MOVEMENT AND ACCUMULATION OF CANDIDATUS LIBERIBACTER SOLANACEARUM IN POTATO PLANTS

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Movement and accumulation of *Candidatus* Liberibacter solanacearum

in potato plants

By

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ABSTRACT

A new disease affecting potatoes was first detected in Mexico in 1993. Affected plants had aerial symptoms similar to those caused by potato purple top and psyllid yellows, but tubers had internal brown discoloration when sliced and dark stripes and streaks when processed to produce potato chips. The disease has been found in many potato production areas in Guatemala, Mexico, Honduras, New Zealand and the United States. The disease, termed Zebra Chip (ZC), has been associated with the presence of heavy infestations of the potato-tomato psyllid (*Bactericera cockerelli*). In 2009, a research group in New Zealand discovered that a new disease in tomato and pepper plants was caused by *Candidatus* Liberibacter solanacearum (Lso) and subsequently this same bacterium was associated with ZC in potato samples from Texas.

The objectives of this study were: to assess the accumulation of Lso in various potato organs, to determine the effect of plant age on detection of Lso, symptom development and plant death, and (*iii*) to determine the effect of phosphorous acid on the development of ZC.

Results from these studies showed significant differences in Lso populations between above and below ground tissues of the potato plant, with Lso populations in stolons and tubers being three to four times higher than those of leaf tissue and over seventy times greater than in stems. Time for detection of Lso by PCR in potato leaves of different ages at the time of inoculation ranged from 21 to 26 days after inoculation, symptoms development took 23 to 36 days. Plant death, took 24 to 47 days in plants of different age groups at the time of inoculation. In plants 15 weeks old at the time of inoculation, Lso was detected after 14 days in one plant out of 18; in plants 16 weeks

old at the time of inoculation, Lso was detected after seven days in two plants out of 18. Phosphorous acid applications had no effect on the populations of Lso in potato tubers, onset of symptoms or plant death. All tubers showed ZC symptoms, making them unacceptable for the market.

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DEDICATION

I want to dedicate this work to my family, my father Francisco; my mother Lucky; my wife Rachel, my brother Francisco, my grandmother Yolanda († - ppnp), and to my uncles and cousins. Without their support, encouragement and sacrifices throughout my educational endeavors I couldn't have made it. Thank you very much. I am what I am because of you.

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LITERATURE REVIEW

Zebra Chip disease of potato

In the mid 1990s a defect in potato tubers produced in Mexico was observed with internal brown discoloration when sliced and dark stripes and streaks when the affected tubers were processed to produce potato chips (Secor and Rivera, 2004). The term Zebra Chip (ZC) was coined to describe the disease, based on the characteristic dark stripes in infected tubers, and since then the designation has become established (Goolsby *et al*., 2007). ZC of potato was first found in potato fields in Saltillo, Mexico in 1994 (Secor and Rivera, 2004) and has since spread to several potato production areas in Mexico (Munyaneza *et al.,* 2009a), Honduras, Guatemala and the western United States. In the United States, the disease was first reported in potato fields in Pearsall and Lower Rio Grande Valley in Texas (Secor and Rivera, 2004; Munyaneza *et al.* 2007b) and since then has spread to Nebraska, Colorado, New Mexico, Arizona, Nevada, California, Wyoming and Kansas (Munyaneza *et al.*, 2007a; Munyaneza *et* al., 2009b; Crosslin *et al.,* 2009a; Rehman *et al.*, 2010). In 2008, a new disease affecting tomato and pepper plants was found in New Zealand, and later in the same year, a the disease was found in potato plants, which were showing ZC symptoms in Auckland, NZ. (Liefting *et al.*, 2008; Liefting *et al.*, 2009), and in 2011, the disease was found in potato fields in Idaho, Oregon and Washington (Crosslin *et al*, 2011a; Crosslin *et al*, 2011b). This disease poses a serious economic threat to the processed and fresh potato industry. Disease incidence and severity fluctuates from year to year, but the disease has caused millions of dollars in losses and it is not uncommon that whole fields are left abandoned (Munyaneza *et al.*, 2007a). In Texas, direct losses in the value of potato

production due to ZC have been calculated at $25.86 million in the 2003-2005 period, and potential losses in business activities associated with potato in the neighborhood of $100 million (CNAS, 2006). Affected potatoes are not marketable in fresh, processing markets, and export potato industries (Gudmestad and Secor, 2007; Secor *et al*., 2009), and are therefore left in the field or sold for starch at reduced prices (CNAS, 2006). ZC affects marketability of potato tubers affected since it causes unsightly dark stripes, particularly when they are processed into chips. Fresh potatoes with ZC taste sweet, but no edibility or health issues have been found with diseased potatoes.

Etiology

The putative causal agent of the ZC disease has been recently identified as *Candidatus* Liberibacter solanacearum (Jagoueix). The identification of this bacterium had been an elusive matter for several years. Initially phytoplasmas were suspected to be the causal agent due to the resemblance of symptoms to those caused by these pathogens, but their presence could not be confirmed by polymerase chain reaction (Secor *et al*., 2005). Bacterium-like organisms (BLO's) were also suspected based on microscopic evidence of affected plants. Transmission electron microscopy (TEM) revealed bacterial-like particles in phloem of roots, stems and stolons. They were found only in the phloem of symptomatic potato and tomato plants and not in the associated companion or mesophyll cells. All particles were pleomorphic and similar in appearance with tri-laminar envelope, an electron dense cell wall, and resemble TEM images of other BLO's, including Liberibacter species infecting citrus (Secor *et al.,* 2009).

In 2009, Liefting *et al.* reported a new disease affecting tomato and pepper in New Zealand associated with a new species of *Candidatus* Liberibacter. This was subsequently named *Candidatus* Liberibacter solanacearum (Liefting *et al, 2009b)*. In 2009 Secor *et al.*, described the association of *Ca.* L. solanacearum with ZC using electron microscopy and molecular characterization. Successful transmission of the pathogen from infected to healthy potato plants using graft transmission has been documented (Secor *et al.*, 2009, Crosslin *et al.,* 2009b), but no mechanical transmission has been documented.

Taxonomy and morphology

Candidatus Liberibacter solanacearum (Lso) is a non-culturable, phloem-limited bacterium that belongs to the α subdivision of the class *Proteobacteria*. Members of this class are Gram negative. This subdivision is a diverse group of microorganisms that include plant pathogens, symbionts and human pathogens (Bové, 2006). Individuals in this subdivision live in intimate association with eukaryotic cells Transmission electron microscopy indicates that *C.* L. solanacearum is 0.2 μm in width and 4 μm in length with rounded ends (Liefting *et al.*, 2009a; Tanaka *et al.*, 2007). Lso is classified as a *Candidatus*, which is a scientific classification for a bacterium that is well characterized but as yet uncultured and cannot be maintained in a bacteriology culture collection (Stackebrandt *et al.*, 2002). Since Liberibacters cannot be cultured, detailed information about their etiology, physiology and mode of pathogenesis are lacking, and their detection, identification and classification are based largely on molecular techniques and specific signatures, particularly in the 16S rRNA gene (*Li et al.,* 2009; *Secor et al*, 2009; Lin *et al.,* 2009). Based on phylogenetic analysis of the

16S rRNA gene, the 16S/23S rRNA spacer region and the rplKAJL-rpoBC operon, Liefting *et al.* (2009a) concluded that the organism is a new species of the *Candidatus* Liberibacter genus. This species is phyllogenetically distinct from the three currently described Liberibacter species ('*C.* L. asiaticus', '*C.* L. africanus', '*C.* L. americanus') and the first Liberibacter species known to naturally infect plants outside the *Rutaceae* family, family that includes citrus, in which Liberibacter is an important pathogen. This bacterium was first named "Liberobacter", from the Latin Liber (= bark) and bacter (=bacterium) (Jagoueix *et al*, 1997); Liberobacter was subsequently replaced by Liberibacter because `bacter' is of masculine gender and thus the connecting vowel between "Liber" and "bacter" should be `i' when the preceding term is of Latin origin (Garnier *et al.*, 2000).

Lso is classified as follow (Cavalier-Smith, 2002; Jargoeix, 1997; Liefting *et al.,* 2009):

Kingdom:	Bacteria
Subkingdom:	Negibacteria
Phylum:	Proteobacteria
Class:	Alphaproteobacteria
Order:	Rhizobiales
Family:	Rhizobiaceae
Genus:	*Candidatus* Liberibacter (Jagoeix)
Species:	*Candidatus* Liberibacter solanacearum

The name *Candidatus* Liberibacter psyllaurous is considered to be synonymous to *Candidatus* Liberibacter solanacearum, because the 16s rRNA sequence of *C.* L. psyllaurous is identical to the 16s rRNA sequence of *C.* L. solanacearum associated with ZC in the United States and in New Zealand (Secor *et al.*, 2009). At about the same time that Liefting *et al.* (2008) reported the new *Candidatus* Liberibacter species

affecting potatoes in New Zealand, Hansen *et al*. (2008) reported an association of *C*.L. psyllaurous with psyllid yellows affecting tomato and potato in the United States. The name *C*.L. solanacearum was preferred and has become the more widely used name for the pathogen because it was validly published by Liefting *et al.* (2008) according to the rules of the Code of Nomenclature for Bacteria, and the name *C*.L. psyllaurous was not (Secor *et al.*, 2009).

Vector

Surveys of insects associated with potato fields affected with ZC in the southwestern United States found that *Bactericera cockerelli* was the most abundant and common pest (Goolsby *et al.,* 2007; Munyaneza *et al.,* 2007a). It has been demonstrated that the potato psyllid, *B. cockerelli* is the major vector of Lso, the causal agent of ZC (Munyaneza *et al.*, 2007a; Secor *et al.,* 2009; Sengoda *et al.,* 2010).

Bactericera cockerelli (Sulc.) (Homoptera: Psyllidae) was originally described by Sulc in 1909, and classified as *Trioza cockerelli* and assigned to the genus *Paratrioza* by Crawford (Crawford, 1911). Recently the potato psyllid has been reassigned to the genus *Bactericera* (Burkhardt and Laureter, 1997; Miller *et al.,* 2000). The genus Bactericera includes 28 species.

The taxonomic classification of *B. cockerelli* is as follows (Ruggiero *et al.,* 2011):

Domain:	*Eukaryota*
Kingdom:	*Animalia*
Subkingdom:	*Bilateria*
Branch:	*Protostomia*
Infrakingdom:	*Ecdysozoa*
Superphylum:	*Panarthropoda*
Phylum:	*Arthropoda*
Subphylum:	*Mandibulata*
Infraphylum:	*Atelocerata*

Superclass:	*Panhexapoda*
Epiclass:	*Hexapoda*
Class:	*Insecta*
Subclass:	*Dicondylia*
Infraclass:	*Pterygota*
Superorder:	*Condylognatha*
Order:	*Hemiptera*
Suborder:	*Sternorrhyncha*
Infraorder:	*Psyllomorpha*
Superfamily:	*Psylloidea*
Family:	*Psyllidae*
Genus:	*Bactericera*
Specifies:	*cockerelli* - (Sulc)
Scientific name:	*Bactericera cockerelli* (Sulc)

B. cockerelli is polyphagous phloem feeder, and can successfully reproduce on a wide variety of hosts, that include plant species in 21 families (*Amaranthaceace, Asclepiadaceae, Asteraceae, Brassicaceae, Chenopodiaceae, Convolvulaceae, Fabaceae, Lamiaceae, Lycophyllaceae, Malvaceae, Menthaceae, Pinaceae, Poaceae, Polygonaceae, Ranunculaceae, Rosaceae, Salicaceae, Scrophulariaceae, Violaceae* and *Zygophyllaceae*), but plants in the *Solanaceae* family are the preferred host, and has been a pest of potato and tomato for many years (Hansen *et al.,* 2008; Wallis, 1955; Gao *et al.,* 2009). The potato psyllid originated in North America, and is naturally distributed in Canada (Alberta, British Columbia, Ontario, Quebec, Saskatchewan), Mexico, the United States (Arizona, California, Colorado, Idaho, Kansas, Minnesota, Montana, Nebraska, Nevada, New Mexico, North Dakota, Oklahoma, South Dakota, Texas, Utah, Wyoming), Guatemala and Honduras. Texas, southern New Mexico, Arizona, California and northern Mexico are desert breeding areas of *B. cockerelli* (Al-Jabr, 1999). *B. cockerelli* has not been found in Europe, Asia, the United Kingdom or

Australia (Morris *et al.,* 2009). The potato psyllid was recently introduced to New Zealand, where it was first discovered in 2006 (Liefting *et al.,* 2009a; Teulon *et al.,* 2009). It is important to note that Lso has not been found in areas where *B. cockerelli* is absent, since there is no mechanism for bacterial spread (Morris *et al.*, 2009), but samples showing ZC symptoms and positive for Lso were evaluated by Wen *et al.* (2009). Munyaneza *et al.* (2010) reported the first known association of Lso with a non-solanaceaous crop in Finland, where it was found to affect carrot (*Daucus carrota* L.). In this case, Lso appears to be vectored by the carrot psyllid (*Trioza apicalis* Förster), which is a serious pest in north and central Europe. This was also the first report of Lso outside North and Central America and New Zealand (Munyaneza *et al.*, 2010). In 2011 Lso was found in carrots in Sweden and Norway (Munyaneza *et al* 2011a; Munyaneza *et al*, 2011b) and in 2012, in carrots in the Canary Islands associated with *Bactericera trigonica* (Hodkinson) (Alfaro-Hernandez *et al.*, 2012).

Bactericera cockerelli biology

B. cockerelli is a very prolific insect, and females lay very small eggs, usually less than 2 mm in length, but in large numbers, around 500 eggs per female, but have been reported to lay as many as 1300 eggs (Knowlton and Janes, 1931; Teulon, *et al.,* 2009; Abdullah, 2008). In greenhouse conditions, the vast majority of eggs are laid in the edge of the leaf, but it is not uncommon to find them on the leaf surface; in field conditions, however, most eggs are laid on the lower leaf surface (Pletsch, 1947). Eggs have an average incubation period of 6.7 days. After hatching, they pass through five nymphal stages, with the main structural change being the increase of body size and development of wings. Wing pad development is first noted in the third instar and

becomes obvious in fourth and fifth instars, and an added number of marginal wax glands (Pletsch, 1947). Upon hatching, nymphs have a yellowish-brown color that changes to green after the third molt (Lehman, 1930). Passage through all five nymphal stages can last up to 22 days before emerging as adults. Psyllid nymphs (immature) are more frequently found feeding on the underside of leaves**.**

Adults are light yellow to pale green in color in the beginning, until they eventually darken to a grey or black color. Adult potato psyllids can be separated from other insects by its distinctive white stripes in the dorsal side of the abdomen, a trifurcate branching on the basal vein of the forewing (other species it is bifurcated), and by the number of inner apical spurs on the tibiae of the hind legs; adult psyllids have a tendency to jump if disturbed (Teulon *et al.,* 2009). Adult potato psyllids are small (2 mm long) and look like small cicadas with clear wings. Males and females can be distinguished by the shape of the apex of the abdomen (Figure 1). The mature female abdomen, which terminates with a short ovipositor, is well rounded and more robust than the male abdomen and has five abdominal segments, plus the genital one. The male genitalia present a more blunt appearance at the tip, and the abdomen is divided in six segments, plus the genital one. (Pletsch, 1947; Abdullah, 2008). Male and females can also be distinguished when they are immature, when the yellow coloration of developing testes is strong enough in the fourth and fifth instar nymph stages. The mycetome is a yellowish-orange roughly U-shaped body found in the basal half of the nymph abdomen. In male nymphs, spindle-shaped testes extend along and beyond the mycetome lobe, and forming with the mycetome a H-shaped structure. In females, a pair of hyaline bodies can be seen posterior to the mycetome. With this

method, over 95% of males can be correctly identified with some ease (Carter, 1961).

Mating can occur several times and initiates once adults become mature and gain their dark color (Knowlton and Janes, 1931). The total development period of the psyllid ranges between 25 and 33 days, with an average lifespan of 41 days (Abdullah, 2008). Pletsch (1947) found that when potato psyllids were given a choice to feed on four host plants (potato, tomato, pepper and eggplant), most psyllids were recovered from tomato and potato, then eggplant and finally, pepper. When females were offered the same hosts, oviposition on potato was preferred.

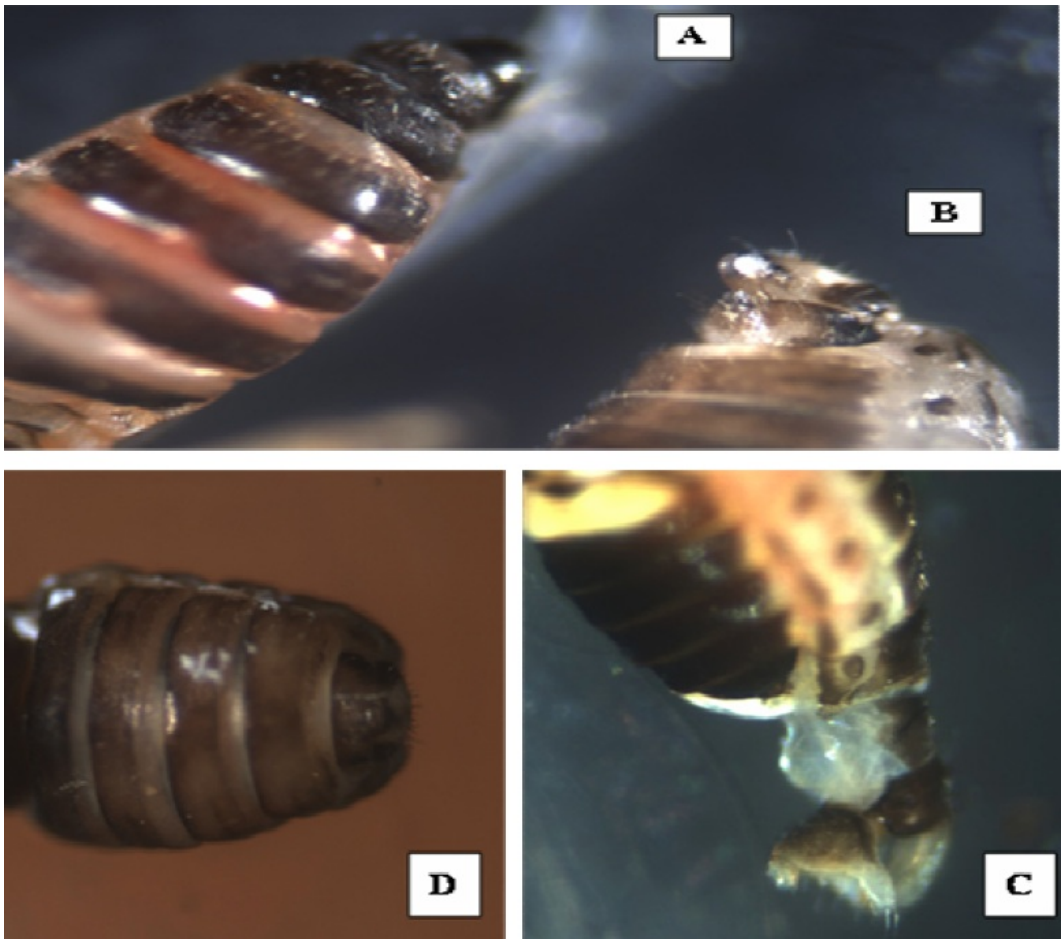

Figure 1. Abdominal segments of *B. cockerelli* adults illustrating differences between male (A and C) and female (B and D) (Abdhulla, 2008)

Zebra Chip symptoms

Infected potato plants show a variety of symptoms, which resemble those of potato purple top disease (PPT), caused by clover proliferation group phytoplasma (Lee *et al.*, 2004), and psyllid yellows (PY), whose causal agent(s) remain unknown (Sengoda *et al.,* 2010).

ZC is differentiated from PPT and PY in that tubers affected by ZC show tuber necrosis; neither PPT nor PY induce necrosis. In some cases PPT aerial symptoms may be confused with aerial ZC symptoms, but vascular discoloration caused by PPT does not affect tuber tissue (Secor *et al*., 2009; Sengoda *et al.*, 2010). Potato plants affected by ZC tend to die more rapidly than plants affected by PY, and often times, plants affected by PY can recover and regrow once the psyllid pressure is eliminated from the plants; some can even appear completely recovered at the end of the season (Sengoda *et al.,* 2010). All classes and cultivars of table stock, seed and chip potatoes are affected by the disease (Wen *et al.,* 2009).

Foliar symptoms

Aerial symptoms include chlorosis, yellow or purple discoloration, leaf rolling, leaf scorch, twisted stems with a zigzag appearance, proliferated axillary buds, swollen nodes and production of aerial tubers (Figure 2). Vascular discoloration (browning) is also observed. Plants wilt, senescence early and may die suddenly (Secor *et al.*, 2009).

Underground symptoms

Underground tuber symptoms include brown discoloration of the vascular ring and medullary rays that is visible through the entire length of the tuber when it is cut. This symptom is diagnostic and used to differentiate from other potato diseases.

Figure 2. Zebra Chip foliar symptoms on potato. Photos: Gary Secor

In both raw and after frying, potato slices show dark blotches, stripes or streaks, which reduces the value and quality of all market classes of potato (Figure 3). Tubers are of reduced size and may show necrosis, enlarged lenticels, vascular tissue browning of the parenchymatic medullary region and cortex which affects the entire tuber from the stem end to the bud end, and may show necrotic flecking that resemble symptoms of net necrosis of potato tubers caused by potato leaf roll virus (Slack, 2001; UNL, 2009; Miles *et al.*, 2010a). Stolons may collapse and show vascular discoloration and, if severe, discoloration on the surface (Secor *et al.*, 2009, Crosslin *et al*., 2010). Tubers show necrotic flecking and seldom sprout, and if they do, they produce hair sprouts and weak plants (Munyaneza *et al*, 2007a). Plants often show vascular discoloration that resembles symptoms caused by *Fusarium* spp.

