THESIS

UNDERSTANDING THE DISEASE ECOLOGY OF THE CORN BACTERIAL LEAF STREAK PATHOGEN *XANTHOMONAS VASICOLA* PV. *VASCULORUM*

Submitted by

Mary Carmen Ortiz Castro

Department of Bioagricultural Sciences and Pest Management

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2019

Master's Committee:

Advisor: Jan Leach
Co-advisor: Kirk Broders

Amy Charkowski
Meagan Schipanski

ABSTRACT

UNDERSTANDING THE DISEASE ECOLOGY OF THE CORN BACTERIAL LEAF STREAK PATHOGEN *XANTHOMONAS VASICOLA* PV. *VASCULORUM*

Bacterial leaf streak, caused by *Xanthomonas vasicola* pv. *vasculorum* (*Xvv*), is an emerging disease of corn in North and South America. Based on the combined $52.4 billion value of the corn industry, early reports of *Xvv* disease severity, and lack of management methods, this emerging pathogen represents an economic threat to corn production in the United States. The primary goal of this research is to provide a basic understanding of the infection ecology and survival of the corn bacterial leaf streak pathogen. Through genetic transformations of the bacteria with fluorescent proteins and confocal microscopy, we were able to show the localization of the bacteria within plant leaves. In addition, we found that there is a significant interaction between *Xvv* isolates and two corn varieties. By evaluating the bacterial fitness across representative isolates of *Xvv*, we showed that 22ºC is the optimal temperature for bacterial growth in culture. We also evaluated the interaction of *Xvv* with the endophyte *Pantoea ananatis* and found that the presence of the endophyte significantly decreases *Xvv*'s disease response. Finally, through litter studies at multiple locations, we demonstrated that infected residue left on the surface of the soil harbored significantly greater quantities of *Xvv* than infected residue buried 10 cm below the surface. These findings will be useful to understand the bacterial leaf streak disease cycle and aid in the development of management strategies that may limit the distribution of *Xvv* within corn fields and prevent its spread to other corn producing regions.

ACKNOWLEDGMENTS

I would first like to thank God for being my strength and company and for giving me a lifetime of learning experiences in a new country and culture.

I would like to thank my advisor, Dr. Kirk Broders, for his support, encouragement, and mentorship throughout my time as a graduate student. He is an inspiration for young scientists and I will always be grateful to him for giving me the training I need to accomplish my future professional goals. I would also like to thank Dr. Jan Leach for her helpful discussions and for welcoming me into her lab. Many thanks to my committee members, Dr. Amy Charkowski and Dr. Meagan Schipanski, for their valuable advice during my research project and for instilling confidence in me to become a better scientist. I also want to acknowledge past and present members of the Broders Lab, especially Dr. Tessa Albrecht, Gloria Broders, and Gabby Lavden who helped me with my experiments and always brought a smile to my face.

I would also like to thank our collaborators at the University of Nebraska and Iowa State University, Dr. Tamra Jackson-Ziems, Terra Hartman, and Dr. Alison Robertson, for sending me samples and helping me with my field survival studies. Many thanks to the Colorado Corn growers who were interested in supporting and learning more about my research, especially Mr. Brett Adler who let us use his fields for our study.

I feel very grateful for the support of my family in Colombia and in Colorado. To my parents, Waldo Ortiz and Sara Castro, thank you for always being there for me and teaching me the values of discipline, honesty, and hard work which were fundamental in completing this degree. To my sisters Maria Claudia and Mariana Carolina, thank you for bringing joy and laughter to my life. Thank you to my entire family - grandparents, cousins, aunts, and uncles -

who have always celebrated my academic accomplishments. Thank you to the Sullivan and Diamant families who have accompanied me from the beginning of this degree and even went with me to collect samples in the field in inclement weather conditions. Last but not least, thank you to Bryan Sullivan for his support and honest criticism of my figures and presentations. You are my biggest fan and have been with me since I first started my scientific career. Thank you for pushing me to do my best and not doubting for a second that I could do this, even when I did. This degree is as much yours as it is mine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Emerging plant diseases

One important issue in plant health is the emergence and re-emergence of pathogens. Since plant populations are unable to quickly adapt to pathogens, emerging plant diseases represent a risk to food security and the health and integrity of natural ecosystems (Garrett et al., 2010). An often complicating factor of new plant pathogens is that scientists do not possess information on the basic biology of the organism. Hence, this lack of knowledge results in an inability to anticipate, respond, and manage the emerging diseases.

How and why do new pathogens emerge? This may occur by 1) natural or accidental introduction of species into new geographic regions with naïve hosts, 2) evolution of a novel race within the same pathogen species, and 3) evolution of a pathogen to infect a wider range of hosts. In any case, a plant pathogen that is introduced to different susceptible plants with a suitable environment can quickly generate an emerging disease event. Examples of emerging diseases of crops include: Asian soybean rust (Fletcher et al., 2010), bakanae of rice (Singh & Sunder, 2012; Gupta et al., 2015), bacterial leaf streak of corn (Korus et al., 2017), zebra chip of potato (Lin & Gudmestad, 2012) and wheat stem rust (Singh et al., 2015). The Asian soybean rust pathogen was naturally introduced through wind currents from Asia to South America and then to North America (Fletcher et al., 2010). Bakanae of rice and bacterial leaf streak of corn pathogens were likely accidentally introduced through infected plant tissues and seeds (Gupta et

al., 2015; Broders, 2017; Korus et al., 2017). Wheat stem rust and zebra chip of potato pathogens, on the other hand, evolved multiple races and infect a wider host range, respectively (Lin & Gudmestad, 2012; Singh et al., 2015).

Governmental agencies have established pre-and post-introduction actions when a new plant disease is identified in a certain region. Pre-introduction strategies are based on inspecting plant material at the port of entry and ensuring plants are accompanied with a phytosanitary certificate from the exporting country. Post-introduction actions include early detection, surveillance, and priorization of the emergent pathogen (Fletcher et al., 2010).

One critical post-introduction task is the priorization of new plant pathogens. Priority ranking is given to diseases afflicting a high value commodity. For example, corn (*Zea mays* L.) is the most widely produced grain crop in the United States with an approximate 81,740,000 acres harvested in 2018 at an estimated value of $52.4 billion (USDA-NASS, 2018a). Due to its high revenue, diseases of corn would have a higher ranking and urgency for research than diseases of less valued crops. One emerging disease of corn present in the United States in recent years is bacterial leaf streak (Korus et al., 2017; Lang et al., 2017).

Bacterial leaf streak of corn (BLS) is caused by the pathogen *Xanthomonas vasicola* pv. *vasculorum* (*Xvv*) (Lang et al., 2017) . In the United States, BLS was first observed in Nebraska in 2014, but became widespread by 2016 (Korus et al., 2017). Since then, the disease has been reported in at least nine other states including the major corn producing states (Damicone et al., 2018; Jamann et al., 2019; Korus et al., 2017; Lang et al., 2017; Smith et al., 2018). Given that the bacterial pathogen has spread rapidly in the last three years and that there are a large number of acres of corn production, the understanding of its ecology and epidemiology is crucial to prevent future losses.

1.2 Symptoms, impact and current distribution of bacterial leaf streak of corn

Symptoms of BLS include: dark, water-soaked, linear lesions with wavy-edged margins that range from brown to yellow streaks and occur between the leaf veins anywhere on the leaf blade or close to the midrib. Lesions range from less than an inch to several inches in length and appear translucent when backlit (Korus et al., 2017; Lang et al., 2017) (Figure 1.1). Early in the growing season, symptoms are mostly observed in the lower canopy of the plant, but in mid to late season, symptoms are observed in the upper canopy of the plant (above ear leaf). In 2016, farmers reported disease incidence levels above 90% and disease severity greater than 50% of leaf area infected. These levels denote that the disease has reached epidemic proportions (Broders, 2017; Korus et al., 2017).

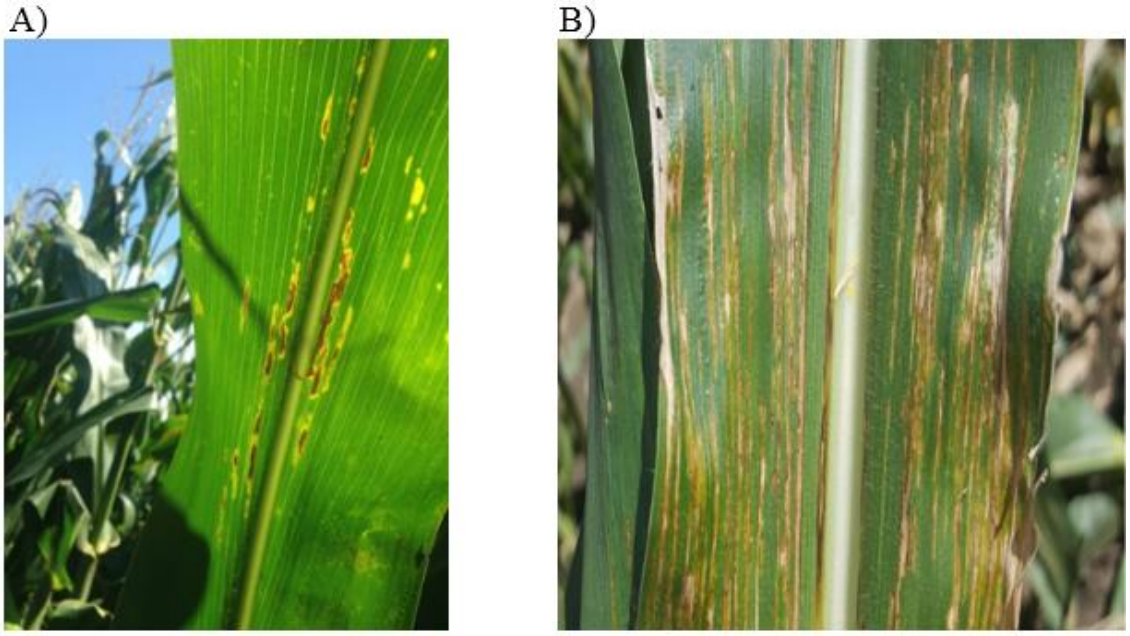

Figure 1.1: ***Symptoms of bacterial leaf streak of corn.*** *Bacterial leaf streak symptoms were observed both (A) early and (B) later in the growing season. Photo credit: Dr. Kirk Broders*

Although no formal studies on the yield impact due to BLS have been completed, it is predicted this disease will have a negative effect on corn yield if the bacterium reaches the upper

canopy. At this point, the pathogen can colonize leaves of important photosynthetic activity directly related to yield potential. Another foliar disease with similar intervenial streak symptoms to BLS is gray leaf spot. Symptoms of this fungal disease, caused by *Cercospora zeae-maydis* (Hyre, 1943), also begin to appear on the lower leaves and travel up the plant (de Nazareno et al., 1992). When not controlled with fungicides during the growing season, gray leaf spot has the potential to reduce yields up to 65% in susceptible hybrids (Ward et al., 1999). Because of the similarity of the symptoms and behavior, and considering there are currently no bactericides available for BLS, these two diseases may have a similar effect on corn yield.

Examples of yield impact caused by other species of pathogenic *Xanthomonas* can range from 20-50% in the crop they infect (Hirano & Upper, 1983). Foliar pathogens like *X. axonopodis* pv. *allii* infecting onion and *X. translucens* pv. *undulosa* infecting wheat and barley have both been found to affect yield. Reports of the yield loss caused by *X. axonopodis* pv. *alli* can be 20% or greater depending on infection time, weather conditions, and cultivar susceptibility (Schwartz & Gent, 2011). Yield losses caused by *X. translucens* pv. *undulosa* can be anticipated to be about 20% if 50% of the flag leaf of wheat is infected (Adhikari et al., 2011). Other reports have also described yield losses as high as 40% in Idaho winter wheat for bacterial leaf streak of wheat (Sapkota et al., 2018).

Moreover, closely related species to *Xvv* such as *X. vasicola* pv. *musacearum* (*Xvm*) (causal agent of bacterial wilt of banana and plantain) and *X. oryzae* pv. *oryzae* (*Xoo*) (causal agent of bacterial blight of rice) can result in either total economic loss or up to 70% loss, respectively (Mew et al., 1993; Mwebaze et al., 2006; Reddy et al., 1979; Studholme et al., 2019). Based on this information, it is likely that *Xvv* causes a yield reduction on corn production; however evidence of this decrease is still pending.

Bacterial leaf streak was first described in corn in 1949 in South Africa (Dyer, 1949a), and prior to 2016 it had not been documented in the United States (Korus et al., 2017). Currently, the disease has been reported in Nebraska, Colorado, Kansas, Iowa, Illinois, Minnesota, South Dakota, Texas, Oklahoma, and Wisconsin (Damicone et al., 2018; Jamann et al., 2019; Korus et al., 2017; Lang et al., 2017; Smith et al., 2018) (Figure 1.2). Recently, the disease has also been reported in Argentina (Plazas et al., 2018) and Brazil (Leite Jr. et al., 2018) (Figure 1.2), however, symptoms of BLS in Argentina have been present since at least 2010 in the Córdoba province (personal communication, Maria Cristina Plazas).

The first symptoms of BLS in the United States were reported in Nebraska, Colorado, and Kansas. Interestingly, these three states, and the regions of South Africa and Argentina where BLS is most prevalent, share the same type of cold semi-arid climate (BSk classification) (Earth-Data, 2018) (Figure 1.2). Therefore, it is possible this type of climate favored the initial settlement of the pathogen and since then, it has spread to other regions with higher humidity conditions like those in the Corn Belt states (humid, continental climate with hot summers, Dfa classification) (Earth-Data, 2018) (Figure 1.2). It is not known yet if the other three corn producing countries sharing this same semi-arid climate, Mexico, China, and Australia, have this disease, but it will not be surprising if the disease appears in these regions in the future. The potential arrival of *Xvv* in these countries will depend on the stringency of plant material transport measures and restrictions in each of those countries.

