seed tubers are normally used for potato propagation and are grown in all geographical locations where potato production occurs. Although of poorer quality, smaller tubers harvested from the previous crop year are often used for seed potatoes. To obtain good quality seed however, tubers need to be grown specifically for that purpose (Van Loon, 2007). According to Van Loon (2007), the conditions for good quality seed potatoes include the "use of healthy (certified) initial seed, land free from soil-borne diseases and special care being taken in the control of diseases and pests during the growing season". Before being sold to producers the seed product must then pass an inspection from a certified agency (Van Loon, 2007). Despite these measures, difficult climatic conditions, aphid populations and soil-borne diseases are persistent problems for the production of seed potatoes. These risk factors underline the importance of establishing properly organized certification agencies as many countries will not import seed potatoes across their borders if a quarantine disease or pest is found to have contaminated them

(Van Loon, 2007). The traditional method of multiplying potato plants for agriculture is to plant the tubers soon after the end of their dormancy period, when they undergo apical dominance to produce sprouts from their eyes (Barry *et al.*, 2001; Delanoy *et al.*, 2003; Struik, 2007). Shoots develop from these sprouts while roots are developed from the sprouts' primordia. From the shoots, the stems, more roots, stolons, foliage, inflorescences and the new generation of tubers are formed. The physiological age and size of the tuber planted will invariably have a strong effect on the above-ground and below-ground development of the potato plant (Struik, 2007). Although tubers are by far the most common way of propagating the potato plant, individual cells, meristems, tissues, sprouts, leaf or stem cuttings and true botanical seeds may also be used. The type of propagule used (if other than a seed tuber for instance) will also have an effect on the morphology of the developing potato plant (Struik, 2007). Potatoes can be successfully grown during months where minimum temperatures are above 3^{o}C (below this there is a high risk of frost) and the average temperature remains below 22^{o}C (above which tuberization is strongly reduced) (Hijmans *et al.*, 2000).

Just as in centuries past when potatoes helped provide essential nutrients to vitamin-C deprived sea travellers, potatoes continue to be an excellent source of vitamins (especially B and C), carbohydrates, good quality (albeit somewhat low levels of) dietary protein, fibre (particularly from the thick cell walls of the peel), minerals (magnesium, phosphorus and potassium among others) and other useful phytochemicals such as carotenoids and their derivative xanthophylls (Donnelly and Kubow, 2011). There is a common belief among consumers that potatoes are quite fattening and should be avoided to make room for carbohydrate alternatives such as rice or pasta. This is not true: there is almost no fat in potatoes and they have an energy density very similar to that of legumes (Donnelly and Kubow, 2011). Because the potato has a tremendous yield per unit area (more than rice or wheat), it should be considered an important source of carbohydrates for developing countries (Hossain *et al.*, 2014). Overall, the potato can be considered a low-fat, energy-rich carbohydrate food source that also contains important levels of minerals and vitamins. As such, it can play an important role in maintaining good health and preventing diseases (Donnelly and Kubow, 2011).

2.2 Late blight

2.2.1 Pathogen: *Phytophthora infestans*

Late blight is a plant disease which affects several members of the Solanaceae family (most notably the potato and tomato) and is caused by the plant pathogen *Phytophthora infestans* (Mont.) De Bary (Ristaino, 2002). *Phytophthora infestans* is classified as an oomycete belonging to the kingdom Stramenopila, phylum Oomycota and family Pythiaceae. Being morphologically similar, the microorganism was long believed to be a fungus. However, it is phylogenetically related to brown algae (Mizubuti *et al.*, 2007). *Phytophthora infestans* has always been considered an extremely aggressive pathogen and as such merits its name: "Phyto" comes from the Greek word "phyton" meaning "plant" and "Phthora" translates to "destroyer" (Mizubuti *et al.*, 2007). What makes the oomycete so aggressive is its arsenal of sophisticated weaponry. Avirulence genes code for effector molecules that can rapidly infect host tissue and begin colonization. A complex set of proteins (metallopeptidase, cutinase among others) are then activated causing either disease in the plant or a resistance response. A total of 10 avirulence proteins have been identified as being involved in this process (Mizubuti *et al.*, 2007). Furthermore, *P. infestans* is capable of producing several protease inhibitors to combat defence proteins released by the plant (Mizubuti *et al.*, 2007). Another success trait is the pathogen's very effective reproduction mechanisms: both in sexual and asexual forms. Fig. 2-2 outlines the various reproductive structures of *P. infestans* and its life cycle. During asexual reproduction, thousands of sporangia (the enclosure in which spores are formed) are produced per lesion on sporangiophores (dispersal-aiding structures that use the passive movement of wind, rain, etc.) (Fig. 2-2). After making contact with the plant, sporangia can begin to germinate host tissue by quickly developing mycelial growth on leaves, stems and other potato plant structures. Subsequent sporangia are formed from mycelial growth and the cycle continues (Nowicki *et al.*, 2012). The pathogen is able to complete its life-cycle (from infection to sporulation; Fig. 2-2) in as little as three days (Mizubuti *et al.*, 2007). However, moist conditions are required for a minimum of 7-10 hours for sporulation to occur; highlighting the significance of environmental influences to the development of disease (Aburjai *et al.*, 1998). When temperatures are below

optimal (< 15°C), sporangia forgo mycelial growth (direct germination through the development of germ tubes) to produce asexual zoospores. These are then released, dispersed and able to germinate new hosts causing infections to occur at an even quicker pace (indirect germination) (Peters *et al.*, 2014). *Phytophthora infestans*, a heterothallic (sexes residing in separate individuals) pathogen, has two mating types: A1 and A2. Before 1980, A1 mating types were exclusively responsible for disease incidences in North America. With the introduction of A2 mating types from Mexico in the 1980s, sexual reproduction now became possible and more aggressive (often fungicide-resistant) populations resulted (Peters *et al.*, 2014). When mycelia of an A1 mating type meet mycelia of an A2 mating type, their interaction can result in sexual reproduction and oospore formation. Oospores, large and thick-walled spores, are able to survive for long periods of time (even years) in the absence of host tissue (in soil or plant debris, for e.g.) and under extreme temperatures (Mizubuti *et al.*, 2007). *Phytophthora infestans* can also survive from season to season without sexual reproduction through infected tubers and sometimes crop debris (Mizubuti *et al.*, 2007). Until the 1980s, since only A1 mating types were found outside of Mexico, it was believed that sexual reproduction did not play a significant role in the *P. infestans* disease cycle (Nowicki *et al.*, 2012).

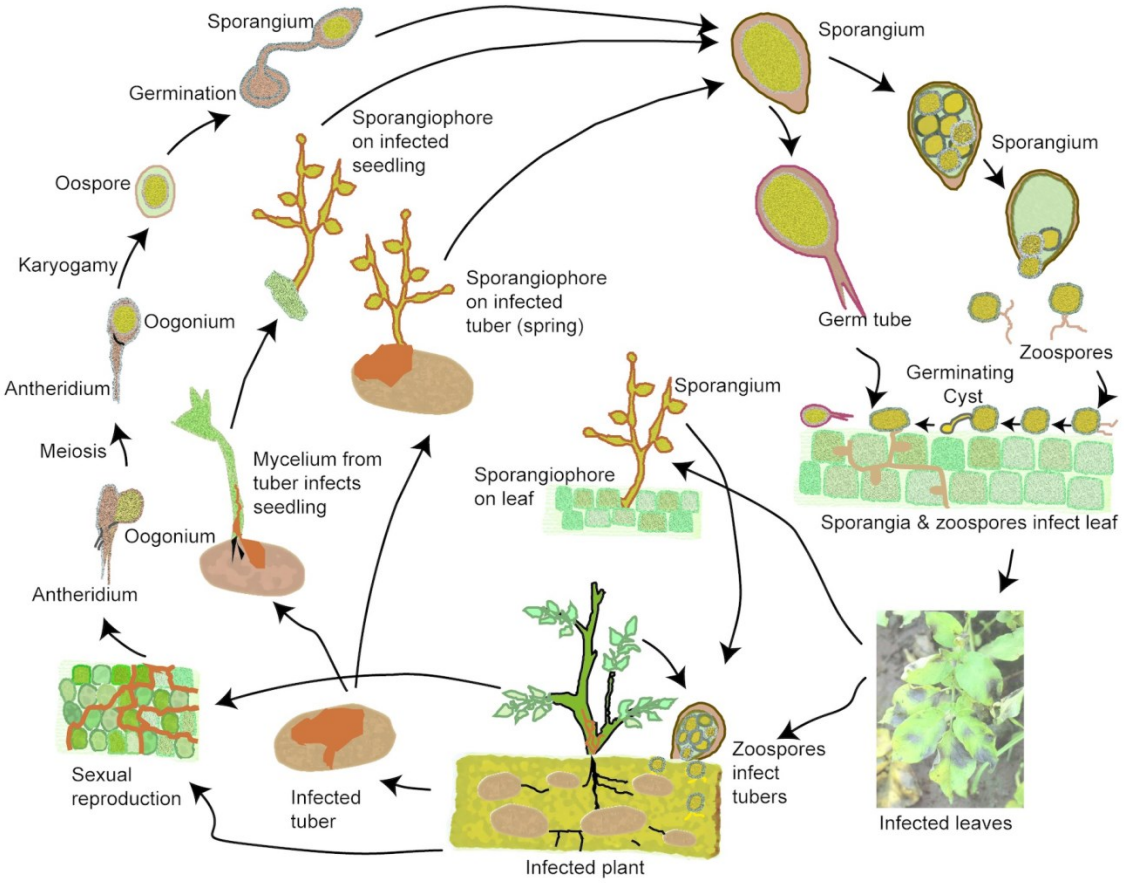

Figure 2-2. ***Phytophthora infestans*** **life cycle.**
This diagram (Vetukuri *et al.*, 2012) illustrates the asexual and sexual reproductive pathways of *P. infestans*. During sexual reproduction, mycelia of different mating types can interact in response to hormones and form a large, thick-walled oospore from the oogonium of one sex and the antheridium of the other (Nowicki *et al.*, 2012; Nemose, 2014).

The presence of both mating types now found around the world has made it increasingly difficult to manage late blight disease – especially when more aggressive, fungicide-resistant genotypes are more common and the pathogen's ability to go dormant in soils, during potato transportation or in storage warehouses through the production of oospores is increasingly prevalent (Nowicki *et al.*, 2012).

2.2.2 Population origins and diversity

Two competing theories place the pathogen's origin in either central Mexico or the Andes. However a study by Goss *et al.* (2014) which tested both theories using microsatellite markers and four nuclear gene sequences sampled from *P. infestans*, concluded that their analysis supports a Mexican origin. Aided by the development of resistant *P. infestans* genotypes to synthetic fungicides and the emergence of new genotypes, potato late blight is still a reemerging disease today, over 150 years after the great Irish potato famine (Ristaino, 2002).

In many parts of Canada since 2009, potato and tomato production have experienced increasing levels of late blight diversity. For the past 15 years, the *P. infestans* genotype group US-8 (A2 mating type) has dominated national populations but a comprehensive survey conducted in 2012 has revealed the emergence of new genotypes in many parts of the country (Alkher *et al.*, 2015). Table 2-1 lists the genotype group of *P. infestans* isolates collected from various Canadian locations during the 2012 season as determined by Alkher *et al.* (2015). Although the A2 genotype group US-22 was detected on tomato in the province of Ontario, all other isolates found across the country of other genotype groups were of the A1 mating type. US-23 was especially prevalent for 2012; 100 of the 119 isolates collected being identified as belonging to that genotype group (Alkher *et al.*, 2015). *Phytophthora infestans* isolates belonging to the genotype groups CA-11, CA-13, CA-14, US-11, US-22, US-23, US-24 are now found in provinces across Canada. This increasing diversity demonstrates the rapid shifts in *P. infestans* populations due to migration of genotypes via infected plants and/or tubers, mutations, climate change and sexual recombination (Alkher *et al.*, 2015). Although it remains limited in Canada, sexual *P. infestans* recombination appears to have occurred in the British Columbia and Prince Edward Island populations of late blight. This is supported by the presence of both mating types in BC over several years which may have resulted in the creation of new genotypes (Alkher *et al.*, 2015). Having a good understanding of the spread of *P. infestans* genotypes and the potential for sexual recombination within Canada and elsewhere will be essential to tracking the pathogen`s movement and in developing effective prevention and control strategies (Peters *et al.*, 2014).

Table 2-1. ***Phytophthora infestans*** **isolates collected in Canada during 2012. Adapted from Alkher *et al.* (2015)**.

Province	Host	Mating type	Genotype group	Mefenoxam
British Columbia	Potato	A1	CA-12	Resistant (R)
	Potato	A1	US-11	Intermediate (I)
Alberta	Potato	A1	US-23	I-Sensitive (S)
Saskatchewan	Tomato	A1	US-23	S
	Potato	A1	US-23	S
Manitoba	Potato	A1	US-23	I-S
	Tomato	A1	US-23	I
	Potato	A1	US-24	I
Ontario	Tomato	A2	US-22	I-S
Quebec	Potato	A1	US-23	I
New Brunswick	Potato	A1	US-23	I-R
Prince Edward Island	Potato	A1	CA-13	S
	Potato	A1	CA-14	I
	Potato	A1	US-23	I-R

2.2.3 Symptoms

Phytophthora infestans infects potato plants under high relative humidity (80-100%) and average temperatures of 18 to 20°C (Mizubuti *et al.*, 2007). These favourable weather conditions cause the death of foliage and rapid rot of tubers (Ghorbani *et al.*, 2005). Following infection with zoosporangia, symptoms can first become apparent as early as 3-4 days later depending on the susceptibility of the host and the environmental conditions (Fall *et al.*, 2014). Free water is a necessity for the oomycete to grow; dry conditions halting the appearance of new sporangiophores (Termorshuizen, 2007). Late blight typically first becomes apparent as grey to green spots on the surface of infected leaves. These irregularly shaped spots will appear water-soaked. If adequate moisture and temperature conditions are met, these spots will become necrotic, total loss of leaves will occur and the whole plant will eventually die (Mizubuti *et al.*, 2007; Correa *et al.*, 2009; Al-Mughrabi, 2012). When foliage conditions are moist, a white granular mildew will often appear on the underside of leaves. This white moldy growth contains the specialized hyphae sporangiophores which are responsible for producing sporangia (the asexual propagules). While symptoms are usually most apparent on leaves, late blight disease

affects all parts of the plant; above and below ground. Motile spores can be washed down from the leaves along the stem after rainfall to infect tubers underground (Mizubuti *et al.*, 2007; Correa *et al.*, 2009; Al-Mughrabi, 2012). Once an unprotected potato crop is infected, the whole crop can be devastated by *P. infestans* within 7-10 days (Nowicki *et al.*, 2012). *Phytophthora infestans* can only survive on living host tissue. When the plant dies, so does the pathogen. It must then be transmitted to a new host in order to continue infection (Al-Mughrabi, 2012). Fig. 2-3 demonstrates symptoms of late blight on potato leaves.

Figure 2-3. **Symptoms of late blight on potato foliage.**

2.2.4 Measurements of disease severity

James (1971) developed a disease assessment key for potato late blight in order that the disease severity of *P. infestans* could be measured at regular intervals after the epidemic had started. His method involves surveying the crop to estimate the number of foci per acre or hectare, determining the average area of the foci, and using the disease assessment key to assess

the percentage of leaf area affected within the foci sampled so that an estimate for the whole crop can be made. James' key in question – seen in Fig. 2-4 - illustrates disease severity (blackened areas) on affected leaves at different percentage levels (James, 1971). Modified versions of James' key are still widely used today. An analysis of variance for one such key demonstrated significant differences in absolute error among keys and individual evaluations. However, differences between evaluators were not found. Although the use of grading keys unavoidably introduces errors of measurement, their standardization and speed of use outweigh these shortcomings (Correa *et al.*, 2009).