The importance of tuber infections in the epidemiology and spread of the Lso has been studied, with somewhat mixed results. Henne *et al.* (2010) found that 20 to 40 percent of ZC affected seed tubers sprouted and Lso-free psyllids failed to acquire the bacterium from those plants. Sprouted plants did not have a significant effect on the surrounding potato plants, and they concluded that seed tubers infected with Lso do not pose a significant threat to production since they do not contribute to ZC incidence and spread in the field. In contrast, Pitman *et al.* (2011) found that infected tubers could play a role in the life cycle of Lso in New Zealand. In their study, 93.6 percent of ZC infected tubers successfully sprouted. Of these infected tubers that sprouted, 70 percent of the plants did not show zebra chip symptoms despite being positive to Lso by PCR. Transmission of Lso from infected tubers to progeny was demonstrated in this study, however, no studies were performed to determine how efficiently *B. cockerelli* could acquire Lso from this plants. Pitman concludes that distribution of seed tubers infected with Lso could enhance the spread of this disease.

Figure 3. Zebra Chip necrotic browning in medullary ray tissues. Left: fresh cut. Right four panels: after frying. Photos: Gary Secor

Tubers with ZC have significantly higher levels of glucose (Gao *et al.*, 2009), which results in the darkening of fried potatoes. Navarre *et al.* (2008) found that infected tubers had higher concentrations of the phenolic compounds, tyrosine and salicylic acid; the latter is a key regulator of systemic acquired resistance and also involved in R-gene mediated resistance. The marked increase in tyrosine concentration is most likely contributing to the browning observed in tubers after slicing and frying. Their results also indicate that carbohydrate metabolism in general is being disrupted. Recently, it was determined that levels of ion leakage (conductance) were significantly higher in potato tubers affected by ZC than those not affected, indicating cell death; this, in conjunction with optical, fluorescent and electron microscopy, as well as the regulators of plant defenses, strongly suggest that a hypersensitive response leading to programed cell death (death of specific cells of an organism, whose initiation and execution is mediated by an intracellular program) is induced in affected potato tubers (Miles *et al.,* 2010a). ZC affected tubers have also been found to have significantly higher concentrations of phosphorous, potassium, zinc and calcium than tubers without ZC. These minerals have also been shown to be significantly altered by various potato related diseases, but to fully understand the implications of nutrition in the ZC disease process more work needs to be done (Miles *et al.,* 2010b).

Management

Management of ZC is mainly achieved through the use of insecticides targeting the vector, the potato psyllid, as controlling the vector is easier that controlling the pathogen itself. In the case of certain groups of pathogens, like viruses or fastidious bacteria, vector control is more helpful when the control measures are carried in the

areas on which the vector overwinters (Agrios, 2005). In cases where the pathogen has already been established, control of the disease by means of controlling the vector is seldom successful. The polyphagous nature of *B. cockerelli* makes it difficult to control, given that it can migrate to potato fields from many other sources.

Many strategies have been investigated to manage the spread of ZC. Currently, the only effective means of control of *B. cockerelli* is the application of insecticides, but alternative methods are also being investigated, including plant resistance, biological control, mineral oils and repelling compounds (Butler *et al*., 2010, Lacey *et al*, 2010, Zens *et al*, 2010). A recent study looked into the efficacy of using Kaolin particle film on potatoes as a repellent of the potato psyllid (Peng *et al.*, 2011). Kaolin is an aluminosilicate mineral that when applied on the plant surface, it creates a film that provides a protective physical or mechanical barrier against pests and pathogens. Results from this study showed that when psyllids had no feeding choice and landed on plants treated with kaolin, they laid fewer eggs than psyllids in plants treated with water controls. When the psyllids had a choice between plants treated with kaolin and non-treated plants, they avoided treated plants.

Insecticide applications to control *B. cockerelli* often need to be done on a regular basis due to the high fecundity and short life period of the psyllid, and farmers usually apply more insecticide than necessary in order to achieve season long protection against ZC. This not only increases the costs to the producers, but also has triggered environmental concerns and potential damage to beneficial insect populations. Spray programs are rotating with products with different insecticide classification groups, in order to avoid development of resistance in populations of

psyllids. Groups commonly utilized by farmers include pyrethroid, neonicotinoid, spinosyns, avermectins and feeding blockers (Bynum *et al*., 2010).

Butler *et al.* (2010) found two potato lines, 463-4 from the Idaho potato breeding program and NY-138 from the Texas potato breeding program, showed significantly lower feeding duration and the time that psyllid avoidance time was also significantly longer. In this study, Butler also found several natural enemies of the potato psyllid in southern California, including *Orius tristicolor* and mirids that were predators of nymphs, and several other insect species that predate nymphs and adults (*Hippodamia convergens, Coccinella septempunctata, Harmonia axyridis and Nabis spp.*). Levy *et al.* (2011) used two advanced potato selections that have been identified as tolerant to ZC, NY138 and BTX1749-1W/Y. It is believed that psyllid feeding preference and behavior are contributing factors for tolerance or avoidance of ZC in these cultivars; psyllids spent less time feeding and more time resting in these selections than they did in other cultivars and selections. Using these selections, Levy *et al.* (2011) found that susceptible and tolerant plants did not significantly differ from one another in the time for detection on Lso and found that translocation patters in both groups were similar. However, earlier disease onset and greater severity was observed in susceptible cultivars Atlantic and Russet Norkotah than in the tolerant ones.

Lacey *et al.* (2010) studied the use of entomopathogenic fungi to control the potato psyllid, both in laboratory and field conditions. Results from this work showed that use of *Isaria fumosorosea* (Pfr 97, Certis, USA) and *Metarhizium anisopliae* (F52, Novozymes Biologicals, USA) provided psyllid control comparable to use of Abamectin (Agri-Mek, Syngenta, USA).

Detection of *C.* L. solanacearum

A requirement for the control of plant diseases is the proper identification of the causal agent (Schaad and Frederick, 2002). Some limitations to accurately diagnose a diseased plant include lack of experience to recognize symptomatology of the disease, atypical symptomatology, asymptomatic tissues, or insufficient symptoms. In cases where the pathogen is impossible to isolate and identify, as is the case with ZC because Lso cannot yet be cultured or other limitations, sensitive techniques using molecular tools for the detection of pathogens have been developed to allow reliable identification.

In the case of Lso, molecular techniques are used to identify and characterize the pathogen. Electron microscopy has been used in the past to visualize the presence of Lso in phloem cells in ZC affected plants (Secor *et al.*, 2009), but the preferred method to detect the presence of Lso in plants is polymerase chain reaction (PCR) and real-time polymerase chain reaction (rtPCR) or quantitative real time (qPCR) (Munyaneza *et al,* 2007a; Hansen *et al*, 2008; Lin *et al,* 2009; Secor *et al*, 2009; Wen *et al*, 2009). Since real time PCR has become commercially available and more accessible, it has become the most accurate and sensitive way to detect the presence of Lso in plant tissue.

Detection of Lso begins with the extraction of total DNA from the plant, usually using extraction kits commercially available (DNeasy Plant Mini Kit, Qiagen, Valencia, CA) due to the resulting quality and purity of the DNA, which is necessary for PCR and qPCR to work properly. In case of conventional PCR, sufficient DNA quality is obtained by using the CTAB protocol (or a modified protocol) (Gawel and

Jarrett, 1991). After the DNA is extracted, the concentration and quality is estimated using a micro-volume spectrophotometer and the concentration is adjusted for the particular PCR assay.

OBJECTIVES

The general objectives of these studies are:

1. To determine the accumulation of *Candidatus* Liberibacter solanacearum in organs of potato plants
2. To determine the effect of plant age on movement of *Candidatus* Liberibacter solanacearum to tubers.
3. To determine the effect of phosphorous acid on the populations of *Candidatus* Liberibacter solanacearum in potato tubers.

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CHAPTER I. ACCUMULATION OF C. L. SOLANACEARUM IN POTATO PLANT ORGANS

Hypotheses

H_o: The data collected will not show differences in accumulation and detection of *C.* L. solanacearum in different potato organs.

H_a: The data collected will show differences in accumulation and detection of *C.* L. solanacearum in different potato organs.

Introduction

Only limited studies have been made about the *in planta* distribution of Lso in potatoes, Tatineni *et al.* (2008) conducted studies of the Huanglonbing disease of citrus (formerly known as citrus greening), which is caused by the three species of *Candidatus* Liberibacter (*C.*L americanus, *C.*L. africanus and *C.*L asiaticus) to understand the distribution and movement of the pathogen in the infected citrus tree using conventional PCR and RT-PCR. Results from this study concluded the pathogen was present in all floral and fruit parts of the plant and that *in planta* distribution of the pathogen is uneven, ranging from 14 bacteria per µg of total DNA in pistils, to 137,031 bacteria per µg of total DNA in fruit peduncles. Even though the pathogen was found at varying levels in the plant, there was no significant difference in the concentration present in most of the sampled tissues (root, leaf midrib, petal, pistil, stamen, columella, seed coat, endosperm, young whole fruit and bark of the citrus trees). In the case of a comparison between pistil and peduncle, there was a 10,000-fold difference in bacterial population density among those tissues. This indicates that peduncles are

preferred targets for detecting the pathogen and might help to avoid the occurrence of false negatives in samples from infected plants.

In the case of the Haunglongbing pathogen, the distribution from inoculation point to different organs, particularly young tissues, strongly suggests movement through the phloem given that *C.* Liberibacter asiaticus was found in sink organs of the plant, such as young leaves, fruit, flowers and roots. Further evidence is the systemic movement of the pathogen from the site of infection to different parts of the plant following graft inoculation; this indicates that the pathogen is transferred systemically through the vascular system. Electron microscopic observations of phloem sieve element pores showed that *C.* L. asiaticus could move through the sieve plate pore, implying that it can move with assimilate flow from leaves to sugar consuming organs of the plant.

Similar results were obtained by Levy *et al.* (2011) who looked at the movement of the ZC pathogen (Lso) in potato and tomato. They found that there is a source to sink movement towards newly differentiating leaves, although they caution that this pattern might change depending on the developmental stage of the potato plant when inoculated. A limitation of this study is that it only detected the presence or absence of Lso in different potato tissues, but there was no information about the bacterial population in those tissues. Wen *et al.* (2009) studied the distribution of Lso in twelve potato plants collected from commercial fields in Texas that showed severe ZC symptoms. Results from this study indicated that Lso populations were generally higher in below ground tissues than it was in aboveground tissues. Detection of Lso ranged from 91% to 100% of the samples tested from stolons and from 0% to 83% in

aboveground tissues (leaf, midvein, petiole and stem). In aboveground tissues sampled, Lso was detected more frequently in stems.

In this study, the differential accumulation of Lso in different organs of potato plants was tested using experimentally infected plants. Plants were inoculated under homogenous conditions in a growth chamber. After the plants had been exposed to Lso infected psyllids, they were transferred to growth chambers and remained in the growth chambers for a period of six weeks until evaluated.

Information regarding bacterial numbers in different plant tissues will help in understanding the movement and distribution of Lso in potato plants. This will provide a tool for potato breeders to compare different selections and cultivars quantitatively in order to make an informed decision about the existing level of resistance in breeding materials. This study will also provide guidelines and recommendations for potato plant organ testing to facilitate accurate and repeatable test results.

Materials and Methods

Plant material

Potatoes, cultivar Atlantic, were grown from disease-free potato mini-tubers obtained from Sklarczyk Seed Farms LLC (Johannesburg, MI). This chipping cultivar was used because it is highly susceptible to ZC and commonly grown in regions where ZC is found (Munyaneza *et al.*, 2007a; Munyaneza *et al.*, 2007b; Munyaneza *et al.*, 2008). Potatoes were grown in individual six-inch pots containing Sunshine Mix (Sun Gro Horticulture, Vancouver, BC). A plant fertilizer (17:17:17 N-P-K) was incorporated into the potting soil prior to planting. All potatoes were planted and

grown in a single isolated greenhouse room maintained at 25 ºC – 28 ºC, with photoperiod cycles of 16 hours light / 8 hours dark until the time of inoculation.

Psyllid colonies

A psyllid colony established using psyllids provided by Dr. Joseph Munyaneza (USDA-ARS, Wapato, WA) was used for all inoculations. Munyaneza psyllids were initially collected in Texas in 2007 and have been reared for multiple generations in his laboratory. Colonies were grown in commercial rearing cages (Bug Dorm-2, BioQuip, Rancho Dominguez, CA, USA) consisting of an aluminum frame covered with insect-proof mesh with enough space to contain four plants in six-inch pots.

The potato psyllid colonies were maintained on potato plants at 25 ºC with photoperiod cycles of 16 hours light / 8 hours dark in a greenhouse and growth chambers. Plants were replaced periodically to sustain psyllid colony growth. Insects were reared for multiple generations and used as needed. To confirm the presence of Lso in both of the psyllid colonies, subsamples of psyllids were tested periodically by PCR according to Secor *et al.* (2009) (Appendix III).

Psyllid colony establishment and maintenance

Psyllid colonies in rearing cages are often contaminated with other pests, among which, thrips and aphids are the most common. When infestations occurred after colony establishment colonies were re-established by transferring psyllids to clean cages on non-infested plants. Colonies were re-established by collecting individual nymphs from potato leaflets with the aid of a dissecting microscope. Nymphs were identified and observed to insure the absence of contaminating insects, and collected by removing a 5-mm leaf disk containing a single nymph (Figure 1.1).

Leaf disks containing individual nymphs were transferred to cages and distributed on potato plants.

Figure 1.1. Leaf disks containing individual nymphs

Inoculations

Plants were inoculated six weeks after emergence by exposing 15-25 cm pathogen free Atlantic potato plants to infected adult psyllids from Munyaneza colonies. Adult psyllids from infective colonies were collected using a hand held insect aspirator and storing in a 15 ml collection tube prior to release into cages. Nine potato plants were enclosed in commercial rearing cages. Sixty adult potato psyllids were released into each cage and allowed to feed on the plants for seven days. Inoculations were conducted at the USDA-ARS greenhouse entomology research complex because no insecticides applications take place in that complex. After feeding for seven days, cages containing the inoculated plants were transported to the Plant Pathology greenhouse complex for the remainder of the experiment and psyllids killed with insecticide sprays (M-Pede Insecticidal Soap, Dow AgroSciences, IN). After insect removal, regular greenhouse pesticide was carried thought the remainder of the

experiment. All potato plants were maintained in a greenhouse room at a temperature of 25 ºC with light cycles of 16 hours light / 8 hours dark.

Experimental design

This experiment was arranged as a completely randomized design (CRD) and was composed of three potato plants per replicate, with three replicates per treatment (potato organ). The experiment was conducted twice.

Plant DNA extraction

Five hundred milligrams of leaf, stem, stolon and tuber tissue was ground in liquid nitrogen with a mortar in an Agdia sample bag with mesh (Agdia, Elkhart, IN), and total DNA extraction was performed using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions.

The concentration and quality of total DNA was estimated with a 2-µL volume in a micro-volume spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA concentration for real time PCR assays was not adjusted.

Psyllid DNA extraction

Psyllid DNA extraction was done according to Hung *et al.* (2004). In this protocol, individual psyllids are homogenized in 2 ml of lysing matrix tubes (MP Biomedicals, LLC, Fountain Parkway, Solon, OH) in which the ceramic sphere has been removed and replaced with four to five 2.5 mm glass beads (BioSpec Products, Bartlesville, OK). To the tube was added 300 µl of extraction buffer (Appendix II) and the sample homogenized in a FastPrep FP120 cell disrupter (Qbiogene, Inc., Carlsbad, CA). After homogenization, the sample is incubated at 65 ºC for 30 to 60 minutes, and an equal volume of phenol/chloroform/isoamyl alchohol (25:24:1) was added to each

sample, vortexed (Vortex Genie-2, Bohemia, NY) and centrifuged at 12,000 rpms at 4ºC for ten minutes. The supernatant was transferred to a new 1.5 ml Eppendorf microcentrifuge tube and 500 µl of 95% ethanol is added, inverted gently several times and then centrifuged at 12,000 g's for ten minutes. The resulting DNA pellet at the bottom of the tube was then washed with 70% ethanol, air dried and suspended in 15 µl of molecular grade water (Sigma, St. Louis, MO), and stored at -20 ºC.

The concentration and quality of total DNA was estimated with a 2-µL volume in a micro-volume spectrophotometer (NanoDrop Technologies, Wilmington, DE). Real time PCR was performed without adjusting the DNA concentration.

PCR primers

Lso specific primers developed by Liefting *et al.* (2009) were used to detect Lso in plant and psyllid samples. These primers are: forward primer CLi.po.F (5'–TACGCCCTGAGAAGGGGAAAGATT-3') that was empirically designed from the 16S rDNA sequences in the NCBI GenBank for '*C.* L. asiaticus', '*C.* L. africanus', '*C.* L. americanus', and '*C.* L. solanacearum' from ZC infected potato plants in New Zealand (accession number EU834130) and the United States (accession numbers EU884128 and EU884129); reverse primer O12c (5'-GCC TCG CGA CTT CGC AAC CCA T-3').

PCR conditions

PCR amplification was performed in 25 µl reactions containing 1X PCR buffer (Applied Biosystems, Foster City, CA), 2.0 mM $MgCl_2$, 0.4 mM dNTPs, 1 µM of each primer, and 1 U of AmpliTaq Gold *Taq* polymerase (Applied Biosystems, Foster City, CA). The PCR conditions were: an initial cycle at 94 ºC for 1 min., 30 cycles of 94 ºC

for 1 min., 95 ºC for 5 sec., and 68 ºC for 30 sec., plus an additional cycle of 10 min at 72 ºC. All amplifications were performed in a PTC-200 Peltier thermal cycler (MJ Research, Ramsey, MN). Primer pair CLi.po.F and O12c amplifies a 1070 bp fragment from ZC infected potato tissue. Aliquots of 5 µL PCR product were analyzed by agarose gel electrophoresis in 1.5% agarose gels (1X TAE) containing Gel Red (Biotium CA, cat. no. 41004).

Real-time PCR conditions

All real time PCR assays were performed using a Stratagene Mx3005P Real-Timer PCR system (Stratagene, La Jolla, CA) in accordance with Li *et al.* (2009). Primers used in this study were ZCf forward primer (5'–CGAGCGCTTATTTTTAATAGGAGC–3'), reverse Primer HLBr (5'-GCGTTATCCCGTAGAAAAAGGTAG–3') and the Liberibacter universal TaqMan Probe HLBp (5'–/56-FAM/AGACGGGTGAGTAACGCG/3BHQ_1/ –3'). Cytochrome oxidase primers for the host plant as internal positive control targeting plant DNA, were: forward primer COXf (5'-GTATGCCACTGCGCATTCCAGA–3'), reverse primer COXr(5'–GCCAAAACTGCTAAGGGCATTC– 3') and TaqMan probe COXp (5' – /5TET/ ATCCAGATGCTTACGCTGG /3BHO_2/ –3').

The real-time PCR mix contained 1X PCR buffer, 1 unit of Gold Taq polymerase (Invitrogen, Carlsbad, CA), 6.0 mM MgCl2, 240 µM each dNTPs, 240 µM each primer, 120 µM each probe, and 2 µl of DNA sample. Real-time PCR program was: 1 cycle for 20 seconds at 95 ºC with optics off, 40 Cycles at 95 ºC with optics off, 58 ºC 40 seconds with optics on. For real-time PCR assays, a threshold (Ct) of ≤ 35 was used to determine that a sample is positive. Lso populations (expressed as genome

copy number) were calculated using the following standard curve for quantification (Figure 1.2) provided by Dr. Aimin Wen (personal communication):

$$y = -2.546 \bullet \log (X) + 33.35$$

Where

y = Ct value, and

X = Lso genome copies number (population density)

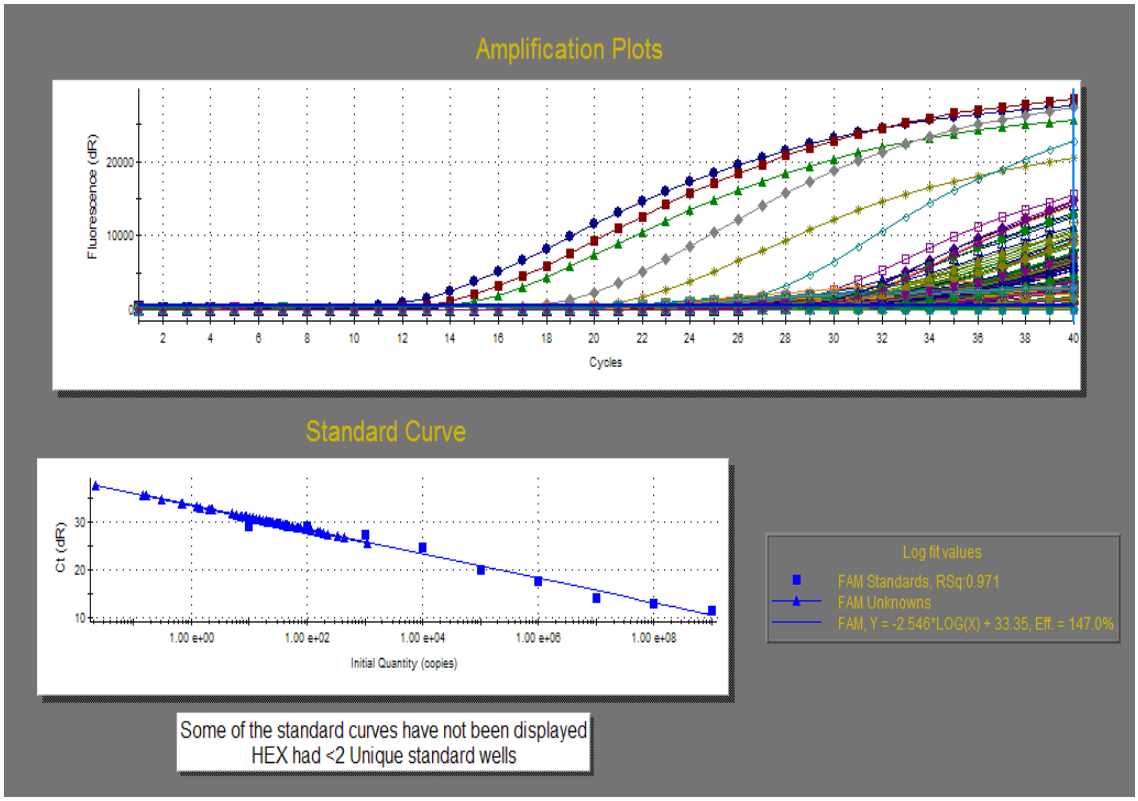

Figure 1.2. Standard curve for quantification of Lso using real time PCR (ZCf/HLBr+HLBp). Aimin Wen, personal communication

Evaluation

Four weeks after inoculation, samples were collected from each inoculated plant, and total DNA was extracted from leaf, stem, stolon, and tuber, using a DNeasy

Plant Mini Kit (Qiagen, Valencia, CA). Resultant DNA was quantified and tested for the presence of Lso by conventional and real-time PCR as previously described.