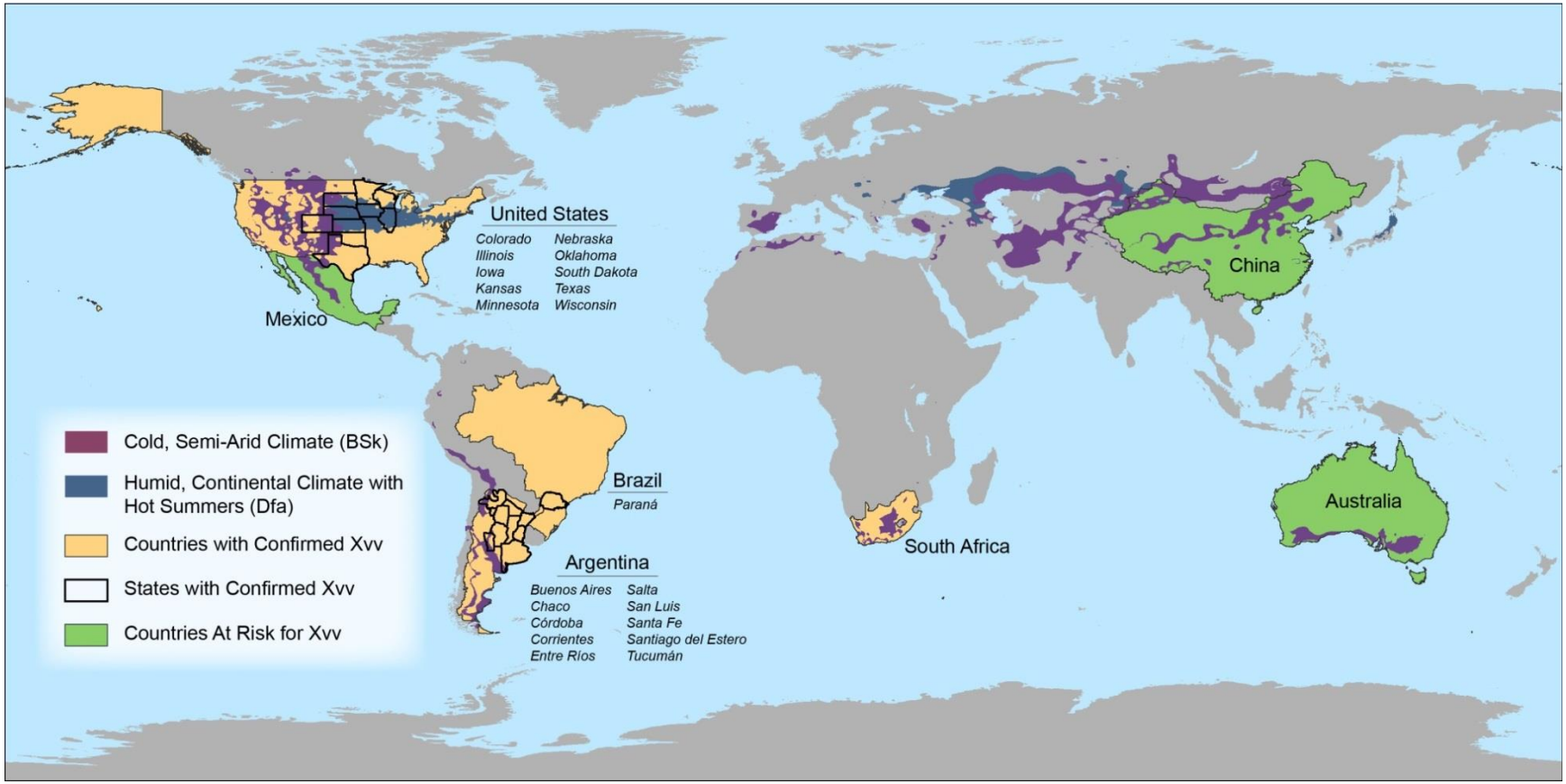

Figure 1.2: ***Current distribution of bacterial leaf streak of corn.*** *Global map shows countries and states where Xvv is confirmed (yellow), countries at risk for* Xvv *(green), cold, semi-arid regions (purple) and humid continental climate with hot summers (blue). Purple and blue regions correspond to the Koppen Climate Classification BSk and Dfa, respectively (Earth-Data, 2018).*

It is unclear how *Xvv* was introduced into the United States. There are several hypotheses for the arrival of *Xvv*. First, it is possible *Xvv* was introduced from either South Africa or Argentina. The latter is more plausible as many American agri-businesses have winter corn nurseries in South America with year-round transportation of plant materials. Thus, a movement of infected plants or contaminated seed could have brought the disease to the United States. A second possibility is that there was an ancestor of the pathogen present in North America and it evolved to cause disease on corn. However, there is limited evidence to support this possibility. At this point, any of the above hypotheses may have introduced the pathogen and it is crucial to conduct research to identify the entry route or change in virulence and host conditions of this bacterial pathogen, so that farmers can take the appropriate measures to stop its spread.

1.3 Disease ecology of *Xanthomonas* species

All strategies of plant disease management and their efficacy are founded on the understanding of the ecology of plant pathogens (Morris et al., 2017). In this context, ecology of a disease refers to optimal conditions for disease development, mechanism of survival, dissemination, host range, and mechanism of infection regulated by biotic and abiotic factors.

The ecology of *Xanthomonas* species is very diverse including a collective host range of at least 124 monocotyledonous and 268 dicotyledonous plant species (Jacques et al., 2016; Leyns et al., 1984). Hosts of *Xanthomonas* spp. include: citrus (lime, orange, lemon, and pomelo), rice, crucifers (cabbage, broccoli, cauliflower, and radish), cassava, sugarcane, and banana among others (Hayward, 1974; Leyns et al., 1984). Since this bacterial genus has a wide variety of hosts, and can colonize stems, twigs, leaves, flowers, buds, fruits, and seeds (Bart et al., 2012),

the ecology and evolution of host specificity of *Xanthomonas* species has long been an active topic of research among many plant pathology laboratories.

Xanthomonas species are gamma proteobacteria, gram-negative, aerobic, and rod-shaped with a polar flagellum (Jacques et al., 2016). Colonies of *Xanthomonas* species usually are mucoid, convex, and yellow when grown on yeast dextrose calcium carbonate (YDC). All members of this genus are catalase positive and oxidase negative (Adhikari et al., 2011). Optimum temperature for growth and symptom development is 25-30ºC (Esgalhado et al., 1995). There is no quantitative data on optimum temperatures for the growth of *Xvv* but it is likely similar to the reported temperatures as this range often occurs during the corn growing season (NCEI, 2018).

Xanthomonas species have several mechanisms of survival. For example, *X. campestris* pv. *campestris* (Randhawa & Schaad, 1894) and *X. malvacearum* (Rothrock et al., 2015; Wang et al., 2019) can survive in diseased crucifers and cotton seeds, respectively. *X. citri* pv. *citri*, on the other hand, may overwinter in holdover cankers or blight twigs of perennial woody plants (Shiotani et al., 2009). Several *Xanthomonas* species including *X. phaseoli* pv. *fuscans* (Darsonval et al., 2008b), *X. euvesicatoria* (Jones et al., 1985), *X. campestris* pv. *phaseoli* (Gilbertson et al., 1990), and *Xanthomonas translucens* pv. *undulosa* (Adhikari et al., 2011) survive associated with their respective hosts residue. Finally, *Xanthomonas* may also survive in the soil; *X. vasicola* pv. *musacearum*, for example, persists longer in soil than in banana debris (Mwebaze et al., 2006). Other bacteria such as *X. axonopodis* pv. *allii* may survive in between susceptible crops, in association with contaminated seed, infested crop debris, and epiphytically or pathogenically on volunteer onion, weeds and leguminous plants (Gent et al., 2005). To date, it is unknown how *Xvv* survives from one corn production season to the next.

Dispersion of pathogen propagules is another aspect related to the ecology of disease. The direction of disease spread is sporadic, due to variability in wind speed and duration, rainfall incidence and duration, temperature variability, just to name a few. This results in an uneven distribution of the pathogen across a geographic region. For example, *X. axonopodis* pv. *allii* (Schwartz et al., 2003) and *X. translucens* pv. *undulosa* are dispersed by wind-driven rain events (Curland et al., 2018). Leaf abrasion by wind and windblown sand may favor infection as well. It is likely *Xvv* uses similar mechanisms of transmission; however, no study has been conducted to formally investigate the extent to which wind-driven rain affects bacterial leaf streak development.

After bacteria have successfully dispersed and landed on a susceptible host and if environmental conditions are conducive, bacteria will multiply to form large populations and attempt to gain entry into the tissues through natural openings (stomata, hydathodes, lenticels, and nectaries) or wounds (Figure 1.3). The ability of a particular *Xanthomonas* species to infect and colonize a host is dependent on the possession and use of virulence factors. Some of these factors include microbe-associated molecular patterns, type III effectors, lipopolysaccharides, transcriptional regulators, and chemotactic sensors (Jacques et al., 2016; Wasukira et al., 2014). Once the bacteria have entered, the virulence sensors will perceive the environment as favorable or not, and eventually start colonizing the intercellular or vascular tissue depending on the type of bacteria species (Karamura et al., 2015; McFarlane & Coutinho, 2010; Ricaud & Autrey, 1989).

The type III effectors, which are injected directly into the host cell through the type III secretion system, constitute the major factors involved in the pathogenicity of the *Xanthomonas* genus. This has been demonstrated as numerous type III effectors suppress immunity or trigger a

hypersensitive response that will eventually result in the total suppression of the effector-triggered immunity response of the plant cell. As a consequence, the pathogen has the perfect condition for proliferation. Based on genotypic information available for *Xvv*, there is evidence for the sequences of the type III secretion system (Lang et al., 2017; Wasukira et al., 2014); however no effectors of *Xvv* have been studied yet and nothing is known about the specific infection mechanism of *Xvv* on corn.

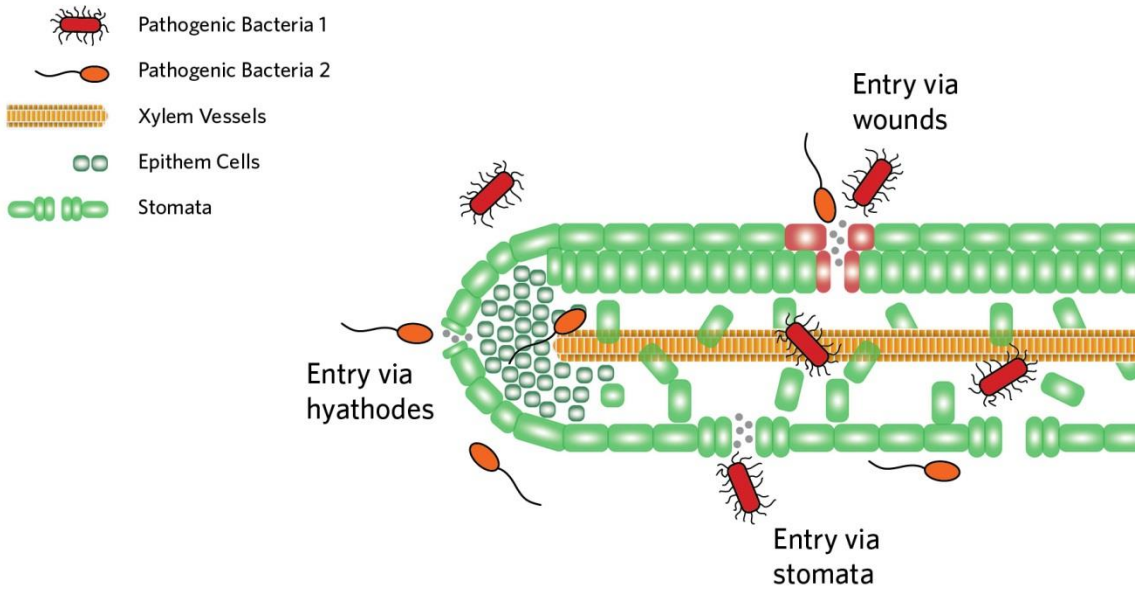

Figure 1.3: ***Colonization of leaf surface by pathogenic bacteria.*** *Bacteria may enter leaf tissue via hydathodes, stomata, and wounds (Figure adapted from Jacques et al. (2016))*

One last aspect pertaining to ecology of plant pathogens is the role of agronomic practices on disease development. Recent studies by Hartman (2018) investigated agronomic factors contributing to incidence of bacterial leaf streak of corn. Using Classification and Regression Tree (CART) (Breiman et al., 1984) and Random Forest (Breiman, 2001) analyses (Kim et al., 2002; Paul & Munkvold, 2004; Langemeier et al., 2017), they found that irrigation, crop rotation, growth stage, tillage, and planting date were the most important predictors of a sample testing

positive for the presence of *Xvv* (Hartman, 2018). Out of these factors, irrigation appears to be the most relevant factor in creating a conducive environment for disease development. This is because corn fields in Colorado and Nebraska, where bacterial leaf streak has a high incidence, frequently use center pivot irrigation (Kranz et al., 2008; Lichtenber, 1989; Turkington et al., 2004) leading to high humidity conditions which would favor the spread and colonization of *Xvv*.