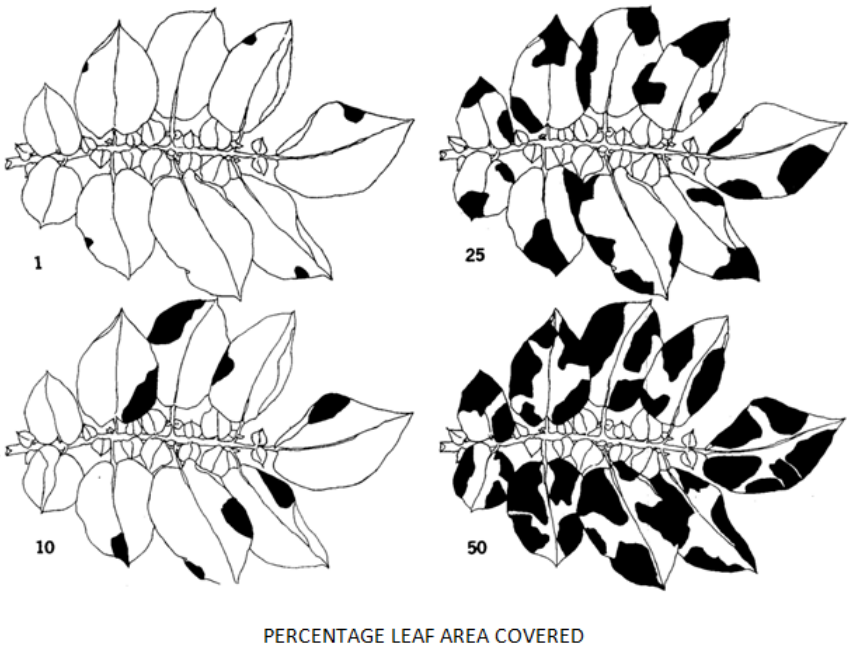

Figure 2-4. **James (1971) potato late blight disease assessment key.**
Darkened areas represent the level of disease severity of the affected leaf. Adapted from James (1971).

2.3 Economic impact of potato late blight

Conservative estimates place the worldwide economic burden due to potato late blight at over six billion United States dollars annually (Johnson *et al.*, 2015). Because the disease can spread rapidly via wind movements, an entire crop can be killed within a few weeks. Should this happen early in the season, potato production yields can be reduced by up to 80% (Haverkort *et*

al., 2009). In developed countries such as those found in Europe and North America, synthetic fungicides are used to reduce yield losses due to *P. infestans* and account for about 10-20% of the total potato production costs. In developing countries where commercial fungicide products are not available and/or affordable, yields tend to be 25% of those in developing countries (Haverkort *et al.*, 2009). During the year 2009, total potato losses in the United States were estimated to have attained a cost of 3.5 billion USD. Of these losses, up to half could be attributed to *P. infestans* (Nowicki *et al.*, 2012). In order to extrapolate figures concerning the economic importance of late blight in Canada, statistics from the Netherlands are examined. The total costs of late blight in the Netherlands amounts to 124.9 million €; or about 15.8% of the total farm gate price (Haverkort *et al.*, 2009). In 2012, the total farm game value for the potato industry in Canada was placed at 1.120 billion Canadian Dollars. If we assume that a similar amount of yield losses and control costs took place here in Canada as for the Nertherlands (both are northern, developed countries), then the total cost attributed to late blight in Canada during 2012 is of approximately 177 million CAD (15.8% of the total farm gate value) (Agriculture and Agri-Food Canada, 2014).

2.4 Methods of control

2.4.1 Synthetic fungicides

Control of *P. infestans* through cultural practices alone can be quite challenging, especially in agricultural regions where both potatoes and tomatoes are grown. Synthetic fungicides have thus become the standard method of control and are relied on heavily by growers all around the world to minimize crop losses (Nowicki *et al.*, 2012). Two main types of compounds are used routinely: protectant (including chlorothalonil, dithiocarbamates, and triphenyltin hydroxide) and systemic (including aliphatic nitrogen fungicides such as cymoxanil; phenylamides, such as metalaxyl/mefenoxam; and morpholine fungicides such as dimethomorph) (Nowicki *et al.*, 2012). The British Columbia Ministry of Agriculture lists 26 fungicides registered for use against potato late blight. These include fungicides with active

ingredients mancozeb, metiram, captan, chlorothalonil, pyraclostrobin, fenamidone, cymoxanil, cyazofamid, zoxamide, propamocarb, fluazinam, mono-/di-potassium salt of phosphorous acid, dimethomorph, mandipropamid and products using metalaxyl as one of its components (Sabaratnam, 2014).

In Canada, the heavy use of metalaxyl in previous years has resulted in metalaxyl-resistant *P. infestans* genotypes, and thus the loss of the only truly systemic fungicide that could be used to thwart late blight epidemics (Agriculture and Agri-Food Canada, 2013). A strict program of repeated protectant fungicide applications are what growers must now rely on. In addition, a mancozeb-based treatment of seed potatoes and a foliar application of cymoxanil at 80% emergence are often recommended but there exists little data to support these management approaches (Agriculture and Agri-Food Canada, 2013). The use of synthetic pesticides in Canada is regulated under the Pest Control Products Act by the Department of Justice Canada (2002) (last amended 2006). Prince Edward Island, Canada's smallest province, is the country's largest producer of potatoes (1,186,825 tonnes harvested in the 2014 crop year) (Agriculture and Agri-Food Canada, 2015). Despite the installation of buffer zones; because the province is heavily concentrated with agricultural land, the distance from cropland to rivers and wetlands is often minimal. Between 1962 (shortly after pesticides were introduced to Prince Edward Island) and 2011, there has been 50 sets of fish kills in the province – the worst of which arguably occurred in 1995 when 40,000 salmon smolts were lost (Macphail Ecological Woods Forestry Project, 2011). Although the cause of several of these kills remains unknown to this day, those investigations which reached evidence-based conclusions all state pesticides as the major cause. Mancozeb and Chlorothalonil – both used in late blight control – dot the list of causal agents (Macphail Ecological Woods Forestry Project, 2011). Human health risks arising from the use of pesticides in agriculture have also been questioned. In 2009, Garron *et al.* released a study examining downwind air concentrations of synthetic pesticides used in potato agriculture. Concentrations of carbofuran, methamidophos, mancozeb and diquat dibromide were observed following spray drift within 24 hours of their application, within 100 meters of potato fields. The authors found maximum downwind concentrations to be higher than levels previously observed during similar studies and that some were above the published air quality guidelines (Garron *et al.*, 2009). Moreover, synthetic treatments can sometimes be ineffective, especially when

environmental conditions are highly favourable for *P. infestans* development (Nowicki *et al.*, 2012).

2.4.2 Organic fungicides

A number of organic fungicides exist which, if coupled with good plant health management strategies, can help curb the effect of phytopathogens such as *P. infestans*. These include: sulfur (purported to prevent fungal spores from germinating), copper (somewhat effective at killing fungi and bacteria but can be quite toxic to host plants) and bicarbonates (sodium bicarbonate, ammonium bicarbonate or potassium bicarbonate which can be effective to some small degree - especially if used in combination with oils) (Beckerman, 2008). In many regions of Canada, copper hydroxide can be the only tool available for controlling late blight in organic potato production and is sprayed on average every 5-7 days (Glover *et al.*, 2011). Although essential to many biological processes, excess copper can be a significant problem ecologically and to the potato plant. Reactive oxygen species (ROS) can be produced from excess copper and trigger an important reorganization of a plant's root system (Ferreira *et al.*, 2014). In addition, ROS are highly reactive and toxic to various plant proteins, lipids, carbohydrates and DNA. This ultimately leads to increased oxidative stress for the plant (Ferreira *et al.*, 2014). Furthermore, it quite simply may not be possible to control potato late blight with even the best organic fungicides; especially when conditions favor *P. infestans* activity or a highly aggressive isolate is present (McGrath, 2010).

2.4.3 Disease resistant cultivars

Following potato late blight epidemics in Europe during the mid-1840s, the race to find resistant sources has been ongoing. During the 1960s, eleven major resistance genes (R-genes) were identified from *Solanum demissum* (a wild hexaploid tuber-bearing plant from Mexico) using conventional methods (Bains and Howard, 1950; Sharma *et al.*, 2013). These proved instantly successful but it was not long before the resistance provided by these genes was

defeated by new and more virulent *P. infestans* genotypes. It turned out the resistance was vertical; i.e. that the resistance was strain- or race-specific and therefore easily overcome by pathogen mutagenesis (Sharma *et al.*, 2013). Breeders were forced to abandon this approach. The search for minor genes providing durable race-non-specific resistance (deemed horizontal) to late blight was therefore initiated. Aided by the availability of new molecular tools, it was discovered that both major (vertical) and minor (horizontal) R-genes may be similar at the molecular level. This sparked renewed interest in breeders to reexamine the abandoned major R-genes (Sharma *et al.*, 2013). Eight major genes have since been mapped: *R1* on chromosome 5 by Leonards-Schippers *et al.* (1992); *R6* and *R7* on chromosome 11 by El Kharbotly *et al.* (1996); *R2* on chromosome 4 by Li *et al.* (1998); *R3a* and *R3b* by Huang *et al.* (2004), *R10* and *R11* by Bradshaw *et al.* (2006) – all on chromosome 11; and most recently *R8* on chromosome 9 by Jo *et al.* (2011). Four of these eight genes have subsequently been cloned: *R1* by Ballvora *et al.* (2002), *R3a* by Huang *et al.* (2005), *R2* by Lokossou *et al.* (2009), and *R3b* by Li *et al.* (2011) (Sharma *et al.*, 2013). Around 20 late blight resistant genes from sources other than *Solanum demissum* have also been cloned. Furthermore, this led to the development of effective Marker Assisted Selection (MAS) by closely linking molecular markers to gene sequences (Sharma *et al.*, 2013). There is several potato cultivars registered in Canada and the United States that have resistive properties against late blight. For example: Defender, a russet variety, has performed well against pressure from *P. infestans* in the United States. However it does not have the fresh market appeal that distributors and consumers look for (Glover *et al.*, 2011). In Canada, several other varieties such as AC Brador, Accent, Butte, Cherokee, Fundy, Island Sunshine, Keswick, Krantz, Nooksack, Northstar, Pungo, Redsen, Rosara, Umatilla Russet and Van Gogh have been reported to possess late blight resistant capabilities of the foliage, tuber or both (Canadian Food Inspection Agency, 2015). Glover *et al.* (2011) tested late blight disease severity in Island Sunshine and Krantz against the marketable variety Norkotah. Island Sunshine was found to have significantly lower levels of disease development than Norkotah or Krantz. However, even though Norkotah was not the most resistant variety to late blight, it still had the strongest yield (i.e. greatest number of plants and tubers at harvest and the largest weight of said tubers) (Glover *et al.*, 2011). This is a good example of the difficulties involved in marketing late blight resistant varieties. Resistance is not enough; cultivars must also be profitable for the farmer in terms of yield and appealing for the consumers in terms of appearance, taste and familiarity. In addition,

producing a new cultivar through genetic breeding can require a very long time. Although genetic engineering allows this process to be sped up by transferring individual genes encoding for resistance from other plant sources, the target plant also acquires many new unpredictable traits which warrant the careful consideration of using genetically modified plants (Zakharchenko *et al.*, 2011).

2.4.4 Crop production practices

The Canadian Horticulture Council recommends various crop production practices to include in any late blight management program to reduce infection rates and minimize disease severity. These include recommendations for seed potatoes, properly managing cull piles, removing volunteer plants, reporting suspected incidences of *P. infestans* infection to agricultural authorities, top killing or roguing an area twice the size of that infected, harvesting crops under dry conditions whenever possible and being aware of upcoming weather conditions favorable to late blight development (Canadian Horticultural Council, 2011). Applying mulches or soil mounds overtop tuber seeds has also been used to help filter out spores from the soil water suspension in order to reduce direct contact. However these practices have been demonstrated to exhibit moderate effectiveness at best – especially with the rise of aggressive genotypes (Olanya *et al.*, 2009). Haulm destruction prior to harvest to eliminate the foliage source of tuber blight inoculum and irrigation management to minimize the microclimate conditions favorable to late blight activity are also recommended practices (Olanya *et al.*, 2009). As easy to implement and relatively inexpensive as these practices may be, late blight control via crop production practices alone can be extremely challenging – especially in areas where other nightshades such as the tomato are also being cultivated (Nowicki *et al.*, 2012). Their effectiveness is also highly dependent on the time and duration that practices are implemented relative to the amount of *P. infestans* inoculum present which is almost impossible to measure in natural field situations before the infection becomes symptomatic. Additional control measures are almost always recommended and required (Olanya *et al.*, 2009; Nowicki *et al.*, 2012).

2.5 Biocontrol

Biological control can be defined simply as the use of microorganisms and/or their metabolites to protect plants against pathogenic threats (Tomar *et al.*, 2013). Because the biotic control of potato late blight can offer an effective alternative to environmentally-taxing synthetic fungicides without the need for developing potentially less marketable varieties, the study of bacteria with antifungal properties has become of great interest for potato pathologists and producers all over the world (Cao and Forrer, 2001). In addition to combating potato late blight, many of these microorganisms produce phytohormones (plant growth regulators), fix atmospheric nitrogen and improve plants' phosphorus nutrition (Zakharchenko *et al.*, 2011). Biocontrol treatments may have another advantage over synthetic applications in that they can colonize the plant host and therefore have the potential to increase in activity over time; research with *Bacillus cereus* showing that plant colonization on the potato surface increased until 61 days after planting (Wharton *et al.*, 2012). Several microorganisms have already been tested for their ability to prevent late blight infection, often with significant levels of success.

2.5.1 Modes of action

Biocontrol treatments stemming from living microorganisms or their metabolites can provide protection against plant pathogens through one of the following mechanisms:

- Through the production of antibiotics, biosurfactants or other molecules with the ability to deteriorate the pathogen's viability and/or disease development.
- By competing with the pathogen for nutrients and/or space.
- By inducing systemic resistance of the host plant (Daayf *et al.*, 2003).

Some microbes have the ability to antagonize pathogens directly by producing low molecular weight antifungal compounds. One example includes lipoproteins from *Bacillus subtilis* consisting of a lipophilic fatty acid chain and a hydrophilic peptide ring which have been

shown to contain powerful biocontrol properties (Chen *et al.*, 2008). Serenade, whose active ingredient is a *B. subtilis* strain, stops the germination of plant pathogen spores through three groups of lipoproteins. These bacterial metabolites also disrupt the pathogen's mycelial and germ tube growth, and inhibit the attachment of the pathogen to the leaf's surface (Stephan *et al.*, 2005). In addition, biosurfactants are naturally-occurring compounds produced by microorganisms that reduce surface and interfacial tension (Tomar *et al.*, 2013). These amphiphilic materials are produced extracellularly or as part of the cell membrane by several bacteria. Their high specificity, biodegradability and ability to inhibit fungal pathogens have made them good candidates for late blight biocontrol agents (Tomar *et al.*, 2013). On the other hand, some molecules do not combat the pathogen directly but act indirectly through the activation of the host plant's innate immune defense system prior to infection by the pathogen (Cao and Forrer, 2001). Induced systemic resistance (ISR) or systemic acquired resistance (SAR) in a variety of host-pathogen systems is a phenomenon which has been well documented. Heller and Gessler (1986) were the first to demonstrate this event for late blight protection in tomatoes and Doke *et al.* (1987) for potatoes (Cao and Forrer, 2001). The defense-related phytoalexins and/or other pathogenesis-related proteins produced by the plant are excellent defense mechanisms that can be used to respond to pathogenic stresses. Many bacterial metabolites (such as curdlan, a linear water-insoluble β-1,3-glucan produced by the fermentation of *Agrobacterium* sp.) can act as triggers for activating the plant's defense response before the pathogen makes its unwanted arrival (Li *et al.*, 2014). Direct antagonists or inducers can be either curative (the treatment is most effective when or after the plant is infected) or protective (the treatment is most effective when applied before the plant is infected) (Stephan *et al.*, 2005).