Data analysis

Levene's test of homogeneity of variances was performed to determine if data from the two runs of the experiments could be combined. If data were combined, analysis of variance was performed with PROC GLM using SAS (SAS Institute, Cary, NC) using Ct values from real time quantification and Lso copy number data for each of the organs. Mean comparisons were conducted using Fisher's Least Significant Difference (LSD).

Results

Psyllids from the colony used in this study were tested periodically for Lso. Results show that 87% to 100% of individual psyllids tested were positive to Lso (Appendix III). All plant inoculations were successful resulting in high frequencies of plant infection in the trials. Plants showed both foliar and tuber symptoms consistent with ZC infections and tested positive for Lso by PCR. Non-inoculated control plants did not show zebra chip symptoms and tested negative for Lso by PCR.

Levene's test for homogeneity of variances for CT values of qPCR for different tissues was performed and results showed no significant differences among variances between trials, so data from both trials were combined for further analysis (Table 1.1).

Significant differences in Lso populations in organs as expressed by cycle thresholds (CT) values were observed for the combined experiments (Table 1.2). Mean CT values ranged from a low of 25.7 in stolons to a high of 30.6 in stems (Table 1.3, Figure 1.3).

Table 1.1. Levene's test of homogeneity of variances for real time PCR CT values from different potato organs.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	0.380	0.380	0.1232	0.7289^{NS}
Error	22	67.839	3.083		

^{NS} Non Significant differences at p<0.05

Table 1.2. Source of variation, degrees of freedom, mean squares, and F value for CT values on potato organs.

Source	DF	Sum of Squares	Mean Squares	F Values	Pr > F
Run	1	0.429	0.429	0.50	0.4902^{NS}
Organ	3	93.179	31.059	26.49	0.0116*
Run x Organ	3	3.517	1.172	1.36	0.2901^{NS}
Error	23	110.901			

* Significant difference at p<0.05 Coefficient of variation=3.385
^{NS} Non Significant differences at p<0.05

Table 1.3. Mean CT values for detection of Lso in potato organs.

Plant Organ	Mean CT Values	t Grouping
Stem	30.6	a
Leaf	27.3	b
Tuber	26.0	c
Stolon	25.7	c

Means followed by the same letter are not significantly different at p <0.05.
LSD=1.135

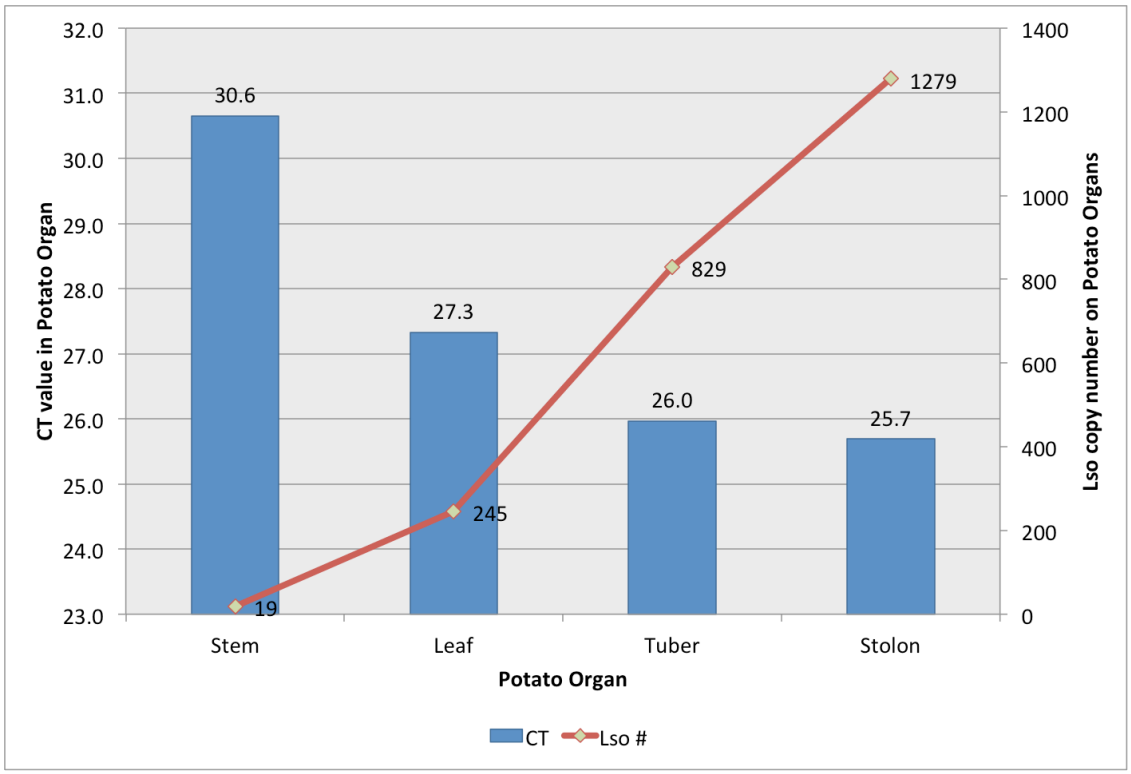

Figure 1.3. Real time PCR CT values and Lso numbers in potato organs infected with Lso

Based on the absolute standard curve, the concentration of Lso 16s rDNA extracted from different organs was estimated. When CT values were converted to Lso copy numbers using the correlation equation, significant differences in Lso concentration between organs were not found (Table 1.4), with Lso copy numbers ranging from 19 copies for stem tissue to 1279 in stolons (Table 1.5, Figure 1.3), a seventy-fold difference between tissues. Differences were found between leaves and stems, and stolons and tuber portions of the plant, with the highest Lso copy number found in aboveground parts (Table 1.5, Figure 1.3).

Table 1.4. Source of variation, degrees of freedom, mean squares, and F value for Lso copy numbers of potato organs.

Source	DF	Sum of Squares	Mean Squares	F Values	Pr > F
Trial	1	498031	498032	2.23	0.1545 ^{NS}
Organ	3	5862095	1954032	4.39	0.1277 ^{NS}
Trial x Organ	3	1333925	444642	1.99	0.1556 ^{NS}
Error	16	3568475	223029		

* Significant difference at $p<0.05$ Coefficient of variation=79.655
^{NS} Non Significant differences at $p<0.05$

Table 1.5. Mean Lso copy number in potato organs.

Plant Organ	Mean Lso Copy number	t Grouping
Stolon	1279	a
Tuber	829	a
Leaf	245	b
Stem	19	b

Means followed by the same letter are not significantly different at $p <0.05$.
LSD=578.01

Discussion

CT values from the real time PCR assays showed significant differences among organs of the potato plant, with the highest CT values in leaves and stems, which were significantly different from the CT values on stolons and tubers. CT values in stolons and tubers were not significantly different from each other. However, when Lso copy numbers were calculated based on the CT values obtained from the real time PCR assay, analysis of variance did not show significant differences among potato tissues, even though Lso copy numbers in different organs were greatly different. Even though

an analysis of variance showed that no differences in Lso populations were found among potato organs, results from a non-protected Fisher's Least Significant Difference (LSD) test did show significant differences among potato organs. Being that significant differences were found among organs using CT values and that Lso copy number is calculated as a function of CT values, the results from the Fisher's LSD test will be considered in the interpretation of these results.

Lso populations were significantly different between above and below ground organs, indicating an uneven distribution in the plant. This is not unexpected, as similar results were found with potato (Li *et al.*, 2009, Wen *et al.,* 2009) and in the Huanglongbing disease on citrus, caused by different species of *Candidatus* Liberibacter (Jagoueix *et al.*, 1996; Li *et al.*, 2006; Teixeira *et al.*, 2008). The Lso population in stolons and tubers were three to four times higher than those of leaf tissue and over seventy times that of stem tissue, which is also in agreement with Li *et al.* (2009). In a previous study, Lso was more frequently detected in stems when above ground portions of the plants were tested (Wen *et al.*, 2009). In a different study by Li *et al*. (2009), Lso population on stems was slightly lower than those on the rest of the plant, but not significantly different. This is in contrast with results obtained in this study, which found that Lso copy numbers were significantly lower on stems with an average copy number of 19 copies. The causes for such a marked difference are not known, but it could be due to the fact that plants used in previous studies were collected in potato fields in Texas and selected because of the severity of their symptoms. Under these conditions, it is possible that those plants had a longer period of time to develop higher titers of Lso and therefore more severe symptoms. In this

study, plants were sampled four weeks after inoculation, given that Lso can be detected by PCR three weeks after infection. Lso populations are markedly lower in this study, and may mean that that Lso needs a longer than three weeks after inoculation to increase to maximum titer in potato plants.

Underground organs, stolon and tubers, had the highest copy numbers of Lso. This coincides with empirical observations that suggested that detecting Lso was easier and more consistent using stolon tissue, and also with studies indicating that Lso detection in stolons was successful with conventional PCR in 91.7 to 100 percent of available samples, compared to zero to 83 percent in other organs (leaf, midvein, petiole and stems (Wen *et al.*, 2009). Lso populations in stolon and tuber were not significantly different, but detection from stolons in conventional PCR assays has been more consistent. This has been the case in a number of studies (Buchman, 2011; Odokonyero *et al.*, 2010), in which nearly 50 percent of all symptomatic tubers PCR detection of Lso has failed. This has been attributed to several possible causes, including low Lso titers in tubers, the presence of inhibitors of PCR reaction in tuber tissues that hamper cPCR detection, effectiveness of PCR diagnostic and Lso population changes over time, but no definitive answer to this issue has been found (Buchman, 2011). Among the possible explanations for the lack of detection of Lso by PCR in tuber samples that have clear ZC symptoms, the presence of PCR inhibitors in this tissue seems to be the most plausible one. ZC affected tubers have been found to have significantly higher levels of polyphenolic compounds, particularly salicylic acid, and ZC causes an overall change in phenolic metabolism in ZC positive plants (Navarre *et al.,* 2008). Polyphenolic compounds are a class of plant constituents,

consisting of a number of organic acids and their derivatives (John, 1992), and have been implicated in the inhibition of PCR reactions (Singh and Singh, 1996). The class and composition of polyphenolic compounds varies greatly between plant species and cultivars (John, 1992), and these compounds have been implicated in the inhibition of PCR detection of mycoplasma like organisms in several plant species showing classical MLO disease symptoms (Gibb and Padovan, 1994).

The higher populations in the underground parts of the potato plant may be associated with the availability of higher amounts of sugars in these tissues, particularly sucrose, fructose and glucose that may enhance Lso growth and population (Karley *et al.*, 2002; Viola *et al.*, 2001). Analysis of the complete genome sequence of Lso indicates that this bacterium lacks a set of genes that would allow it to utilize fructose and sucrose as sources of carbon (Lin *et al.,* 2011). Instead, it is hypothesized that glucose and malate might be the main sources of reduced carbon (Lin *et al.,* 2011). It is interesting that the higher concentration of Lso was found in stolons, given that in this tissue in particular, the concentrations of glucose show a similar pattern in all developmental stages along the longitudinal axis of the stolon (Viola *et al.*, 2001). Starch is the main polysaccharide used by plants to store glucose for later use as energy and the most important reserve material in the potato tuber (Artschwager, 1924). Given that potato tubers are composed of an average of 18% of starch (Grubb and Guilford, 1912), it may be a preferred organ in potato plants for Lso to survive In ZC affected plants, as both glucose and sucrose were found to have highly elevated concentrations in potato tubers compared with non-infected ones (Gao *et al.,* 2009; Buchman *et al.*, 2011). In a study done in the citrus Huangonbling, a disease caused by three species of

C. Liberibacter, Kim *et al.* (2009) found that sucrose levels were higher (11.51 ± 1.81 mg/g) in HLB-affected leaves compared with healthy leaves (8.47 ± 1.81 mg/g). Anatomical analyses showed that HLB infection caused phloem disruption, sucrose accumulation, and plugged sieve pores

Based on these results, the preferred organ for testing for Lso is the stolon, given that it has significantly higher Lso populations, and testing of samples from this organ in affected plants provided consistent results in contrast with tubers or above ground organs. This information can be used by potato breeders to test for resistance or tolerance in potato accessions or cultivars and will be useful for monitoring infections in epidemiological studies.

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CHAPTER II. EFFECT OF PLANT AGE ON ZEBRA CHIP DEVELOPMENT

Hypotheses

H_o: The data collected will not show differences in the development of zebra chip as consequence of plant age.

H_a: The data collected will show differences in the development of zebra chip as consequence of plant age.

Introduction

The effect of plant age on the expression of zebra chip (ZC) has not been fully investigated, while other aspects of ZC disease have been studied, including distribution of the disease in time and space, chemical control strategies, vector biology and behavior and epidemiology among others (Wen et al., 2009). A study by Gao *et al.* (2009) was conducted to understand the relationship between potato psyllid infection timing and expression of zebra chip. In this study, potato plants were infected at various times after plant emergence with infective psyllids reared on four different solanaceous hosts, and maintained on the plants throughout the remainder of the experiment. Results from that study showed that ZC incidence in potato was affected by the timing of psyllid infestation, but the host in which the psyllids were reared had no effect on severity of ZC infection. In their study, healthy potato plants were infected by exposure to potato psyllids 4, 6 and 10 weeks after germination. Plants exposed to the potato psyllid four weeks after germination had the most severe ZC expression; these plants showed visible and severe symptoms both in the foliage and tubers. In contrast, plants infected at six weeks after germination did not express foliar symptoms and only some of the tubers had the characteristic ZC symptoms. The same was true

for plants inoculated ten weeks after germination, but even fewer tubers showed tuber symptoms. However, it is important to note that tubers were only visually evaluated for symptoms, and no other diagnostic tests were performed. In all cases, plants produced fewer and smaller tubers. Reducing sugars and photosynthetic rates of infected plants were measured, and it was found that ZC plants had significantly reduced photosynthetic rates and glucose and sucrose concentrations in leaves, but starch concentrations were higher. In tubers, ZC caused a significant increase in glucose concentration, starch levels were not significantly lower from those in healthy controls and dry matter contents were significantly lower than in healthy plants.

Troxclair and Rowland (2010) studied the physiological response of potato plants to ZC under two irrigation regimes: full irrigation, and 70% deficit irrigation. Results showed that ZC had little effect on plant physiological function when compared with healthy non-inoculated plants. There was a trend for up-regulation of photosynthesis and stomatal conductance (the speed at which water evaporates from pores in a plant) in plants subjected to full irrigation. Water use patterns in plants that received the 70% irrigation deficit was significantly reduced in infected plants; under full irrigation, water use was not significantly different than control plants (not inoculated with Lso), which indicates that effects of ZC on potato plants are exacerbated when plants are water stressed. This study also compared the photosynthesis rate of plants inoculated with Lso at two growth stages, emergence and bloom, and determined that photosynthesis of plants infected with Lso at emergence presented a 25% reduction in photosynthetic rate than control measurements; plants inoculated at bloom had a 75% decrease in photosynthesis compared to baseline

readings in control plants. They hypothesize that this may be due to a more plastic response or genetic up-regulation of defense responses early in development as developmental plasticity often varies with plant age.

In a recent study by Munyaneza *et al.* (2011), plants were exposed to Lso infected psyllids in different years at different growth stages. In 2010, plants were inoculated at bloom and in 2011 plants were inoculated four weeks after bloom. Results from this study showed that when plants were inoculated at a later stage, symptoms and disease onset were slightly delayed and frequency of ZC symptoms in tubers were lower. They also looked at development of ZC in exposed tubers stored at 10 °C, and found that after four and eight weeks in storage ZC symptoms in tubers could not be observed, but after 16 weeks ZC symptoms became apparent.

The objective of this study was to evaluate the time to first detection of Lso by PCR, time to onset of ZC symptoms, and time to plant death after inoculation by Lso of potato plants at different growth stages (plant age). Results from this study provide information about the timeframe of the infection process and how it affects the expression of symptoms and detection of the pathogen as plants age. This information will benefit epidemiological studies and may help in the management of the vector as means to control the disease.

Materials and Methods

Plant material

Potatoes, cultivar Atlantic, were grown from disease-free potato mini-tubers obtained from Sklarczyk Seed Farms LLC (Johannesburg, MI). This chipping cultivar was used because it has been reported that it is highly susceptible to ZC and commonly

grown in regions where ZC is found (Munyaneza *et al.*, 2007a; Munyaneza *et al.*, 2007b; Munyaneza *et al.*, 2008).

All the potato plants necessary for this study were planted simultaneously and grown for up to 16 weeks in individual six-inch pots containing Sunshine Mix (Sun Gro Horticulture, Vancouver, BC). The period of 16 weeks was selected because plants of this cultivar reach maturity at 16 weeks of age (112 days after planting), and are generally ready for harvest under field conditions. A total of 650 plants were used in this study. A plant fertilizer (17:17:17 N-P-K) was incorporated into the potting soil prior to planting. All potatoes were planted and grown in a single greenhouse room maintained at 25 ºC – 28 ºC, with photoperiod cycles of 16 hours light / 8 hours dark until the time of inoculation. For each inoculation period, 18 plants were removed from the greenhouse and inoculated using infected psyllids at a separate location. No insecticide was applied in any of the plants used for these experiments. The experiment performed twice.

Psyllid colonies

A psyllid colony established with psyllids provided by Dr. Joseph Munyaneza (USDA-ARS, Wapato, WA) was used for all inoculations. Munyaneza psyllids were initially collected in Texas in 2007 and have been reared for multiple generations in his laboratory. Colonies were grown in commercial rearing cages (Bug Dorm-2, BioQuip, Rancho Dominguez, CA, USA) consisting of an aluminum frame covered with insect-proof mesh with enough space to contain four plants in six-inch pots.

The potato psyllid colonies were maintained on potato plants at 25 ºC with photoperiod cycles of 16 hours light / 8 hours dark in a greenhouse and growth

chambers. Plants were replaced periodically to sustain psyllid colony growth. Insects were reared for multiple generations and used as needed. To confirm the presence of Lso in both of the psyllid colonies, subsamples of psyllids were tested periodically by PCR according to Secor *et al.* (2009) (Appendix III).

Inoculations

The inoculation cycles were initiated when potato plants began to emerge, between seven and ten days after planting, and were performed at weekly intervals for 16 weeks after emergence. In this manner, at the end of the experiment, all plants were exposed to Lso at different growth stages.

On a weekly basis, 18 plants were removed from the greenhouse room in which they were grown and transferred to an insect proof cage. Plants were inoculated by transferring adult psyllids collected from Munyaneza colonies to the plants contained in cages. Plants were exposed to sixty infective psyllids per cage, with the exception of the group of plants one and two weeks old, which did not have enough foliage to sustain that number of psyllids, and to prevent the possibility of high disease pressure at this growth stages. These small plants were inoculated with four psyllids from Lso positive colonies and covered with a plastic cup to restrict the movement of psyllids to other plants.

A hand-held aspirator was used to collect adult psyllids from the infective colonies. These insects were then released onto the potato plants. All potato plants were inoculated and maintained inside mesh cages in a greenhouse room set at a temperature of 25 ºC with a photoperiod cycle of 16 hours light / 8 hours dark. Lso infected psyllid adults were released and allowed to feed on the plants for a period of

seven days and then killed with insecticides (M-Pede Insecticidal Soap, DowAgroSciences, IN). After psyllids were killed, potato plants were removed from the cage in which they were inoculated, and moved to an open bench in a greenhouse room for the remainder of the experiment.

Plants of 15 and 16 weeks of age (105 and 112 days after planting) were inoculated and exposed to infective psyllids for a seven-day period. Under field conditions, plants at this growth stage are at the end of the growing season and close to harvest (110-120 days after planting). Therefore, plants of 15 weeks of age were vine killed two weeks after inoculation, and plants 16 weeks of age were vine killed one week after inoculation. Inoculations of plants 15 and 16 weeks of age were conducted in order to determine if late infections of potato plants that are close to harvest will show ZC symptoms in tubers, and if translocation of Lso to underground parts of the potato plant is possible at this late stage of development.

Sampling of inoculated potato plants

Plants were tested by PCR at weekly intervals, beginning one week after psyllids were killed, and continued until each of the inoculated plants tested positive to Lso. If a plant tested negative to Lso by PCR, it was sampled a second time to ensure that the negative result was due to the absence of Lso or because Lso was not at a high enough titer to allow its detection, and not because of the sampling method. If a plant tested negative to Lso by PCR, sampling and testing was repeated weekly until Lso could be detected.

Even though results from chapter one of this work indicate the best tissue to sample and detect Lso are stolons, the destructive nature of sampling this tissue and the

limited number of plants available for the study prevented the use of stolons, so sampling was conducted by collecting tissue from the top, middle and bottom part of the foliage, using both leaflets and attached petiole. Root samples were also collected for testing, and as potato plants aged, stolon and tuber samples were also collected when possible.

Experimental design

This experiment was arranged as a completely randomized design (CRD) and was composed of three potato plants per replicate, with three replicates per treatment. The experiment was conducted twice.

Response variables

Different parameters were used to compare treatments (plant age at time of inoculation): (a) Time between inoculation and detection of Lso by PCR in different potato parts, (b) days to development of first ZC foliar symptoms after inoculation expressed as DAI, and (c) Days after inoculation to plant death.