1.4 Host range of *Xanthomonas vasicola* species

Host range and host range expansion often drives the emergence of a new disease. A host jump occurs when there is a colonization of a new host species that is phylogenetically distinct to species of the contemporary host range (Schulze-Lefert & Panstruga, 2011). There are two examples of *Xanthomonas vasicola* species having made a host shift resulting in a novel host-pathogen interaction. The first of these cases was reported in 2004 with the appearance of Xanthomonas wilt on banana and plantain in East Africa (Tushemereirwe et al., 2004). It is believed there was a host jump from sugarcane to banana (Karamura et al., 2015; Studholme et al., 2019). The second reported example is the emergence of blight and dieback of *Eucalyptus* spp. (Coutinho et al., 2015). Coutinho et al. (2015) proposed this host jump from a monocotyledonous plant such as sugarcane to a dicotyledonous plant like eucalyptus in South Africa.

X. vasicola species have an extensive host range, which includes several monocots grown in the United States (Hartman, 2018). Previous reports have shown that *X. vasicola* can infect sugarcane (*Saccharum* spp.), corn (*Zea mays* L.), banana (*Musa* spp.), hurricane palm (*Dictyosperma album*), tiger grass (*Thysanolaeana maxima*), Cuban royal palm (*Roystonea*

regia), betel palm (*Areca catechu*), and broom bamboo (*Thysanolaena maxima*) (Karamura et al., 2015; McFarlane & Coutinho, 2010; Ricaud & Autrey, 1989).

Of particular interest to this research is the group of *X. vasicola* pv. *vasculorum* that causes gumming disease in sugarcane (North, 1935). This strain of *Xvv* causes both leaf blight and vascular wilting symptoms that affect sugarcane production regions around the world, however there are no reports of this disease on sugarcane in the United States or Mexico. One genetic distinction between *Xvv* strains from corn and sugarcane is the lack of the *XopAF* effector in *Xvv* from corn (Lang et al., 2017; Wasukira et al., 2014). Although there has not been an experimental characterization of this effector on sugarcane, it is reasonable to suppose that the *Xvv* protein is likely to be a type III effector and a potential avirulence factor, given the 86% identity between this protein and the experimentally characterized *X. euvesicatoria XopAF* (Wasukira et al., 2014; Astua-Monge et al., 2000).

Lang et al. (2017) demonstrated that *Xvv* from corn can infect sorghum (*Sorghum bicolor*) as well, although this has not been observed in the field. Recently, Hartman (2018) evaluated the ability of *Xvv* to infect potential alternate hosts found in or near corn fields. By measuring the percentage of leaf tissue infected under greenhouse conditions, researchers found that *Xvv* can colonize sixteen hosts, 13 of them were symptomatic and three were asymptomatic. Symptomatic hosts included crops such as oat (*Avena sativa*) and rice (*Oryza sativa*), as well as the prairie grasses orchard grass (*Dactylis glomerata*), indiangrass (*Sorghastrum nutans*), big bluestem (*Andropogon gerardii*), little bluestem (*Schizachyrium scoparium*), timothy (*Phleum pratense*), sand bluestem (*Andropogon hallii*), green foxtail (*Setaria viridis*), and bristly foxtail (*Setaria verticillata*). This study also confirmed infection of the weeds johnsongrass (*Sorghum halepense*), shattercane (*Sorghum bicolor*), and yellow nutsedge (*Cyperus esculentus*). From all

tested species, symptoms were least severe on yellow nutsedge with 4% leaf area infected, and most severe on johnsongrass at 27% of leaf area infected. Asymptomatic hosts encompassed downy brome (*Bromus tectorum*), tall fescue (*Festuca arundinacea*), and western wheatgrass (*Pascopyum smithii*).

Field studies with these hosts found that infection in big bluestem and bristly foxtail was possible but with low incidence levels. The study by Hartman (2018) is the first estimation of *Xvv* host range, and has multiple implications for the management of bacterial leaf streak of corn. If *Xvv* persist in grass weeds from one season to the next one, management should be focused not only on corn but also on these potential hosts.

1.5 Taxonomic classification of *Xanthomonas vasicola* species

A recent overview of the different evolutionary lineages of *X. vasicola* species has divided them into five groups: 1) *X. vasicola* pv. *vasculorum* infecting corn and sugarcane, 2) *X. vasicola* pv. *holcicola* (*Xvh)* infecting sorghum, 3) *X. vasicola* pv. *musacearum* (*Xvm*) infecting enset and banana, 4) *X. vasicola* strains isolated from *Tripsacum laxum*, and 5) *X. vasicola* strains isolated from areca nut (previously *X.campestris* pv. *arecae*) (Studholme et al., 2019). The taxonomic description of the first group, where *Xvv* belongs, has been in a state of flux for over 30 years. Several changes have been made to the taxonomic nomenclature from *X. campestris* pv. *zeae* to *X. vasicola* pv. *zeae* to its current designation as *X.vasicola* pv. *vasculorum* (Bradbury, 1986; Coutinho & Wallis, 1991; Lang et al., 2017; Qhobela et al., 1990; Sanko et al., 2018). Studholme et al. (2019) stated that *X.campestris* pv. *zeae* fall within a clade of *X.campestris* pv. *vasculorum* (Cobb 1894) that belongs within the species *X. vasicola* pv. *vasculorum* (Vauterin et al., 1995).

One distinction among *Xvv* is that the sugarcane infecting strains can infect vascular tissue while corn infecting strains are limited to foliar tissue (Karamura et al., 2015). This difference in colonization resulted in the sugarcane pathogen being erroneously described as *X. axonopodis* pv. *vasculorum* (Lewis Ivey et al., 2010). Fortunately, more recent phylogenetic comparisons, through average nucleotide identity calculations and pathogenicity tests, solve this ambiguity demonstrating that sugarcane and corn *Xvv* strains are the same pathogen (Karamura et al., 2015; Lang et al., 2017; Wasukira et al., 2014).

The second group of *X. vasicola* species comprised *X.vasicola* pv. *holcicola* (*Xvh*). This xanthomonad infects sorghum (Navi et al., 2002) but is rarely found on corn (Moffett, 1983; Péros et al., 1994). Lang et al. (2017) showed that *Xvh* can cause disease on both corn and sugarcane after infiltration experiments in the greenhouse. In the same study, it was determined that *Xvv* strains from Nebraska belonged to *Xvv* and not *Xvh* as they only shared 98.6% of average nucleotide identity (ANI) with *Xvh* compared to 99.3% ANI with *Xvv* from South Africa. Although *Xvh* can cause symptoms on all tested hosts, symptoms were not as prominent as when *Xvv* was inoculated on to corn.

The third, fourth, and fifth groups include strains previously assigned to *X. campestris* (Young et al., 1991) pathovars *musacearum* and *arecae* and some strains collected from *T. laxum* grass that have not been previously assigned to species nor pathovar. Molecular sequence and biochemical data for these groups indicate that these strains are much closer to *X. vasicola* than to *X. campestris* (Studholme et al., 2019).

1.6 Predictions of bacterial leaf streak disease cycle

All infectious-disease causing agents follow a disease cycle that includes the stages of development of the pathogen and the effects of the disease on the host plants (Coakley et al., 1999). Even though no formal studies have described the disease cycle of bacterial leaf streak of corn, the following represents a description of its main points based on observations from the last three corn growing season and literature comparing BLS behavior to other foliar diseases (Figure 1.4).

Xvv may overwinter in infected residue from the previous growing season, but this has not yet been experimentally demonstrated. Since corn is often grown continuously in Colorado and in a corn-soybean-corn rotation in Nebraska (USDA-NASS, 2018b), infected residue likely is the primary inoculum for disease development. Other xanthomonads are also residue-borne pathogens. For example, *X. axonopodis* pv. *alli*, causal agent of Xanthomonas leaf blight of onion, is residue-borne (Gent et al., 2005) and has a similar distribution across the plant as bacterial leaf streak of corn.

Then, in the following growing season, as corn seedlings emerge, bacteria from the residue are splashed onto new tissue through early season rain and irrigation. After the bacterium has established its niche, it may enter the plant through natural openings or wounds. Once inside, bacteria will reproduce and cause characteristic intervenial streaks on corn leaves.

Symptoms are typically first observed on the lower leaves of the plant, and begin to appear in the mid to upper canopy usually after wind-driven rain events as the season progresses (personal communication, Tamra Jackson-Ziems & Kirk Broders). These events provide the environmental conditions for secondary inoculum formation. This is consistent with previous reports that wind-

driven rain events can disperse inoculum and aggravate diseases such as Xanthomonas leaf blight of onion (Schwartz et al., 2003). From mid to late season, bacteria may continue to spread between fields until corn is harvested and residue is left again on the surface of the soil, providing the principal inoculum for the next growing season (Figure 1.4). Aspects related to potential alternative hosts, persistence of *Xvv* on seeds, and irrigation water were not included in the predicted disease cycle because there is no evidence of these behaviors occurring in the field yet.

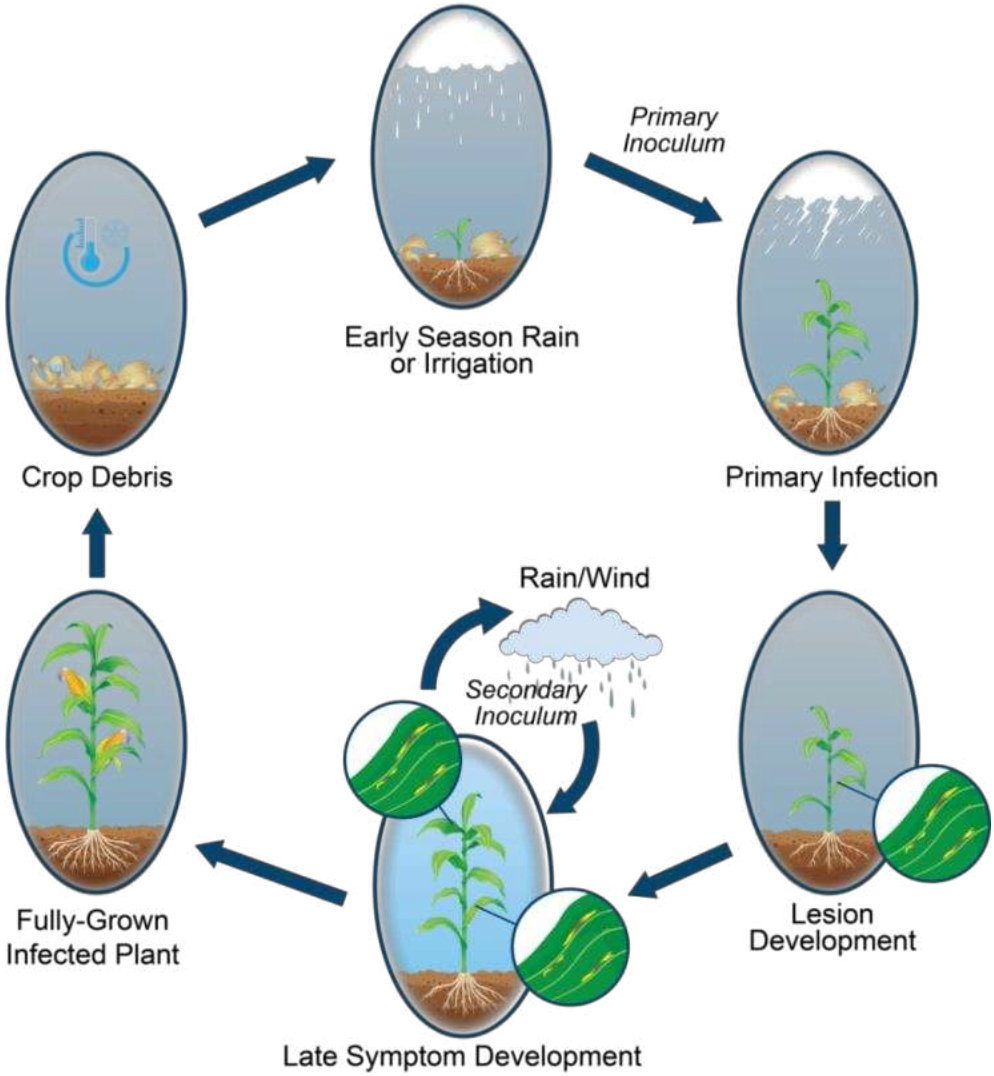

Figure 1.4: ***Predicted disease cycle of bacterial leaf streak of corn***

1.7 Thesis objectives

Due to its emergence and lack of effective mitigation methodologies, there is no doubt that bacterial leaf streak will be a challenging disease to manage in the upcoming crop production years. Even though there are a lot of questions related to this disease, the elucidation of its disease cycle is an urgent topic of study. Understanding the major aspects of the disease cycle will provide information that can be utilized by growers to make management decisions as well as preventing its further spread to other corn producing regions.

The primary goal of this research is to provide a basic understanding of the ecology of the corn bacterial leaf streak pathogen (*Xanthomonas vasicola* pv. *vasculorum*) in the United States. To this end, the specific objectives of this research are to: **1)** Characterize the infection ecology of *Xvv* on corn and determine the optimal conditions for disease development (Chapter 2). **2)** Characterize the survival process of *Xvv* across different regions in the United States (Chapter 3).