2.5.2 Bacteria

Pierson and Pierson (1996) demonstrated that phenazine antibiotics produced by *Pseudomonas aureofaciens* are primarily responsible for the bacteria's competitive fitness in the rhizosphere; giving rise to its ability to act as a biocontrol agent. *Pseudomonas putida* and *Bacillus subtilis* were shown to suppress the development of *P. infestans* zoospores during *in vitro* tests in Russia (Cao and Forrer, 2001). Yan *et al.* (2002) used two strains of plant growth-

promoting bacteria, *Bacillus pumilus* and *Pseudomonas fluorescens*, to elicit systemic protection against late blight in greenhouse tomatoes at a level equivalent to systemic acquired resistance induced by *P. infestans* itself or induced local resistance by a chemical inducer. Forty-three bacteria were isolated and screened for their biocontrol abilities against a *P. infestans* isolate of the genotype group US-8 in potato by Daayf *et al.* (2003). Bacteria were tested using three systems: *in vitro* culture media, detached leaves and whole plant bioassays. This complementarity of testing proved beneficial and it was determined that bacteria with biocontrol activity were from the genera *Bacillus*, *Pseudomonas*, *Rahnella* and *Serratia* (Daayf *et al.*, 2003). Stephan *et al.* (2005) demonstrated that the metabolites of *Bacillus subtilis* and not the microorganism itself were responsible for its biocontrol abilities. Júnior *et al.* (2006) screened 208 epiphytic microorganisms and 23 rhizobacteria using *in vitro* inhibition of sporangia and detached leaflet bioassays for antagonistic activity against late blight in tomatoes. Four epiphytic microorganisms (*Aspergillus* sp., *Cellulomonas xavigena*, *Candida* sp., and *Cryptococcus* sp.) were then selected and one rhizobacterium (*Bacillus cereus*) for further disease severity testing which demonstrated a reduction in severity using *B. cereus* in combination with *C. xavigena*, *Candida* sp. and *Cryptococcus* sp. but not when plants were treated with the rhizobacteria alone (Júnior *et al.*, 2006). Tran *et al.* (2007) showed that the compound massetolide A produced by *Pseudomonas fluorescens* was effective in preventing tomato late blight infection and that the expansion of existing late blight lesions was also significantly reduced. The study determined that induced systemic resistance was responsible for their results (Tran *et al.*, 2007). Zakharchenko *et al.* (2011) demonstrated increased protection against phytopathogens including *P. infestans* when plants were colonized with a strain of *Pseudomonas aureofaciens*. Wharton *et al.* (2012) found that potato seed pieces treated with *Bacillus subtilis* exhibited reduced late blight disease incidence and severity. Tomar *et al.* (2013) showed that biosurfactants extracted from five strains of *Pseudomonas aeruginosa* were effective at inhibiting *P. infestans* growth by up to 85.33% when tested *in vitro* (Tomar *et al.*, 2013).

Recent late blight biocontrol research includes the works of Hunziker *et al.* (2015), Van Der Voort *et al.* (2015) and Bengtsson *et al.* (2015). Hunziker *et al.* (2015) demonstrated that cyanogenic *Pseudomonas* strains completely inhibited *P. infestans* growth while noncyanogenic

strains also produced anti-oomycete compounds (Hunziker *et al.*, 2015). In 2015 Van Der Voort *et al.* further characterised their previously isolated *Pseudomonas* sp. SH-C52 from the rhizosphere of sugar beets. Its antifungal properties – at least in part – were attributed to the production of the chlorinated 9-amino-acid lipopeptide thanamycin. Seven structural variants of the newly isolated lipopeptide thanapeptin were then also found to possess varying degrees of antifungal activity against *P. infestans* (Van Der Voort *et al.*, 2015). Bengtsson *et al.* (2015) observed a significant decrease in late blight severity and an induced secretion of proteins such as pathogenesis-related protein 1 within the leaf apoplast after treatment with the biosurfactant-producing strain *Pseudomonas koreensis* 2.74 (Bengtsson *et al.*, 2015).

In Canada, several biopesticide products are registered for use against *P. infestans* or have pending registrations including Actinovate (*Streptomyces lydicus*), Sonata (*Bacillus pumilus*), Rhapsody (*Bacillus subtilis*) and three formulations of Serenade (*Bacillus subtilis*) (Glover *et al.*, 2011; Reuters, 2012). However as of yet, these products have not been able to demonstrate a sufficient and consistent level of late blight antagonism in order to significantly curb the heavy use of synthetic and copper-based fungicides – Glover *et al.* (2011) finding little (if any) late blight severity difference between untreated control plants and those treated with Actinovate or Sonata for example.

Chapter 3 : Materials and methods

3.1 Selection of *Phytophthora infestans* isolates

3.1.1 *Phytophthora infestans* isolates

AAFC isolates originating from AAFC Dr. Ken Conn were kept growing on sterile Rye B agar (RBA) plates (Rye seeds originating from nearby AAFC "Jean-Charles Chapais Farm" in Lévis, QC) or on sterile Russet Burbank tuber cores (potatoes originating from Patate Dolbec Inc., St-Ubalde, QC). All nine isolates (Table 3-1) were re-plated approximately every month or inoculated onto fresh tubers or rye seeds approximately every two months in order to avoid nutrient deprivation, restricted growth and decreased pathogenicity. Plates and tuber cores were kept under sterile conditions in a growth room maintained at 22^{o}C.

Table 3-1. Nine *Phytophthora infestans* isolates in the AAFC Québec City collection.

CA-9 (A1)	US-22_1[z] (A2)	US-22_2 (A2)	CA-10_1 (A1)
US-22_3 (A2)	US-22_4 (A2)	CA-10_2 (A1)	US-8 (A2)
US-23 (A1)			

[z] Note that numerical subsets are given to the isolate names for those belonging to the same *P. infestans* genotype group as others in the collection.

3.1.2 Detached fed-leaf bioassays using nine *Phytophthora infestans* isolates

The identification of virulent *P. infestans* candidates was determined using a detached fed-leaf bioassay experiment – a method developed by Dr. Susan Boyetchko's and Dr. Patrice Audy's laboratories at Agriculture and Agri-Food Canada (AAFC). A detached fed-leaf bioassay setup consists of detached potato leaves placed in separate nutrient solution tubes and enclosed in a high humidity box (85-95% relative humidity) (Fig. 3-1). Leaves can then be sprayed with a *P. infestans* solution for infection and remain in an environment extremely favourable for late blight development.

Figure 3-1. **Detached fed-leaf bioassay box.**
Detached leaves are placed in 50 mL falcon tubes containing nutritive Hoagland solution and enclosed in a high humidity box (85-95% RH) in order to favour pathogenic growth.

3.1.2.1 Pathogen suspension

Ten days before leaf infection, three dedicated RBA plates for each *P. infestans* isolate were inoculated and cultures grown so that sporangia solutions could be prepared for infection. Upon infection day, the 27 RBA plates containing the nine pathogen isolates were removed from incubation. Distilled water (15 mL per plate) was pipetted (pipettor: IBS Integra Biosciences, Hudson, NH, USA; 10 mL pipettes: SARSTEDT, Nümbrecht, Germany) onto each RBA plate. A glass spreader was then used to rub the top of the agar in order to bring the sporangia into solution. The 45 mL of pathogen solution from each isolate was then transferred in turn to a clean 1000 mL glass beaker (Kimble Chase Life Science and Research Products LLC,

Rockwood, TN, USA) and placed on a stir plate (VWR Scientific Products, Radnor, PA, USA). The solution was mixed using a magnetic stir bar for five minutes. Using a hemocytometer (American Optical, Buffalo, NY, USA) and a ZEISS AX10 microscope (Carl Zeiss MicroImaging, Göttingen, Germany), the concentration of sporangia per millilitre was determined for each isolate and the volume adjusted with distilled water accordingly so that each pathogen solution was of 10,000 sporangia/mL. Each pathogen solution was then transferred to dedicated spray bottles (Symak Sales co., Montréal, QC).

3.1.2.2 Detached fed-leaves

Upon infection day, a total of 27 mature leaves were cut with a sharp metal blade just above the stipule from potato plants grown from seed potatoes (McKenzie co., Brandon, MB) in one of the AAFC research greenhouses (Soils and Crops Research and Development Centre, Québec City, QC). Leafs were thoroughly rinsed twice with distilled water and dried using brown paper towel (Kimberly-Clark, Irving, TX, USA) to absorb excess moisture. Excess leaflets were then cut so that each leaf contained five leaflets only. Meanwhile, a plastic box setup was constructed for each nine isolate in order that the detached leaves could occupy a relatively enclosed area during the course of the experiment. For each box setup, two 6L plastic shoe boxes (a.b.m. Canada Inc., Milton, ON) were placed one on top of the other in order to form an enclosed space (Fig. 3-1). A back hinge where the boxes meet was made using standard duct tape so that the boxes were held together but could still swing open. Two brown paper towels were placed on the inside bottom of the lowest box and wet with 250 mL distilled water in order to help achieve very humid starting conditions (85-95% RH). A 4*9 test tube tray (Magenta corp., Chicago, Illinois, USA) was then placed overtop the paper towels. Three 50 mL falcon tubes (SARSTEDT, Nümbrecht, Germany) were placed at equal distances apart in the tray and 45 mL of 10% Hoagland solution prepared as described by Hoagland and Arnon (1938) was added to each of the three tubes. All nine box setups were prepared in the same way for all *P. infestans* isolates being tested for pathogenicity. Three detached leaves per box of the 27 leaves prepared were randomly selected and placed into the falcon tubes containing Hoagland solution.

3.1.2.3 Pathogen inoculation on detached fed-leaves

All three leaves in each box setup were mist sprayed until runoff occurred with the appropriate pathogen solution. The top inside surface of each box setup was heavily mist sprayed with distilled water in order that the starting conditions remained very humid (85-95% RH). All nine box setups were then transported to the growth room kept at 22^{o}C and under 34W fluorescent lighting (Sylvania, Danvers, MA, USA) for 16 continuous hours of every 24h cycle. Box location in the growth room was randomly assigned. At the beginning of each new day, the inside of all box setups were heavily misted with distilled water in order to maintain high humidity conditions (85-95% RH). Disease severity ratings were read for all 27 leaves using a modified version of James' (1971) disease assessment key (James, 1971) after seven days in which each leaf was given a score of 0 to 100% in increments of 5% disease severity.

3.1.2.4 Selection criteria

Phytophthora infestans isolates selected for use in subsequent biocontrol experiments displayed strong levels of pathogenicity (those with higher median disease severity scores were favoured), were of both mating types A1 and A2 and were representative of the *P. infestans* genotypes found in Canadian fields at that time – as observed by Peters *et al.* (2014). Median disease severity ratings were calculated from each isolate box.

3.2 Selection of bacterial candidates

3.2.1 Detached fed-leaf bioassay of 46 bacterial strains

Prior to the research completed for this Master's thesis degree, the identification of six good bacterial candidates for late blight biocontrol was determined using a series of detached fed-leaf bioassay experiments conducted at the Agriculture and Agri-Food Canada research centre (Québec City, QC). Forty-six different strains of bacteria (isolated by AAFC scientist Dr.

Susan Boyetchko's team from Saskatchewan soils) were tested against *P. infestans* isolate US-8 obtained from Dr. Ken Conn. This series of experiments tested whole culture and filtrate bacterial fermentation solutions for their antagonistic behaviour against late blight in order that six candidates from the 46 total be chosen for further biocontrol testing.

Each bacterial treatment was composed of three experimental box setups: one for the whole culture, one for the filtrate solution, and one for an untreated control. Because of space constraints, not all bacteria were able to be tested simultaneously but over the course of many months. However, the growth room was maintained at a constant humidity level, 22^{o}C and with the same light (16h) to dark (8h) cycle. Disease severity ratings were read for all leaves using a modified version of James' (1971) disease assessment key (James, 1971) after seven and ten days in which each leaf was given a score of 0 to 100% in increments of 5% disease severity. From these results, six bacterial strains were selected by research supervisors and AAFC staff for further biocontrol testing based on strength of late blight biocontrol and as possessing reasonably good characteristics (as they relate to ease of production/storage, non-pathogenic for humans and the environment, etc.) for being mass-produced as a commercial biofungicide.

3.2.2 Detached fed-leaf bioassays using four *Phytophthora infestans* isolates and six bacterial strains

The further narrowing down of good bacterial candidates was completed using detached fed-leaf bioassay experiments. The six bacterial candidates identified in section 3.2.1 were: 189 (*Pseudomonas chlororaphis*), WAUSV36 (*Bacillus subtilis*), KENGFT3 (*Pseudomonas fluorescens*), UW01 (*Pseudomonas fluorescens*), OXWO6B1 (*Pantoea stewartii*) and OY3WO11 (*Arthrobacter phenanthrenivorans*). These six bacterial strains, which had already shown strong levels of protection against *P. infestans* isolate US-8, were tested against the four *P. infestans* isolates identified in section 3.1 (US-8, CA-10_1, US-22_2 and CA-9). Whole cultures only were tested. All six bacterial strain whole cultures were tested in triplicate (three boxes) across four periods against all four *P. infestans* isolates in turn. Three control box setups were also tested each period in order that potential differences between the six bacterial treatments and an untreated control could be determined. For each isolate:

Figure 3-2. **The experimental breakdown of detached fed-leaf bioassays using four *Phytophthora infestans* isolates and six bacterial strains.**
Each bacterial whole culture (trt1: 189, trt2: WAUSV36, trt3: KENGFT3, trt4: UW01, trt5: OXWO6B1 and trt6: OY3WO11) was tested for antagonism against late blight in triplicate (three box setups) alongside three untreated controls (trt7) for each four periods. Note that box locations were randomly allocated within the growth room. This was repeated for each *P. infestans* isolate identified as strongly pathogenic.

3.2.2.1 Pathogen suspension

Ten days before leaf infection, between 20 to 40 RBA plates (isolate-dependent) were inoculated and grown (22^{o}C) with the *P. infestans* isolate being tested during that experiment so that a sufficient amount of sporangia could be prepared at 7,000 sporangia/mL for infection. Note that the concentration of sporangial solution was reduced from 10,000 sporangia/mL (section 3.1.2.1) due to the fact that certain *P. infestans* isolates produced less sporangia than others after 10 days. To keep the concentration across isolates equal, a concentration of 7,000 sporangia/mL was used, ensuring that sufficient sporangia were always produced for all isolates.

Upon infection day, 20-40 RBA plates containing the specific pathogen isolate were then removed from incubation. The pathogen suspension was prepared as described in section 3.1.2.1.

3.2.2.2 Detached fed-leaves

A total of 126 mature leaves (18 per treatment, 18 for the control) were cut from potato plants grown from seed potatoes (McKenzie co., Brandon, MB) in one of the AAFC research greenhouses and prepared as described in section 3.1.2.2. Six falcon tubes containing Hoagland solution with six leaves each were placed in each box setup. Twenty-one box setups were constructed; three per bacterial treatment and three for the untreated control.