Development of ZC symptoms was monitored on a daily basis on all inoculated plants. Early ZC symptoms include yellowing of the base of young leaflets followed by curling of the leaf edge. As disease progresses, the yellowing at the base of leaflets turns a pale shade of pink that later on progresses to a deep purple. Time (in days) to onset of ZC symptoms was recorded for each plant in this experiment. Plant death was recorded in the same manner.

Plant DNA extraction

Total DNA extraction was performed using a CTAB protocol (Gawel *et al.* 1991 – Appendix I). Five hundred milligrams of plant tissue was collected from

inoculated plants, ground in liquid nitrogen with a mortar in an Agdia sample bag with mesh (Agdia, Elkhart, IN).

The concentration and quality of total DNA was estimated with a 2-µL volume in a micro-volume spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the concentration adjusted to 10 ng/µL for all PCR assays. Resulting DNA was tested for the presence of Lso by polymerase chain reaction (PCR) using specific primers OA2/OI2c and CLi.po.F/OI2c (Secor *et al.*, 2009).

Data analysis

Levene's test of homogeneity of variances was performed to determine if data from the two runs of the experiments could be combined. If data were combined, analysis of variance was performed with PROC GLM using SAS (SAS Institute, Cary, NC) using time for first detection of Lso by PCR after inoculation, days to symptom onset and days to plant death after inoculation. Mean comparisons were conducted using Fisher's least significant difference (LSD).

Linear regression analysis was performed for combined data of days to first PCR detection of Lso after inoculation using PROC REG. Regressions were calculated for the period of time comprising growth stage I, growth stage II and growth stages II, IV and V of the potato plant (Johnson, 2008).

Results

Psyllids from the colony used in this study were tested periodically for Lso. Results show that 87% to 100% of individual psyllids tested were positive to Lso (Appendix III). No quantification of Lso populations in tested psyllids was performed.

Results of this study are presented in two sections. The first part includes data from plants inoculated when they were one week of age through 14 weeks of age at time of inoculation, and the second part includes plants of 15 and 16 weeks of age at the time of inoculation exclusively. They are separate because plants that were 15 and 16 weeks of age at the time of inoculation were mature and under field conditions they would be vine killed shortly after the time of inoculation in this experiment and only allow a short access period for the psyllids. This was simulated in this study in order to determine if an infective psyllid access period of one week would be sufficient time for Lso to migrate from the foliage at the point of inoculation to tubers and cause ZC symptoms after an incubation period of 14 days for plants that were 15 weeks of age at the time of inoculation, and seven days for plants that were 16 weeks of age at the time of inoculation. This is important to determine if late infections can have adverse effects on the potato crop to be harvested shortly after.

Days to first PCR detection

Levene's test for homogeneity of variance for days to first PCR detection of Lso after inoculation was performed and results showed that there were no significant differenced among variances so data from both trials were combined for further analysis (Table 2.1).

Table 2.1. Levene's test of homogeneity of variances for days to first PCR detection of Lso after inoculation.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	15.851	15.851	2.400	0.1252^{NS}
Error	81	534.842	6.602		

^{NS} Non Significant differences at $p<0.05$

Significant differences in the time needed to detect Lso by PCR in potato plants were found (Table 2.2). Mean days for detection of Lso by PCR ranged from 21 days up to 26 days after inoculation (Table 2.3, Figure 2.1). In plants that were 13 and 14 weeks of age at the time of inoculation, Lso was first detected after 21 days of inoculation, but these plants were not actively growing, also were showing signs of senescence and damage due to thrips. In these instances, senescence can be confounding ZC symptoms and it was not clear if plant death was due to ZC or plant age, even though they were positive for Lso. In general, plants in the late stages of development were etiolated, weak and their canopy was not compact and vigorous as was the case in younger plants. They were also damaged by thrips since none of the plants used in this experiment were sprayed with insecticides. Other factors that affected the development of potato plants at the later stages of this experiment was the size of pot in which they were grown, but because of space limitations in the greenhouse room and the large number of plants needed, bigger pots could not be used.

Table 2.2. Source of variation, degrees of freedom, mean squares, and F value for days to first PCR detection of Lso after inoculation.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	14.087	14.087	2.93	0.0927^{NS}
Plant age	13	216.746	16.672	4.03	0.0087*
Run x plant age	13	53.755	4.135	0.86	0.5985^{NS}
Error	56	269.560	4.813		

* Significant difference at $p<0.05$ Coefficient of variation=9.198
^{NS} Non Significant differences at $p<0.05$

Table 2.3. Mean number of days to first PCR detection of Lso after inoculation.

Plant Age (in Weeks)	Mean Days	t Grouping
5	26	a
7	26	ab
6	26	ab
8	25	abc
9	25	abc
1	25	abc
2	24	bcd
10	23	cde
12	23	cde
4	23	cde
13	23	cde
11	23	cde
3	22	de
14	21	e

Means followed by the same letter are not significantly different at p <0.05.
LSD=2.537

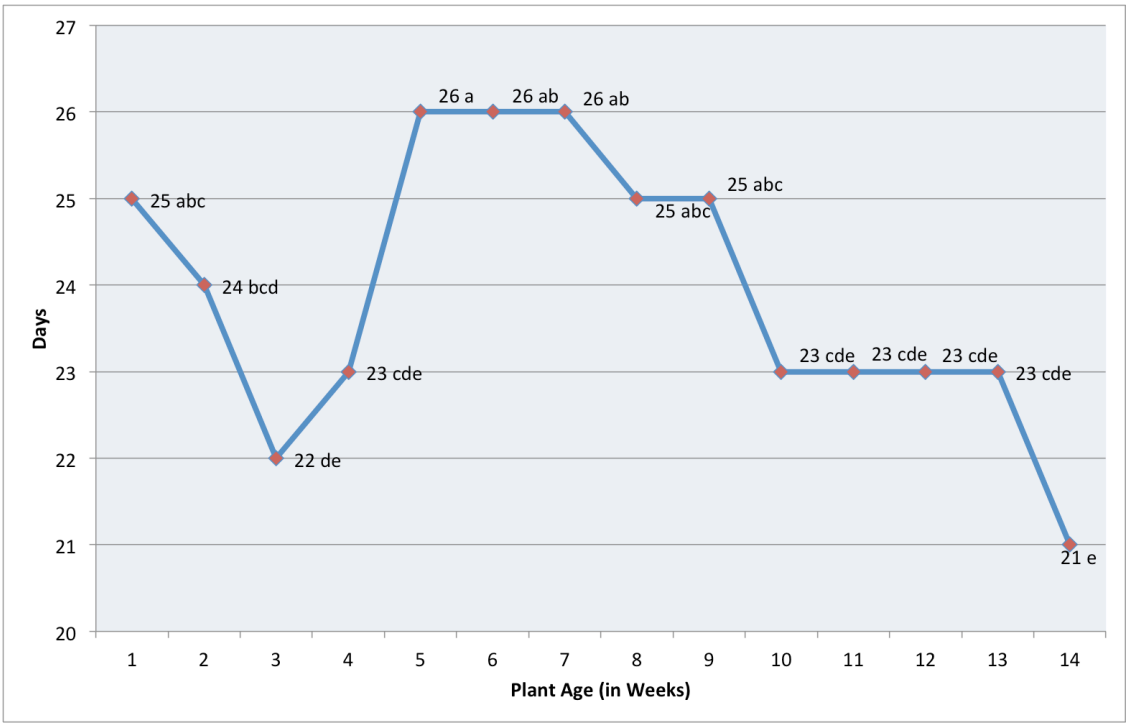

Figure 2.1. Days to first detection of Lso by PCR after inoculation. LSD=2.5.

Of the total number of plants tested for Lso by PCR, 0.4 percent tested positive after 7 days of inoculation, 1.2 percent were positive 14 days after inoculation, 63.9 percent tested positive after 21 days, 92.9 percent after 28 days of inoculation and 100 percent of the total plants sampled was positive for Lso after 35 of inoculation (Figure 2.2). It is important to note that the only instance in which Lso was first detected in less than 21 days after inoculation was in plants that were 15 and 16 weeks of age at the time of inoculation with Lso, in which psyllids had an access period of only one week.

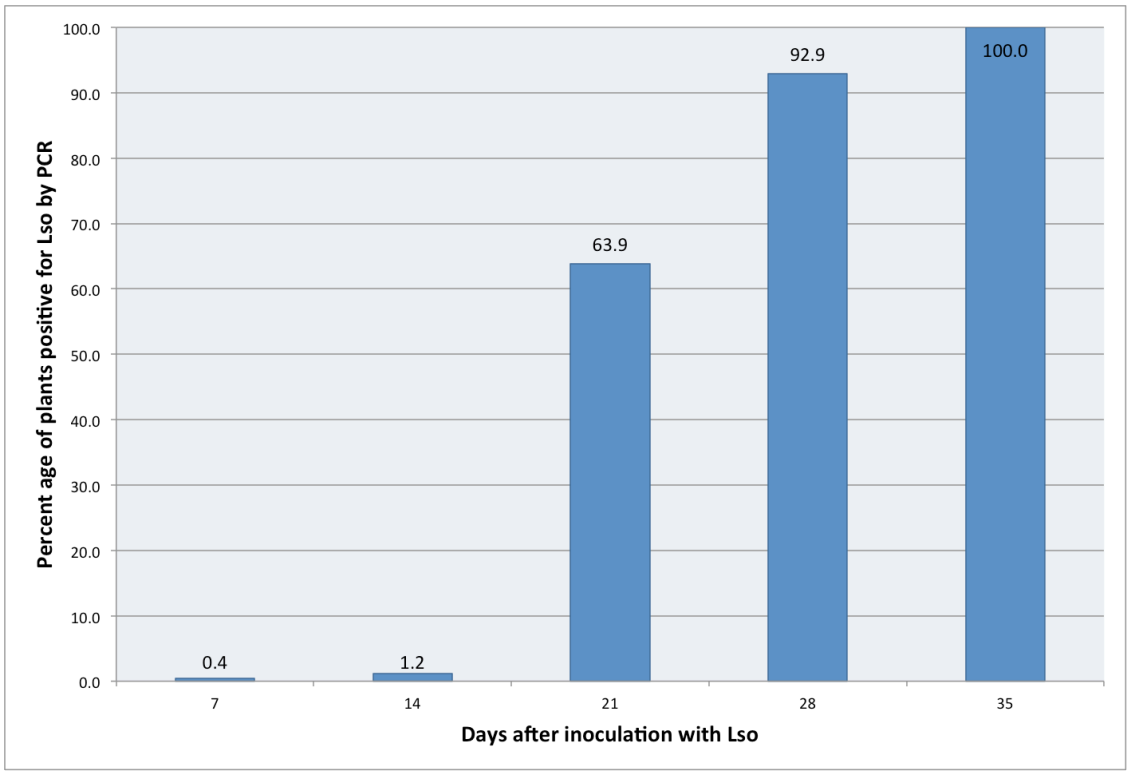

Figure 2.2. Percent age of plants positive for Lso by PCR after 7, 14, 21, 28 and 35 days from time of inoculation with Lso

Regression analysis

Linear regression analysis was performed for the time to detect Lso by PCR on three growth stages of the potato plant development. The first linear regression analysis

comprises the first three weeks of the potato plant development, which correspond with the sprout development stage. The second period corresponds with the vegetative growth stage of potato development, and the third one, the rest of the potato development after tuber initiation, which includes stages III, IV and V (Johnson, 2008). This was done to determine if there is a correspondence of the time in days to detect Lso by PCR and physiological stages of the potato development.

Results indicate that when using the period of time comprising sprout development (Stage I), there was a significant relationship between plant age, and number of days to first detection of Lso by PCR ($Y = -1.361X + 26.056$ $R^2 = 0.765$ $P=0.022$) (Figure 2.3)

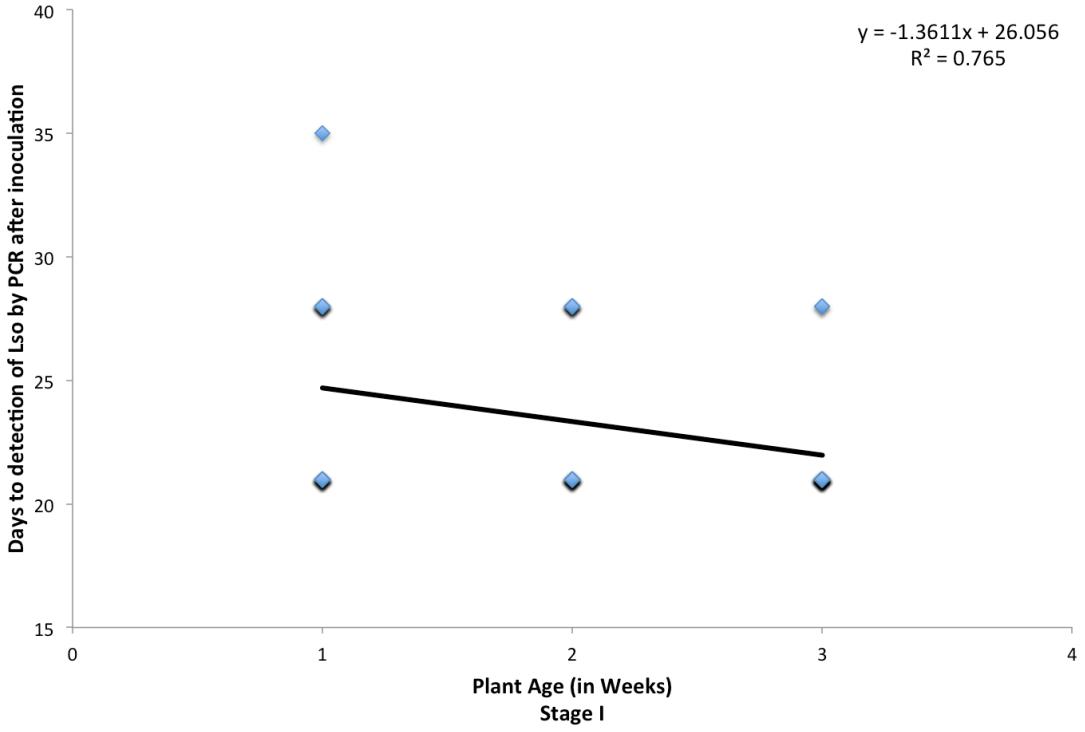

Figure 2.3 Regression analysis of days to first PCR Detection of Lso after inoculation at potato growth stage I. Data points in each week are the average of 18 readings

Linear regression analysis of days to PCR detection after inoculation with Lso in the vegetative growth stage (Stage II) shows a non-significant positive tendency to increase time of detection of Lso by PCR as plant ages (Y = 1.555X + 17.370 R^2= 0.3529 P=0.2137) (Figure 2.4).

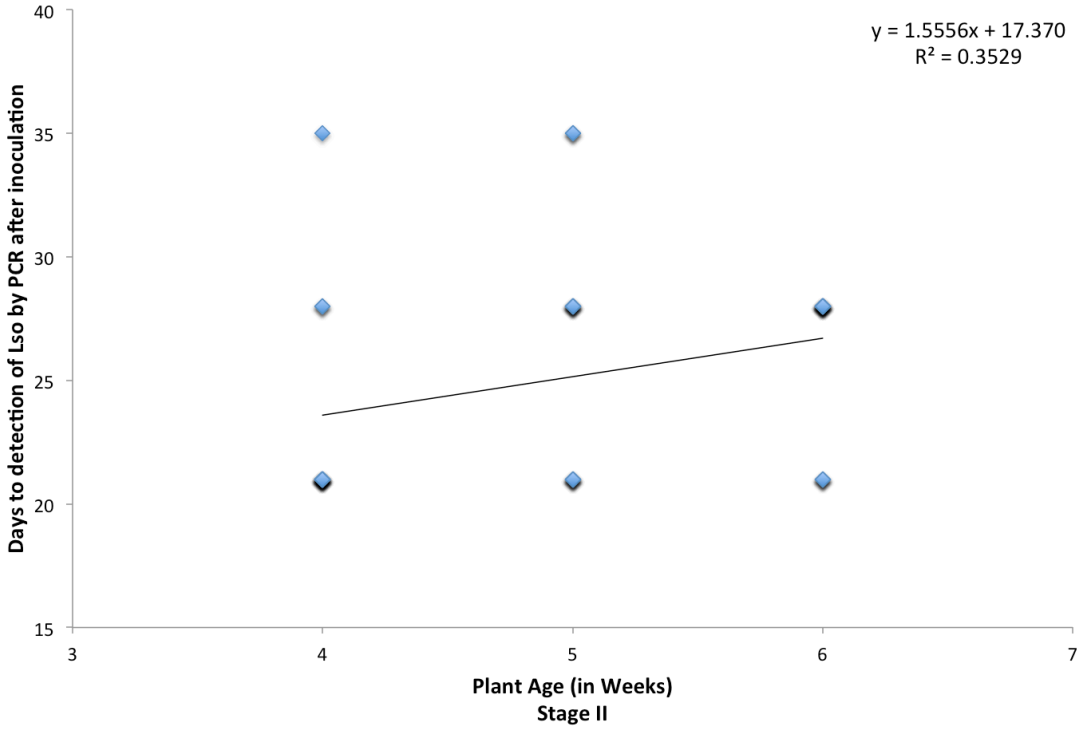

Figure 2.4 Regression analysis of days to first PCR Detection of Lso after inoculation at potato growth stage II. Data points in each week are the average of 18 readings

When growth stages III, IV and V are considered together for calculation of a linear regression, a significant relationship between plant age and days to first detection of Lso by PCR was found (Y = -0.6019X + 30.042 R^2= 0.628 P=0.0002) (Figure 2.5)

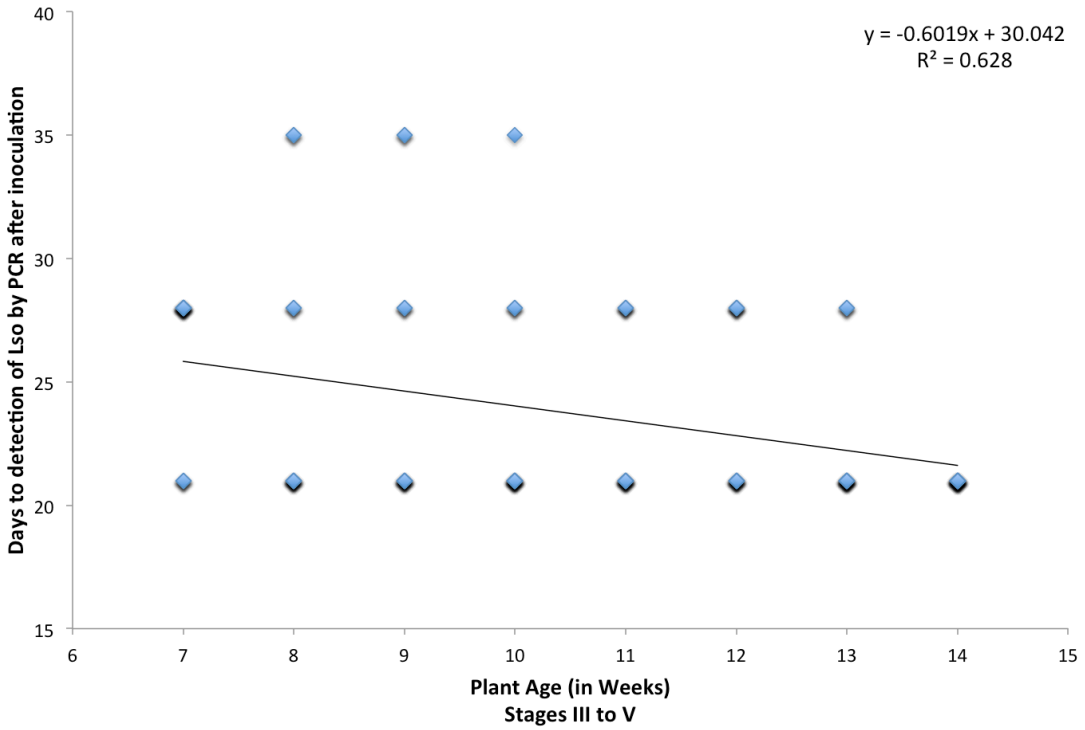

Figure 2.5 Regression analysis of days to first PCR Detection of Lso after inoculation at potato growth stages III, IV and V. Data points in each week are the average of 18 readings

Days to first ZC symptoms after inoculation of potato plants with Lso

Levene's test for homogeneity of variances for days to expression of first ZC symptoms after inoculation of potato plants with Lso was performed and results show there were no significant differences in variances among trials, so the data from both trials were combined for further analysis (Table 2.4).

Table 2.4. Levene's test of homogeneity of variances for days to first symptom detection of ZC.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	1.166	1.166	0.1128	0.7378^{NS}
Error	82	848.156	10.343		

^{NS} Non Significant differences at $p<0.05$

Significant differences were found in days required for expression of ZC foliar symptoms (Table 2.5), with a minimum of 23 days required for plants 14 weeks of age when inoculated with Lso and a maximum of 36 days for plants 11 weeks of age at the time of inoculation with Lso (Table 2.6). In general, it can be observed that there was a tendency toward an increase in the number of days necessary to observe initial symptoms of ZC on foliage as the plant ages, but ZC symptomatology can be confounded with the declining appearance of senescing in plants of 13 and 14 weeks of age at the time of inoculation. In these cases, discoloration of stolons and wilting could be observed three weeks after inoculation and yellowing of leaves was also apparent, but there was also a general decline in plant appearance due to aging. Lso could be detected in foliar samples three weeks after inoculation in plants of 13 and 14 weeks of age at the time of inoculation, even though there was a sharp decrease in the number of days observed for symptom development, foliar ZC symptomatology was confounded by plant senescence, particularly in plants that were inoculated at 14 weeks of age.

Table 2.5. Source of variation, degrees of freedom, mean squares, and F value for Days to first detection of ZC symptoms.