CHAPTER 2

UNDERSTANDING THE INFECTION ECOLOGY OF THE EMERGING DISEASE BACTERIAL LEAF STREAK OF CORN

2.1 INTRODUCTION

Bacterial leaf streak of corn, caused by the pathogen *Xanthomonas vasicola* pv. *vasculorum* (*Xvv*), is an emerging disease in North and South America. This disease was first reported in the United States in 2014 and became widespread in 2016 affecting grain corn, sweet corn, and popcorn (Korus et al. 2017). Currently, bacterial leaf streak of corn is present in ten states: Colorado, Kansas, Minnesota, South Dakota, Texas, Oklahoma, Wisconsin, and the top three corn-producing states of Illinois, Iowa, and Nebraska (Korus et al., 2017; Damicone et al., 2018; Jamann et al., 2019; Lang et al., 2017; Smith et al., 2018). In 2017, bacterial leaf streak was reported on corn in Argentina (Plazas et al., 2018) and in 2018 in Brazil (Leite Jr. et al., 2018). Prior to 2016, the only reports of *Xvv* infecting corn came from South Africa in 1949 (Dyer, 1949b) and 1987 (Coutinho, 1987). It is unknown how the pathogen was introduced to the Americas or if it was already present in a latent state. Due to its recent emergence and the economic importance of corn in the United States, there is a need to understand the ecology and epidemiology of this bacterial pathogen.

The most prevalent symptoms of bacterial leaf streak on corn have been reported in western Nebraska, western Kansas, and eastern Colorado (Broders, 2017). These three states and the regions of South Africa and Argentina, where the disease is also present, share the same type of

cold semi-arid climate (BsK classification). Hence, it is possible this type of climate favored the initial settlement of the pathogen in North and South America. Since then, *Xvv* has spread to other regions with higher humidity levels, like those in eastern Nebraska, Iowa and Illinois. Humid conditions that favor disease development are often observed during the summer growing season with rain and center-pivot irrigation (Lichtenber, 1989; Turkington et al., 2004). Whether in a dry or humid environment, *Xvv* has persisted on corn thanks to the microclimate formed within each field through rain or irrigation and continuous corn production systems.

Splashing water may disperse the bacterial pathogen from leaf to leaf and even to other plants. After landing on leaves, plant pathogenic bacteria will likely multiply to form aggregates that will protect them from external stressors such as desiccation, ultraviolet (UV) radiation, and nutrient limitation (Beattie & Lindow, 1995; Darsonval et al., 2008b; Lindow & Brandl, 2003). Then, under high moisture, bacteria will gain access inside the plants through natural openings, most likely through the stoma structure (Hartman, 2018).

The ability of *Xvv* to infect and colonize its host depends on the possession and use of virulence factors. Important factors that most *Xanthomonas* species use to colonize their hosts are type III effectors. These effectors are directly inserted into the host cell through the type III secretion system (Darrasse et al., 2013; Darsonval et al., 2008b). To date, the mechanisms of how *Xvv* enters and colonizes the corn plants as well as the potential effectors involved in virulence have not yet been characterized.

Xvv belongs to a group of *X. vasicola* species that affect different crops worldwide. For example, *X. vasicola* pv. *holcicola* (*Xvh*), which is the closest phylogenetic relative of *Xvv*, causes bacterial leaf streak of sorghum (Navi et al., 2002) and *X. vasicola* pv. *musacearum* (*Xvm*) is a vascular pathogen of banana and enset (Tushemereirwe et al., 2004). Recently,

Studholme et al. (2019) provided an overview of the taxonomy of *X. vasicola* species dividing it into five groups: 1) *Xvv* infecting corn and sugarcane, 2) *Xvh* infecting sorghum, 3) *Xvm* infecting enset and banana, 4) strains isolated from *Tripsacum laxum*, and, 5) strains isolated from areca nut (previously *X. campestris* pv. *arecae*).

Perez-Quintero et al. (2019) complemented this research by demonstrating that *Xvv* isolates from the United States tended to group together and seemed to be more closely related to strains from Argentina than to historic strains from South Africa. Five genomic regions were overrepresented in *Xvv* populations and one region specifically, comprising a prophage, was found to be shared with *Xvh* and North and South American *Xvv* isolates while absent in South African *Xvv* isolates and *Xvv* from sugarcane. They hypothesized that this prophage region was transferred horizontally to an ancestor of the American *Xvv* populations (Perez-Quintero et al., 2019).

Horizontal gene transfer refers to the acquisition of foreign genes in different organisms (Jain et al., 2003). By allowing the transfer of genes between related and unrelated species, horizontal gene transfer increases genetic diversity of the recipient species (Jain et al., 2003). In this context, it is possible that the prophage region present in *Xvv* isolates from the Americas contains genes that play a role in the phenotypic disease response or provide a potential fitness advantage allowing the bacterial pathogen to infect and colonize corn. Prophages have diverse effects on the bacteria harboring them in their genomes, including increased aggressiveness by enhanced transport of virulence factors, enhancement of fitness, and even decreasing virulence (Ahmad et al., 2014; Brüssow et al., 2004; Figueroa-Bossi et al., 2001; Bossi et al., 2003).

Phenotypic variation is an adaptive mechanism that may lead to increased colonization, persistence, spread, and survival of organisms (Shrestha et al., 2013). Although Perez-Quintero

et al. (2019) showed the variability of *Xvv* at the genome level, it is unknown whether or not the horizontally transferred region had an effect on the bacterial leaf streak disease response.

Increasing fitness is another potential effect of gene transfer on bacterial pathogens (Darsonval et al., 2008a). Fitness is described as the ability of bacteria to replicate and survive under a given environmental condition (Pope et al., 2010). The growth rate of bacteria in culture medium is a commonly used model for evaluating fitness as a measure of reproductive potential (Pope et al., 2010; Bennett et al., 1990; Lenski et al., 1994; Nguyen et al., 1989). For example, HrpG and HrpX proteins increased replication and survival capabilities in *X. fuscans* subsp. *fuscans*, which in turn, enabled higher transmission rates for the pathogen that causes bacterial blight of bean (Darsonval et al., 2008b).

Moreover, Perez-Quintero et al. (2019) also found another region in *Xvv* that contains eight genes found in *Pantoea ananatis.* This may be of importance as *P. ananatis* has been found in corn leaves infected with *Xvv* (Lang et al., 2017), but its role in disease development has not yet been explored. It is possible that *Xvv* and *P. ananatis* have either established an interaction to act as a disease complex and infect corn plants at a higher rate, or are antagonistic to each other. It is also possible that *P. ananatis* has no effect on bacterial leaf streak disease. Establishment of this relationship is important because in some cases, *P. ananatis* has been proven to be beneficial to the host by having antimicrobial activity (Coutinho & Venter, 2009; Iimura & Hosono, 1996) or by inducing systemic resistance (Kang et al., 2007). In other cases, *P. ananatis* has been described as an unconventional pathogen infecting eucalyptus in South Africa (Coutinho & Venter, 2009). Therefore, the interaction between these bacteria may lead to solutions for reducing the occurrence of this new disease or to further research into whether there is a complex of two pathogens affecting corn.

Although genetic analyses proposed important differences between American and South African *Xvv* strains as well as the acquisition of genes from *Xvh* and *P. ananatis*, there are no experimental studies focused on the correlation of genetic and phenotypic information related to bacterial leaf streak of corn. In this research, we aimed to describe several aspects of the infection ecology of emerging *Xvv* populations on corn in the United States and Argentina, in comparison to isolates of *Xvv* from South Africa, putative genome donor *Xvh*, and interaction with *P. ananatis*. This research was guided by the following objectives: 1) determine how *Xvv* enters, colonizes and multiplies in the corn leaf, 2) characterize the phenotypic diversity among isolates of *Xvv* and *Xvh* on corn, sorghum, and sugarcane, 3) evaluate fitness characteristics of representative *Xvv* and *Xvh* isolates, and 4) estimate the effect of *P. ananatis* on the ability of *Xvv* to infect and colonize corn leaves.

2.2 MATERIAL AND METHODS

2.2.1 Isolation and molecular identification of isolates causing bacterial leaf streak of corn

Isolation:

Isolation of *Xvv* from corn leaves was performed as in Lang et al. (2017) with minor modifications. Instead of placing the tissue in distilled water, fresh tissues were dissolved in 1 mL of 10 mM $MgCl_2$, macerated with sterile pellet pestle and incubated for at least 1.5 h at room temperature. For bacterial isolation, one loop-full (10 µL) of solution was spread onto nutrient agar (NA). Plates were incubated at 28ºC for two days. Single characteristic bright yellow colonies were selected, and re-streaked for further isolation until pure colonies were obtained. Samples from United States were collected across several fields in Colorado, Iowa, Kansas and

Nebraska. *Xvv* isolates from Argentina were collected from fields located in San Luis, Córdoba, and Santa Fe provinces and were a kind gift from our collaborator Maria Cristina Plazas at the Universidad Católica de Córdoba, Argentina.

Molecular identification:

For colony PCR, a single colony from NA was suspended in 10 µL of sterile water and boiled at 95ºC for 5 min. Molecular identification of *Xvv* was performed following one of these procedures: first, colony PCR of suspected *Xvv* samples were performed using diagnostic Xvv3 or Xvv5 primers as described by Lang et al. (2017). To further confirm isolates identity, the 16S rRNA gene and a central metabolism gene, *atpD* (ATP synthase β chain), were sequenced.

PCR reactions for 16S rRNA (50 µL) contained 2 µL of boiled DNA template, 0.2 µM of each primer (Table 2.1), 1X GoTaq reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 0.25 unit/µL GoTaq DNA polymerase enzyme (Promega). The cycling conditions were as follows: initial denaturation at 94ºC for 3 min, following 35 cycles of 94ºC for 45 s, 50ºC for 1 min and 72ºC for 1:30 min, and the final extension period at 72ºC for 10 min. PCR fragments were separated in a 1.5% agarose gel for 45 min at 90 V, and fragments were extracted and purified using the DNA clean & concentrator kit (ZYMO Research). Sequencing was performed with 5 ng/µL of each PCR product at Quintara Biosciences (Fort Collins, CO) and analyzed using Geneious software (version 10.0.7). Sequence identities to the genus level were determined using BlastN from the NCBI database.

Genomic DNA for the *Xanthomonas* positive samples was extracted using Easy-DNA kit (Invitrogen) and PCR amplification of the *atpD* gene was carried out for further confirmation to the species level. PCR reactions (40 µL) contained 25 ng/µL of DNA template, 0.4 µM of each primer (Table 2.1), 1X GoTaq reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.1 unit/µL

GoTaq DNA polymerase enzyme (Promega). Cycling conditions were performed as described by Fargier et al. (2011). Furthermore, molecular identification with real-time PCR was performed with all positive *Xvv* isolates to confirm the protocol designed by Stulberg et al. (2019).

Table 2.1: Description of primers used for the molecular identification of *Xvv*

Target gene	**Primer name**	**Primer Sequence (5`-3`)**	**Product size (bp)**	**Ta* (ºC)**
16S rRNA	515-F	GTGCCAGCMGCCGCGGT	290	50
	806-R	GGACTACHVGGGTWTCTAAT		
ATP synthase β chain (atpD)	atpD-F	GGGCAAGATCGTTCAGAT	512	60
	atpD-R	GCTCTTGGTCGAGGTGAT		

Ta*: annealing temperature

2.2.2 Transformation and conjugations of *Xvv* with fluorescent proteins

Transformation and conjugations of *Xvv* were performed using reference isolate CO-5. CO-5 was grown overnight at 28ºC, 220 rpm in nutrient broth media (NB, 5 mL). Cells were harvested at 4000 rpm for 10 min and subsequently washed with cold, sterile water four times. After the final wash, cells were resuspended in 100 µL of water and incubated on ice for 30 min. Electrocompetent cells were transformed with 250 ng of plasmid GFP pJN105 (Leach Lab Collection) containing gentamycin resistance gene. Cells were recovered for three 3 h at 28ºC, 220 rpm and 10 µL were plated into nutrient agar with respective antibiotic. Transformants were allowed to grow for two days at 28ºC and one fluorescent single colony was selected for further isolation.

For conjugations through bi- and tri-parental mating, CO-5 was grown overnight at 28ºC, 220 rpm in NB (5 mL) while *Escherichia coli* Rho3 pTNS3 (Ampicillin resistance: 2,6-Diaminopimelic Acid (ApR: DAP)) (Choi et al., 2005), *Escherichia coli* Rho3 mCherry

(Spectinomycin resistance: 2,6-Diaminopimelic Acid (SpecR; DAP)) (Bartetzko et al., 2009), and *Escherichia coli* pNEO GFP (Kanamycin resistance (KmR)) (Chicaybam et al., 2017) were grown at 37ºC, 220 rpm in Luria Bertani media (LB, 5 mL). The next day, 1.5 mL of CO-5 was spun down at 8000 rpm for 10 min; the supernatant was removed carefully without suspending the pellet. Next, 250 µL of *E. coli* Rho3 pTNS3 and 250 µL of *E. coli* Rho3 mCherry were added to the CO-5 pellet. This was the mating step in which the Tns3 transposase helped introduce the mCherry gene into the genome of CO-5. In addition, *E.coli* pNEO GFP was added to a second CO-5 pellet. Each solution was spun down at 8000 rpm for 10 min. The supernatant was again carefully removed and each pellet was resuspended in 250 µL of NB. Cells were plated on NB plus DAP but without antibiotics and grown at 28ºC overnight. After the overnight incubation, *E. coli-Xvv* mixture were scraped with 250 µL of NB and placed into NA plates with appropriate antibiotics, either spectinomycin for mCherry or kanamycin for GFP. Finally, conjugants were grown at 28ºC for four days and two fluorescent colonies were chosen for further experiments.