3.2.2.3 Bacterial treatments

Bacterial whole culture treatments were prepared according to the method developed by Boyetchko *et al*. (personal communication, 2014) to reach concentrations in CFU/ml (log 10) of: 9.64 ± 0.05 for 189, 8.13 ± 0.04 for WAUSV36, 9.66 ± 0.02 for KENGFT3, 9.62 ± 0.06 for UW01, 9.59 ± 0.04 for OXWO6B1 and 9.71 ± 0.01 for OY3WO11. Bacteria were stored in microtubes at -80^{o}C (SANYO E&E America Company, Bensenville, IL, USA) until five days before infection. At this point, all six bacterial cultures were streaked onto separate sterile Pseudomonas Agar F (PAF) plates using a flame-sterilized inoculation loop in such a way as to produce isolated colonies. All six plates were incubated at room temperature in the dark. After 48 hours or when isolated colonies had appeared, two isolated colonies were transferred aseptically from each plate using a flame-sterilized inoculation loop to two separate glass tubes containing 3 mL of sterile Nutrient Broth (EMD Chemicals Inc. Gibbstown, NJ, USA) each. A total of twelve tubes (two per bacteria) were incubated in a MaxQ400 shaker incubator (Thermo Scientific, Waltham, MA, USA) for 24 hours at 22^{o}C and 200 rpm. The next day, the contents of one glass tube per bacteria (the second being disposed of) were transferred aseptically to separate 500 mL baffled flasks (Corning, Tewksbury, MA, USA) containing 125 mL sterile Yeast Extract Glucose Medium broth (containing 2 g/L Yeast Extract, 2.5 g/L Dextrose, 0.25 g/L KH_2PO_4, 0.25 g/L K_2HPO_4, 0.1 g/L $MgSO_4$. $7H_2O$, 0.015 g/L $MnSO_4$. H_2O, 0.05 g/L NaCl and 0.005 g/L

$FeSO_4$. $7H_2O$) labelled with the name of the bacteria and incubated in the MaxQ400 shaker incubator at 22°C and 200 rpm for 48 hours. Upon infection day, the bacterial whole culture solution was transferred to a 1000 mL glass beaker (Kimble Chase Life Science and Research Products LLC, Rockwood, TN, USA). One by one, all 18 leaves from the three box setups of that respective bacterium were submerged directly into the bacterial solution so that the entirety of the leaf had been treated. After treatment, each leaf was returned to its respective Hoagland solution tube and the box setups closed. In the same manner, all remaining leaves (except the 18 for the untreated control) were treated with their respective bacterial treatment.

3.2.2.4 Pathogen inoculation on detached fed-leaves

After the treated leaves had dried, all 126 leaves in all 21 box setups (3*6 whole culture bacterial treatments and the three untreated controls) were mist sprayed with the pathogen solution until-runoff occurred. Treatment-to-infection time for all treatment leaves was 2 hours. The top inside surface of each box setup was then heavily mist sprayed with distilled water in order that the starting conditions were very humid (85-95% RH). All twenty-one box setups were then transported to the growth room kept at 22°C and under 34W fluorescent lighting (Sylvania, Danvers, MA, USA) for 16 continuous hours of every 24h cycle. Box location in the growth room was randomly assigned. At the beginning of each new day, the inside of all box setups were heavily misted with distilled water in order to maintain high humidity conditions (85-95% RH). Disease severity ratings were read for all leaves using a modified version of James' (1971) disease assessment key (James, 1971) after seven and ten days in which each leaf was given a score of 0 to 100% in increments of 5% disease severity.

3.2.2.5 Data analysis

Raw data consisted of the disease severity percentage of each leaf (sampling unit) within each box (experimental unit). Traditionally, to obtain a correct ANOVA and determine if treatments are statistically significant, mean values of the sampling unit are calculated for each experimental unit (The Pennsylvania State University, 2015). Because the disease severity scores

were measured on an ordinal scale (i.e. non-continuous in increments of 5%), there was a need for data to be analyzed non-parametrically. In the case of ordinal scales, neither differences between scores nor means are defined (Kluth *et al.*, 2010). The median values, typically used with non-parametric tests, of the sampling units (leaves) for each experimental unit (box) were therefore calculated in place of the means (Pappas and DePuy, 2004). All statistical analysis for this section was performed using SAS 9.4 (SAS Institute). Median values from each experimental unit were analyzed using the non-parametric method described by Shah and Madden (2004) for a two-way factorial design (treatment x period). An ANOVA-type statistic (ATS) was performed on both fixed effects plus the interaction. LSMEANS statements were used to estimate the relative effects and multiple comparisons between treatments using Scheffé's adjustment.

3.2.2.6 Selection criteria

Results obtained from section 3.2.2 were used in addition to the professional feedback provided by research supervisors Dr. Patrice Audy and Dr. Susan Boyetchko to select two of the best bacterial candidates for further testing. Microorganism characteristics (as they relate to ease of production/storage, non-pathogenic for humans and the environment, etc.) were again taken into consideration during the selection process.

3.3 Performance of potential biocontrol bacteria

3.3.1 Bacteria, synthetic fungicide and *Phytophthora infestans* isolate selection

For the two bacterial candidates selected in section 3.2.2 (189 and WAUSV36), detached fed-leaf bioassay tests were conducted in order to compare their level of control against late blight to that of a commercial synthetic fungicide used by Canadian potato producers today. The synthetic fungicide selected for comparison was Dithane^{TM} DG 75 (Dow AgroSciences Canada Inc., Calgary, AB, Canada) which is one of the few fungicides used against late blight indoors

and contains the active ingredient mancozeb at 75%. This experiment was conducted using *P. infestans* isolate CA-9; identified in section 3.1 as one of the strongest if not the strongest isolate among the nine tested. According to the latest survey released at that time, it was also known to be problematic in Canada (Peters *et al.*, 2014).

3.3.2 Detached fed-leaf bioassays using *Phytophthora infestans* isolate CA-9, two bacteria and one synthetic fungicide

Both bacterial strain whole cultures 189 and WAUSV36 and the commercial synthetic fungicide Dithane™ DG 75 were tested in septuplicate (seven boxes) during one period against *P. infestans* isolate CA-9. Seven control box setups were also tested in order to determine if potential differences between the bacterial and synthetic fungicide treatments and an untreated control existed (Fig. 3-3).

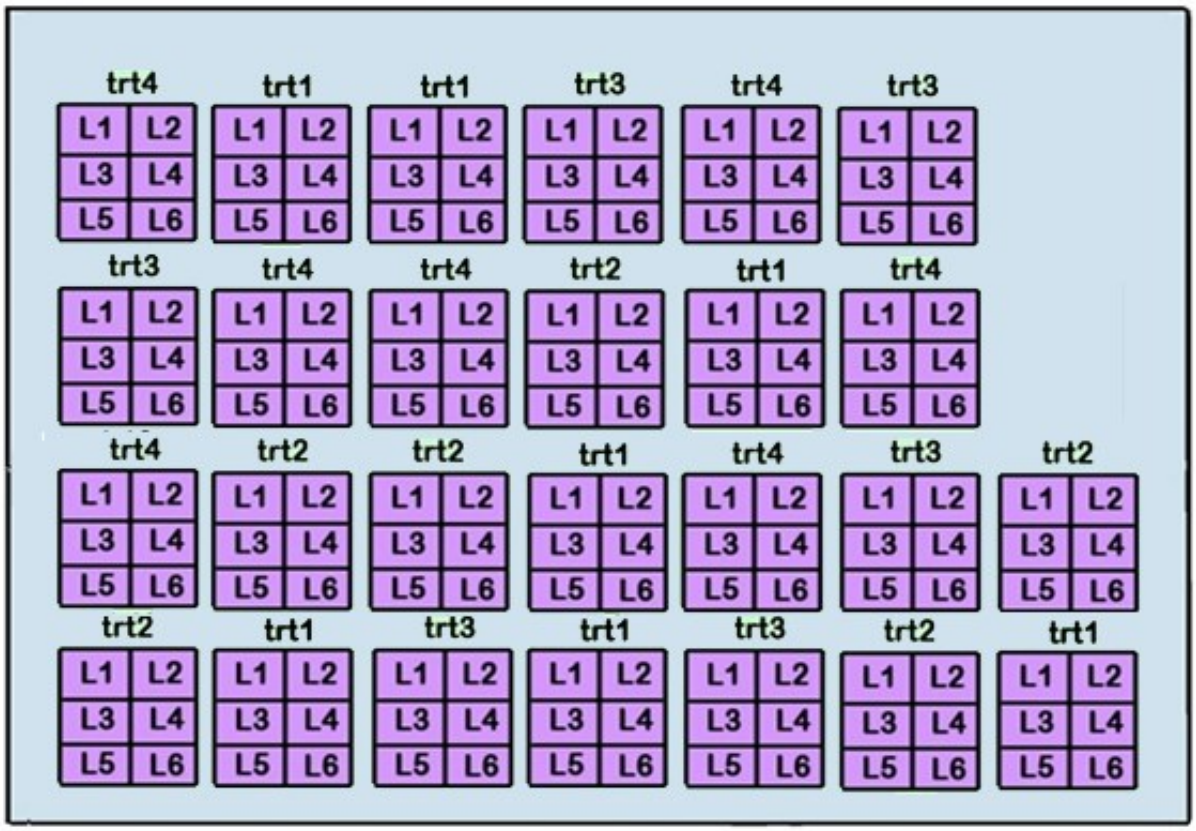

Figure 3-3. **The experimental breakdown of detached fed-leaf bioassays using *Phytophthora infestans* isolate CA-9, two bacteria and one commercial synthetic fungicide.**
Both bacterial strain whole cultures 189 (trt1) and WAUSV36 (trt2) in addition to the commercial synthetic fungicide Dithane^{TM} DG 75 (trt3) were tested for antagonism against *P. infestans* isolate CA-9 alongside control boxes (trt4). Each box was randomly allocated a location in the growth room.

3.3.2.1 Pathogen suspension

Ten days before leaf infection, 20 RBA plates were inoculated and grown (22^{o}C) with the *P. infestans* isolate CA-9 so that a sufficient amount of sporangia could be prepared at 7,000 sporangia/mL for infection. Upon infection day, the 20 RBA plates containing the pathogen isolate CA-9 were removed from incubation and the *P. infestans* solution was prepared as described in section 3.1.2.1.

3.3.2.2 Detached fed-leaves

A total of 168 mature leaves (42 per bacterial or synthetic fungicide treatment, 42 for the untreated control) were cut and prepared as described in section 3.1.2.2. Six falcon tubes containing Hoagland solution with six leaves each were placed in each box setup. Seven plastic box setups per bacterial or synthetic fungicide treatment and seven for the control were constructed.

3.3.2.3 Bacterial and synthetic fungicide treatments

Bacterial solutions 189 and WAUSV36 were prepared in accordance with the procedure described in section 3.2.2.3. Three baffled flasks containing 125 mL broth each were prepared for each bacterium in order that a sufficient amount of liquid be available for treatment. Upon infection day, the total liquid from the bacterial whole culture solution 189 (3*125 mL) was transferred to a 1000 mL glass beaker (Kimble Chase Life Science and Research Products LLC, Rockwood, TN, USA). One by one, all 42 leaves from the seven box setups of the bacterial whole culture 189 were treated as described in section 3.2.2.3. In the same manner, all leaves for the second bacterium WAUSV36 were treated. Leaves for the untreated control boxes were left untouched. Meanwhile, the synthetic fungicide solution Dithane^{TM} DG 75 was prepared under a chemical fume hood at a concentration of 80 g/L by mixing 500 mL distilled water with 40 g of Dithane^{TM} DG 75 powder (Dow AgroSciences Canada Inc., Calgary, AB, Canada) in a 1000 mL beaker. Always under the chemical fume hood, the 42 leaves from the seven boxes dedicated to the synthetic fungicide treatment were dipped one by one and returned to their respective Hoagland solution tubes.

3.3.2.4 Pathogen inoculation on detached fed-leaves

All 168 leaves in all 28 box setups (7*2 whole culture bacterial treatments, 7*1 synthetic fungicide treatment and seven untreated controls) were mist sprayed with the pathogen solution until-runoff occurred. Treatment-to-infection time for all treatment leaves was 2 hours. The top

inside surface of each box setup was heavily mist sprayed with distilled water in order that the starting conditions were very humid (85-95% RH). All 28 box setups were then transported to the growth room kept at 22^{o}C and under 34W fluorescent lighting (Sylvania, Danvers, MA, USA) for 16 continuous hours of every 24h cycle. Box location in the growth room was randomly assigned. At the beginning of each new day, the inside of all box setups were heavily misted with distilled water in order to maintain high humidity conditions (85-95% RH). Disease severity ratings were read for all leaves using a modified version of James' (1971) disease assessment key (James, 1971) after seven and ten days in which each leaf was given a score of 0 to 100% in increments of 5% disease severity.

3.3.2.5 Data analysis

All statistical analysis for this section was performed using SAS 9.4 (SAS Institute). Median values from each experimental unit were analyzed using the non-parametric method described by Kruskal and Wallis (1952) for a one-way experimental design. The EXACT WILCOXON / MC statement was used to produce Monte Carlo estimates for the Kruskal-Wallis exact test. LSMEANS of the ranked data were used to estimate multiple comparisons between treatments using Bonferroni's adjustment.

3.4 Time-delay between bacterial inoculation and pathogen infection

3.4.1 Detached fed-leaf bioassays using *Phytophthora infestans* isolate CA-9 and two bacteria

For the two bacterial candidates selected in section 3.2.2, 189 and WAUSV36, detached fed-leaf bioassay tests were conducted in order to compare biocontrol levels when the time between bacterial treatment and *P. infestans* infection differed from 2 hours up to 24 hours. These tests were important to ensure that potential biofungicide treatments would still protect potato plants even when infections occur up to a day later than treatment. This series of

experiments was conducted across two time periods (due to spatial limitations in the growth room) and used *P. infestans* isolate CA-9 for the same reasons as those listed in section 3.3.1.

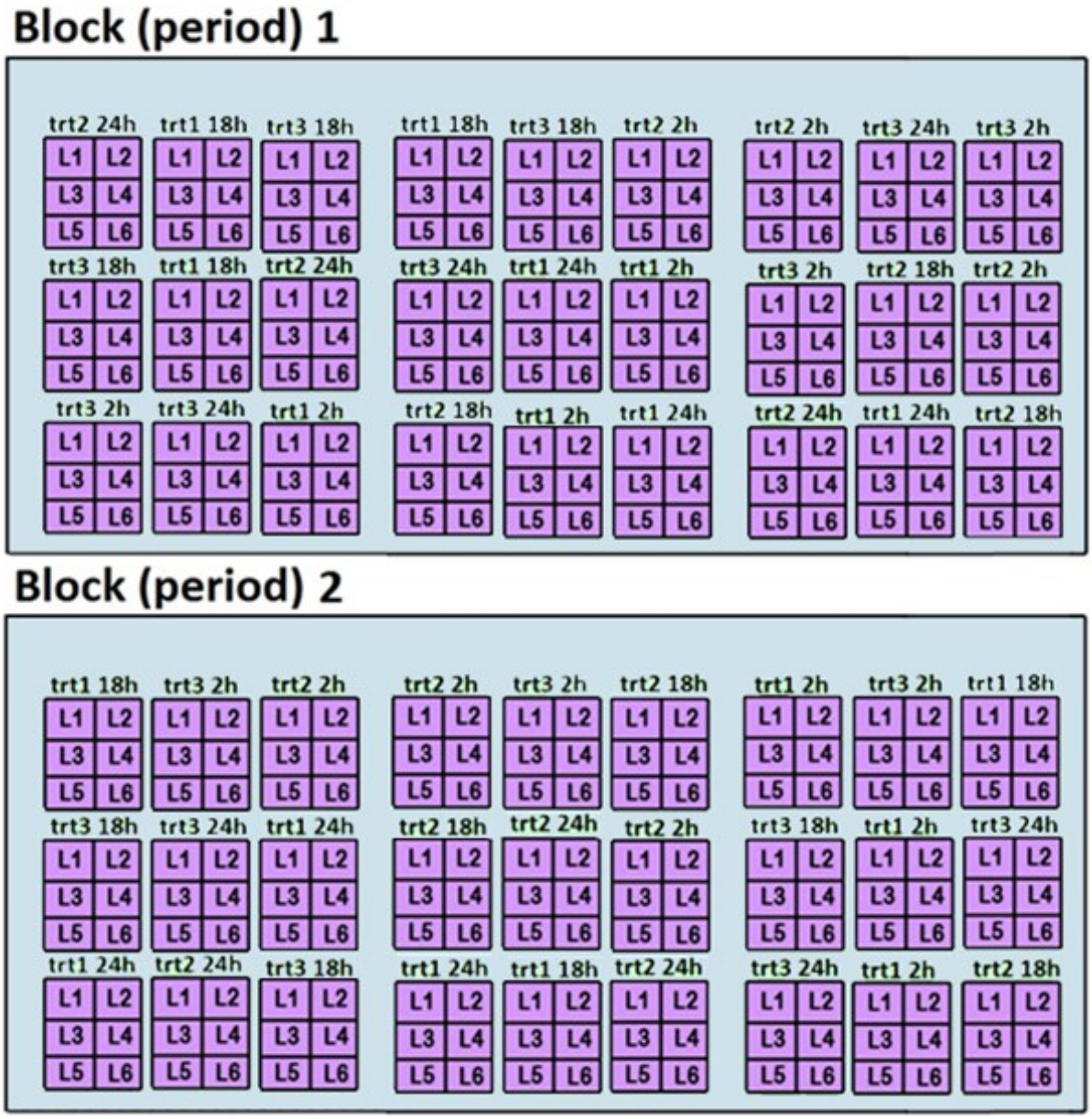

Figure 3-4. **The experimental breakdown of detached fed-leaf bioassays using *Phytophthora infestans* isolate CA-9, two bacteria and three treatment-to-infection time-delays.**
Bacterial strain whole cultures 189 (trt1) and WAUSV36 (trt2) alongside control boxes (trt3) were tested for antagonism against *P. infestans* isolate CA-9 at different treatment-to-infection delay times (2 hours, 18 hours, 24 hours). Each treatment/time-delay combination was run in triplicate. Each box was randomly allocated a location in the growth room. This was repeated across two periods in order to collect ample data for each treatment/time-delay combination.