Source	DF	Sum of Squares	Mean Square	F Values	Pr > F
Run	1	1.166	1.166	1.64	0.2056^{NS}
Plant age	13	798.324	61.409	79.90	<0.0001*
Run x plant age	13	9.991	0.768	1.08	0.3943^{NS}
Error	56	39.840	0.711		

* Significant difference at $p<0.05$ Coefficient of variation=2.490
^{NS} Non Significant differences at $p<0.05$

Table 2.6. Mean number of days to first foliar symptom detection.

Plant Age (in Weeks)	Mean number of days to first foliar symptoms	t Grouping
11	36	a
12	36	ab
8	36	ab
10	35	bc
9	35	bc
5	35	bc
6	35	cd
7	35	cd
4	34	cd
1	34	d
2	34	de
3	34	de
13	33	e
14	23	f

Means followed by the same letter are not significantly different at $p < 0.05$.
LSD=0.975

Days to plant death after Lso inoculation

Levene's test for homogeneity of variances for days to plant death after Lso inoculation was performed and results showed there were not significant differences in variances among trials, so the data from both trials were combined for further analysis (Table 2.7).

Table 2.7. Levene's test of homogeneity of variances for days to plant death after Lso inoculation.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	0.038	0.038	0.0010	0.9751^{NS}
Error	82	3225.663	39.337		

^{NS} Non Significant differences at $p<0.05$

Significant differences were found in the number of days necessary for plants to die due to ZC after inoculation (Table 2.8). Time to plant death ranged from a

minimum of 24 days for plants 14 weeks of age at the time of inoculation to a maximum of 47.1 days for plants of 11 weeks of age at the time of inoculation (Table 2.9). As it was in the case for days to first PCR detection and to onset of ZC symptoms, death of plants due to ZC after being inoculated at 13 and 14 weeks of age was not as clear. It was assumed that the cause was ZC since Lso was detected by PCR in these plants, but the general appearance of the plants did not allow for a clear distinction to be made.

Table 2.8. Sources of variation, degrees of freedom, mean squares, and F values for days to plant death after Lso inoculation.

Source	DF	Sum of Squares	Mean Square	F Values	Pr > F
Run	1	0.038	0.038	0.05	0.8305^{NS}
Plant age	13	3170.645	243.895	382.40	<0.0001*
Run x plant age	13	8.291	0.637	0.76	0.6927^{NS}
Error	56	46.726	0.834		

* Significant difference at $p<0.05$ Coefficient of variation=2.123
^{NS} Non Significant differences at $p<0.05$

Table 2.9. Mean number of days to plant death after Lso inoculation.

Plant Age (in Weeks)	Mean days to plant death	t Grouping
11	47	a
10	47	ab
12	47	ab
9	46	bc
7	46	c
8	46	c
6	45	cd
4	45	cd
2	44	d
3	44	d
5	44	d
1	44	d
13	34	e
14	24	f

Means followed by the same letter are not significantly different at $p < 0.05$.
LSD=1.056

PCR detection, foliar symptom development and plant death of plants 15 and 16 weeks of age at time of inoculation

Plants that were inoculated at 15 and 16 weeks of age showed different results compared with plants inoculated when they were one through 14 weeks of age. For plants that were 15 weeks of age at the time of inoculation, one out of 18 (5.5 percent) tested positive for Lso 14 days after inoculation. For plants that were inoculated when they were 16 weeks of age, two out of 18 (11.1 percent) tested positive for Lso seven days after inoculation (Figure 2.6). No foliar symptoms were observed in these plants,

with the exception of one apical leaf in a plant inoculated when it was 15 weeks old and exposed to Lso for a period of 14 days.

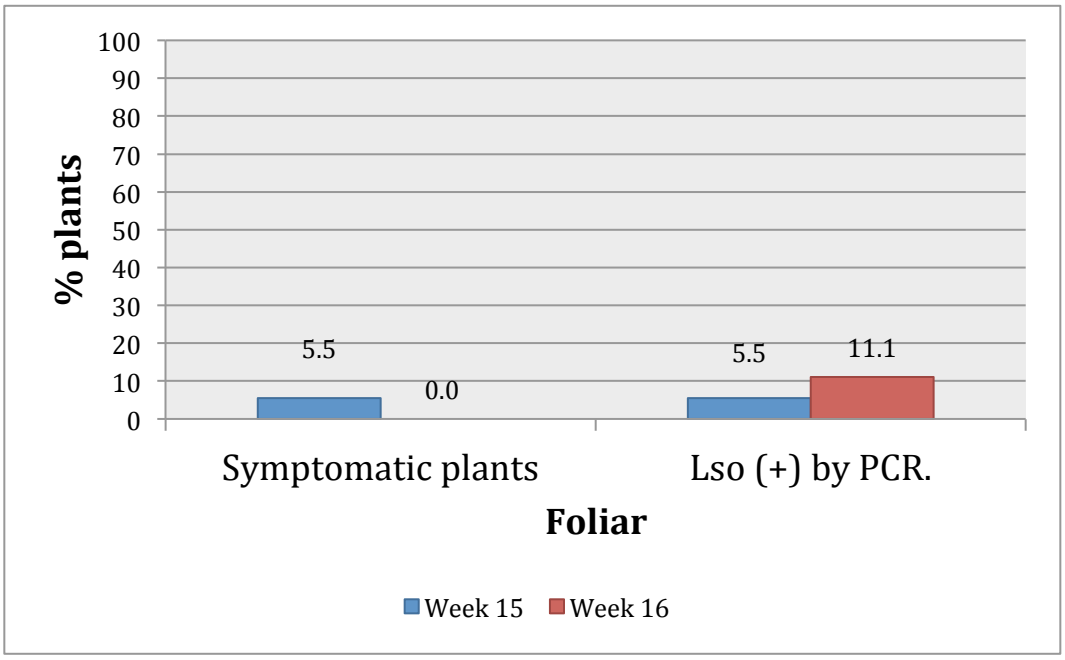

Figure 2.6. Lso detection by PCR and foliar ZC symptoms in plants inoculated at 15 and 16 weeks of age

Results show that of the plants that were inoculated when they were 15 weeks of age and exposed to Lso for 14 days, 78 percent of the tubers showed ZC symptoms three weeks after the stem was cut, however, of the tubers that had visual ZC symptoms, only 39 percent tested positive to Lso by PCR (Figure 2.7).

For the plants that were 16 weeks of age at the time of inoculation and that were exposed to Lso for a period of seven days before vine killing, 100 percent of the tubers showed ZC symptoms three weeks after stems were cut to simulate vine killing, and 56 percent of these ZC symptomatic tubers were positive for Lso by PCR (Figure 2.7). This is in contrast with plants that were one to 14 weeks of age when inoculated;

tubers from those plants were checked for ZC symptoms after plant death and invariably showed the characteristic ZC damage.

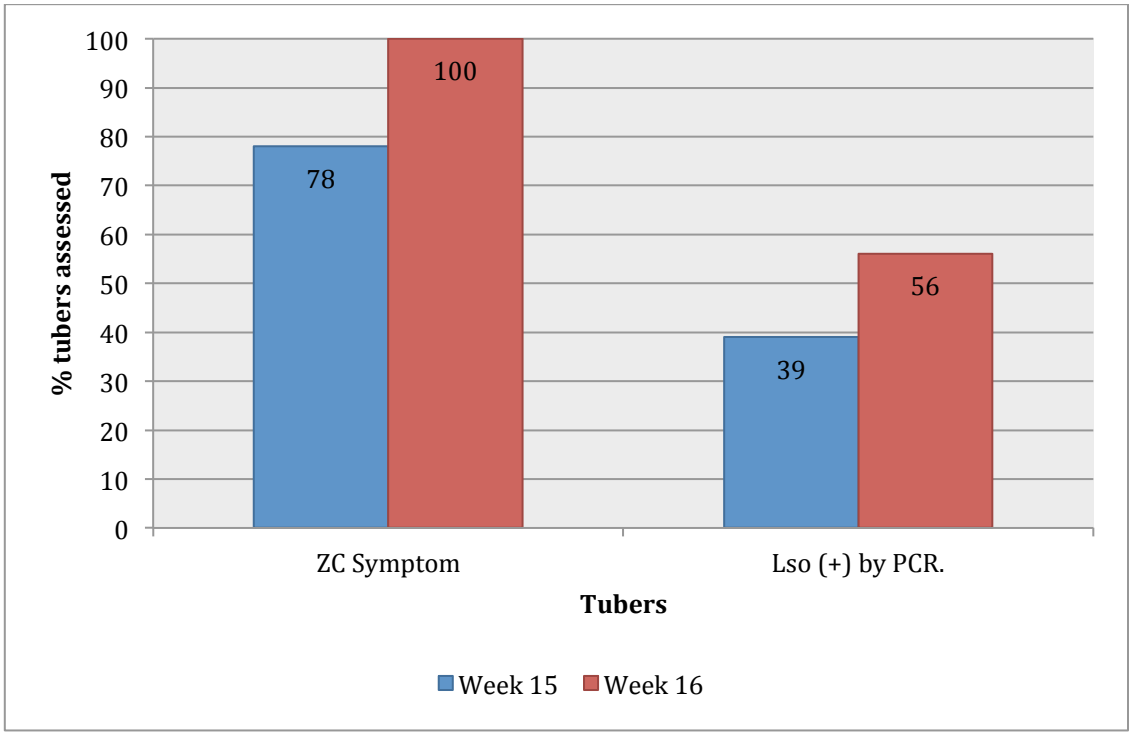

Figure 2.7. Lso detection by PCR and tuber ZC symptoms in plants inoculated when they were 15 and 16 weeks of age

Discussion

Time for detection of Lso by PCR in plants that were inoculated at ages ranging from one to 14 weeks after emergence varied significantly. The earliest that Lso could be detected with PCR was in plants that were three and 14 weeks old at the time of inoculation, with detection at an average of 21 days post-inoculation; plants that were five to seven weeks of age at time of inoculation required significantly more days for Lso to be detected by PCR, with a mean of 25 to 26 days post-inoculation. Plants that were inoculated when they were 13 and 14 weeks old required significantly less time Lso to be detected, but in in all cases through this study (with the exception of plants

inoculated at 15 and 16 weeks of age) detection of Lso was only possible after at least 21 days from the time of inoculation. This is consistent with other studies showing that Lso detection takes at least three weeks after the initial observation/infestation of plants with Lso carrying psyllids (Levy *et al*, 2011, Munyaneza, *et al.,* 2012). Lso can be translocated within the plant following the source to sink movement of carbohydrates, and can be distributed through the whole plant in as little as seven days from the time of inoculation, but titer levels of Lso high enough to allow detection by PCR are not reached until three to four weeks after inoculation (Levy *et al,* 2011). Based on the pattern of detection of Lso in potato plants, Levy *et al.* (2011) hypothesized that Lso moves in a fashion similar to those of viruses and concluded that if this is the case, once Lso reaches the phloem it would follow a source to sink pattern and could be distributed and detected in plant parts away from the site of infection in as little as one week. No precise measure of the translocation of Lso from the point of inoculation to potato tubers have been made given the destructive nature of the sampling necessary for a study like this.

In plants inoculated when they were in early stages of development (one, two and three weeks of age at time of inoculation), Lso could be detected by PCR in average after 25, 24 and 22 days after inoculations respectively. A regression analysis of the time necessary for detection of Lso in this range of time (1-3 weeks of age), showed a significant negative correlation between plant age at the time of inoculation and the number of days it took to detect Lso ($Y = -1.361X + 26.055$ $R^2= 0.765$ $P=0.022$). This may be due to the physiology of the growing potato plant at this stage. At this time in the development of the plant, the main sink are the newly developing

foliar portions of the plant, which the major source of energy moving upward from the seed piece.

When plants were inoculated when they are four to six weeks of age, the time necessary to detect Lso by PCR went from a low of 22 days after inoculation to a high of 26 days after inoculation. In this period of time, a non-significant positive tendency to increase time of detection of Lso by PCR as plant ages ($Y = 1.555X + 17.370$ $R^2= 0.3529$ $P=0.2137$) is observed. The time to detection of Lso shows a slight trend to increase as plant grows, particularly after tuber initiation, which takes place roughly after 40 days of planting. During the tuber initiation and tuber bulking stages (plants ages seven to 12 weeks old at the time of inoculation), it takes longer to detect Lso by PCR in foliage of inoculated plants that it does in the vegetative and maturation stages. A regression analysis of the time necessary to detect Lso by PCR after inoculation when plants are seven weeks old and older shows a significant relationship between plant age and days to first detection of Lso by PCR ($Y = -0.601X + 30.041$ $R^2= 0.628$ $P=0.0002$). This could be due to the fact that when tubers are being formed, sugars and photosynthates are being translocated and used in the strongest sink, which would be downward to the tubers. This may be responsible for the delay in detection in foliage at this stage, but in previous studies, detection of Lso in different potato plant parts has been variable as it was in this study (Lin *et al,* 2009; Wen *et al.* 2009).

The time necessary for detection of Lso in foliage as plant ages declines when plants were 13 and 14 weeks of age at the time of inoculation. In this case, Lso could be detected in an average of 21 or 22 days after inoculation with Lso, a similar timeframe as when plants were in the sprout development and vegetative growth stage.

It is not clear why plants that are rapidly growing at the earlier stages of development show similar times for Lso detection as plants that are in the maturation stage. This suggests that, in general, minimum time for translocation, titer increase of Lso and subsequent detection in plant is 21 days from the time of inoculation, with some exceptions in which the pathogen was detected earlier (3 samples out of 576 total samples, less than 1 percent). Variations in detection of Lso can be attributed to several factors, among which we can include plant age, point of inoculation, frequency of feeding by the psyllid, titer of Lso being transmitted by the psyllid in each feeding event, nutritional stage of the plant, water availability (Troxclair and Rowland, 2010) and environmental factors.

In the case of plants that are 15 and 16 weeks of age at the time of inoculation, detection of Lso by PCR in foliage was not possible in most of the cases, with the exception of one plant of 15 weeks old at the time of inoculation that tested positive for Lso by PCR 14 days after inoculation. Of plants that were inoculated when they were 16 weeks of age, two tested positive for Lso after seven days of inoculation, however, no symptoms were observed. These plants were inoculated in the same way as others during this study, with the only difference being plant age, but as mentioned previously, the same multiple factors can influence disease development and symptom expression. In the case of plants 15 weeks of age at the time of inoculation that had a two-week incubation period before being vine killed, that was enough time for Lso to be translocated to the whole plant and multiply. Two of the plants inoculated when they were 16 weeks of age tested positive to Lso by PCR after an incubation period of only seven days. Previous studies have indicated that seven days are enough for Lso to

be translocated, but it is not clear if this is enough time to allow multiplication of the bacterium to a level that allows its detection by PCR. Levy *et al.* (2011) was able to detect Lso in an upper tier potato leaf using real time PCR in one plant seven days after inoculation, but not with conventional PCR. Detection in such short period is possible, but uncommon, as translocation patterns of photosynthates and consequently, Lso, are variable and dependent on several factors in the plant. In the case of the plants that were inoculated when they were 15 weeks of age, 78 percent of the tubers had clear ZC symptoms after three weeks of incubation, but only 39 percent of the symptomatic tubers tested positive for Lso by PCR. For plants 16 weeks of age at the time of inoculation, 100 percent of the tubers showed ZC symptoms, and 56 percent of those tubers were positive for Lso by PCR. This is a common occurrence when working with potato tubers, and has been reported in several studies (Odokonyero, 2010, Levy *et al.*, 2011, Li *et al*, 2009, Wen *et al.*, 2009, Buchman *et al.* 2011). There is no definitive explanation as to why Lso detection in tubers is so erratic, a possible explanation is the presence of inhibitors in tubers that play a role in the detection of this bacterium by PCR, and also the possibility of the involvement of another pathogen or complex involved in ZC has been suggested (Odonkoyero, 2010, Pitman *et al.*, 2011). As explained in Chapter I, the presence of PCR inhibitors in tuber tissue seems to be the more plausible explanation for the lack of detection in ZC symptomatic tubers. ZC affected tubers have been found to have significantly higher levels of polyphenolic compounds, particularly salicylic acid, because ZC causes an overall change in phenolic metabolism in ZC positive positive plants (Navarre *et al.,* 2008). These compounds have been implicated in the inhibition of PCR detection of mycoplasma

like organisms in several plant species showing classical MLO disease symptoms (Gibb and Padovan, 1994).

Symptom development in potato plants shows significant differences with plant age; older plants take longer to show foliar zebra chip symptoms. As in the case of days necessary for the first detection of Lso by PCR, onset of ZC symptoms was significantly less for plants 13 and 14 weeks of age at the time of inoculation, but plants in the late stages of development were etiolated, weak and their canopy was not compact and vigorous as it was with younger plants, and often had thrips damage. These factors may have obfuscated symptom expression and affected the results. Other factors that affected the development of potato plants at the later stages was the size of pot in which they were grown, but because of space limitations in the greenhouse bigger pots could not be used.

This is consistent with results from Munyaneza *et al.* (2011), that observed a slight delay on symptom onset when plant were inoculated four weeks after bloom, which could be due to ontogenic resistance or because an older plant has more biomass as it ages, and therefore, it takes longer for the pathogen to develop to a high enough titer and cause damage to the plant. Munyaneza *et al.* (2011) also found reduced tuber infection rates in plants inoculated four weeks after bloom that in plants inoculated at bloom, which was not the case in this study, as all tubers sampled after plant death had obvious ZC symptoms. Other factors undoubtedly play a role in the onset and development of symptoms. Development of ZC symptoms is apparently influenced by light intensity (Pitman *et al.,* 2011), and it has been observed that psyllid yellows symptom development in plants under greenhouse conditions is not uniform (Richards,

1931). Of all the ZC symptoms that are typically observed in potato plants under field conditions, we could not observe the production of aerial tubers, axillary buds or zigzagging of the stem in this study, while in plants that are described as having severe ZC symptoms from field samples, these are frequently observed.

The whole range of factors affecting the expression of ZC symptoms in potato have not been elucidated, but in the citrus huanglongbing system, infection by Lsa has been found to significantly affect the expression of 624 genes in sweet orange (Kim *et al.*, 2009), with over 10 percent of them related to plant defense and stress. Among plant defense genes that were activated, several encode products that are classified as pathogenesis related proteins, which may be an indication of the activation of defense mechanisms that lead to callose deposition in and around phloem tissues, that could indicate that phloem blockage results from plugged sieve pores and not aggregation of bacteria. In fact, sieve pores are large enough (15 μm or more) to allow free passage of Lso (Bové and Garnier, 2002). They also found up-regulation of genes involved in sugar metabolism, phytohormone and cell wall metabolism. Of particular interest is the fact that among the genes whose expression was affected in sweet orange, three key starch biosynthetic genes were up regulated, which in turn contributed to starch accumulation in leaves. Gao *et al.* (2009) found that in ZC affected potato plants, starch concentrations were significantly higher than in non-infected plants, and that this accumulation was higher in plants that were infected at earlier stages. In general, Lso affects carbohydrate metabolism in plants, resulting in the accumulation of reducing sugars, particularly glucose, and influencing ZC symptom expression.

Plant death was also significantly delayed, as plants were older at the time of inoculation. There is a distinctive trend for increase in the number of days necessary for plants to die after inoculation as plants age, with the exception of plants inoculated at 13 and 14 weeks of age. The reason may be than when plants 13 and 14 weeks of age were inoculated they were already in the tuber maturation phase (growth stage V) and by the time symptom developed and progressed, they were already showing signs of senescence and plant death due to their maturity. Lso was detected by PCR in plants in these two age groups, but death due to ZC was less apparent.

In conclusion, the detection of Lso by PCR in plants occurs between 21 and 26 days on average. A clear trend in time for Lso detection was not apparent, as first detection of Lso was variable in all age groups, so it seems that Lso detection depends on other factors in addition to plant age. The number of days necessary for symptom onset and plant death show a tendency to increase as plant age at the time of inoculation increases, but when plants are reaching maturity and are old, this relationship is not as clear as it is in younger plants and can be confused with signs of plant senescence, particularly if the plant is stressed by other factors.

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CHAPTER III. EFFECT OF PHOSPHOROUS ACID ON THE POPULATIONS OF C. LIBERIBACTER SOLANACEARUM IN POTATO TUBERS

Hypotheses

H_o: The data collected will not show differences in Lso populations and ZC expression as a direct consequence of the application of phosphorous acid in potato plants.

H_a: The data collected will show differences in Lso populations and ZC expression as a direct consequence of the application of phosphorous acid in potato plants.

Introduction

The use of phosphonate salts has been used to induce systemic protection against several pathogens in different crops by activating defense pathways in the plant (Agrios, 2005). Systemic acquired resistance (SAR) is a defense mechanism in plants that confers long lasting resistance against a wide variety of pathogens, which can be elicited by natural and synthetic chemicals.

Plants can defend themselves against pathogen infection through a wide variety of mechanisms that can be local, constitutive, or inducible (Franceschi *et al*. 2000). Inducible resistance mechanisms such as systemic acquired resistance is a generalized resistance in response to the infection by avirulent forms of the pathogen, hypovirulent pathogens and non-pathogens, or by the use of simple chemical substances, either natural or synthetic (Agrios, 2005).

Inducers of SAR seem to sensitize the plant to respond rapidly after infection. At least nine gene families are activated in non-infected leaves of inoculated plants.

Some genes in these gene families have direct antimicrobial activity or are closely related to antimicrobial proteins (Ward, 1991). These proteins include β-1,3-glucanases, chitinases (Busam *et al.*, 1997), cysteine-rich proteins, and PR-1 proteins (Anfoka and Buchenauer, 1997), and general responses such as phytoalexin accumulation, and lignification (Gottstein and Kuć, 1989).