2.2.3 Growth curves in culture and *in planta* of fluorescent *Xvv*

Growth curves in culture were performed with three independent replicates of CO-5 wildtype, one colony of CO-5 pJN105, two colonies of CO-5 mCherry (mch1 and mch2), and two colonies of CO-5 GFP (GFP1 and GFP2). Colonies were grown overnight in NB (5 mL) with respective antibiotics. The next day, cell suspensions were pelleted for 10 min at 4500 rpm. Cell pellets were then washed three times with sterile water at 4500 rpm for 5 min. Pellets were resuspended in 5 mL of sterile water and bacterial concentration was adjusted to 10^8 CFU/mL. Serial dilutions were performed until obtaining 10^4 CFU/mL and 200 µL of each sample were loaded by triplicate in a flat bottom 96-well plate (Corning Incorporated). The plate was sealed

with breathable film (Breathe-easy, Diversified Biotech) and placed into microplate spectrophotometer (Power Wave HT, BioTek). This instrument measured the optical density (OD) at 600 nm and 630 nm every hour for 72 h with a constant temperature of 28ºC.

For the growth curve *in planta*, each strain was grown to 10^8 CFU/mL. Then, they were inoculated by infiltration into healthy three-week-old corn plants at five points in each leaf. At 0, 24, 72, 120, and 168 h the infected tissues were collected, surface sterilized with 10% bleach for 30 s, and rinse with sterile water three times. Infected tissues were placed into 2 mL tubes containing one stainless steel bead (5 mm, QIAGEN) and 1 mL water. Then, solution tubes were lysed for 1 min and serially diluted to 10^{-8}. From here, 5 µL of each dilution was plated in nutrient agar and grown at 28ºC for 24 h. After each time point, colonies were counted under light microscope and CFU/mL was plotted against time.

For statistical analyses, growth curves were fitted to a nonlinear logistic growth model using self-starting function in R (version 3.5.2), SSlogis. Each fitted curve provided three parameters: Asym, Xmid, and Scal. Out of those, Xmid and Scal were biologically relevant for this study. This is because Xmid represents the time of fastest bacterial multiplication (inflection point) and Scal corresponds to the inverse of the slope, which is an indirect measure of the growth rate at Xmid value. After calculating the inverse of Scal (1/Scal), the growth rate during exponential phase was determined for each curve (Zwietering et al., 1990; Hall et al., 2014). Pairwise comparisons were done with the gnls function from the nlme package in R (version 3.5.2) using CO-5 as reference strain. Each of the growth curves represents the average of three independent replicates per strain. Independent replicates were achieved with the average of three technical replicates in each sample.

2.2.4 Confocal laser scanning microscopy

Spray inoculations were performed on three week-old popcorn plants as described by Hartman (2018). Fluorescence in epidermal cells of the abaxial corn leaf side was assessed after three days post inoculation. Three pieces of infected leaves (2 cm) showing water soaking streak symptoms were sampled and mounted in water for observation under the microscope. In these experiments an Olympus IX81 Inverted Confocal laser scanning Microscope (Olympus, Microscopy Solutions) was used, and images are displayed as maximum projections of picture stacks. GFP and mCherry were excited with a 20 mW laser at 488 and 559 nm respectively, and the emission filter wavelengths were 497 to 526 nm for GFP and 570 to 615 nm for mCherry.

2.2.5 Disease phenotyping of *Xvv* on corn, sorghum, and sugarcane

Corn hybrids (Pioneer P0302, Pioneer P1151, and popcorn), sorghum (Super Sugar), and sugarcane (L-99-226) were grown in a 1:1 mix of Promix-BX Biofungicide + Mycorrhizae (Quakertown, PA) under greenhouse conditions (28ºC ± 1 ºC, 16 h day length, and 80% relative humidity). Bacterial isolates were cultured in peptone sucrose agar (PSA) for 24 h at 28ºC and then suspended to 10^8 CFU/mL in sterile, distilled water. Three weeks after planting, plants were infiltrated as described by Lang et al. (2017). Five *Xvv* isolates, three from Colorado and two from Iowa, were inoculated by triplicate into two grain corn varieties (P1151, P0302 (Dupont Pioneer)) and one popcorn variety. To complement this study, inoculations by infiltration on corn, sorghum, and sugarcane were performed with 25 *Xvv* isolates and one *Xvh* strain (Table 2.2). Two leaves were inoculated on at least seven individual plants, except with sugarcane, where only three individual plants were inoculated by isolate. Sterile, distilled water was used as a negative control in all inoculations. Infiltration experiments were repeated three times and data

was combined to perform statistical analysis. Quantification of the lesion length was done by measuring the expansion distance beyond the infiltration site at seven days post inoculation (dpi).

For statistical analysis, a one-way ANOVA using appropriate transformation to each cases were performed in R (version 3.5.2) with the lm and anova functions. For the analysis including three corn varieties, Log (0.5+ lesion length) transformation was done to satisfy ANOVA assumptions with two predictors (isolate, variety) and one response (lesion length). Analysis of individual corn hybrids inoculations was done with square root transformation. All pairwise comparisons were done using emmeans function from the emmeans package with Tukey adjustment test (HSD, honestly significant difference) and $\alpha = 0.05$.

Table 2.2: Description of isolates of *Xvv* and *Xvh* used for leaf inoculations on corn, sorghum, and sugarcane

Bacteria Identity	Lab Name	Original name	Location	State	Country	Year
Xvv	Arg-1A	M10	Adelia María	Córdoba	Argentina	2016
Xvv	Arg-2B	M017	Rosario	Santa Fe	Argentina	2015
Xvv	Arg-3B	M062	Villa María	Córdoba	Argentina	2017
Xvv	Arg-4A	M088	Villa Mercedes	San Luis	Argentina	2017
Xvv	Arg-5B	M152	Monte de los Gauchos	Córdoba	Argentina	2017
Xvv	Arg-6B	M162	Villa María	Córdoba	Argentina	2017
Xvv	Arg-7A	M188	Villa Mercedes	San Luis	Argentina	2017
Xvv	CO-5	16-CO-044-4	Phillips	Colorado	United States	2016
Xvv	CO-6	16-CO-044-2	Phillips	Colorado	United States	2016
Xvv	CO-8	16-CO-044-3	Phillips	Colorado	United States	2016
Xvv	IA-2	Xv-Har 1-2	Harcourt	Iowa	United States	2016
Xvv	IA-3	Xv-Har 1-3	Harcourt	Iowa	United States	2016
Xvv	KS-1	201600004	Finney	Kansas	United States	2013
Xvv	NE-1	201600027B	Lincoln	Nebraska	United States	2016
Xvv	NE-11	201600332	Wayne	Nebraska	United States	2016
Xvv	NE-3	201600380	Red Willow	Nebraska	United States	2016
Xvv	NE-4	201600257	Harlan	Nebraska	United States	2016
Xvv	NE-5	201600118	Keith	Nebraska	United States	2016
Xvv	NE-7	201600018	York	Nebraska	United States	2016
Xvv	NE-8	201600058	Phelps	Nebraska	United States	2016

Xvv	NE-9	201600029	Dixon	Nebraska	United States	2016
Xvv	SAM 113	SAM 113	Klerksdorp	North West	South Africa	1988
Xvv	SAM 118	SAM 118	Klerksdorp	North West	South Africa	1988
Xvv	SAM 119	SAM 119	Klerksdorp	North West	South Africa	1988
Xvh	Xvh 482	Xvh-L	Lancaster	Nebraska	United States	2016

2.2.6 Effect of temperature on growth rate of *Xvv* and *Xvh*

Growth curves in culture of seven *Xvv* isolates (CO-5, NE-3, Arg-3B, Arg-7A, SAM 113, SAM 118, SAM 119) and one *Xvh* isolate (Xvh 482) were performed as described in section 2.2.3 under three different temperatures: 22ºC, 28ºC, and 34ºC. Absorbance measurements (OD at 600 nm and 630 nm) were taken every hour for 48 h at 28ºC and 72 h for temperatures 22ºC and 34ºC with continuous shaking in the middle speed setting of the microplate spectrophotometer (Power Wave HT, BioTek).

Statistical analyses were done with a subset of the data, until 48 h, to first ensure a logistic model could fit all the data without considering values at which the absorbance decrease due to cell death and second, to have consistency among all curves. Statistical analyses were done as described in section 2.2.3 using 28ºC and CO-5 as reference groups for temperature and isolate, respectively.

2.2.7 Interaction between *Pantoea ananatis* and *Xvv in vitro* and *in planta*

Molecular identification of *Pantoea ananatis*:

To test the role of endophytic bacteria, *Pantoea ananatis,* on bacterial leaf streak disease progression, a positive identification of *P. ananatis* was first performed to differentiate these bacterial species from all other *Pantoea* species isolated from the corn fields. PCR using *gyrB* gene (DNA gyrase, RNA polymerase β subunit) was carried out according to Brady et al. (2008) with minor modifications. Each PCR reaction (50 µL) contained 2 µL of DNA template, 0.3 µM

of each primer (gyrB 01-F, gyrB 02-R), 1X GoTaq reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.25 unit/µL GoTaq DNA polymerase enzyme (Promega). The cycling conditions were as described by Brady et al. (2008) with 50ºC as annealing temperature instead of 55ºC. PCR fragments were separated in a 1.5% agarose gel for 45 min at 90 V, and fragments were extracted and purified using the DNA clean & concentrator kit (ZYMO Research). Sequencing was performed with 5 ng/µL of each PCR product at Quintara Biosciences (Fort Collins, CO) and analyzed using Geneious software (version 10.0.7). Sequence identities to the genus level were determined using BlastN from the NCBI database. Positive strain CO-2 (referred to as *P. ananatis*), was chosen for further experiments.

Co-inoculations of *Xvv* and *P. ananatis* on corn

Co-inoculation experiments were performed with *Xvv* (CO-5 reference isolate) and *P. ananatis*. Popcorn was grown as described in section 2.2.5. Bacterial strains were cultured in PSA for 24 h at 28ºC and then suspended to 10^8 CFU/mL in sterile, distilled water. Equal quantities of both bacteria were mixed for co-inoculation. Three week-old plants were infiltrated as described by Lang et al. (2017) using ten individual plants per each treatment (*Xvv*, *P. ananatis*, *Xvv* + *P. ananatis*). Sterile, distilled water was used as a negative control in all inoculations. Infiltration experiments were repeated three times and data was combined to perform statistical analysis.

Statistical analysis was done in R (version 3.5.2) using two-sample t-test (t.test function) of 1/(Lesion Length + 0.5) by strain (α=0.05). Transformation was performed to satisfy two-sample t-test assumptions of normality of data and equality of variances. *P. ananatis* measurements were not included in the analysis since this bacterium never resulted in disease symptoms.

Spray inoculations were also performed with strains mentioned above. Bacterial suspensions were sprayed (~15 mL per plant) onto nine popcorn plants per each treatment. Plants were left under high relative humidity (>90% relative humidity) for 48 h in growth chamber and then transferred to standard greenhouse conditions (30 ± 1ºC, 16 h day length). Symptom development was assessed seven days post inoculation and severity was ranked by counting the number of leaves infected over the total number of leaves on a plant. Statistical analysis was done using two-sample t-test (t.test function) of severity by strain ($\alpha = 0.05$) in a completely randomized design.

Inhibition experiments and growth curves of *Xvv* and P. *ananatis*

To test inhibition of *Xvv* by *P. ananatis* or vice versa, both bacterial strains were grown on NA plates. Three scenarios were set up: in the first scenario, *Xvv* was grown with a streak until the middle of the plate and *P. ananatis* on the other half of the plate. In the second *Xvv* (100 µL) was spread onto the entire plate and then 10 µL of *P. ananatis* was placed into the center of the plate. Third scenario was the opposite of the second one, where *P. ananatis* was spread on the entire plate and 10 µL of *Xvv* was placed in the center of the plate. Growth of each bacterium was monitored for 24 h at 28ºC. Individual growth curves of *Xvv* and *P. ananatis* were performed in NB medium at 28ºC for 72 h as described in section 2.2.3

2.3 RESULTS

Three *Xvv* isolates were recovered in 2016 (CO-5, CO-6, and CO-8) from different plants across the same location in Phillips, Colorado. The isolation from surveyed fields was first done by using the protocol designed by Lang et al. (2017). The recovery of *Xvv* from field corn leaves

was improved by 54% when using $MgCl_2$ instead of water. One issue with the isolation method was that the bacteria from the genus *Pantoea* were isolated at a high frequency instead of *Xanthomonas*. These two genera were confused as they possess similar morphology with bright yellow colonies when grown in general media. From the isolates recovered from the 140 survey samples collected, at least 40 were *Pantoea* spp.

Xvv isolates were identified using the Xvv3 and Xvv5 diagnostic assay (Lang et al., 2017) and all isolates were verified by sequencing the *atpD* gene (Fargier et al., 2011). Moreover, several of our collaborators at Iowa State University, University of Nebraska-Lincoln, University of Illinois, and Universidad Católica de Córdoba (Argentina) sent putative *Xvv* isolates for diagnostic confirmation. Following the previous procedure, 40 *Xvv* positive isolates were obtained, 32 of them from five different regions of the United States and seven from three provinces across Argentina (Table 2.3). Additionally, Koch's postulates were completed for the Colorado and Argentina isolates on corn to confirm the emergence of this pathogen in both countries.

Table 2.3: Isolation of *Xvv* across different corn fields in United States and Argentina

Country	**Location**	**Number of *Xvv* isolates**
United States	Colorado	17
	Nebraska	10
	Iowa	2
	Illinois	2
	Kansas	1
Argentina	Córdoba	4
	Santa Fe	2
	San Luis	2

Localization of *Xvv* at the plant cellular level and the possible entry/exit mode from the corn plant was assessed through confocal laser microscopy. To this end, constructs of the bacteria with two different reporter molecules, green fluorescent protein (GFP) (Chalfie et al., 1994;

Chicaybam et al., 2017) and red fluorescent protein (mCherry) (Bartetzko et al., 2009) were developed. Out of those, five transformants, CO-5 pJN105, CO-5 GFP1, CO-5 GFP2, CO-5 mch1, and CO-5 mch2, were confirmed to possess the fluorescent reporters.