3.4.1.1 Pathogen suspension

Ten days before leaf infection, 20 RBA plates were inoculated and grown (22^{o}C) with the *P. infestans* isolate CA-9, so that a sufficient amount of sporangia were prepared at 7,000 sporangia/mL for infection. Upon infection day, the 20 RBA plates containing the pathogen isolate CA-9 were removed from incubation and the *P. infestans* solution was prepared as described in section 3.1.2.1.

3.4.1.2 Detached fed-leaves

A total of 162 mature leaves (54 per bacterial treatment, 54 for the control) were cut and prepared as described in section 3.1.2.2. Six falcon tubes containing Hoagland solution with six leaves each were placed in each box setup. Nine plastic box setups per bacterial treatment and nine for the control were constructed.

3.4.1.3 Bacterial treatments

Bacterial treatments 189 and WAUSV36 were prepared in accordance with the procedure described in section 3.2.2.3. Three baffled flasks containing 125 mL broth each were prepared for each bacterium in order that a sufficient amount of liquid be available for treatment. Upon infection day, the total liquid from the bacterial whole culture solution 189 (3*125 mL) was transferred to a 1000 mL glass beaker (Kimble Chase Life Science and Research Products LLC, Rockwood, TN, USA). One by one, all 54 leaves from the nine box setups of the bacterial whole culture 189 were treated as described in section 3.2.2.3. In the same manner, all leaves for the second bacterium WAUSV36 were treated. Leaves for the untreated control boxes were left untouched.

3.4.1.4 Pathogen inoculation on detached fed-leaves

All 54 leaves for bacterial treatment and control boxes subject to a 2 hour time-delay only (one third of total leaves within nine of 27 boxes) were then mist sprayed with the pathogen solution until-runoff occurred. The top inside surfaces of all 27 box setups were heavily mist sprayed with distilled water in order that the starting conditions were very humid (85-95% RH). All 27 box setups were then transported to the growth room kept at 22^{o}C and under 34W fluorescent lighting (Sylvania, Danvers, MA, USA) for 16 continuous hours of every 24h cycle. Box location in the growth room was randomly assigned. Pathogen inoculation was repeated for all 54 leaves subject to an 18 hour time-delay and again for all 54 leaves subject to a 24 hour time-delay. A fresh pathogen solution (always prepared identically and from the same *P. infestans* isolate stock) was prepared for each time-delay infection. At the beginning of each new day, the inside of all box setups were heavily misted with distilled water in order to maintain high humidity conditions (85-95% RH). Disease severity ratings were read for all leaves using a modified version of James' (1971) disease assessment key (James, 1971) after seven and ten days in which each leaf was given a score of 0 to 100% in increments of 5% disease severity.

3.4.1.5 Data analysis

All statistical analysis for this section was performed using SAS 9.4 (SAS Institute). Median values from each experimental unit were analyzed using the non-parametric method described by Shah and Madden (2004) for a three-way factorial design (treatment * time * period). An ANOVA-type statistic (ATS) was performed on all fixed effects plus the interactions. LSMEANS statements were used to estimate the relative effects and multiple comparisons amongst treatments and times using Bonferroni's adjustment.

Chapter 4 : Results

4.1 Detached fed-leaf bioassays of nine *Phytophthora infestans* isolates

Nine *P. infestans* isolates in the AAFC Québec City research center collection were tested for pathogenicity using detached fed-leaf bioassays (Table 4-1).

Table 4-1. Median disease severity ratings of nine *Phytophthora infestans* isolates.

P. infestans isolate	Mating type	Median disease severity rating[Z]
US-23	A1	0 ± 0%
CA-10_2	A1	0 ± 3%
US-22_1	A2	30 ± 8%
US-22_3	A2	60 ± 8%
CA-10_1	A1	60 ± 14%
US-8	A2	75 ± 14%
US-22_4	A2	90 ± 3%
CA-9	A1	100 ± 0%
US-22_2	A2	100 ± 0%

[Z] The median of disease severity scores from each experimental unit (boxes) was calculated for each isolate alongside standard errors.

It can be clearly observed in Table 4-1 that great variability exists in the level of disease severity exhibited by these nine *P. infestans* isolates. US-23 and CA-10_2 caused virtually no symptoms on detached fed potato leaves, with median disease severity ratings of 0%. US-22_1 and US-22_3 induced moderately greater disease severity levels, with median disease severity ratings of 30% and 60% higher (respectively) than US-23 and CA-10_2. However, the other two US-22 isolates US-22_4 and US-22_2 caused at least 30% more median disease severity than US-22_1 and US-22_3 with median ratings of 90% and 100% respectively. Similarly, median disease severity for CA-10_1 was 60% higher than that of CA-10_2. US-8 induced significant disease severity on detached potato leaves, with a median rating greater than five of the nine isolates tested. CA-9 induced a very strong median disease severity rating of 100%, a score which was at least 40% greater than all other A1 isolates tested.

4.2 Bacterial candidates

4.2.1 Detached fed-leaf bioassays using six bacteria and four *Phytophthora infestans* isolates

See Appendix, Fig. A-1 and Fig. A-2, for the results of detached fed-leaf bioassay experiments screening 46 bacterial candidates against *P. infestans* isolate US-8. The testing of bacterial strain whole cultures 189, WAUSV36, KENGFT3, UW01, OXWO6B1 and OY3WO11 (selected from amongst 46 overall candidates by research supervisors Patrice Audy and Susan Boyetchko) for biocontrol against four *P. infestans* isolates encompassed a great bulk of the research conducted for this Masters project. Manipulations spanned 16 individual two-week detached fed-leaf bioassay experiments conducted from June 2014 to May 2015. Results are divided by the *P. infestans* isolate that was tested against in Table 4-2 thru to 4-5. Results obtained from data acquired on both measurement days (7 and 10) post-infection are listed. Because the Trt * Period interaction was almost always statistically significant for every isolate/measurement day combination (CA-9 day 10 being the exception), statistically significant differences ($P \leq 0.05$) between treatments were analyzed period-by-period the vast majority of the time. The simple effect of the bacterial treatments after ten days for CA-9 was analyzed broadly.

Results for US-8 biocontrol infections, perhaps one of the weaker four isolates as determined by section 4.1 results, are compiled in Table 4-2. The interaction effect Trt * Period was significant for both measurement days with P values of 0.0009 (Day 7) and 0.0067 (Day 10). Fixed effects Trt and Period were always highly significant at $P < .0001$ across both measurement days. 189 was statistically significantly different than the control during seven of eight measurements (there were two measurements per period, with four periods for each bacterial treatment against US-8). This treatment-control difference occurred for 189 more often than for any other bacterial treatment tested against US-8. WAUSV36 was the only bacterial treatment statistically significantly different than the control during measurement: Period 2 Day 7. However WAUSV36 was only significantly different than the control during three of eight measurements. KENGFT3 was significantly different than the control during four measurements,

OXWO6B1 during three, UW01 during two and OY3WO11 during only one. Overall, bacteria were statistically significantly different than the control during 20 of the 48 measurements (there were eight measurements per bacterial treatment, with six bacteria, thus 48 measurements total).

Table 4-2. Relative disease severity rates following treatment with six bacterial biocontrol agents after seven and ten days post-inoculation with *Phytophthora infestans* isolate US-8.

		Day 7			Day 10	
			Relative disease severity rate			Relative disease severity rate
Trt (Bacterial strain)		189	19		189	16
		WAUSV36	36		KENGFT3	39
		KENGFT3	37		WAUSV36	40
		OY3WO11	41		OY3WO11	42
		UW01	46		UW01	43
		OXWO6B1	47		OXWO6B1	44
		Control	72		Control	73
Period		P3	21		P3	22
		P2	39		P2	35
		P1	49		P1	52
		P4	62		P4	61
Trt * Period	P3	189	14 a[z]	P3	189	13 a
		WAUSV36	14 a		KENGFT3	13 a
		KENGFT3	14 a		UW01	13 a
		UW01	14 a		OXWO6B1	13 a
		OXWO6B1	14 a		OY3WO11	24 ab
		OY3WO11	20 ab		WAUSV36	25 ab
		Control	54 b		Control	57 b
	P2	WAUSV36	14 a	P2	189	13 a
		189	23 ab		WAUSV36	19 ab
		KENGFT3	31 ab		OY3WO11	29 abc
		UW01	40 ab		KENGFT3	32 abc
		OY3WO11	43 ab		UW01	38 bc
		OXWO6B1	45 b		OXWO6B1	38 bc
		Control	77 b		Control	78 c
	P1	189	14 a	P1	189	17 a
		KENGFT3	37 b		KENGFT3	46 ab
		OY3WO11	37 b		OY3WO11	51 abc
		OXWO6B1	57 b		UW01	52 abc
		WAUSV36	57 c		OXWO6B1	57 abc
		UW01	59 c		WAUSV36	59 bc
		Control	79 c		Control	79 c
	P4	189	26 a	P4	189	22 a
		WAUSV36	57 ab		WAUSV36	58 ab
		KENGFT3	65 ab		OY3WO11	66 ab
		OY3WO11	65 ab		OXWO6B1	67 ab
		UW01	70 b		KENGFT3	67 b
		OXWO6B1	72 b		UW01	68 b
		Control	78 b		Control	78 b

Table 4-2. continued

P value (ATS)	Trt	<.0001	Trt	<.0001
	Period	<.0001	Period	<.0001
	Trt * Period	0.0009	Trt * Period	0.0067

[z] Differing alphabetical letters between two bacterial strains within a given period indicate a significant difference between those two treatments ($P \leq 0.05$).

Results obtained from detached fed-leaf bioassays using *P. infestans* isolate CA-10_1, another of the two weaker isolates according to section 4.1 results, are compiled in Table 4-3. *P* values of 0.0007 (Day 7) and 0.0010 (Day 10) were determined for the interaction effect Trt * Period. It was therefore significant across both measurement days. Fixed effects Trt and Period were always highly significant at $P < .0001$ across both measurement days. 189 and WAUSV36 were both significantly different than the control during six of their eight measurements each. For period 4, no treatments were significantly different than the control across both measurement days. UW01 was significantly different than the control during three of eight measurements. All other treatments were never different than the control across all periods and measurement days.

Table 4-3. Relative disease severity rates following treatment with six bacterial biocontrol agents after seven and ten days post-inoculation with *Phytophthora infestans* isolate CA-10$_1$.

		Day 7			Day 10	
			Relative disease severity rate			Relative disease severity rate
Trt (Bacterial strain)		189	22		189	19
		WAUSV36	26		WAUSV36	25
		UW01	39		KENGFT3	40
		KENGFT3	40		UW01	41
		OXWO6B1	47		OXWO6B1	48
		OY3WO11	56		OY3WO11	57
		Control	66		Control	67
Period		P3	17		P3	17
		P1	35		P1	38
		P2	55		P2	56
		P4	61		P4	58
Trt * Period	P3	189	6 a	P3	189	3 a
		UW01	6 a		WAUSV36	6 a
		WAUSV36	8 a		UW01	9 a
		KENGFT3	14 ab		KENGFT3	12 ab
		OXWO6B1	21 ab		OXWO6B1	22 b
		OY3WO11	25 ab		OY3WO11	28 b
		Control	42 b		Control	40 b
	P1	WAUSV36	12 a	P1	189	16 a
		189	21 a		WAUSV36	19 a
		OXWO6B1	33 ab		KENGFT3	33 ab
		KENGFT3	33 ab		OXWO6B1	33 ab
		UW01	38 ab		UW01	44 ab
		OY3WO11	47 b		OY3WO11	53 b
		Control	63 b		Control	70 b
	P2	189	18 a	P2	189	16 a
		WAUSV36	33 ab		WAUSV36	33 a
		UW01	52 abc		UW01	58 b
		KENGFT3	61 bc		KENGFT3	63 bc
		OXWO6B1	63 bc		OXWO6B1	65 bc
		OY3WO11	79 c		OY3WO11	80 c
		Control	80 c		Control	80 c
	P4	189	43 a	P4	189	42 a
		WAUSV36	53 a		WAUSV36	44 a
		KENGFT3	53 a		KENGFT3	51 a
		UW01	60 a		UW01	54 a
		OY3WO11	71 a		OY3WO11	67 a
		OXWO6B1	72 a		OXWO6B1	72 a
		Control	77 a		Control	77 a

Table 4-3. continued

P value (ATS)	Trt	<.0001	Trt	<.0001
	Period	<.0001	Period	<.0001
	Trt * Period	0.0007	Trt * Period	0.0010

Results for US-22_2 biocontrol infections, a very strong isolate according to section 4.1 results, are compiled in Table 4-4. The interaction effect Trt * Period was statistically significant for both measurement days with *P* values of 0.0100 (Day 7) and 0.0330 (Day 10). Fixed effects Trt and Period were always highly significant at $P < .0001$ across both measurement days. 189 emerged as having the most treatment-control differences against US-22_2, with seven out of eight measurements being statistically significantly different than the control. WAUSV36 was the runner-up by exhibiting significantly lower disease severity rates than the control during half of the eight measurements. KENGFT3 displayed significant levels of biocontrol during three measurements while UW01 did for two. OXWO6B1 and OY3WO11 were never successful in producing significantly lower disease severity rates than the control for US-22_2.

Table 4-4. Relative disease severity rates following treatment with six bacterial biocontrol agents after seven and ten days post-inoculation with *Phytophthora infestans* isolate US-22$_2$.