Ray (1901) was the first one to study systemic acquired resistance (SAR) while working on *Botrytis cinerea* in Begonia. He found that heat or cold treated isolates of *B. cinerea* could cause a variation in virulence. Begonia plants were then inoculated, and independent of which isolate was used, plants developed resistance to the more virulent strains of *B. cinerea.* After Ray, no other study of SAR was done for nearly sixty years until Ross (1961) demonstrated that inoculations of single tobacco leaves with TMV reduced the severity of symptoms on other parts of the tobacco plant. Other pathogens have shown to be capable of inducing SAR, such as *Rheynoutria sachalinensis* in several crops, and *Bacillus subtilis* for powdery mildew in barley (Kessmann *et al.*, 1994). Other well studied systems include resistance in watermelon, cucumber and muskmelon against *Colletotrichum lagenarium* (Caruso and Kuc, 1977), cucumber resistance to anthracnose and leaf spot (Caruso and Kuc, 1977), Fusarium wilt (Gessler and Kuc, 1982), SAR development to *C. lagenarium* as a result of inoculation with Tobacco Necrosis Virus in cucumber (Jenns and Kuc, 1977, Jenns and Kuc, 1980), and cross resistance against *Cladosporium susumerinum* and *C. lagenarium* in cucumber plants (Staub and Kuc, 1980).

Chemical inducers of SAR have also been extensively documented. According to Kessmann *et al.* (1994), a chemical can be considered and activator of SAR if (a) it

induces a resistance response to the same spectrum of pathogens as biological models, (b) the chemical has no direct antimicrobial activity, and (c) the same biochemical processes are induced as in plant tissues after the activation of SAR by biological agents. Chemical compounds that induce SAR include salicylic acid, 2,6-dichloroisonicotinic acid, jasmonic acid and phosphorous acid (Kessmann *et al.*, 1994). Disease control using phosphonates has been shown to be effective in several crops, including cucumber, pepper, grapevines, rice and barley (Agrios, 2005).

Phosphorous acid

Phostrol™ (Nufarm Americas, Inc.) is a phosphite-based fungicide with the active ingredients mono- and dibasic-sodium, potassium, and ammonium phosphites. According to the label, this product is composed of a 53.6% of phosphorous acid (H_3PO_3) by weight. This fungicide belongs the Fungicide Resistance Action Committee (FRAC) group 33, and it is considered to have a low risk of resistance development, but potential for appearance of resistant isolates exists if sufficient selection pressure is applied via heavy use of phosphonates. Vegh *et al.* (1985) reported the appearance of a naturally occurring isolate of *Phytophthora cinnamomi* isolate in a nursery in France that was no longer controlled by applications of phosphonates, and resistant isolates of *Bremia lactucae* have been reported in lettuce in California (Brown *et al.*, 2004). Bower and Cofey (1985) reported the recovery of three isolates of *Phytophthora capsici* from zoospores that after exposure to the chemical mutagen *N*-methyl-*N*-nitro-*N*-nitro-soguanidine were stable and pathogenic to pepper seedlings treated with six times the amount of phosphonates needed to control sensitive isolates.

Phosphorous acid releases the phosphonate ion (HPO_3^{2-}), also called phosphite, upon disassociation and phosphorous acid is often referred to as phosphonate, phosphite, and phosphonic acid. Phosphonates are highly selective, non-toxic fungicides with activity against numerous fungal pathogens, and provide both protective and curative responses against plant diseases caused by the genera of *Phytophthora*, *Rhizoctonia*, *Pythium*, and *Fusarium*, and others plant diseases (Cohen and Coffey, 1986; McGrath, 2004)

Phosphorous acid (K_3PO_3) is known to have powerful antifungal activity, and has been shown to have direct effect on fungal pathogens by inhibiting fungus growth and by changing the nature of the fungal cell walls, and an indirect effect. The indirect effect stimulates host defenses by activating the plants own defense response through rapid cytological action, and triggering cellular phytoalexin accumulations and metabolic changes and other resistance inducers (Agrios, 2005; Nemestothy and Guest, 1990; Guest and Grant, 1991).

Phosphorous acid is systemic in both acropetal and basipetal directions in the plant, moving in both the xylem and phloem (Cohen and Coffey, 1986) and seems to be active in plants for several weeks (Ouimette and Coffey, 1989). Phosphorous acid moves systemically around the plant unreacted unlike phosphates (H_3PO_4), which form adenosine diphosphate (ADP) and adenosine triphosphate (ATP) (Ouimette and Coffey, 1989). Phosphonates are easily taken up and translocated inside the plant. Phosphorous acid (and it's derivatives) does not get converted into phosphate, because there are no plant enzymes that can oxidize phosphonate into phosphate, the primary

source of P for plants, and thus, does not contribute to the plant as a nutrient (Schilder, 2005). This is the reason phosphonate is stable in plants (Smillie *et al.*, 1989).

The precise mode of action of phosphorous acid is unknown, but it is believed to inhibit phosphorous metabolism by interfering with oxidative phosphorylation in the metabolism of Oomycetes (McGrath, 2004; Guest *et al*, 1995). In Phytophthora species, phosphorous acid triggers the accumulation of polyphosphate and pyrophosphate, and inhibits key pyrophosphorylase reactions essential for the pathogen anabolism (Guest and Grant, 1991; Niere *et al*, 1990; Niere *et al,* 1994). Phosphorous acid rapidly accumulates in the plant and the pathogen where it cannot be assimilated into ATP and ADP energy compounds. This accumulation weakens the pathogen sufficiently to release stress metabolites, which are detected by the plant. The plant will then protect itself by eliciting a defense response and kill the pathogen (Guest *et al*, 1995).

Phosphorous acid to control plant pathogenic bacteria

Even though reports of the use and effectiveness of phosphorous acid in the control of fungi are abundant (Cohen *et al,* 1986; Agostini *et al*, 2003; McGrath, 2004), particularly in the control of diseases caused by Oomycetes, reports about their use to control bacterial diseases are limited. Wen *et al* (2009) demonstrated that phosphonates have a direct effect on bacterial spot of tomato pathogen, *Xanthomonas perforans*, but no direct confirmation of their effect in activating plant defense responses. Norman *et al.*, 2006 found that phosphorous acid inhibited in vitro growth of *Ralstonia solanacearum*, by acting as a bacteriostatic compound in the soil, halting bacterial growth and hindering its ability to naturally infect geranium roots. In both

cases, the exact mechanism of action of this compound is unknown. French-Monar *et al.* (2010) found that among different treatments to control Lso in potato, including antibiotics and compounds that could trigger plant defense responses, plants treated with phosphorous acid had significantly lower yield than untreated controls. In this study it is mentioned that good psyllid pressure was present and that zebra chip was present, but no data was presented regarding the severity of ZC in their trials.

The objective of this study was to evaluate the effect of applications of foliar applications of phosphorous acid (Phostrol™) to potato plants of the Atlantic on population of Lso in potato tubers, symptom development and control of ZC of potato.

Materials and Methods

Plant material

Potatoes, cultivar Atlantic, were grown from disease-free potato mini-tubers obtained from Sklarczyk Seed Farms LLC (Johannesburg, MI). This chipping cultivar was used because it has been reported to be highly susceptible to zebra chip and commonly grown in regions where ZC is found (Munyaneza *et al*, 2007a, Munyaneza *et al*, 2007b, Munyaneza *et al*, 2008). Potatoes were grown in individual six-inch pots containing Sunshine Mix (Sun Gro Horticulture, Vancouver, BC). A pre-plant fertilizer (17:17:17 N-P-K) was incorporated into the potting soil prior to planting. All potatoes were planted and grown in a single isolated greenhouse room maintained at 25 ºC - 28 ºC, with photoperiod cycles of 16 hours light / 8 hours dark until the time of inoculation.

Psyllid colonies

Psyllid colonies were established with psyllids provided by Dr. Joseph Munyaneza (USDA-ARS, Wapato, WA). Munyaneza psyllids were initially collected in Texas in 2007 and have been reared for multiple generations in his laboratory. Colonies were grown in commercial rearing cages (Bug Dorm-2, BioQuip, Rancho Dominguez, CA, USA) consisting of an aluminum frame covered with insect-proof mesh with enough space to contain four plants in six-inch pots.

The potato psyllid colonies were maintained on potato plants at 25 ºC with a photoperiod cycle of 16 hours light / 8 hours dark of 16 hours of light and 8 hours of darkness in a greenhouse and growth cambers. Plants were replaced periodically to sustain psyllid colony growth. Insects were reared for multiple generations and used as needed. To confirm the presence of Lso in colony psyllids, subsamples of psyllids were tested periodically by PCR according to Secor *et al.* (2009).

Inoculations

Plants were inoculated after six weeks from emergence and plant heights ranging from 15-25 cm by transferring psyllids from established colonies to healthy potato plants. Adult psyllids were collected using an insect aspirator. Psyllids trapped in collection tubes were then used to inoculate healthy potato plants.

Pathogen-free potato plants were exposed to potato psyllid adults collected from an Lso infected psyllid colony as described above. Nine plants were enclosed in commercial rearing cages. Sixty adult potato psyllids were released into each cage and allowed to feed on the plants for seven days. Inoculations were conducted at the USDA-ARS greenhouse entomology research complex because no insecticides are

applied in that complex. After feeding for seven days, cages containing the inoculated plants were transported to the Plant Pathology greenhouse complex for the remainder of the experiment and psyllids killed with insecticide sprays (M-Pede Insecticidal Soap, Dow AgroSciences, IN). After insect removal, standard greenhouse insecticide application was used during the remainder of the experiment. All potato plants were maintained in a greenhouse room at a temperature of 25 ºC with a light cycle of 16 hr light/8 hr dark.

Experimental design

This experiment was arranged as a completely randomized design (CRD) and was composed of three potato plants per replicate, with three replicates per treatment. The experiment was conducted twice.

Treatments

Treatments consisted of applications of phosphorous acid (Phostrol™), one week before and one week after psyllid inoculations, or both, at full label and half label rates. Applications of Phostrol™ were done in a spraying booth (DeVries Manufacturing, Hollandale, MN) at the AES research greenhouse complex to insure accuracy of product application. The product was diluted in water at 109 liters ha^{-1} and applied at 275.79 kPa with a cone jet nozzle.

Treatments were as follow:

1. Phostrol™ at 0.5X before inoculation (5.8 liters ha^{-1})
2. Phostrol™ at 1X before inoculation (11.6 liters ha^{-1})
3. Phostrol™ at 0.5X after inoculation (5.8 liters ha^{-1})
4. Phostrol™ at 1X after inoculation (11.6 liters ha^{-1})

5. Phostrol™ at 0.5X before and after inoculation (5.8 liters ha^{-1})
6. Non-treated inoculated control
7. Non-inoculated and non-treated plants.

Response variables

Three response variables were recorded to compare treatments: a. quantification of bacterial populations in potato tubers by qPCR four weeks after inoculation, b. days after inoculation (DAI) to development of first ZC foliar symptoms, c. days after inoculation to plant death.

Development of ZC symptoms was monitored on a daily basis on all inoculated plants. Early ZC symptoms include yellowing of the base of young leaflets followed by curling of the leaf edge. As disease progresses, the yellowing at the base of leaflets turns a pale shade of pink that later on progresses to a deep purple. Time (in days) to onset of ZC symptoms was recorded for each plant in this experiment. Plant death was recorded in the same manner.

Plant DNA extraction

Five hundred milligrams of a compound sample of tuber tissue from each plant was ground in liquid nitrogen using a mortar and pestle in an Agdia sample bag with mesh (Agdia, Elkhart, IN), and total DNA extraction was performed using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions.

The concentration and quality of total DNA was estimated with a 2-µL volume in a micro-volume spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the concentration adjusted to 10 ng/µL for all PCR assays. DNA concentration for real time PCR assays was not adjusted.

Data analysis

Levene's test of homogeneity of variances was performed to determine if data from the two runs of the experiments could be combined. If data were combined, analysis of variance was performed with PROC GLM using SAS (SAS Institute, Cary, NC) using Ct values from real time quantification and Lso copy number data from potato tubers. Mean comparisons were conducted using Fisher's least significant difference (LSD).

Results

All plant inoculations were successful and high levels of infection were obtained in the experiments. Plants showed both foliar and tuber symptoms, consistent with ZC infections and tested positive for Lso by PCR. Non-inoculated control plants did not show zebra chip symptoms and tested negative for Lso by PCR. Treated plants showed some scorching on the edge of leaves, presumably indicating phytotoxicity due to treatment with phosphorous acid.

CT values for plants inoculated with Lso and treated with Phostrol

Levene's test for homogeneity of variances for CT values of qPCR for different treatments was performed, and results showed that there were not significant differences in variances among trials, so the data from both trials were combined for further analysis (Table 3.1).

Significant differences in Lso populations as expressed by cycle thresholds (CT) values were observed in both trials (Table 3.2). Mean CT values ranged from a low of 24.2 in non-treated and inoculated plants to a high of 27.4 in an application of half a rate of Phostrol prior to inoculation (Table 3.3, Figure 3.1).

Table 3.1. Levene's test of homogeneity of variances for CT values on potato tubers following treatment with Phostrol.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	0.228	0.228	0.0026	0.9596[NS]
Error	40	3526.815	88.170		

[NS] Non Significant differences at p<0.05

Results from treated plants were significantly different when CT values were compared; treatments that included Phostrol at a rate of 0.5X and 1X before inoculation had significantly higher CT values than the remaining treatments, which included Phostrol at a rate 0.5X after inoculation, Phostrol at a rate of 1X before and after inoculation, and applications of Phostrol one week after inoculation. Applications of Phostrol at the recommended rate before and after inoculation were not significantly different from an application of Phostrol one week after inoculation. All treatments were significantly different from non-treated plants that were inoculated with Lso, which had the lowest Ct value of all treatments (24.2) and thus, the highest population of Lso (Table 3.3).

Table 3.2. Source of variation, degrees of freedom, mean squares, and F value for CT values on potato tubers following treatment with Phostrol.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	0.228	0.228	0.66	0.4240 [NS]
Treatment	6	3514.432	585.778	1326.44	<0.0001*
Run x Treatment	6	2.649	0.441	1.27	0.3025 [NS]
Error	28	9.733			

* Significant difference at p<0.05 Coefficient of variation=2.650
[NS] Non Significant differences at p<0.05

Table 3.3. Mean value of CT values in potato tubers after treatment with Phostrol.

Treatment	Mean CT Value	t Grouping
Phostrol 0.5X Before	27.4	a
Phostrol 1X Before	27.3	a
Phostrol 0.5X After	26.3	b
Phostrol 1X Before and After	25.4	c
Phostrol 1X After	25.0	c
Non treated, inoculated	24.2	d
Non treated non inoculated	0	e

Means followed by the same letter are not significantly different at p <0.05.
LSD=0.697

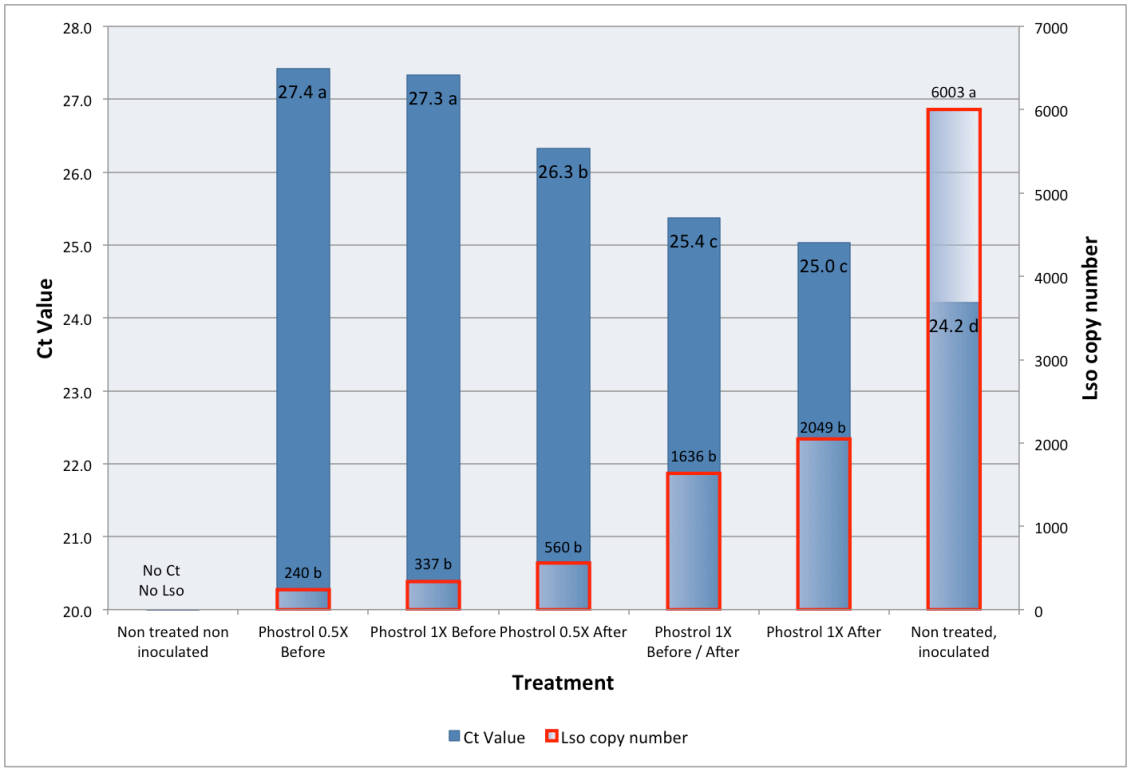

Figure 3.1 Mean CT value and Lso copy number from tubers after treatment with Phostrol

Lso copy number for plants inoculated with Lso and treated with Phostrol

Real time CT values were converted to Lso copy number and analyzed. Levene's test for homogeneity of variances for Lso copy number for different treatments showed there were no significant difference in variance among trials, so the data from both trials were combined for further analysis (Table 3.4).

Table 3.4. Levene's test of homogeneity of variances for Lso copy number values on potato stolons following treatment with Phostrol.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	2845027	2845027	0.3951	0.5332[NS]
Error	40	288054814	7201370		

[NS] Non Significant differences at $p<0.05$

Significant differences in Lso copy number were found only between plants treated with Phostrol and inoculated with Lso, and plants that were not treated and inoculated with Lso (Table 3.5). The differences found between Ct values for all treatments did not translate into significant differences of calculated Lso populations among plants that were treated with Phostrol and inoculated with Lso. (Table 3.6, Figure 3.1). The only significant difference found was between control treatment that was inoculated with Lso, and treatments that included applications of Phostrol before or after inoculation with Lso.

When transformation to Lso copy number was performed, no significant differences were found among treatments. However, Lso populations in plants treated with Phostrol at rates of 0.5X and 1X before inoculation were 25 and 17 times lower, respectively, than mean Lso populations in non-Phostrol treated and inoculated plants (Table 3.6).

Table 3.5. Source of variation, degrees of freedom, mean squares, and F value for Lso copy number on potato tubers following treatment with Phostrol.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	2845027	2845027	0.87	0.3600^{NS}
Treatment	6	159937132	26656189	4.43	0.0465*
Run x treatment	6	36125407	6020901	1.83	0.1287^{NS}
Error	28	91992274	3285438		

* Significant difference at $p<0.05$ Coefficient of variation=117.20
^{NS} Non Significant differences at $p<0.05$

Table 3.6. Mean Lso copy number in potato tubers after treatment with Phostrol.

Treatment	Mean Lso Copy number	t Grouping
Non treated, inoculated	6003	a
Phostrol 1X After	2049	b
Phostrol 1X Before and After	1636	b
Phostrol 0.5X After	560	b
Phostrol 1X Before	337	b
Phostrol 0.5X Before	240	b
Non treated non inoculated	No Lso	…

Means followed by the same letter are not significantly different at $p <0.05$.
LSD=2143.6

Days to first ZC symptoms on plants inoculated with Lso and treated with Phostrol

Levene's test for homogeneity of variances for days to expression of first symptoms on potato plants after inoculation with Lso showed there were not significant differences in variances among trials, so the data from both trials were combined for further analysis (Table 3.7).

Table 3.7. Levene's test of homogeneity of variances for days to expression of first symptoms of ZC on potato plants following treatment with Phostrol.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	0.015	0.015	0.001	0.9960[NS]
Error	40	23386.447	584.661		

[NS] Non Significant differences at $p<0.05$

Significant differences were found among treatments (Table 3.8), but this was only observed between plants that were treated with Phostrol and inoculated with Lso, and negative control ones that were not treated nor inoculated with Lso (Table 3.9). Plants that were treated and inoculated did not show significant differences between treatments, with an overall average of 33 days from the time of inoculation with Lso to expression of first ZC symptoms. The non-inoculated non-treated control did not develop zebra chip, as expected; these plants were destroyed 60 days after inoculation, after plants in all other treatments had died. None of the different treatments was significantly different from the non-treated inoculated control when it comes to symptom development (Table 3.9).

Days to plant death on plants inoculated with Lso and treated with Phostrol

Levene's test for homogeneity of variances for days to plant death on potato plants after inoculation on the different treatments showed there were not significant differences in variance, so the data from both trials were combined for further analysis (Table 3.10).

Table 3.8. Source of variation, degrees of freedom, mean squares, and F value of days to expression of first symptoms of ZC on potato plants following treatment with Phostrol™.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	0.015	0.015	0.02	0.9022 ^{NS}
Treatment	6	23355.019	3892.503	6367.07	<0.0001*
Run x treatment	6	3.668	0.611	0.62	0.7152 ^{NS}
Error	28	27.760	0.991		

* Significant difference at p<0.05 Coefficient of variation=2.357
^{NS} Non Significant differences at p<0.05

Table 3.9. Mean number of days to expression of first symptoms of ZC on potato plants following with Phostrol™.

Treatment	Mean Days to first ZC symptom	t Grouping
Non treated non inoculated	60 (No ZC)	a
Phostrol 0.5X After	33	b
Phostrol 1X Before	33	b
Phostrol 1X After	33	b
Phostrol 0.5X Before	33	b
Non treated, inoculated	32	b
Phostrol 1X Before and After	32	b

Means followed by the same letter are not significantly different at p <0.05.
LSD=1.177

Table 3.10. Levene's test of homogeneity of variances for days to plant death due to ZC on potato plants following treatment with Phostrol.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	1	0.0038	0.0038	0.0001	0.9975^{NS}
Error	40	15796.200	394.905		

^{NS} Non Significant differences at p<0.05

Significant differences were found between inoculated plants treated with Phostrol, and non-treated with Phostrol inoculated plants (Table 3.11). Significant differences were found in the time it took for plants to die due to ZC in all plants that were treated with Phostrol, with an overall average of 45 days from the time of inoculation with Lso. The non-inoculated non-treated control plants did not develop zebra chip, as expected, and were destroyed 60 days post inoculation after all Lso inoculated plants had died. None of the inoculated treatments was significantly different from the non-treated inoculated control when it comes to days to plant death, with the exception of plants treated with half the rate of Phostrol after inoculation. Days from inoculation to plant death in plants treated with the full rate of Phostrol before inoculation was significantly different from that in plants that were treated with the full rate of Phostrol after inoculation (Table 3.12).