Growth curves in culture media and *in planta* were done to compare the fitness of the new transformants to the wildtype CO-5 strain. While all strains had the distinctive growth stages in culture (lag, exponential, and stationary phases) (Figure 2.1A), it was harder to distinguish these phases in the growth curves from the inoculated plants (Figure 2.1B). Based on the growth curves in culture medium, the CO-5 mch1 and CO-5 mch2 strains behaved the most similarly to the wildtype CO-5. This information was supported as the average for the parameters Xmid (time of fastest multiplication) and Scal (inverse of growth rate) of CO-5 mch1 and CO-5 mch2 were not significantly different from wildtype CO-5 (p-values > α=0.05 in Table 2.4). Curves corresponding to the other strains grew slower, spending approximately six more hours in the lag phase than wildtype CO-5.

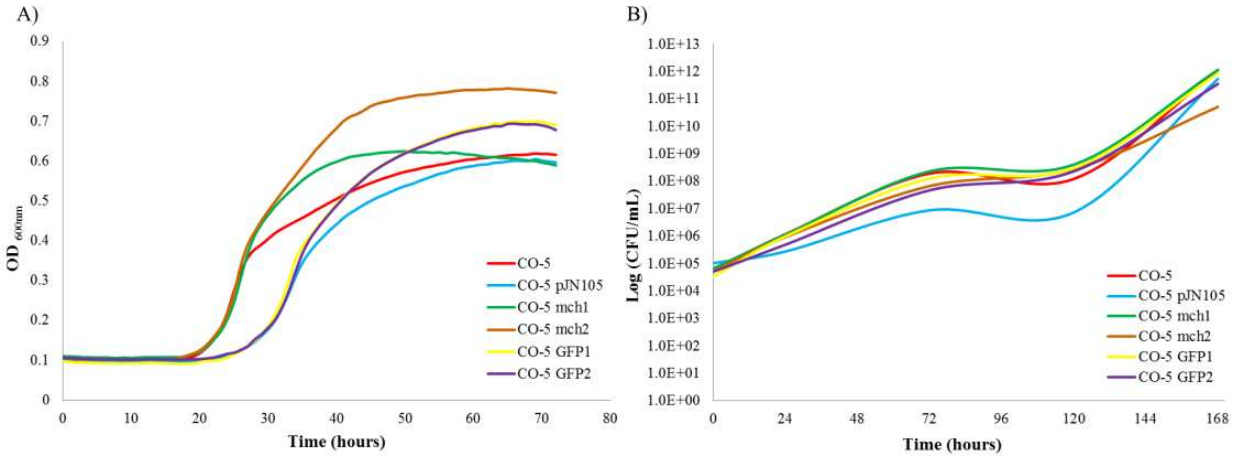

Figure 2.1: ***Growth curve of* Xvv *and fluorescent strains in culture and in planta***. *Growth curves of* Xvv *(CO-5 (red)) and fluorescent strains (CO-5 pJN105 (blue), CO-5 mch1 (green), CO-5 mch2 (brown), CO-5 GFP1 (yellow), CO-5 GFP2 (purple)) were performed either in culture (A) or inside corn plants (B). Each curve represents the average of three independent replicates. In both panels, x-axis represents the time (hours) at which each isolate was grown and y-axis shows either optical density (OD_{600nm}) at 600 nm (A) or Log (CFU/mL) (B).*

Table 2.4: Average parameters and p-values of parameters for growth curves of *Xvv* fluorescent strains in culture

Isolate	Xmid	p-value Xmid CO-5 vs strain	Scal	p-value Scal CO-5 vs strain
CO-5	26.91	-	8.27	-
CO-5 pJN105	55.16	0.0491	15.01	0.0080
CO-5 mch1	26.71	0.8950	6.90	0.1920
CO-5 mch2	29.02	0.1855	7.3	0.3433
CO-5 GFP1	43.61	0.0012	11.21	0.0687
CO-5 GFP2	54.15	0.0156	13.86	0.0073

Additionally, for the bacterial growth curves inside the plant the lag phases were indistinguishable for all isolates and the exponential phases for the majority of the strains, except for CO-5 pJN105, had similar growth rate (p-values > α= 0.05). The stationary phases were also observed in all the evaluated samples but no death phase was present; instead a second exponential phase started. Due to the similarity in fitness of the strains compared to the wildtype CO-5 in culture and *in planta*, it was decided to conduct all downstream experiments with CO-5 mch1 and CO-5 GFP1. Although CO-5 mch1 behaved more similarly to the wildtype than CO-5 GFP1, they were both included to account for the autofluorescent of corn plants when using confocal microscopy.

To simulate natural field infection, selected transformants were sprayed inoculated onto corn under greenhouse conditions. After four days, water soaking symptoms were noticeable and a portion of each lesion, about 2 cm, was observed under a confocal microscope. Images, under green and red lasers respectively, showed that the bacteria surround the plant cells in the apoplast and they did not move across the xylem vessels (Figure 2.2A-B). Moreover, the bacteria were found next to the veins and not in the vascular tissue.

Upon observing a stoma, it appeared bacteria were more clustered at the stomatal opening (Figure 2.2C). This could be possible due to an increase bacterial multiplication around the

opening of the stoma, exit from the interior of the stomatal chamber, or even just random chance. Since we did not captured bacteria moving and stomata were not found opened, the entrance and exit events were not caught in images. It is likely that from the time of lesion excision from the plant to imaging, leaves were exposed to temperature and humidity changes that promoted closure of natural openings in order to maintain leaf viability.

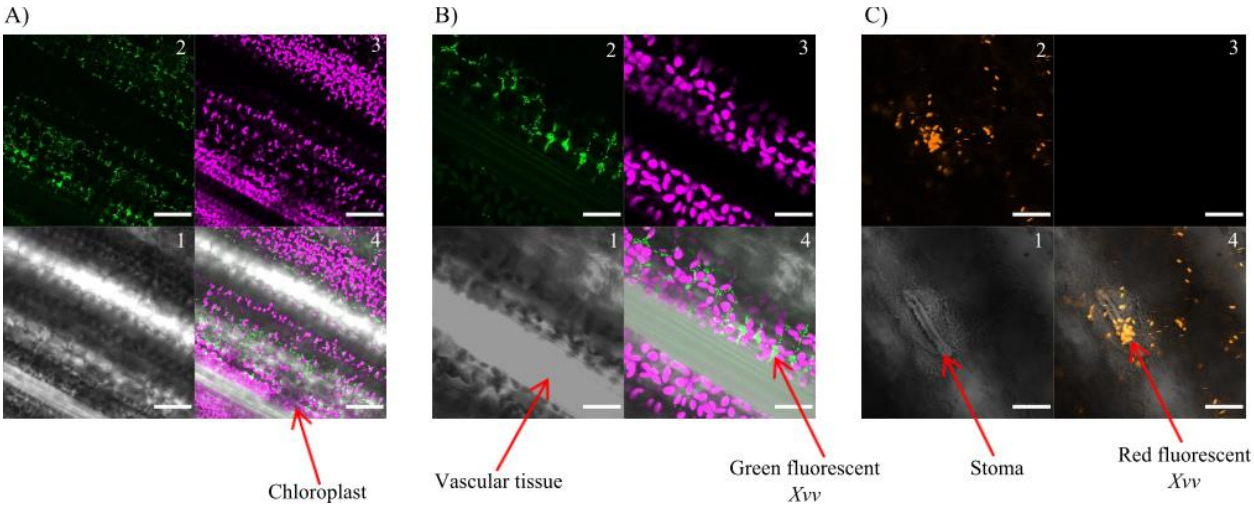

Figure 2.2: ***Images of fluorescent*** **Xvv** ***inside corn plants***. *Plant leaf images were captured with confocal laser scanning microscope (Olympus IX81 inverted) 72 h post inoculation, and fluorescent channels were scanned sequentially. Chloroplasts of plant cells are shown in purple, green fluorescent* Xvv *(CO-5 GFP1) are shown in green (A and B), and mCherry fluorescent* Xvv *(CO-5 mch1) are shown in orange (C). Panel 1 in each image represents the negative control without any fluorescence, panel 2 shows fluorescence by bacteria strains, panel 3 shows chloroplasts, and panel 4 is the merged view of the fluorescence channels without chlorophyll autofluorecence. The scale bar represents 100 pixels (26.5 mm).*

Variation in phenotypic disease response caused by *Xvv* positive isolates was evaluated using an infiltration inoculation method. This method was chosen as it provided a more consistent way to quantify the lesion size caused by the pathogen. Disease symptoms were quantified seven days post inoculation by measuring the expansion length outside of the infiltration site. The first analysis of *Xvv* aggressiveness levels were carried out with Colorado and Iowa strains, CO-5, CO-6, CO-8, IA-2, and IA-3, after inoculation with a suspension of 10^8 CFU/mL. From these

inoculations, bacterial ooze was observed coming out of the infected leaf tissue generally four days after inoculation and lesions became dry three days after that (Figure 2.3A).

Statistical analysis comparing the means of each isolate demonstrated a significant interaction of isolate by variety (F-statistic= 3.1343, p-value = 0.01) (Figure 2.3B). In general, P1151 was the most susceptible variety except when inoculated with isolates CO-6 and IA-2. These strains were recovered from the same fields as the other isolates, CO-5 and IA-3, in Colorado and Iowa, respectively. CO-8, and IA-3 were more aggressive than CO-6 and IA-2 across all corn types. Pairwise comparisons across all isolates and varieties showed that CO-5 was significantly more aggressive on P1151 than when inoculated on P0302 (Figure 2.3B).

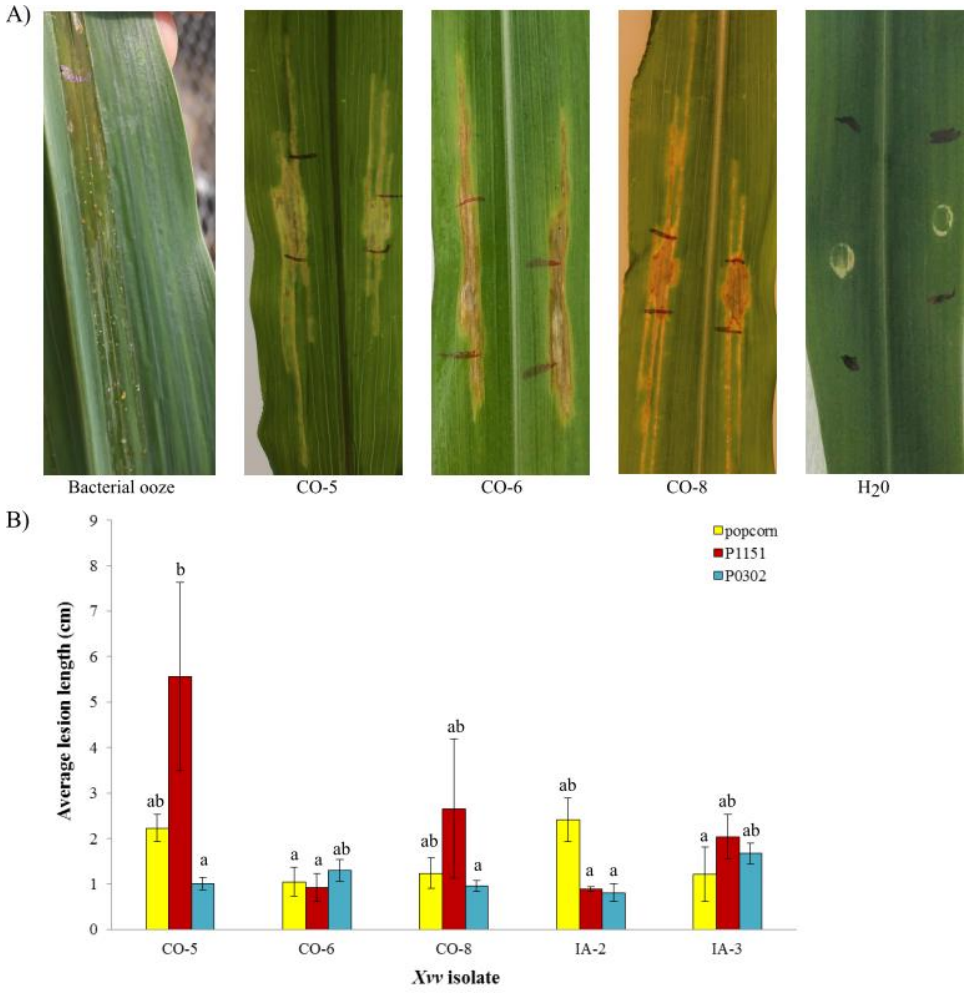

Figure 2.3: ***Phenotypic diversity of bacterial leaf streak on corn.*** *A) Symptom development of* Xvv *four (bacterial ooze) and seven (CO-5, CO-6, CO-8) days post inoculation. H_2O was used*

as negative control for the experiment. B) Disease caused by Xvv *with isolates from Colorado (CO-5, CO-6, CO-8) and Iowa (IA-2, IA-3) on popcorn (yellow) and two grain corn varieties (P1151 (red), P0302 (blue)). Leaves of three-week old plants were infiltrated with each strain at* 10^8 *CFU/mL, and disease was assessed at seven days post inoculation. Lesion lengths indicate expansion beyond the infiltration site. Letters designate significance at p-value* $<$ *0.01 of the interaction of isolates by varieties.*

To complement studies done on genotypic diversity of bacterial leaf streak pathogen (Perez-Quintero et al., 2019), the disease phenotypes of *Xvv* isolates from United States, Argentina, and South Africa were assessed. Disease phenotyping on P1151 showed that contemporary isolates from Argentina and United States tend to cause more severe symptoms than older *Xvv* isolates from South Africa and contemporary *Xvh* (Figure 2.4 top panel). Although the majority of isolates did not have significantly different disease levels, isolates Arg-4B and Arg-5B caused significantly more disease than SAM 113.