		Day 7			Day 10	
			Relative disease severity rate			Relative disease severity rate
Trt (Bacterial strain)		189	16		189	15
		WAUSV36	31		WAUSV36	30
		KENGFT3	34		KENGFT3	34
		UW01	38		UW01	36
		OXWO6B1	53		OY3WO11	53
		OY3WO11	53		OXWO6B1	57
		Control	69		Control	67
Period		P1	20		P1	23
		P2	43		P2	42
		P4	49		P4	50
		P3	55		P3	51
Trt * Period	P1	189	4 a	P1	189	4 a
		UW01	8 a		UW01	6 a
		KENGFT3	9 a		KENGFT3	12 a
		WAUSV36	11 a		WAUSV36	13 ab
		OXWO6B1	20 ab		OXWO6B1	27 ab
		OY3WO11	30 ab		OY3WO11	36 ab
		Control	60 b		Control	66 b
	P2	189	22 a	P2	189	19 a
		WAUSV36	31 ab		WAUSV36	32 a
		UW01	40 abc		UW01	33 ab
		KENGFT3	41 abc		KENGFT3	41 ab
		OY3WO11	45 abc		OY3WO11	43 ab
		OXWO6B1	61 bc		Control	61 b
		Control	64 c		OXWO6B1	66 b
	P4	189	25 a	P4	WAUSV36	23 a
		WAUSV36	26 ab		189	24 a
		UW01	45 abc		KENGFT3	46 a
		KENGFT3	46 abc		UW01	51 a
		OXWO6B1	57 bc		OXWO6B1	67 a
		OY3WO11	65 bc		OY3WO11	69 a
		Control	80 c		Control	74 a
	P3	189	14 a	P3	189	14 a
		KENGFT3	39 ab		KENGFT3	38 ab
		UW01	58 abc		UW01	53 ab
		WAUSV36	58 bc		WAUSV36	54 ab
		OY3WO11	71 bc		OXWO6B1	65 b
		OXWO6B1	74 c		Control	66 b
		Control	74 c		OY3WO11	67 b

Table 4-4. continued

P value (ATS)	Trt	<.0001	Trt	<.0001
	Period	<.0001	Period	<.0001
	Trt * Period	0.0100	Trt * Period	0.0330

Lastly, results obtained from detached fed-leaf bioassays using *P. infestans* isolate CA-9, another very pathogenic isolate according to section 4.1 results, are compiled in Table 4-5. *P* values of 0.0098 (Day 7) and 0.1996 (Day 10) were determined for the interaction effect Trt * Period. This meant that treatments were again analyzed period-by-period for measurement Day 7 but not for Day 10 where differences amongst treatments could be accessed broadly from all four periods. Fixed effects Trt and Period were highly significant at $P < .0001$ for Day 7. Fixed effects were also significant for measurement Day 10 at $P < .0001$ (Trt) and $P = 0.0069$ (Period). Beginning with measurement Day 7 results, 189 and WAUSV36 were statistically significantly different than the control across all four periods. UW01 performed well also, producing lower disease severity rates than the control during three of the four periods. KENGFT3 and OXWO6B1 were different than the control during only one period while OY3WO11 was never. From Day 10 results which can be generalized across all four periods, it was determined that four of the six bacterial treatments significantly lowered disease severity rates versus the untreated control: 189, WAUSV36, UW01 and KENGFT3. 189 and WAUSV36 displayed strong biocontrol abilities by producing relative disease severity rates that were 59% and 51% lower (respectively) than the control. In addition, bacterial treatments UW01 and KENGFT3 produced relative disease severity rates 30% and 26% lower (respectively) than the control. Bacteria OXWO6B1 and OY3WO11 were not found to be statistically significantly different than the control.

Table 4-5. Relative disease severity rates following treatment with six bacterial biocontrol agents after seven and ten days post-inoculation with *Phytophthora infestans* isolate CA-9.

		Day 7		Day 10	
			Relative disease severity rate		Relative disease severity rate
Trt (Bacterial strain)		189	9	189	9 a
		WAUSV36	17	WAUSV36	17 b
		UW01	38	UW01	38 c
		KENGFT3	40	KENGFT3	42 cd
		OXWO6B1	51	OXWO6B1	55 de
		OY3WO11	68	OY3WO11	68 e
		Control	74	Control	68 e
Period		P1	36	P4	38 a
		P4	42	P3	39 a
		P3	43	P1	45 ab
		P2	48	P2	48 b
Trt * Period	P1	189	7 a		
		WAUSV36	9 ab		
		UW01	27 ab		
		OXWO6B1	34 b		
		KENGFT3	36 bc		
		OY3WO11	65 bc		
		Control	73 c		
	P4	189	14 a		
		WAUSV36	24 a		
		UW01	28 ab		
		KENGFT3	39 abc		
		OXWO6B1	54 bc		
		OY3WO11	69 bc		
		Control	69 c		
	P3	189	5 a		
		WAUSV36	13 b		
		KENGFT3	36 c		
		UW01	45 c		
		OXWO6B1	56 cd		
		OY3WO11	68 cd		
		Control	82 d		
	P2	189	11 a		
		WAUSV36	20 ab		
		KENGFT3	48 bc		
		UW01	51 bc		
		OXWO6B1	62 c		
		OY3WO11	70 c		
		Control	75 c		

Table 4-5. continued

P value	Trt	<.0001	Trt	<.0001
(ATS)	Period	<.0001	Period	0.0069
	Trt * Period	0.0098	Trt * Period	0.1996[z]

[z] Estimates for interactions which were not statistically significant are not displayed.

The frequency at which bacterial strain whole cultures were statistically significantly different than the control for all isolates, periods and measurement days was counted (Table 4-6). Bacteria 189 and WAUSV36 exhibited the greatest amount of treatment-control differences, with 28 and 21 significant differences respectively out of 32 (as previously established there were eight measurements per bacterial treatment against each *P. infestans* isolate, thus 32 measurements per bacterium against all four isolates). Albeit somewhat less frequent, UW01 and KENGFT3 exhibited significantly lower disease severity levels than the control during 14 and 12 measurements respectively. Bacteria OXWO6B1 and OY3WO11 exhibited the lowest counts of treatment-control differences when tested against the four *P. infestans* isolates as a whole; with only four and one differences from the control respectively.

Table 4-6. Frequency of statistically significant differences between bacterial strain treatments and the control ($P \leq 0.05$) across all periods and measurement days.

	P. infestans isolates				
Bacterial strains	US-8	CA-10_1	US-22_2	CA-9	Total
189	7/8[a]	6/8	7/8	8/8	28/32[b]
WAUSV36	3/8	6/8	4/8	8/8	21/32
UW01	2/8	3/8	2/8	7/8	14/32
KENGFT3	4/8	0/8	3/8	5/8	12/32
OXWO6B1	3/8	0/8	0/8	1/8	4/32
OY3WO11	1/8	0/8	0/8	0/8	1/32
Total	20/48[c]	15/48	16/48	29/48	80/192[d]

[a] Eight measurements for each bacterial treatment against one *P. infestans* isolate.
[b] Thirty-two measurements for each bacterial treatment against all four *P. infestans* isolates.
[c] Forty-eight measurements for all six bacterial treatments against one *P. infestans* isolate.
[d] One hundred and ninety-two measurements for all six bacterial treatments against all four *P. infestans* isolates.

4.3 Performance of potential biocontrol bacteria compared to a synthetic fungicide

Detached fed-leaf bioassays were used to compare bacterial strain whole cultures 189 and WAUSV36 (selected from amongst the six previous candidates based on section 4.2 results) against the commercial synthetic fungicide Dithane™ DG 75 for late blight control using *P. infestans* isolate CA-9. Because there were fewer treatment types, spatial limitations in the growth room allowed for seven replicates of each treatment simultaneously. Fig. 4-1 illustrates the median disease severity ratings of detached fed-potato leaves subject to bacterial treatments 189 and WAUSV36, the synthetic fungicide Dithane™ DG 75 and untreated controls after seven and ten days.

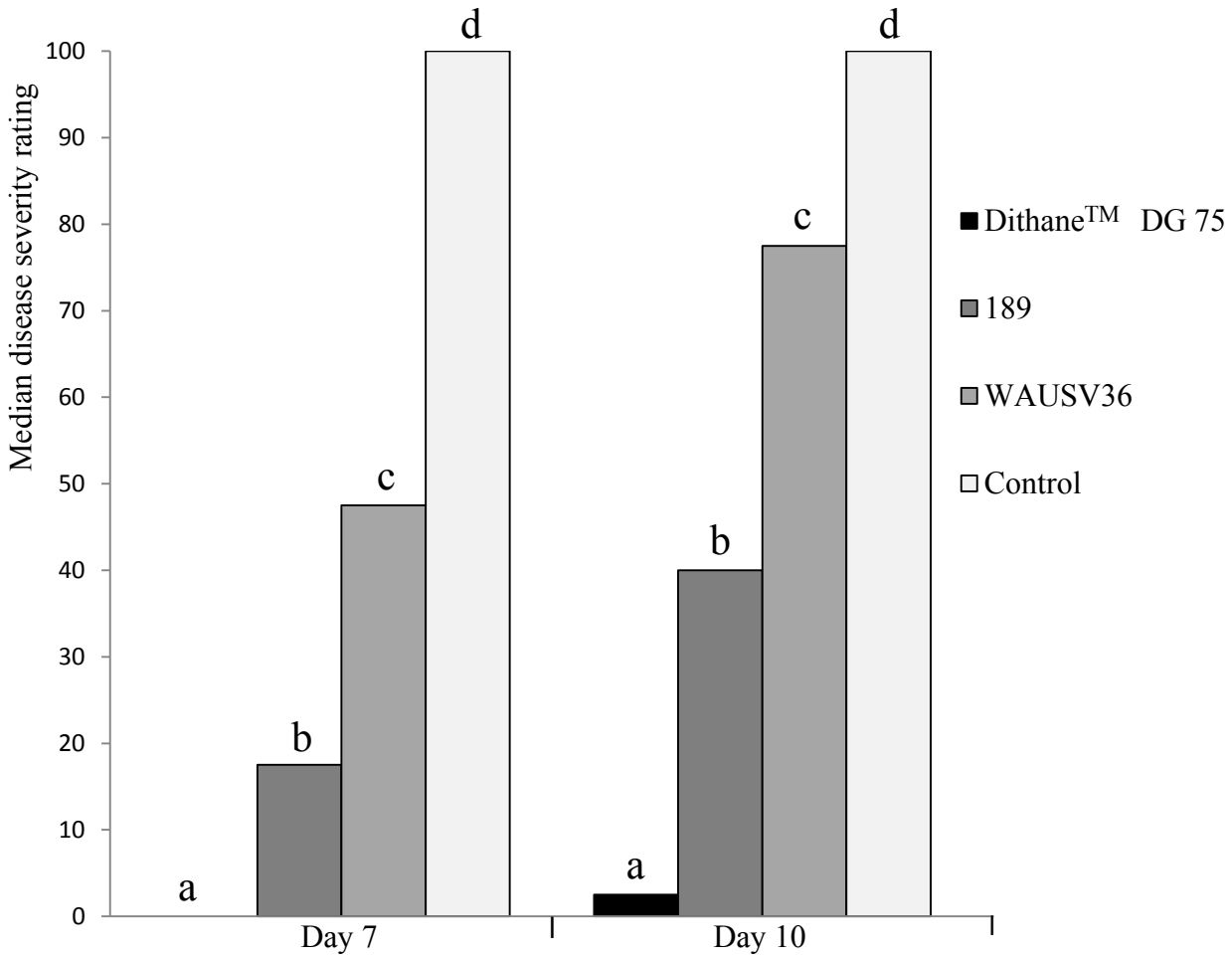

Figure 4-1. Late blight control levels of bacterial candidates compared to a commercial synthetic fungicide.
Median disease severity ratings of detached fed-potato leaves treated with the commercial synthetic fungicide Dithane™ DG 75 prepared at 80 g/L and bacterial strain whole cultures 189 and WAUSV36 against *P. infestans* isolate CA-9 after seven and ten days post-infection.

From Day 7 measurements, a statistically significant difference was found between all treatment types. The commercial synthetic fungicide Dithane™ DG 75 was the strongest late blight control agent with a median disease severity rating of 0%. The bacterial treatment 189 was the second best control agent with a median disease severity rating of 18%. Although to a lesser extent, WAUSV36 exhibited significant levels of biocontrol against the very strong *P. infestans* isolate CA-9 as well with a median disease severity rating of 48% - less than half the median disease severity observed in untreated control boxes (100%). After ten days, potato leaves subject to all treatment types began to show increased symptoms of late blight: Dithane™ DG 75

at 3%, 189 at 40% and WAUSV36 at 78%. The untreated control, having already reached its maximum after seven days, retained a median disease severity rating of 100%. Fig. 4-2 demonstrate how disease severity differences between treatment types were clearly observed visually as well; late blight symptoms being identified by grey to black spots on detached fed-leaves.

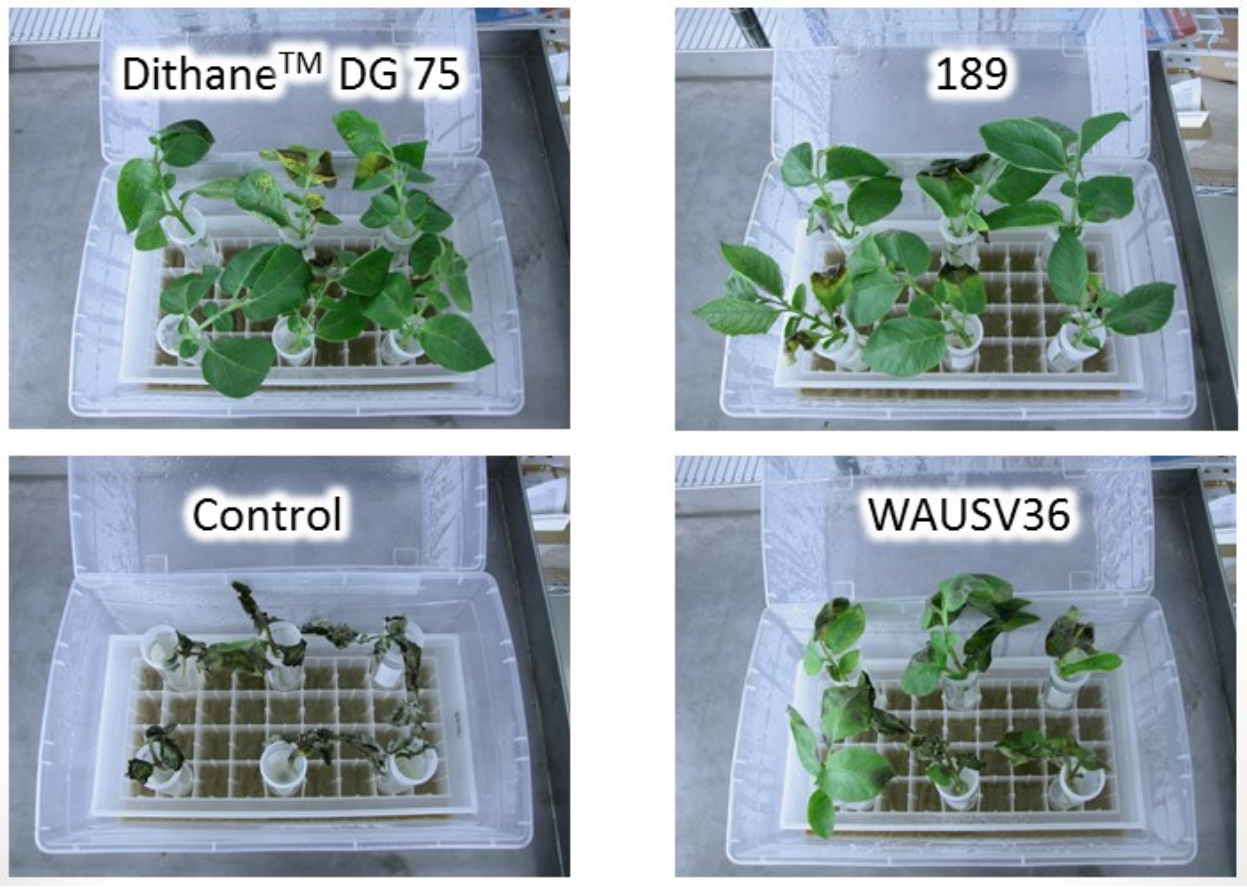

Figure 4-2. Photographs of select detached fed-potato leaves treated with two different bacterial strain whole cultures and a synthetic fungicide against *Phytophthora infestans* isolate CA-9 after ten days post-infection.