Table 3.11. Source of variation, degrees of freedom, mean squares, and F value of days to plant death due to ZC on potato plants following treatment with Phostrol™.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > I
Run	1	0.003	0.003	0.01	0.9403^{NS}
Treatment	6	15769.521	2628.253	1969.67	<0.0001*
Run x treatment	6	8.006	1.334	2.00	0.0992 ^{NS}
Error	28	18.673	0.666		

* Significant difference at $p<0.05$ Coefficient of variation=1.553
^{NS} Non Significant differences at $p<0.05$

Table 3.12. Mean number of days to plant death due to ZC on potato plants following treatment with Phostrol™.

Treatment	Mean Days Plant death due to ZC	t Grouping
Non treated non inoculated	60 (No ZC)	a
Phostrol 1X Before	45	b
Phostrol 0.5X Before	45	bc
Non treated, inoculated	45	bc
Phostrol 1X Before and After	45	bcd
Phostrol 1X After	44	cd
Phostrol 0.5X After	44	d

Means followed by the same letter are not significantly different at $p < 0.05$.
LSD=0.965

Discussion

Results from this study showed that there were significant differences among treatments when the CT values were considered. Plants treated with half and full recommended rates of Phostrol, had CT values that were significantly higher than plants in all other treatments, however, when CT values were used to calculate Lso copy numbers there were no significant differences among treatments in the populations of Lso in tubers of treated plants. Even though the differences were not significant, Lso copy numbers in plants treated with Phostrol at half and full recommended rates before inoculations were markedly lower than control plants that were not treated. Populations in these two treatments were 25 and 17 times lower than the non-treated inoculated control. It is clear that treatment of plants with Phostrol had an effect in the population of Lso. However, this reduction in Lso populations in treated plants did not translate into a reduction of symptoms in plants, as treatments did

not have an effect in days to the appearance of first foliar symptoms, but significant differences were found in time to plant death after inoculation with Lso. Studies done in the past by Norman *et al*, (2006), have shown that the effect of phosphorous acid on the pathogen limit the pathogen capacity to infect the plant, and when in vitro studies were done, the number of culturable cells of *R. solanacearum* decreased over time; however, in this case, phosphorous acid did not have a curative effect, as plants that were systemically infected, wilted and eventually died. In the Norman *et al* (2006) study, applications of phosphorous acid did not result in asymptomatic plants, as is the case in the present study.

In the present study, phosphorous acid application of half the recommended rate and a full-recommended rate before inoculation with Lso were the most effective in reducing the populations of Lso in potato tubers. Half the recommended rate before inoculation treatment resulted in the lowest Lso population. The reason for this is unknown, but Wen *et al,* (2009) had similar results. In that study, a single application of phosphorous acid applied one day before inoculation significantly reduced the severity of bacterial spot of tomato.

Data obtained from the genome sequencing of Lso (Lin *et al*, 2011), indicates that Lso, like *C.* L. asiaticus, has an oxidative phosphorylation pathway that enables it to carry a functional ATP synthesis. It is interesting to note that even though the mode of action of phosphorous acid is still to be clearly determined, it was been found that it inhibits oxidative phosphorylation in other systems (Smillie *et al*, 1989), a system that is present in this bacterium.

The application of phosphorous acid to resistant selections or cultivars may help reduce infection of Lso and severity of ZC in this material, but studies to determine if the effect of a combination of a resistant or tolerant hosts with phosphorous acid are additive and also the cost effective, both monetarily and environmentally, are needed. It would also be interesting to study populations of Lso in plant organs after treatment, given that lower numbers in potato plants could possibly limit the acquisition of the pathogen by its vector, the potato psyllid, and subsequent transmission to neighboring plants.

Further studies are warranted to further understand the best timing of phosphorous applications and effectiveness of multiple applications. Taylor *et al.* (2011) demonstrated that a single application of phosphorous acid provided significant control of *Phytophthora erythroseptica*, but two and three applications were more effective. Other studies have indicated that applications of phosphorous acid should be done in the tuber initiation stage and that efficacy declines if applications begin after row closure. In this study, a single application of phosphorous acid provided a reduction in the populations of Lso, and even though this reduction was not significantly different among Phostrol treatments, it was significantly different from plants that were not treated. The two treatments that had the lowest populations of Lso were those that were applied prior to inoculation; applications of phosphorous acid may provide better results when applied preventively. Taylor *et al.* (2011) showed that applications of phosphorous acid do not provide control of infections that occur prior applications of the product. Clayton *et al.* (2005) also showed that post harvest applications of phosphorous acid to control pink rot in potatoes are meant to keep a

healthy tuber healthy, and do not have a curative effect on tubers that have field infections.

Plant death was significantly delayed in plants that were treated with a full rate of Phostrol before inoculation compared to plants that received a full rate after inoculation and both before, and after inoculation. Reasons for this are unknown, but if high doses in vitro negated the fungicidal activity against *Pythium* (Sanders *et al.,* 1983), the same phenomenon could be at work in this study. It would be interesting to determine if applications and longer intervals could have similar or better effect. It is also strange that the treatment that had the best effect in delaying days to plant death (Phostrol 1X before inoculation) was not the one that reduced populations the most. It has been shown that in tubers showing severe ZC symptoms, the populations of Lso are not as high as they are in tubers in which symptoms are mild or not visible (Wen, personal communication). Under the conditions of this study it is clear that Phostrol failed to provide acceptable control of Lso. Even though populations of Lso were lower in several of the treatments, these differences were not significant or relevant, since any level of infection will result in tuber damage that will make the product unacceptable for the market, unless their use with other management strategies reduce damage to undetectable levels, both in terms of Lso populations and damage to the tuber.

In conclusion, treating plants with phosphorous acid did show an effect on the population of Lso, but no effect on the development of ZC symptomatology. Future studies could focus on determining if there is a population threshold in which damage to the plant is avoidable and correlate Lso populations with ZC symptom severity and if treatment with phosphorous acid could bring the populations of Lso to a level in

which ZC damage is tolerable, if any. Studies using different rates of phosphorous acid and different timings of application should be conducted to better understand its effects and the conditions necessary to maximize the efficacy of this product against Lso and development of ZC.

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APPENDIX I. GENOMIC DNA ISOLATION

N.J., Jarrett R.L. (1991). A modified CTAB DNA-extraction procedure for *Musa* and *Ipomoea*. Plant Mol. Biol. Rep. 91: 262-266

1. Place 500 mg of leaf, stolon or tuber tissue in an Agdia grinding bags, and grind the sample with liquid nitrogen.
2. Add 5 ml of pre-heated (65^{o}C) extraction buffer (2.5% CTAB buffer with 1% PVP-40, 0.2% of 2-mercaptoethanol and 0.5µl of RNase A) to the ground sample from step 1 grind again. Store 1.5 ml of the solution in the freezer -20°C for future testing. A 1.5 ml aliquot is transferred to a 2 ml microcentrifuge tube.
3. Incubate the sample at 65ºC for 30 min to 1 hour and shake the tubes occasionally during the incubation period.
4. Add 500 µl of chloroform:isoamyl alcohol (24:1) mix and incubate the solution for 15 min at room temperature in a horizontal shaker.
5. Centrifuge the solution for 10 min at maximum speed (14,000 rpm) at room temperature.
6. Carefully remove 400 µl of the upper aqueous phase and transfer to a new tube and add an equal volume of ice-cold isopropanol to precipitate the DNA, mix by inversion and incubate the solution for 15 min at -20 ºC.
7. Centrifuge at maximum speed for 10 min at room temperature and remove the supernatant; the pellet contains the DNA.
8. Wash the DNA pellet by adding 500 µl of 70% ethanol and mix by inversion, discard the ethanol and centrifuge at maximum speed for 15 sec and remove the remaining liquid with a pipette.

9. Dry the pellet in a vacuum centrifuge (SpeedVac) or in a hood for 15 minutes by leaving the tubes open. Re-suspend the pellet in 100 µl Sigma water.
10. This sample can be assayed by PCR for Lso.

APPENDIX II. PSYLLID DNA EXTRACTION

Hung, T.H., Hung, S.C., Chen, C.N., Hsu, M.H., and Su, H.J. 2004. Detection by PCR of *Candidatus* Liberibacter asiaticus, the bacterium causing citrus huanglongbing in vector psyllids: application to the study of vector-pathogen relationships. Plant Pathology 53:96-102.

Extraction buffer

100 mM Tris-HCL pH 8.0

50 mM EDTA

500 mM NaCl

1% N-Lauroylsarcosine

Psyllid DNA extraction protocol

1. In a lysing matrix tube in which the ceramic sphere has been removed place one adult psyllid and four to five glass beads.
2. Add 300 µl of DNA extraction buffer and homogenize in FastPrep cell disruption machine at speed 6.5 for 45 seconds.
3. Incubate the mixture at 65ºC for 30 min. to 1 hour and shake the tubes occasionally during the incubation period.
4. Add 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and mix using a vortex.
5. Centrifuge for 10 min at maximum speed (14,000 rpm) at 4 °C.
6. Carefully remove the upper aqueous phase (≈ 200 µl) and transfer to a new tube and add 500 µl of 95% ethanol, gently mix by inversion and centrifuge for 10 min at maximum speed (14,000 rpm) at 4 °C.

7. Wash the DNA pellet by adding 500 µl of 70% ethanol and mix by inversion, discard the ethanol and centrifuge at maximum speed for 15 sec and remove the remaining liquid with a pipette.
8. Dry the pellet in a vacuum centrifuge (SpeedVac) or in a hood for 15 minutes by leaving the tubes open. Re-suspend the pellet in 15 µl of molecular grade water.
9. This sample can be assayed by PCR for Lso.

APPENDIX III. PCR TEST OF PSYLLID COLONIES FOR LSO

Potato psyllids were tested four times during the duration of experiments that involved inoculation of plants with infective psyllids. Results showed that 86 to a 100 percent of tested adult psyllids were carrying Lso. Munyaneza psyllids were used in all inoculations. Psyllids were tested for Lso by PCR using primer pair CLi.po.F/O12c, which amplifies a 1070 bp DNA fragment.

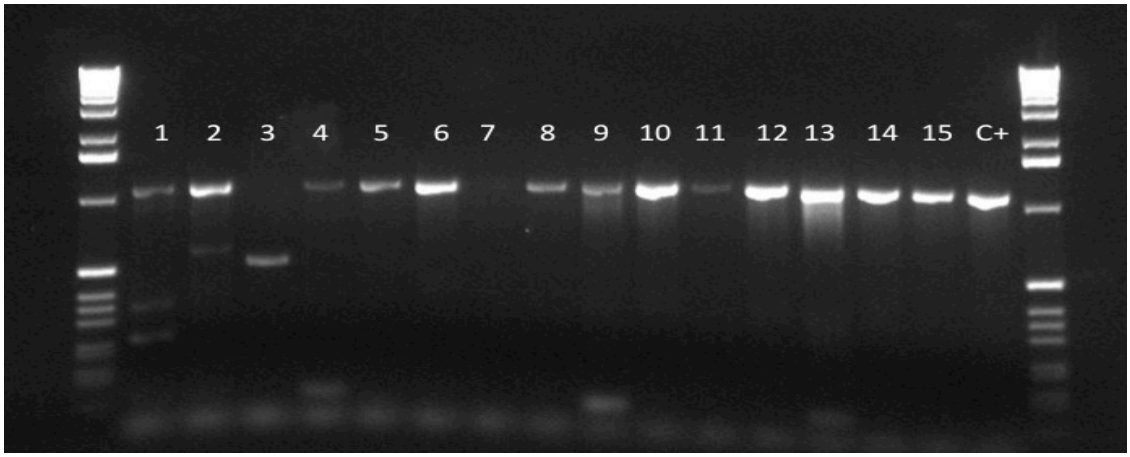

Figure A3.1. Gel electrophoretogram of polymerase chain reaction (PCR) products generated using primer pair CLi.po.F/O12c from adult psyllids (Lanes 1-15) at the beginning of the experiment. Lane 15 is a Lso(+) control

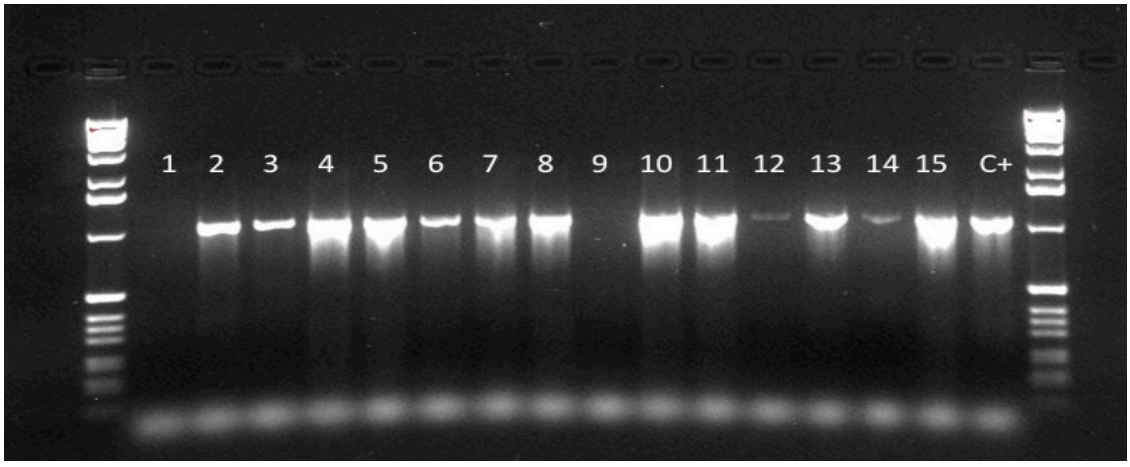

Figure A3.2. Gel electrophoretogram of polymerase chain reaction (PCR) products generated using primer pair CLi.po.F/O12c from adult psyllids (Lanes 1-15) four weeks after beginning of experiment. Lane 15 is a Lso(+) control

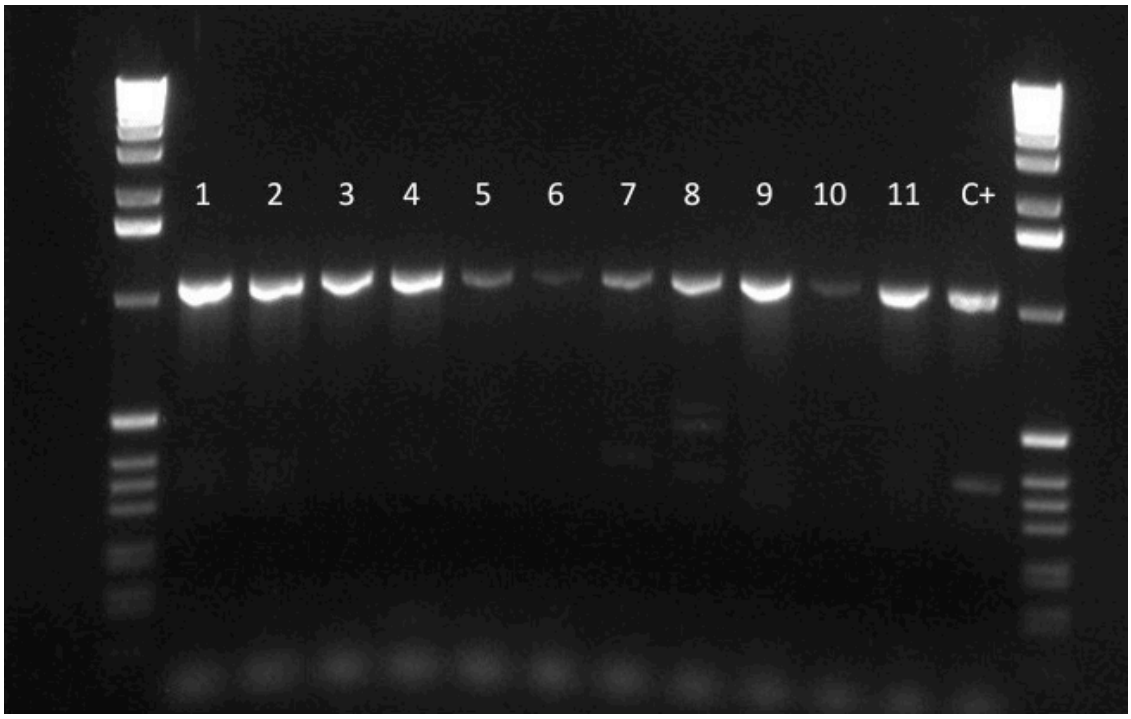

Figure A3.3. Gel electrophoretogram of polymerase chain reaction (PCR) products generated using primer pair CLi.po.F/O12c from adult psyllids (Lanes 1-11)) eight weeks after beginning of experiment.. Lane 12 is a Lso(+) control

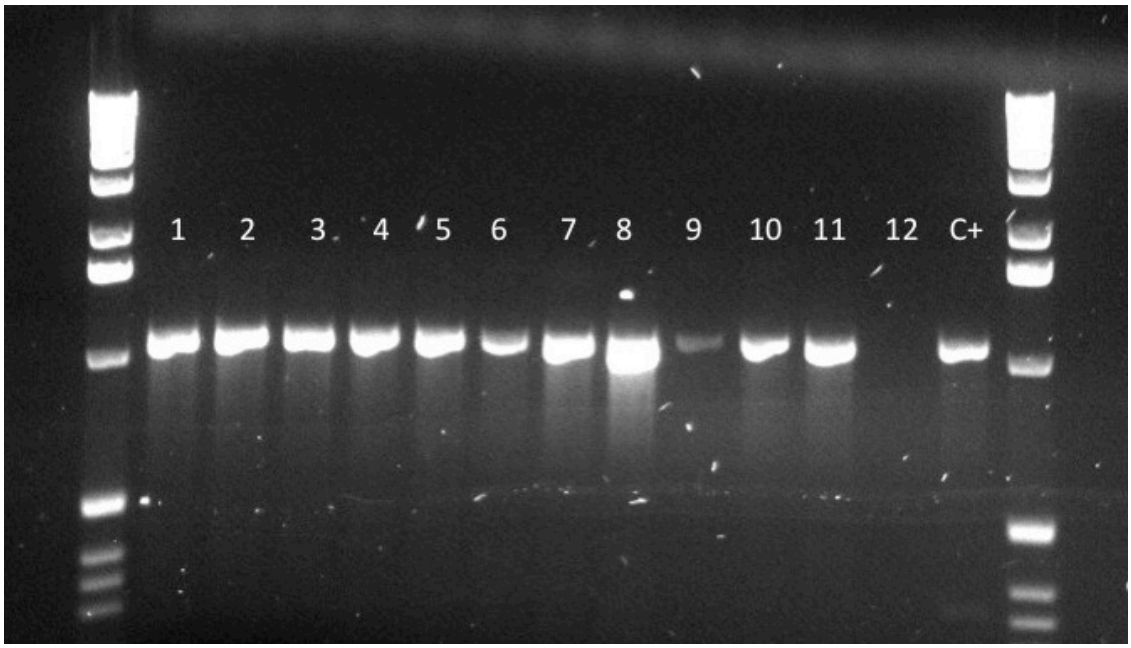

Figure A3.4. Gel electrophoretogram of polymerase chain reaction (PCR) products generated using primer pair CLi.po.F/O12c from adult psyllids (Lanes 1-12) 12 weeks after beginning of experiment.. Lane 13 is a Lso(+) control

APPENDIX IV. BIOLOGICAL SIGNIFICANCE OF HAPLOTYPES OF CANDIDATUS LIBERIBACTER SOLANACEARUM

Hypotheses

H_o: The data collected will show biological differences in ZC expression as a direct consequence of the haplotype used to inoculate the plant.

H_a: The data collected will not show biological differences in ZC expression as a direct consequence of the haplotype used to inoculate the plant.

Introduction

Recently, three haplotypes of *Candidatus* Liberibacter solanacearum have been discovered (Nelson *et* al., 2011, Wen *et al.*, 2009). Two of them, designated haplotype 'a' and haplotype 'b', are corresponding with Clades 1 and 2 from Wen *et al.* (2009), and are associated with zebra chip of potatoes and other plants in the solanaceae family. A third haplotype is associated with diseased carrots in Finland (Nelson *et al.*, 2011). These haplotypes showed distinct patterns of SNP's differences in three gene regions, 16s rRNA, 16s-ISR-23s and 50s rplJ and rplL genes. In other systems, such as Huanglonbing of citrus, this kind of gene variation has provided a starting point for taxonomical and epidemiological studies (Magomere, 2009). So far, besides genomic differences, no biological differences have been found between haplotypes and there is no information available regarding ZC severity when potato plants are inoculated with these distinct haplotypes. Therefore, the objective of this study is to determine if there are any differences in the phenotype of zebra chip infected potato plants, when plants are inoculated with bacterial populations of the different haplotypes and a combination of both haplotypes.