Disease response on popcorn showed lower aggressiveness levels for American *Xvv* isolates than for South African strains (Figure 2.4, bottom panel). On popcorn, isolate SAM 119 was the most aggressive among all other tested samples. The statistical significance analyses were performed with each variety separately, however, when all the data were combined, there was a strong interaction of isolate by variety (p-value $< 2.2*e^{-16}$). Intriguingly, isolates of *Xvv* recovered from the same location on different plants, varied in disease severity. This was the case for CO-5 and CO-6 inoculated on popcorn and Arg-4A and Arg-7A inoculated on P1151. CO-6 was the least aggressive isolate among all tested and Arg-4A was more aggressive than Arg-7A.

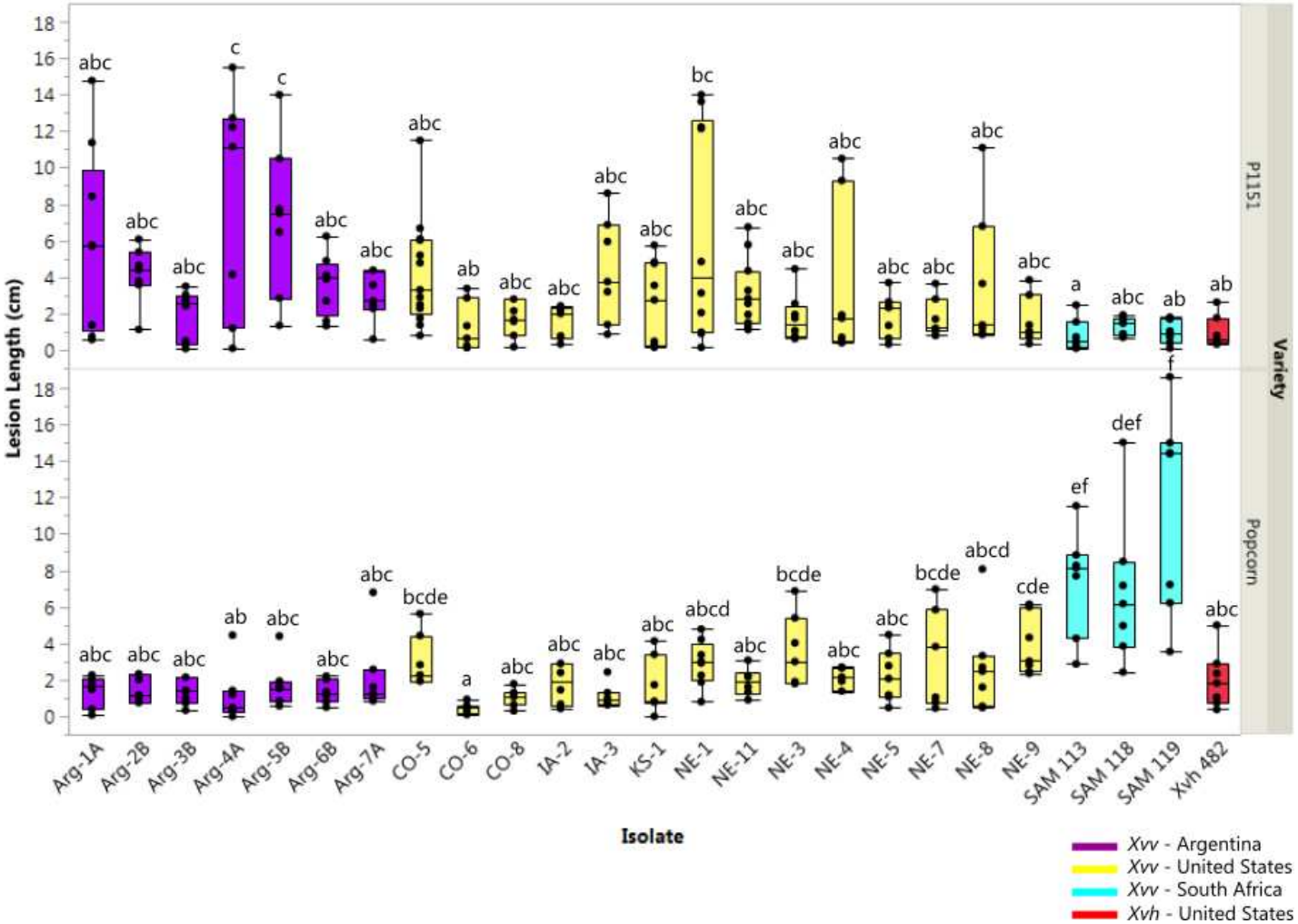

Figure 2.4: ***Phenotypic characterization of*** **Xvv** ***and*** **Xvh** ***on corn.*** *Disease caused by* Xvv *and* Xvh *on corn hybrids P1151 (top panel) and popcorn (bottom panel). Three week-old plants were infiltrated with* 10^8 *CFU/mL of each isolate, and disease was assessed at seven days post inoculation. Lesion length (cm) indicates expansion beyond the infiltration site. The entire experiment was replicated three times and combined data from all replications is shown here. Letters designate significance at p-value <0.0001 using square root transformation of lesion length per each variety and sample size of at least seven replicates per isolate (90% statistical power).*

Disease phenotypes on sorghum and sugarcane were similar to results obtained by Lang et al. (2017). When *Xvv* and *Xvh* isolates were inoculated on sorghum (Figure 2.5), characteristic symptom development for *Xvh* isolate (Xvh 482) was observed, but little to no lesion progression occurred with *Xvv* isolates. Lesions were confined to the point of inoculation for most of the cases and isolate Xvh 482 was significantly different from all *Xvv* samples. However, bacterial ooze was observed on all leaves inoculated with *Xvv*. All samples on sugarcane showed a red coloration with water soaking but no lesion expansion in any of them (data not shown).

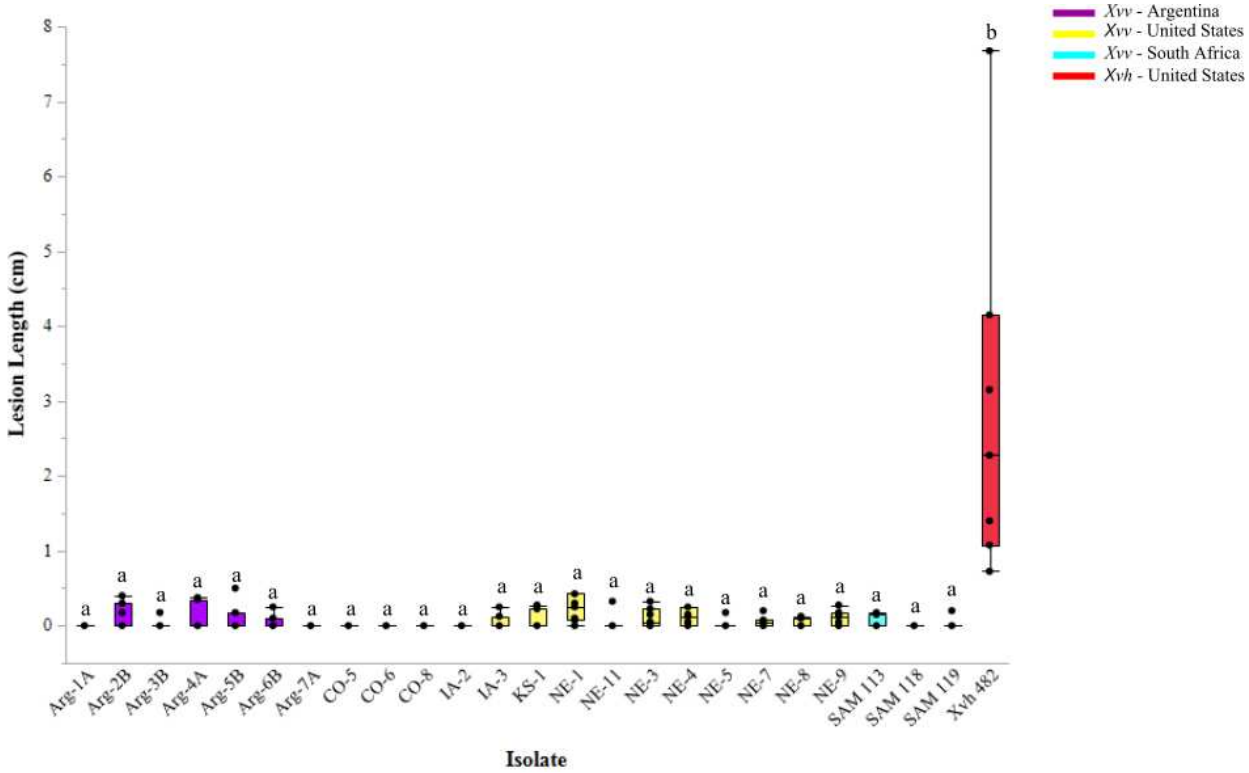

Figure 2.5: ***Phenotypic characterization of*** **Xvv** ***and*** **Xvh** ***on Super sugar sorghum****. Three week-old plants were infiltrated with 10^8 CFU/mL of each isolate, and disease was assessed at seven days post inoculation (dpi). Lesion length (cm) indicates expansion beyond the infiltration site. Letters designate significance at p-value <0.0001 using square root transformation of lesion length and sample size of seven replicates per isolate (90% statistical power).*

All the inoculations by infiltration presented high level of variation. Some of the issues that may have affected the results include: inoculation on one side of the leaf resulted in a long lesion length while lesion on the other side of the mid-vein did not have a significant expansion (Figure

2.6), inoculation on one leaf with both lesions expanding but on the other leaf, in the same plant, did not, and some leaves have minimal lesion expansion but significant amount of bacterial ooze so they could potentially infect other plants.

Figure 2.6: ***Inoculation by infiltration of*** **Xvv** ***on corn.***

To determine whether or not the proposed horizontal gene transfer event from *Xvh* to American *Xvv* strains (Perez-Quintero et al., 2019) provides a fitness advantage, eight representative isolates were analyzed under three different temperatures. To this end, growth curves in culture from 0 to 48 h were performed at 22ºC, 28ºC and 34ºC (Figure 2.7). These temperatures corresponded to high and low averages during the summer growing season. After statistical analysis, two biologically relevant parameters were assessed: time of fastest multiplication (Xmid) and inverse of growth rate at Xmid value (Scal) (Table 2.5). It is relevant to note that the coefficients for strain Xvh 482 at 34ºC were not calculated because the curve did not reach stationary phase after 48 h.

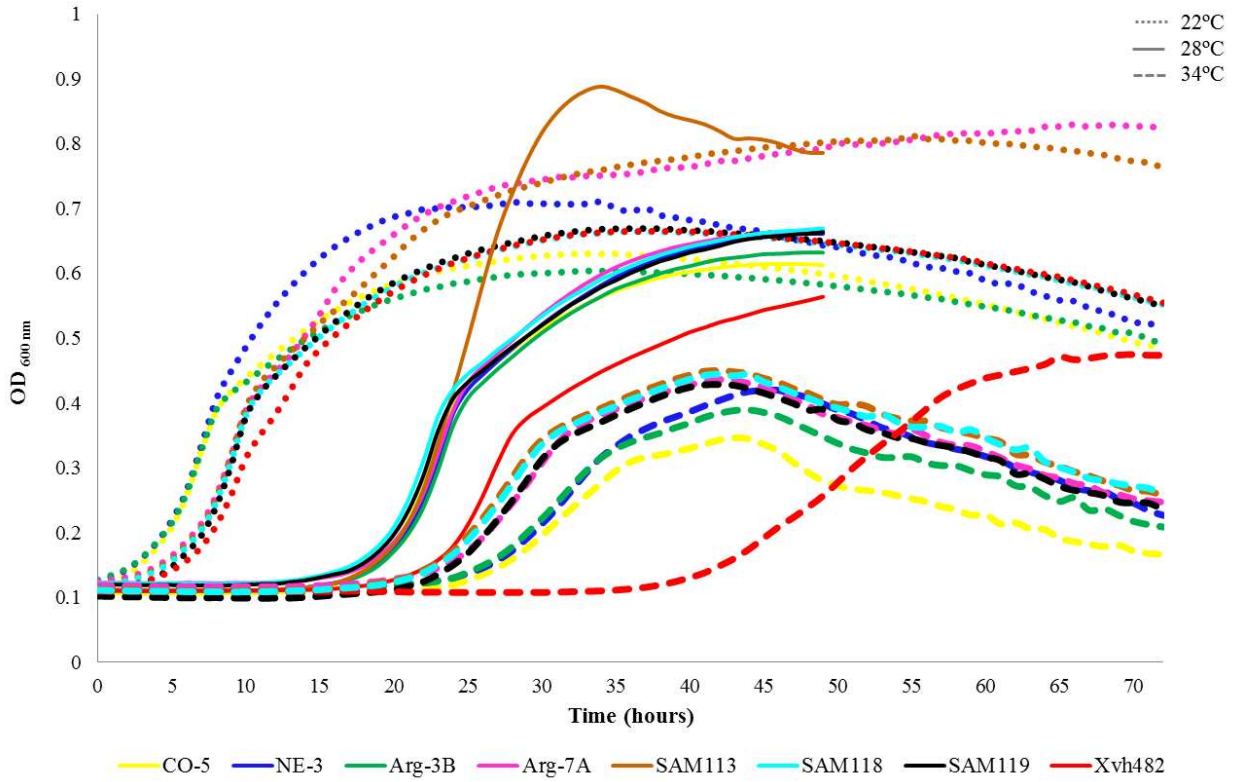

Figure 2.7: ***Growth curves in culture of* Xvv *and* Xvh *isolates at three different temperatures***. *Growth curves were performed in nutrient broth for seven* Xvv *isolates (CO-5, NE-3, Arg-3B, Arg-7A, SAM 113, SAM 118, SAM 119) and one* Xvh *isolate (Xvh 482) under three temperatures (22ºC (small dotted lines), 28ºC (straight lines), and 34ºC (long dotted lines). The experiment was repeated three times and each curve represents the average of three independent replicates. Y-axis represents the optical density at 600 nm (OD $_{600nm}$) while x-axis shows the time (hours) at which each isolate was grown. Absorbance values were recorded every hour during 48 h for 28ºC and 72 h for temperatures 22ºC and 34ºC.*

Pairwise comparisons considering both differences in isolates under the same temperature and temperature changes within the same isolate were done using CO-5 and 28ºC as reference isolate and temperature, respectively (Table 2.5). At 28ºC, Xmid values were not significantly different between any of the isolates versus CO-5 with the exception of Xvh 482 that grew significantly slower (p-value between CO-5 and Xvh 482 was 0.0034, Table 2.5). This significance is evident since all the curves at 28ºC grouped together except for the Xvh 482 curve. On the other hand, the average Scal values showed that there were two significant differences among isolates: first CO-5 and Xvh 482 and second, CO-5 and SAM 113. The first

significance value was expected but the second one was not. It is likely the slope of SAM 113 affected the results because this isolate grew to a much higher optical density than the other ones.