4.4 Performance of potential biocontrol bacteria with different time-delays between bacterial inoculation and pathogen infection

Detached fed-leaf bioassays were used to compare the biocontrol levels of bacterial strain whole cultures 189 and WAUSV36 when the treatment-to-infection time was 2 hours, 18 hours

or 24 hours. *Phytophthora infestans* isolate CA-9 was again used as the pathogen. Due to spatial limitations in the growth room, treatment/delay time combinations were tested in triplicate across two periods. This meant that median disease severity ratings were analyzed as a three-way factorial, with fixed effects Trt, Time and Period. Table 4-7 compiles the results obtained from this series of time-delay bioassay experiments and lists any statistically significant differences observed between fixed effect levels.

Table 4-7. Relative disease severity rates following treatment with two bacterial biocontrol agents subject to three different treatment-to-infection delay times after seven and ten days post-inoculation with *Phytophthora infestans* isolate CA-9.

		Day 7			Day 10	
			Relative disease severity rate			Relative disease severity rate
Trt (Bacterial strain)		189	15		189	15
		WAUSV36	22		WAUSV36	22
		Control	46		Control	46
Time		2 hours	25 a		18 hours	26
		18 hours	26 a		2 hours	27
		24 hours	32 b		24 hours	30
Period		P1	23		P1	22
		P2	32		P2	33
Trt * Period	P1	189	9 a	P1	189	9 a
		WAUSV36	14 a		WAUSV36	13 a
		Control	45 b		Control	45 b
	P2	189	21 a	P2	189	22 a
		WAUSV36	30 a		WAUSV36	30 a
		Control	46 b		Control	47 b
P value (ATS)	Trt		<.0001	Trt		<.0001
	Time		0.0072	Time		0.2140
	Period		<.0001	Period		<.0001
	Trt * Time		0.2489[z]	Trt * Time		0.2714
	Trt * Period		0.0089	Trt * Period		0.0113
	Time * Period		0.8879	Time * Period		0.6986
	Trt * Time * Period		0.5340	Trt * Time * Period		0.6835

[z] Estimates for interactions which were not statistically significant are not displayed.

The interaction effects Trt * Time * Period, Time * Period and Trt * Time were not statistically significant (at $P \leq 0.05$) across both measurement days. However, because the Trt * Period interaction was significant, statistically significant differences between treatments were analyzed period-by-period. As expected, 189 and WAUSV36 exhibited significantly lower median disease severity ratings than the untreated control across all periods and measurement days. Bacterial treatments 189 and WAUSV36 were never significantly different from one another at $P \leq 0.05$. Overall, differences between treatments and the control were congruent with previous results. The single fixed effect Time was statistically significant for disease severity readings measured seven days after infection. The relative disease severity rates due to time-delays of 2 and 18 hours were statistically the same at 25 and 26 respectively. However this rate due to a time-delay of 24 hours was significantly greater at 32 than that of the two shorter delay times. Effectively, when analyzing disease severity readings measured seven days post-pathogen inoculation with CA-9, extending the treatment-to-infection delay time to 24 hours seemed to induce significantly higher disease severity levels among detached fed-potato leaves subject to bacterial treatments 189 and WAUSV36 ($P \leq 0.05$). This was not the case for those measured ten days post-infection as the fixed effect Time was not found to be statistically significant. There was therefore no evidence from Day 10 measurements that extending the treatment-to-infection delay time up to 24 hours might mean significantly reduced levels of biocontrol exhibited by these two bacteria against *P. infestans* isolate CA-9.

Chapter 5 : Discussion

Identifying four *P. infestans* isolates capable of inducing strong late blight infections on potato leaves was essential before biocontrol experiments were performed in order to truly test if bacteria would hold up against extremely aggressive and symptomatic late blight isolates. *Phytophthora infestans* cultures maintained for long periods in the lab after isolation can often lose much of their virulence over time from being continuously transferred plate-to-plate in the absence of true vegetative tissue (Johnson *et al.*, 2015). In addition, the genetic composition of *P. infestans* populations is now evolving at an ever quickening rate; reinforcing the need to screen late blight isolates for pathogenicity before any form of control test is performed (Peters *et al.*, 2014). Although isolates were maintained on either sterilized rye seeds or potato tuber cores to minimize this loss of pathogenicity, bioassay experiments were an important step in order to weed out any non- or low-pathogenic *P. infestans* isolates in the Agriculture and Agri-Food Canada research center collection. Testing biocontrol agents on non-pathogenic isolates would indeed defeat the purpose of developing an effective biopesticide for use outside the laboratory. The four isolates selected (US-8, CA-10_1, US-22_2 and CA-9) for use in our biocontrol tests were therefore those that exhibited the highest levels of disease severity among detached fed-potato leaves; providing they met all other selection criteria.

To arrive at this selection, a total of nine *P. infestans* isolates in the AAFC Québec City research center collection were tested for pathogenicity. US-23 and CA-10_2 caused virtually no symptoms, with median disease severity ratings of 0 ± 0% and 0 ± 3% respectively. US-22_1 and US-22_3 induced mild disease severity levels on detached fed potato leaves, with median disease severity ratings of 30 ± 8% and 60 ± 8% respectively. However these two isolates were the least pathogenic of the four US-22 isolates tested, and as such were easily dismissed from the selection process in order to promote genotypic diversity of the four isolates selected for use in biocontrol tests. This left CA-10_1 at 60 ± 14%, US-8 at 75 ± 14%, US-22_4 at 90 ± 3% and CA-9 and US-22_2 both at 100 ± 0%. Of the two US-22 isolates remaining, US-22_2 was selected over US-22_4 due to its slightly larger disease severity score. Four *P. infestans* isolates thus remained. All four displayed significant levels of pathogenicity (60 ± 14% to 100 ± 0%), were genetically

diverse, and were of both mating types A1 and A2 (two each). In addition, isolates of US-8, CA-10, US-22 and CA-9 genotype groups were known to be problematic in Canada at the time of selection in 2014 (Peters *et al.*, 2014).

Rojas *et al.* (2014) evaluated tuber blight development in response to different *P. infestans* genotypes found in the United States and concluded that although isolates of US-22 were overall less aggressive than those of US-8, US-22 isolates were as virulent as US-8 isolates for some potato cultivars (Rojas *et al.*, 2014). Although our experiment differed in that isolates were tested on potato leaves instead of tubers, two isolates of US-22 were found to elicit weaker disease severity levels than our US-8 isolate (US-22_1 and US-22_3) and two others to elicit stronger ones (US-22_4 and US-22_2). Isolates of the genotype groups CA-9 and CA-10 are believed to be recombinant and were first identified in Ontario. They share a close molecular structure to that of US-22 (Peters *et al.*, 2014). This may explain the relatively strong pathogenicity observed in our CA-10_1 and CA-9 isolates.

Recent surveys have indicated increasing and rapidly changing late blight diversity in Canada, even from one year to the next (Peters *et al.*, 2014). Some of the major forces behind these dramatic shifts include sexual recombination, mutations, climate change and the geographical migration of genotypes via infected plants and/or tubers (Alkher *et al.*, 2015). It was therefore important to not only select four *P. infestans* isolates for use in biocontrol tests which displayed strong levels of pathogenicity on detached fed-leaves but also to select isolates which would be an acceptable reflection of the genotypes known to plague Canadian crops at the time of selection (May 2014). Isolates of genotype groups US-8, CA-10, US-22 and CA-9 were all present in Canada as identified in the 2011 cross-country survey by Peters *et al.* (2014). However, new information released in 2015 sheds light on how quickly changes in late blight diversity can occur. The newer genotype group US-23 now accounts for 100 of the 119 isolates found throughout Canada with isolates from genotype groups US-8, CA-9 and CA-10 being completely absent from the list (Alkher *et al.*, 2015). An additional selection criterion was that the four *P. infestans* isolates chosen be from both mating types A1 and A2 (preferably two from each). Due to the quickly changing landscape of late blight diversity and the presence of both mating types, events of sexual recombination have now become evident in Canada with Alkher

et al. (2015) finding evidence for recombination in isolates collected from both British Columbia and Prince Edward Island. Two of the isolates selected for biocontrol testing during this project were of the A1 mating type (CA-10_1 and CA-9) and two of the A2 mating type (US-8 and US-22_2).

Preliminary screening at the Agriculture and Agri-Food Canada research centre in Québec City using detached fed-leaf bioassays was performed in order to test the biocontrol potential of 46 bacterial strains isolated from Saskatchewan soils by Dr. Susan Boyetchko's lab (Audy *et al.*, 2014). The *P. infestans* isolate US-8 from the AAFC Québec City collection was used as the pathogenic agent because at that time (in 2013) the latest Canadian survey indicated that isolates of the A2 genotype group US-8 were still some of the most problematic in Canada, especially in eastern Canada where they represented 83% of all isolates found (Kalischuk *et al.*, 2012). These preliminary results indicated that great variability existed in the levels of late blight control exhibited by the 92 different bacterial treatments tested (46 whole cultures + 46 filtrates only) ranging from 0 to 100% disease severity after ten days. However one recurring trend was observed: for the large majority of bacterial strains, whole culture treatments were more effective in controlling *P. infestans* than their filtrates only. This was not in agreement with research performed by Tomar *et al.* (2014) where they observed that a culture supernatant of *Pseudomonas aeruginosa* provided increased control over its bacterial cell suspension. However, Tomar *et al*.'s supernatants were not vacuum-filtered like they were during our preliminary screening and *P. aeruginosa* only represents one bacterium whereas the screening tests performed in Québec City involved a wider range of bacteria. The *P. aeruginosa* bacterial cell suspension prepared by Tomar *et al.* (2014), although slightly less inhibitory than its culture supernatant (disease severity: 9.94%), elicited a significantly reduced disease severity (17.96%) in whole potato plants compared to the control (53.96%). Although not frequently, some of the bacterial filtrates tested at the Agriculture and Agri-Food Canada research centre in Québec City did produce lower median disease severity ratings than their corresponding whole cultures. It is therefore not overly surprising that the culture supernatant of *Pseudomonas aeruginosa* provided increased late blight control over its bacterial cell suspension as determined by Tomar *et al.* (2014).

From these preliminary results it was therefore decided to proceed with six whole culture bacterial treatments selected for testing against the four *P. infestans* isolates identified as having strong pathogenicity: US-8, CA-10_1, US-22_2 and CA-9. The six bacterial strains tested as whole cultures were: 189 (*Pseudomonas chlororaphis*), WAUSV36 (*Bacillus subtilis*), KENGFT3 (*Pseudomonas fluorescens*), UW01 (*Pseudomonas fluorescens*), OXWO6B1 (*Pantoea stewartii*) and OY3WO11 (*Arthrobacter phenanthrenivorans*). Because all bacterial whole cultures were prepared from different bacterial strains and (in most cases) representative of different bacterial species, disease severity ratings cannot be and were not concentration-adjusted. Different bacterial strains have different modes of action and produce different compounds (S. Boyetchko, personal communication, 2015).

Because detached fed-leaf bioassays consisted of using three live organisms, some natural variability was expected. To ensure that true significant differences could still be discerned in spite of this, each treatment was tested using a total of 12 replicates across four periods. This was repeated for all four *P. infestans* isolates. These periods were used as blocking factors during the statistical analysis. This proved to be the right approach as the experimental factor Period was significant across all isolates and measurement days. Great variability was observed in the ability of bacteria to control the four aggressive pathogen isolates. This kind of variability has been well documented previously in the literature: El-Sheikh *et al.* (2002) screened 83 bacteria for biocontrol against *P. infestans* in Egypt and found that only 14 were capable of preventing oomycete growth *in vitro*. Furthermore, Tomar *et al.* (2014) determined that only five of 95 rhizosphere bacteria isolated from India and tested in their study exhibited significant biocontrol properties against *P. infestans*. Some of the variability observed amongst biocontrol studies can likely be attributed to differences in climate, altitude and nutrient availability (among others) between the regions from which bacteria were collected. The *P. infestans* isolate(s) used and the varieties of potato plants employed for testing could also contribute to that variability.

For this study, disease severity measurements were not directly compared amongst the four different *P. infestans* isolates. Isolates are representative of different *P. infestans* genotypes and as such have different molecular structures. Therefore from a statistical point of view, results from different pathogen isolates should not be directly compared one to the other. However, the

need to select two bacterial strains for further biocontrol tests remained. The frequency at which bacterial strain whole cultures were statistically significantly different than the control for all isolates, periods and measurement days was therefore counted (Table 4-6) so that two bacteria which exhibited strong levels of biocontrol against multiple *P. infestans* isolates of both mating types could be selected for further evaluation. Collectively, the frequency at which all six bacteria induced lower disease severity ratings differed from one pathogen isolate to the next. Overall, 189 (*P. chlororaphis*) and WAUSV36 (*B. subtilis*) provided the best biocontrol against *P. infestans* isolates; being significantly different than the untreated controls more often than other bacterial treatments. Similarly, Daayf *et al.* (2003) determined that two of their 43 bacterial strain whole cultures (one prepared from a *Serratia plymuthica* strain and another listed only as DF14) distinguished themselves from the others in terms of late blight biocontrol by being the only treatments to inhibit *P. infestans* growth by over 40% on detached potato leaves. The promising results obtained for bacteria 189 (Gram-negative *P. chlororaphis*) and WAUSV36 (Gram-positive *B. subtilis*) fit well with our desire to move forward with one Gram-positive and one Gram-negative bacterium – as each type could potentially present different advantages for biopesticide formulation and eventual commercialization. However it has been argued in the past that Gram-positive bacteria, because their spore-forming abilities can offer heat- and desiccation-resistance, have a natural formulation advantage over Gram-negative bacteria when developing biological control products against plant pathogens (Emmert and Handelsman, 1999).

Although the purpose of this thesis research project was not to identify specific modes of action for any given antagonism observed by the six treatments (this is currently being studied by an AAFC team led by Drs. Ting Zhou in Guelph, ON and Tim Dumonceaux in Saskatoon, SK), it is likely that multiple modes of action were responsible for much of the late blight biocontrol observed. Induced systemic resistance (ISR) is a common mechanism of action for multiple strains of antagonistic bacteria of various genera – including *Pseudomonas* and *Bacillus* (Tran *et al.*, 2007). However because the delay between bacterial treatment and late blight inoculation was a mere 2 hours, it is difficult to believe that the host tissue had sufficient time to produce a powerful ISR response which would be exclusively responsible for some of our more significant biocontrol results (P. Audy, personal communication, 2015). Late blight suppression by bacterial metabolites has been documented in the literature and was likely responsible (in addition to

competition for nutrients and space) for at least some of the biocontrol observed in our study. In fact, Hunziker *et al.* (2015) reported *P. infestans* to be highly susceptible to bacterial volatiles emitted by potato-associated microorganisms.