Literature review

Several pathogens have been suspected to be the causal agent of Zebra Chip (ZC) of potatoes, including phytoplasmas (aster yellows, clover proliferation, stolbur), viruses and bacteria, but *Candidatus* Liberibacter solanacearum (Lso) has been consistently associated with the disorder and is now commonly accepted as the causal agent of ZC (Liefting *et al.*, 2009a; Abad *et al.*, 2009; Crosslin and Bester, 2009; Lin *et al.* 2009; Munyaneza *et al.,* 2009; Secor *et al.,* 2009; Rehman *et al, 2010)*. In the United States a second Liberibacter species, *Candidatus* Liberibacter psyllaurous, was associated with psyllids yellows and zebra chip, but recent studies demonstrated that based on their 16S rRNA gene region, the two organism are identical, even though differences in virulence seem to exist (Wen *et al.,* 2009). Lso from Texas are 99.8% similar to Lso from New Zealand, with only 2 single nucleotide polymorphisms (SNP's) differentiating the North American and New Zealand 'strains'. Wen (2009) found that 'strains' of Lso could be separated in two clusters, C1 and C2. The two clades were identified thanks to two base substitutions in the 16S rRNA gene region (Secor *et al.,* 2009, Wen *et al.,* 2009). Later, Nelson *et al.* (2011) showed that SNP's in three gene regions (16s rRNA, 16s-ISR-23s and 50s rplJ and rplL genes) separated the strains, and the appearance of those SNP's indicated that they are haplotypes. Nelson *et al.* (2011) separated the haplotypes as 'a' and 'b', which correspond to Clade 1 and Clade 2 described by Wen *et al.* (2009). The haplotypes 'a' and 'b' have different geographic ranges. Haplotype 'a' has been found in New Zealand, Honduras, Guatemala, western Mexico, Arizona and California, and in some samples in Texas, Kansas and Nebraska. Haplotype 'b' has been found in eastern Mexico, Texas, and

south central Washington (Figure A4.1). Overlap of some regions exists (Texas, Nebraska and Kansas), but that could be due to movement of infected materials. Even though there are genetic differences in populations of Lso, currently no biological implications have been determined to exist. Recently, intensive testing of potato samples from Washington, Oregon and Idaho has provided a clearer picture of the distribution of haplotypes in these states. Washington samples have been found to be predominantly of the 'a+b' haplotypes, while samples from Oregon and Idaho are predominantly haplotype 'a' (Chris Johnson, personal communication).

Figure A4.1. Distribution of Haplotypes 'a' and 'b' in North and Central America (Nelson *et al.,* 2011)

Candidatus Liberibacter solanacearum

Candidatus Liberibacter solanacearum (Lso) is a non-culturable, phloem-limited bacterium that belongs to the α subdivision of the class *Proteobacteria*. Members of this class are gram negative. This subdivision is a diverse group of microorganisms that include plant pathogens, symbionts and human pathogens (Bové,

2006). Individuals in this subdivision live in intimate association with eukaryotic cells Transmission electron microscopy indicates that *C.* L. solanacearum is 0.2 µm in width and 4 µm in length with rounded ends (Liefting *et al.*, 2009a, Tanaka *et al.*, 2007). Lso is classified as a *Candidatus*, which is a scientific classification for a bacterium that is well characterized but as yet uncultured and cannot be maintained in a bacteriology culture collection (Stackebrandt *et al*, 2002). Since Liberibacters cannot be cultured, detailed information about their etiology, physiology and mode of pathogenesis are lacking, and their detection, identification and classification are based largely on molecular techniques and specific signatures, particularly in the 16S rRNA gene (*L*i *et al.,* 2009, *Secor et al*, 2009, Lin *et al.,* 2009). Based on phylogenetic analysis of the 16S rRNA gene, the 16S/23S rRNA spacer region and the rplKAJL-rpoBC operon, Liefting *et al.* (2009a) concluded that that the organism is a new species of the *Candidatus* Liberibacter genus. This species is phyllogenetically distinct from the three currently described Liberibacter species (‘*C.* L. asiaticus’, ‘*C.* L. africanus’, ‘*C.* L. americanus’) and the first Liberibacter species known to naturally infect plants outside the *Rutaceae* family, family that includes citrus, in which Liberibacter is an important pathogen. This bacterium was first named “Liberobacter”, from the Latin Liber (= bark) and bacter (=bacterium) (Jagoueix *et al*, 1994); Liberobacter was subsequently replaced by Liberibacter because `bacter' is of masculine gender and thus the connecting vowel between “Liber” and “bacter” should be `i' when the preceding term is of Latin origin (Garnier *et al*, 2000).

The name *Candidatus* Liberibacter psyllaurous is considered to be synonymous to *Candidatus* Liberibacter solanacearum, because the 16s rRNA sequence of *C.* L.

psyllaurous is identical to the 16s rRNA sequence of *C*. L. solanacearum associated with ZC in the United States and in New Zealand (Secor *et al.*, 2009). At about the same time that Liefting *et al.* (2008) reported the new *Candidatus* Liberibacter species affecting potatoes in New Zealand, Hansen *et al*. (2008) reported an association of *C*.L. psyllaurous with psyllid yellows affecting tomato and potato in the United States. The name *C*.L. solanacearum was preferred and has become the more widely used name for the pathogen because it was validly published by Liefting *et al.* (2008) according to the rules of the Code of Nomenclature for Bacteria, and the name *C*.L. psyllaurous was not (Secor *et al.*, 2009).

Bactericera cockerelli

It has been demonstrated that the potato psyllid, *B. cockerelli* is the major vector of Lso, the causal agent of ZC (Munyaneza *et al.*, 2007a, Secor *et al.,* 2009, Sengoda *et al.,* 2010).

Bactericera cockerelli (Sulc.) (Homoptera: Psyllidae) was originally described by Sulc in 1909, and classified as *Trioza cockerelli* and assigned to the genus *Paratrioza* by Crawford (Crawford, 1911). Recently the potato psyllid has been reassigned to the genus *Bactericera* (Burkhardt and Laureter, 1997, Miller *et al.,* 2000). The genus Bactericera includes 28 species.

B. cockerelli is polyphagous phloem feeder, and can successfully reproduce on a wide variety of hosts, that include plant species in 20 families (*Amaranthaceace, Asclepiadaceae, Asteraceae, Brassicaceae, Chenopodiaceae, Convolvulaceae, Fabaceae, Lamiaceae, Lycophyllaceae, Malvaceae, Menthaceae, Pinaceae, Poaceae, Polygonaceae, Ranunculaceae, Rosaceae, Salicaceae, Scrophulariaceae, Violaceae*

and *Zygophyllaceae*), but plants in the *Solanaceae* family are the preferred host, and has been a pest of potato and tomato for many years (Hansen *et al.,* 2008, Wallis, 1955, Gao *et al.,* 2009). The potato psyllid originated in North America, and is naturally distributed in Canada (Alberta, British Columbia, Ontario, Quebec, Saskatchewan), Mexico, the United States (Arizona, California, Colorado, Idaho, Kansas, Minnesota, Montana, Nebraska, Nevada, New Mexico, North Dakota, Oklahoma, South Dakota, Texas, Utah, Wyoming), Guatemala and Honduras. Texas, southern New Mexico, Arizona, California and northern Mexico are desert breeding areas of *B. cockerelli* (Al-Jabr, 1999). *B. cockerelli* has not been found in Europe, Asia, the United Kingdom or Australia (Morris *et al.,* 2009). The potato psyllid was recently introduced to New Zealand, where it was first discovered in 2006 (Liefting *et al.,* 2009a, Teulon *et al.,* 2009). It is important to note that Lso has not been found in areas where *B. cockerelli* is absent, since there is no mechanism for bacterial spread (Morris *et al.*, 2009). Munyaneza *et al.* (2010) reported the first known association of Lso with a non-solanaceaous crop in Finland, where it was found to affect carrot (*Daucus carrota* L.). In this case, Lso appears to be vectored by the carrot psyllid (*Trioza apicalis* Förster), which is a serious pest in north and central Europe. This was also the first report of Lso outside North and Central America and New Zealand (Munyaneza *et al.*, 2010). In 2011 Lso was found in carrots in Sweden and Norway (Munyaneza *et al* 2011a, Munyaneza *et al*, 2011b) and in 2012, in carrots in the Canary associated with *Bactericera trigonica* (Hodkinson) (Alfaro-Hernandez *et al.*, 2012).

Psyllid sexing

Sexing of potato psyllids is based on morphological characteristics of the adult

abdomen. The mature female abdomen, which terminates with a short ovipositor, is well rounded and more robust than the male abdomen and has five abdominal segments, plus the genital one. The male genitalia present a more blunt appearance at the tip, and the abdomen is divided in six segments, plus the genital one (Figure A4.2) (Pletsch, 1947, Abdullah, 2008). Male and females can also be distinguished when they are immature, when the yellow coloration of developing testes is strong enough in the fourth and fifth instar nymph stages. The mycetome is a yellowish-orange roughly U-shaped body found in the basal half of the nymph abdomen. In male nymphs, spindle-shaped testes extend along and beyond the mycetome lobe, and forming with the mycetome a H-shaped structure. In females, a pair of hyaline bodies can be seen posterior to the mycetome. With this method, over 95% of males can be correctly identified (Carter, 1961).

Materials and Methods

Plant material

Potatoes, cultivar Atlantic, were grown from disease-free potato mini-tubers obtained from Sklarczyk Seed Farms LLC (Johannesburg, MI). This chipping cultivar was used because it has been reported to be highly susceptible to zebra chip and is commonly grown in regions where ZC is found (Munyaneza *et al*, 2007a, Munyaneza *et al*, 2007b, Munyaneza *et al*, 2008).

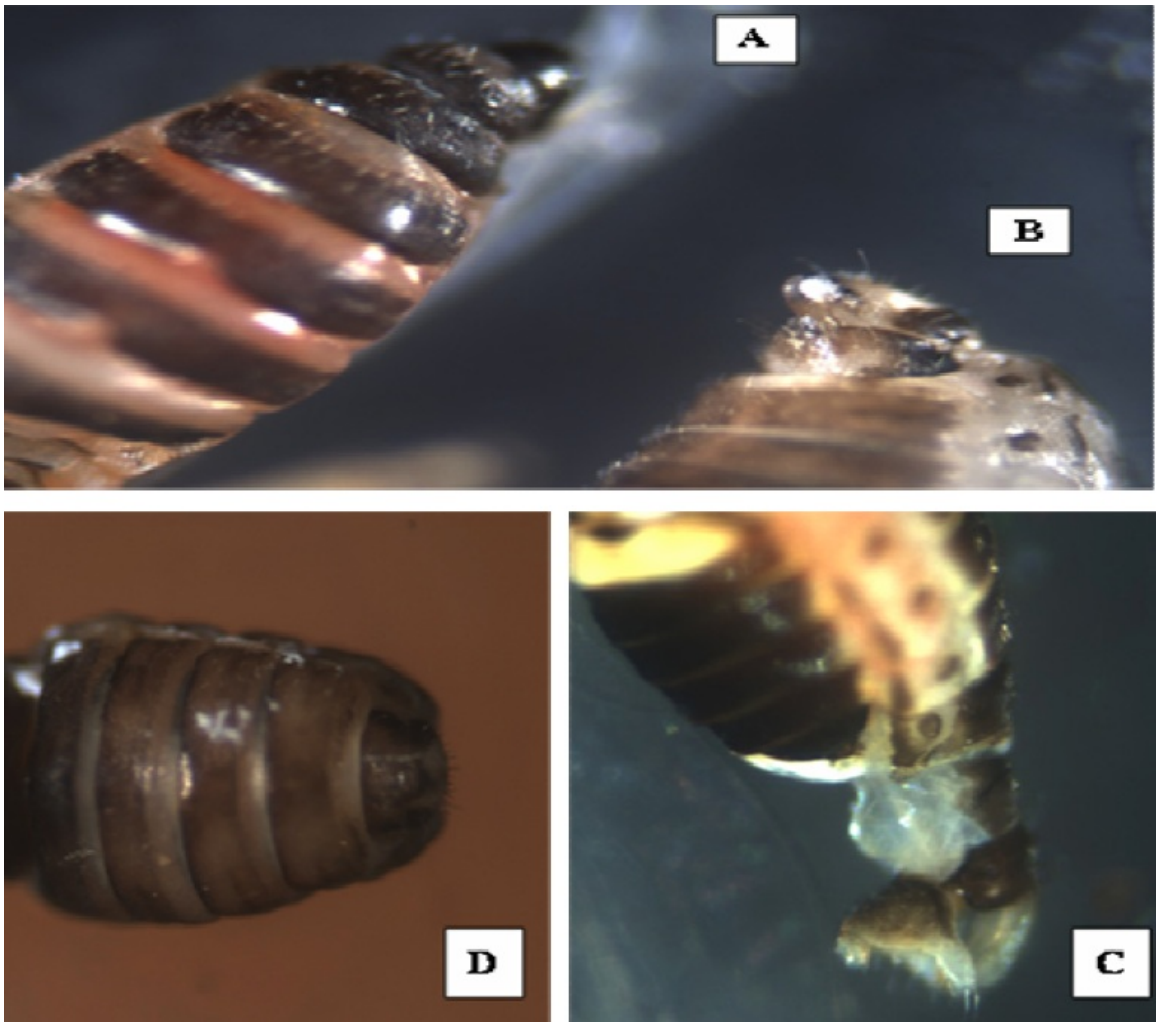

Figure A4.2. Abdominal segments of *B. cockerelli* adults illustrating differences between male (A and C) and female (B and D) (Abdhulla, 2008)

Psyllid colonies

Psyllid colonies were established with psyllids provided by Dr. Joseph Munyaneza (USDA-ARS, Wapato, WA). Munyaneza psyllids were initially collected in Texas in 2007 and have been reared for multiple generations in his laboratory. Colonies were grown in commercial rearing cages (Bug Dorm-2, BioQuip, Rancho Dominguez, CA, USA) consisting of an aluminum frame covered with insect-proof mesh with enough space to contain four plants in six-inch pots.

The potato psyllid colonies were maintained on potato plants at 25˚C with a photoperiod of 16 hours of light and 8 hours of darkness in a greenhouse and growth cambers. Plants were replaced periodically to sustain psyllid colony growth. Insects were reared for multiple generations and used as needed. To confirm the presence of Lso in both of the psyllid colonies, subsamples of psyllids were tested periodically by PCR according to Secor *et al.* (2009).

Biotyping of colonies

Colonies used in this study were established from psyllids provided by Dr. Joseph Munyaneza and have been tested and found to be positive for Lso; furthermore, they have shown to be composed of individuals carrying either haplotype ‘b’ or both haplotypes (a and b), which seems to be prevalent in the population. Primers used to test for haplotypes were developed in Dr. Yong-Ping Duan’s laboratory in Florida. Primer pair 64r and 29r amplify a DNA fragment of 2179 bp and is used to identify haplotype 'a' (or 1), and primer pair 74r and 1r amplify a 1073 bp DNA fragment used to identify haplotype 'b' (or 2). This primers have not been published, thus, the sequence of these primers has not been released.

Biotype separation procedure

Psyllids were collected from the Lso+ colony and sexed in the laboratory based on morphological characteristics (Abdullah, 2008). Isofemale colonies were started with selected individual females transferred to new rearing cages in which they were allowed to lay eggs (Figure A4.3). Once established, a sample of 10 insects from each colony was tested for Lso by PCR. Insects that were Lso+ were tested again to determine which haplotype they carried. If successful, the resulting isofemale colonies

would provide three haplotype conformations: only haplotype ‘a’, only haplotype ‘b’ and haplotypes ‘a’ and ‘b’. A separate colony with psyllids not carrying Lso has also been established.

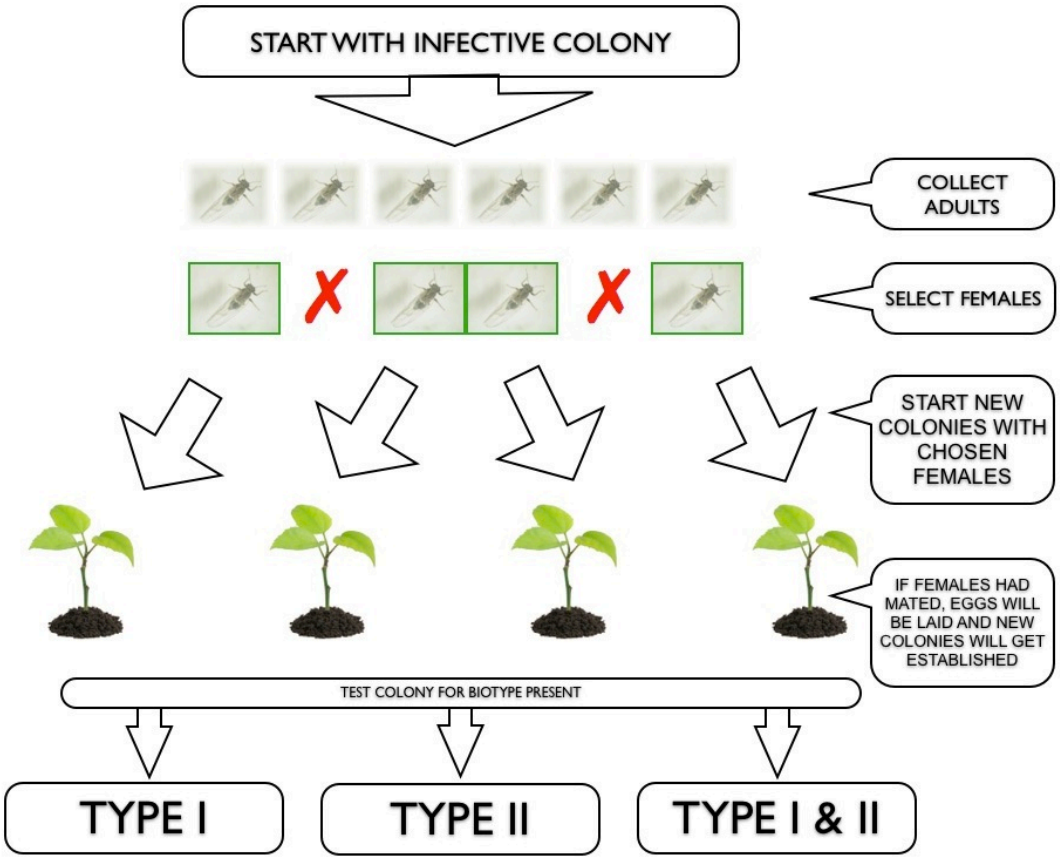

Figure A4.3. Diagram of process followed for separation of Lso biotypes

Inoculations

Once colonies with the three different biotypes are obtained, plant inoculations will be initiated in potato plants. Plants will be inoculated six weeks after emergence and exposed to psyllids for a period of seven days, after which, psyllids will be killed with insecticides. A hand-held aspirator will be used to collect adult psyllids. These insects will be released onto the potato plants. All potato plants will be inoculated in a

greenhouse room set at a temperature of 25ºC with a light cycle of 16 hr. light and 8 hr. dark. Plants will remain in the greenhouse room for the remainder of the experiment.

Experimental design

The experiment will be arranged as a complete random design (CRD) and will have five plants per treatment, with each plant being a single replicate. The experiment will be conducted twice.

Treatments

This experiment will have five treatments, which are as follow:

1. Lso (+) Biotype I psyllids
2. Lso (+) Biotype II psyllids
3. Lso (+) Biotypes I and II psyllids
4. Lso (-) psyllids
5. Non-inoculated control.

Evaluation

Daily visual assessment of ZC symptom development will be performed in the aerial parts of the plant. Total DNA will be extracted from different plant tissues (leaf, stem, stolon, tuber) using the CTAB protocol (Gawel *et al.*, 1991) and will be assessed for the presence of *C.* L. solanacearum by polymerase chain reaction (PCR) using specific primers OA2/OI2c and CLi.po.F/OI2c (Secor *et al.,* 2009). Samples will also be tested for haplotype composition. At the end of the experiment tuber slices will be assessed for ZC symptoms.

Expected results

It is expected that biotypes of Lso will have an effect on the severity of ZC in the inoculated potato plants. Lso colonies of the Biotype I will cause only mild symptoms, while colonies of the Biotype II will cause more severe damage. Colonies with Biotypes I and II will cause the most severe form of ZC.

Results

Thus far, we have been unable to generate a haplotype 'a' only colony via isofemale colony initiation. It has been possible to obtain mixed haplotype ('a+b') and haplotype 'b' only colonies. Psyllid samples from the mixed colony has been tested via haplotype specific quantitative PCR. Initial results indicate that the haplotype ratio is not skewed heavily toward 'b' and should not prevent haplotype 'a' only colony isolation. This may be an indication of variable transmission efficiency between the two haplotypes. Additional transmission experiments are being designed to test for this possibility.

As an alternative strategy for generating a haplotype 'a' only colony, Lso+ potato tubers of haplotype 'a' with strong zebra chip symptoms were planted in insect cages. Once plants were sufficiently large, Lso- psyllids were introduced to the cages. It was hoped that the resulting plants would provide a source of inoculum for psyllids to acquire pure haplotype 'a' Lso. However, plant material and psyllids tested from these cages has been Lso-. Although initial sprouting and plant growth were slow, these plants did not display typical zebra chip symptoms. However, leaf curling and yellowing symptoms have recently become very obvious (plants now 10 weeks old) and hopefully additional testing will result in Lso+ insects and plant tissues. Previous

plantings of zebra chip tubers had resulted in low emergence rate (<50%) and stunted plants with immediate obvious zebra chip symptoms. The resulting plant material also tested Lso+. It is possible that Lso virulence factors may be responsible for differential tuber emergence and zebra chip symptom development. Once haplotype specific colonies are developed, its effects will be studied, both for differential tuber development in Lso+ zebra chip tubers and disease severity.

Possible sources of genetic variability and virulence factors in bacterial pathogens are bacteria phages. A likely phage and prophage were found in the closely related pathogen *Candidatus* Liberibacter asiaticus (Las) (Zhang, *et al.* 2011). A comparison of the phage-like DNA sequences from Las to the published CLso1 genome revealed two regions in CLso1 with high sequence homology and synteny. These two regions, of approximately 40kb each, contain all the necessary genes for function phage in the Podoviridae group. PCR testing has indicated a high degree of variation between the Lso haplotypes in these phage-like regions relative to the genome as a whole. The significance of these regions will be evaluated in future experiments.

In conclusion, it is expected to observe distinct variation in zebra chip disease development relative to bacterial haplotype. Although attempts to produce haplotype specific sources of inoculum have been partially successful, experiments have hinted at haplotype specific variations in transmission efficiency and diseased tuber development. Likely phages present in Lso will be a good place to look for genetic variability and virulence factors responsible for haplotype specific disease phenotypes. Development of pure haplotype colonies will be critical in furthering our understanding of zebra chip disease.

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