In order to convince future buyers that investing in a biopesticide product is money well spent, comparing the late blight control abilities of biological antagonists to those of a commercial synthetic fungicide is an essential marketing step. Bacterial strain whole cultures 189 (*P. chlororaphis*) and WAUSV36 (*B. subtilis*) were tested side by side the commercial synthetic fungicide Dithane™ DG 75 (containing 75% mancozeb) against the strongly pathogenic *P. infestans* isolate CA-9. Daayf and Platt (2003) compared the effectiveness of six commercial synthetic fungicides including Dithane® DF (75% mancozeb) against a *P. infestans* US-8 isolate using potato leaf discs. All fungicides in the study by Daayf and Platt (2003) were prepared at 10 µg a.i./mL. They determined that leaf discs treated with Dithane® DF exhibited mid-range disease severity control levels compared to those treated with the other five synthetic fungicides. Their results revealed that only Bravo® (Chlorothalonil) and Tattoo® C (Propamocarb and Chlorothalonil) performed significantly better than Dithane® DF against their *P. infestans* US-8 isolate (Daayf and Platt, 2003). Dithane™ DG 75 was selected for use in our study not only because mancozeb is a proven late blight control agent but because it was one of the few late blight fungicides registered for use in indoor greenhouse-type applications. In addition, some existing studies have looked at how bacterial treatments measure up to mancozeb-based fungicides when used to manage potato late blight. Weltzien (1991) determined that late blight disease severity in potato foliage treated with compost tea amended with microorganisms including bacteria did not significantly differ from that measured in plants treated with a mixture of metalaxyl and mancozeb. Wharton *et al.* (2012) found that a *Bacillus subtillus* based treatment reduced disease severity by 54.5% on seed tubers while Maxim® MZ (fludioxonil 5 + mancozeb 96 g kg^{-1}) reduced it by 86.5%.

Applying both bacterial treatments 189 (*P. chlororaphis*) and WAUSV36 (*B. subtilis*) resulted in, as expected from our previous results, significantly lower median disease severity ratings on detached fed-potato leaves than leaves left untreated. However Dithane™ DG 75 significantly reduced leaf disease severity to even lower levels – with a median disease severity

rating of only 3% after ten days. This was in stark contrast to the median disease severity ratings measured for untreated controls: 100% (Day 7 and 10). On the other hand Daayf and Platt (2003) determined that although applying the mancozeb-based fungicide Dithane® DF at the relatively weak concentration of 10 µg a.i./mL did significantly reduced disease severity compared to control leaf discs, this reduction was a mere 12% lower than the control. The difference in disease severity results obtained by Daayf and Platt compared to ours can more than likely be attributed to the concentration at which the mancozeb-based treatments were applied. Dithane™ DG 75 was prepared in our study at a concentration of 80 g/L or 60000 µg a.i./mL. This was performed in order to ensure that no edge was given to the bacteria during comparison. However, in retrospect, this may have been an unfair advantage for Dithane™ DG 75 over our bacterial candidates. Mancozeb is usually applied in liquid form for the treatment of vegetable and fruit crops at the concentration of 2-3.5 g/L (Kechrid *et al.*, 2007). Another way bacterial candidates were disadvantaged was the absence of formulation aids in their treatment solutions. Commercial synthetic fungicides often have added ingredients, such as organic solvents, surface-active compounds, stabilizers and/or dyes to make them more effective (University of Minnesota Extension, 2011). Although bacterial strain whole cultures 189 (*P. chlororaphis*) and WAUSV36 (*B. subtilis*) performed quite well compared to the untreated control, with median disease severity ratings of 18% and 48% compared to 100% after seven days, future studies should be performed in which bacteria are amended with formulation aids and compared to a synthetic fungicide treatment at its usual concentration.

Three different treatment-to-infection delay times were tested for both leading bacterial candidates 189 (*P. chlororaphis*) and WAUSV36 (*B. subtilis*) against *P. infestans* isolate CA-9. In order to hint at the possible efficacy length of an eventual biopesticide product stemming from these bacteria, the time-delay experiments were performed in order to ensure that the two bacterial treatments would remain active for at least up to 24 hours after application to detached fed-potato leaves. Again as expected, the bacterial strain whole cultures 189 (*P. chlororaphis*) and WAUSV36 (*B. subtilis*) induced significantly lower disease severity rates in detached fed-potato leaves than the untreated controls across all periods and measurement days. The experimental effects of Trt and Period were always statistically significant as were their interaction. Again this demonstrated that disease severity ratings significantly differed between

periods and that statistically blocking measurements by period was the right approach. Yang *et al.* (2001) found that metabolites from the bacteria *Xenorhabdus nematophilus* were found to be effective at reducing late blight severity in potted potato plants when applied 2 hours before pathogen inoculation. This was in agreement with our results using bacterial strain whole cultures applied 2 hours before infection. In tests performed by Stephan *et al.* (2005) potted potato plants were treated with the biopesticide Serenade (AgraQuest Inc., Davis, CA, USA) (*Bacillus subtillus*) 72 and 24 hours before pathogen inoculation as well as 1 and 24 hours afterwards. They found that the bacterial treatment significantly decreased late blight severity compared to the control throughout all application times except for 24 hours after inoculation with *P. infestans* where no observable effect was detected. However, when the treatment was applied beforehand, biocontrol levels gradually decreased with increasing time between application and pathogen inoculation (Stephan *et al.*, 2005). Our own analysis from Day 7 measurements indicated that increasing the treatment-to-infection delay time to 24 hours did indeed produce significantly higher disease severity ratings than when that interval was 2 or 18 hours. However, this significant difference was not observed when disease severity ratings were measured ten days after pathogen inoculation. Our results seem to suggest therefore that little, if any, evidence exists to say that bacterial strain whole cultures 189 (*P. chlororaphis*) and WAUSV36 (*B. subtilis*) decrease in biocontrol effectiveness when they are applied to detached fed-potato leaves up to 24 hours in advance of infection. Future studies using these bacteria with time-delays of multiple days or even weeks should therefore be conducted in order to determine at which point the whole culture treatments might decrease in biocontrol ability.

Chapter 6 : Conclusion

In summary, four *P. infestans* isolates US-8, CA-10_1, US-22_2 and CA-9 were identified from nine tested as capable of eliciting strong late blight infections among detached fed-potato leaves. These isolates were therefore selected for use in biocontrol tests. In addition, isolates of US-8, CA-10, US-22 and CA-9 genotype groups were known to be problematic in Canada at the time of selection in 2014.

Results from preliminary tests screening whole cultures of 46 bacterial strains and their filtrates were used to select six of the most promising bacterial candidates for further biocontrol testing as whole culture treatments. The variability of these six bacterial strain whole cultures to significantly reduce disease severity due to the four aggressive *P. infestans* isolates was in agreement with other late blight biocontrol studies. Bacterial strains 189 and WAUSV36 displayed the strongest and most consistent levels of antagonism against all pathogen isolates overall. These two strains belonged to bacterial genera which had been identified as having antagonistic effects against late blight in previous studies.

Bacterial strains 189 and WAUSV36 were compared to the commercial synthetic fungicide Dithane^{TM} DG 75 (75% mancozeb) at 80 g/L for late blight control using *P. infestans* isolate CA-9. Although the bacterial strain whole cultures elicited significantly lower median disease severity ratings in detached fed-potato leaves than untreated controls, they were significantly weaker late blight control agents than the synthetic fungicide treatment. However it was postulated that by adding formulation aids to 189 and WAUSV36 and reducing the synthetic fungicide concentration to levels more typically used, bacterial treatments would more closely emulate results found in previous biocontrol/synthetic fungicide comparisons.

These same two bacterial strains were tested for biocontrol against the *P. infestans* isolate CA-9 using three different treatment-to-infection delay times: 2, 18 and 24 hours. A delay time of 24 hours significantly reduced biocontrol levels of 189 and WAUSV36 after seven days compared to 2 and 18 hours. This decrease over time was in agreement with previous studies.

However Day 10 measurements indicated no significant differences amongst the three time-delays. Future tests with 189 and WAUSV36 extending treatment-to-infection time-delays to multiple days or even weeks are therefore warranted in order to determine at what length of time these bacterial treatments may no longer be effective.

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Appendix

A.1 Preliminary results

Forty-six bacteria were tested against *P. infestans* isolate US-8 using whole culture and filtrate solutions in order to move ahead with six promising bacterial treatments for extensive testing against the four *P. infestans* isolates identified as strongly pathogenic. Results of this preliminary screening have been included here in order to provide additional depth to the bacterial candidate selection process. Fig. A-1 and Fig. A-2 show the median disease severity ratings of detached fed-potato leaves subject to 46 bacterial strains against *P. infestans* isolate US-8 after seven and ten days as determined at the Agriculture and Agri-Food Canada research centre in Québec City.

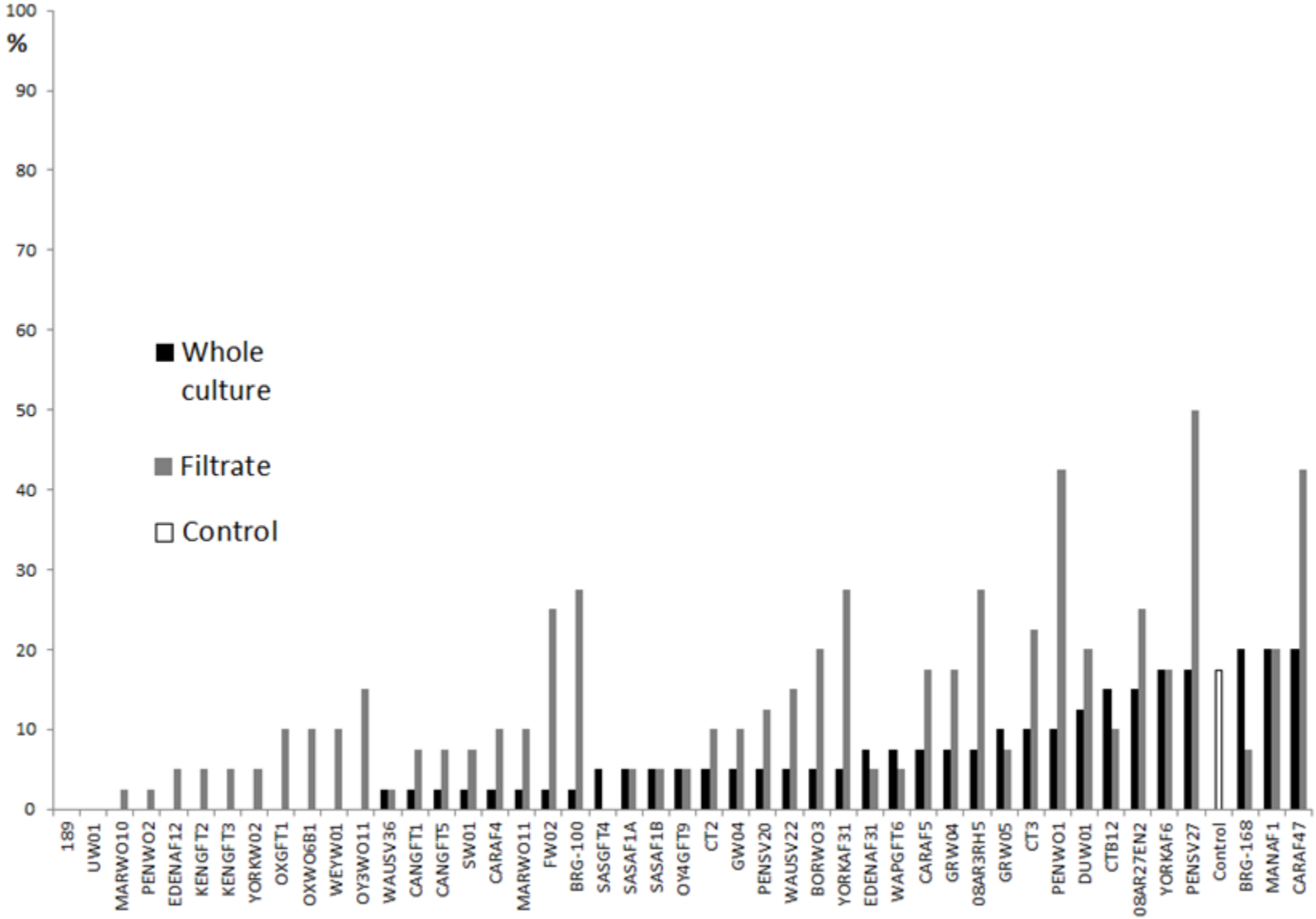

Figure A-1. Median disease severity ratings of detached fed-potato leaves subject to 46 bacterial filtrate and whole culture solutions against *Phytophthora infestans* isolate US-8 after 7 days post-infection.

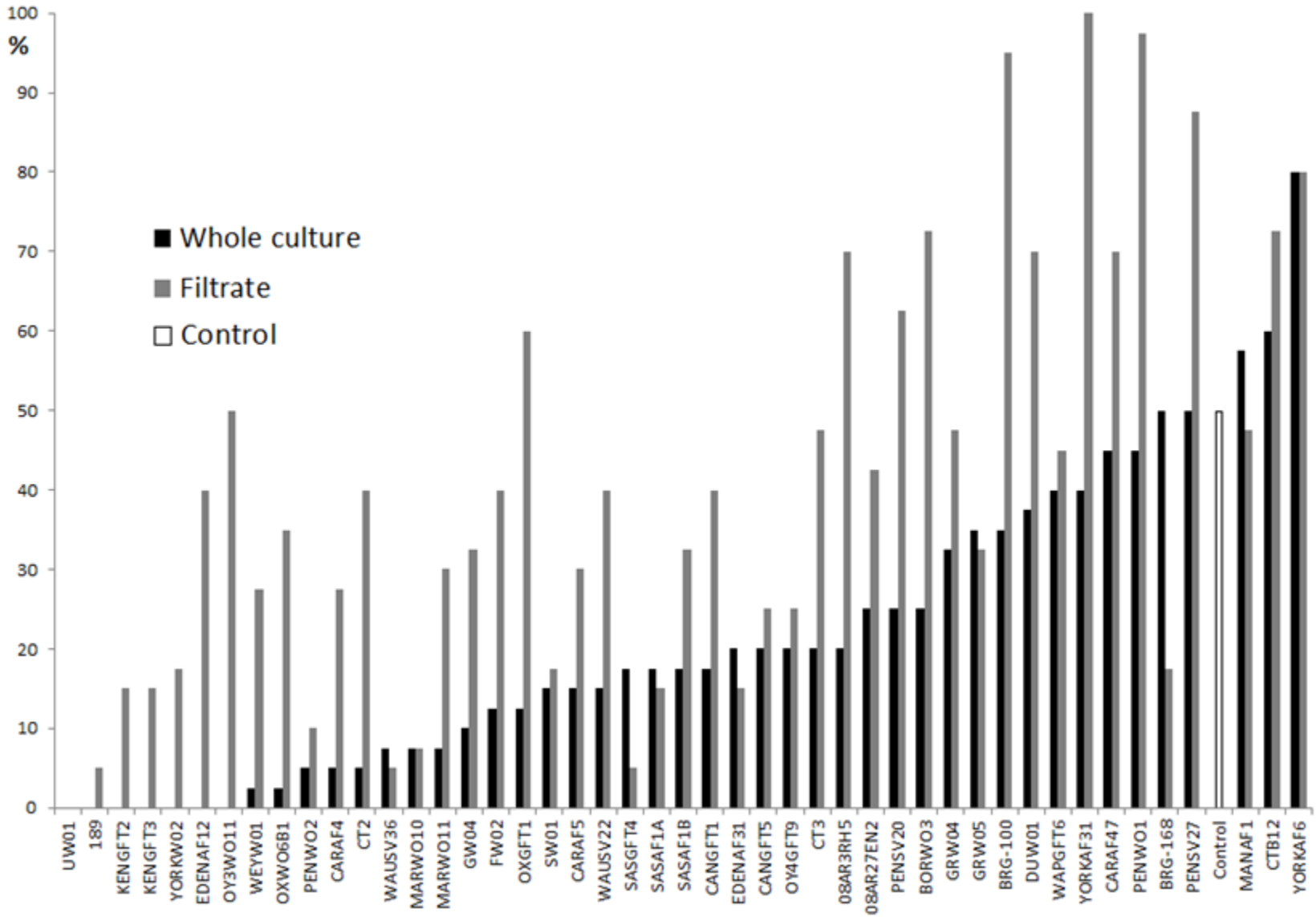

Figure A-2. Median disease severity ratings of detached fed-potato leaves subject to 46 bacterial filtrate and whole culture solutions against *Phytophthora infestans* isolate US-8 after 10 days post-infection.