At 22ºC the time of fastest multiplication (Xmid) was significantly different between North and South American *Xvv* isolates (CO-5, NE-3, Arg-3B) versus South African *Xvv* isolates and contemporary *Xvh* isolates with the exception of Arg-7A that grouped together with the latter samples. Significant differences in Xmid values were between three to six hours for this temperature. Multiplication rates for all of the isolates were significantly different from CO-5 except Arg-3B.

At 34ºC, no significant differences in Xmid or multiplication rate were observed except with Xvh 482 which took longer to reach exponential and stationary phases. Although parameters for this curve were not obtained, it is evident that this isolate multiplied much more slowly than the rest. In fact, the starting of its exponential and stationary phases were 40 and 65 h respectively, while all the other isolates were in stationary phase at his point.

Population growth rates at 28ºC were significantly different from 22ºC and at 34ºC (Table 2.5). The 22ºC and 34ºC growth rates were statistically different from the 28ºC values in opposite directions, indicating that the growth rates between 22ºC and 34ºC varied significantly. Similarly, Xmid values at 22ºC were significantly different from 28ºC but values at 34ºC were not significantly different from Xmid values at 28ºC. This is congruent as curves at 34ºC were closer to 28ºC than 28ºC versus 22ºC. The time of fastest growth between 28ºC and 34ºC were closer, although their slopes were different.

Table 2.5: Average parameters and p-values of parameters for growth curves of *Xvv* and *Xvh* isolates at three different temperatures

Temperature	Isolate	Xmid	Comparison by isolate	Comparison by temperature	Scal	Comparison by isolate	Comparison by temperature	1/Scal
			p-value Xmid*	p-value Xmid*		p-value Scal*	p-value Scal*	
22ºC	CO-5	6.94	-	0.00001	4.09	-	0.00001	0.24
	NE-3	7.09	0.4462	0.00001	3.50	0.0019	0.00001	0.29
	Arg-3B	6.53	0.0583	0.00001	4.23	0.5282	0.00001	0.24
	Arg-7A	10.73	0.00001	0.00001	5.10	0.00001	0.00001	0.20
	SAM 113	11.32	0.00001	0.00001	5.76	0.00001	0.00001	0.17
	SAM 118	9.51	0.00001	0.00001	4.76	0.0016	0.00001	0.21
	SAM 119	9.70	0.00001	0.00001	4.54	0.0283	0.00001	0.22
	Xvh 482	10.68	0.00001	na	4.87	0.0002	na	0.21
28ºC	CO-5	22.66	-	-	6.33	-	-	0.16
	NE-3	23.93	0.3292	-	6.84	0.4814	-	0.15
	Arg-3B	23.73	0.3783	-	6.91	0.2947	-	0.15
	Arg-7A	23.62	0.2345	-	7.21	0.5754	-	0.14
	SAM 113	23.48	0.2949	-	3.05	0.00001	-	0.33
	SAM 118	23.02	0.7439	-	7.43	0.1968	-	0.14
	SAM 119	23.47	0.4773	-	7.54	0.1639	-	0.13
	Xvh 482	29.46	0.0034	na	10.10	0.0038	na	0.10
34ºC	CO-5	40.14	-	0.1267	18.01	-	0.0012	0.06
	NE-3	56.59	0.5769	0.0902	19.62	0.7007	0.0003	0.05
	Arg-3B	42.61	0.8931	0.0758	17.61	0.9414	0.0005	0.06
	Arg-7A	29.65	0.3147	0.0976	12.87	0.2254	0.0017	0.08
	SAM 113	27.20	0.2202	0.1906	10.91	0.0727	0.00001	0.09
	SAM 118	27.52	0.2308	0.0557	11.05	0.0801	0.0071	0.09
	SAM 119	27.80	0.2386	0.0484	10.39	0.0526	0.0245	0.10
	Xvh 482	na	na	na	na	na	na	na

*p-values: pairwise comparison between CO-5 vs each isolate and 28ºC vs each temperature, $\alpha = 0.05$, na: not applicable

Another aspect of bacterial leaf streak disease ecology is related to the interaction of *Xvv* with the proposed corn endophyte *P. ananatis* (Sheibani-Tezerji et al., 2015). Co-infiltrations of *Xvv* and *P. ananatis* resulted in significantly smaller lesions than when *Xvv* was inoculated by itself. Likewise, when *P. ananatis* was inoculated by itself, no disease symptoms developed under the same environmental conditions (Figure 2.8A).

Spray inoculations that simulate natural infection in the field had the same results as the co-infiltrations experiments (Figure 2.8B). Disease incidence was 100% for all plants inoculated with *Xvv* and co-inoculation with *Xvv* + *P. ananatis*, but 0% incidence when inoculated with only *P. ananatis*. Disease severity was assessed by calculating the percentage of leaves infected in a given plant. An average of 40% of leaves were infected on plants inoculated with only *Xvv* compared to an average of 19% of leaves infected in the co-inoculated, *Xvv* + *P. ananatis*, plants. These values were significantly different from each other as with the infiltration experiments (Figure 2.8B).

The lower disease levels observed for the co-inoculation may be the result of inhibition of *Xvv*'s growth through the secretion of toxins or secondary metabolites by *P. ananatis* or by a competition for space between *Xvv* and *P. ananatis*. To test the first option, inhibition experiments were completed with both bacteria grown on the same plates: either on a lawn of *Xvv* with a drop of *P. ananatis* and vice versa. Inhibition was not observed for any of the plates (data not shown).

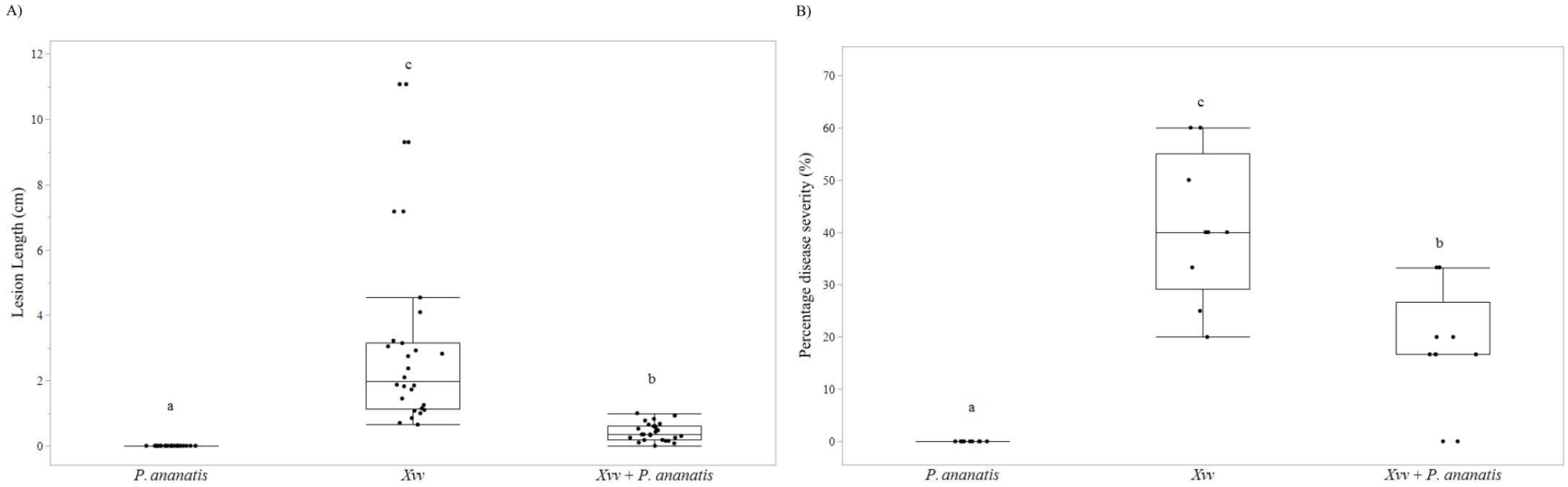

Figure 2.8: ***Interaction of*** **Xvv** ***and*** **P. ananatis** ***on popcorn.*** *Three week-old corn plants were either infiltrated (A) or sprayed (B) with* 10^8 *CFU/mL of* P. ananatis*,* Xvv *and co-inoculum (*Xvv + P. ananatis*). Disease was assessed at seven days post inoculation. The entire experiment was replicated three times and combined data from all replications is shown here. Letters designate significance at p-value <0.0001 using 26 replicates per treatment for (A) and nine replicates per treatment in (B).*

To address the second possibility of space competition, we performed growth curves of both strains and found that *P. ananatis* multiplies almost two times faster than *Xvv* ($Xmid_{P.\ ananatis}$ = 12.17 h versus $Xmid_{Xvv}$ = 20.26 h) in culture (Figure 2.9). It is likely that *P. ananatis* suppresses disease symptoms caused by *Xvv* via colonizing leaf surfaces faster than the corn pathogen. Growth curves *in planta* were not done as *P. ananatis* did not cause any symptom development and we did not develop marked strains to differentiate these bacteria on the plant tissue.

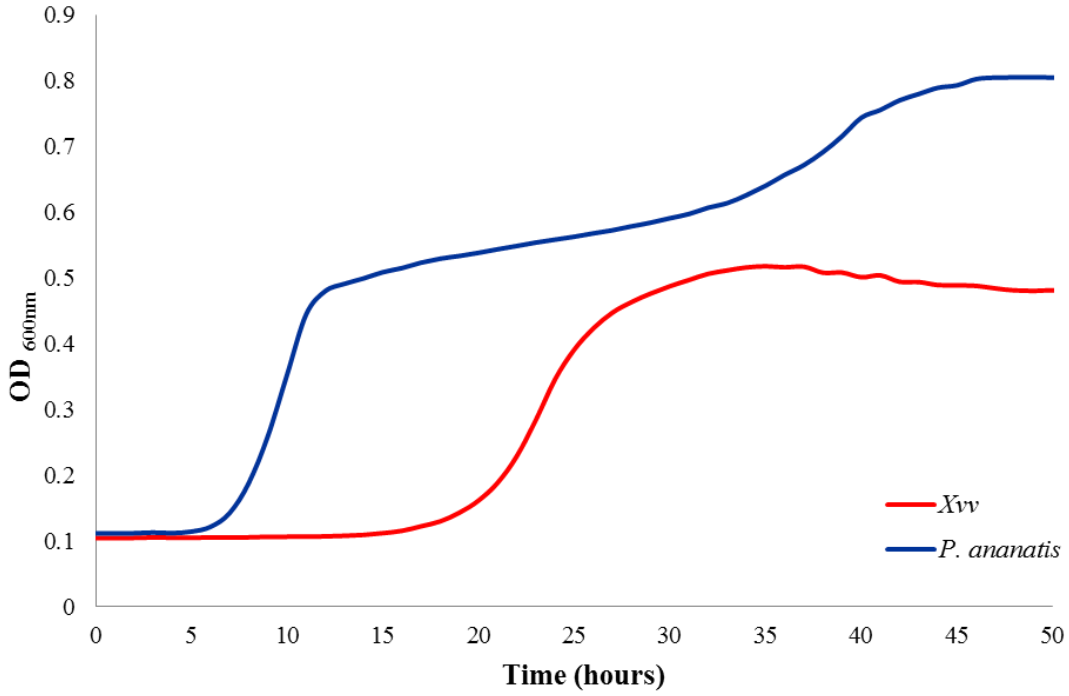

Figure 2.9: ***Growth curve of Xvv (red line) and P. ananatis (blue line) at 28ºC in nutrient broth.*** *Each curve shows the average of three independent replicates. Y-axis represents the optical density at 600 nm (OD $_{600nm}$) while x-axis shows the time (hours) at which each isolate was grown. Absorbance values were recorded every hour during 72 h.*

2.4 DISCUSSION

In this work we studied several aspects of the infection ecology of the emerging disease bacterial leaf streak of corn. All strategies that manage plant diseases depend on the knowledge of the ecology and biology of the pathogens causing the disease (Morris et al., 2017). Hence, by

understanding how the bacterial leaf streak pathogen enters, exits, interacts with different hosts and with other microbes, growers could make informative fact-based decisions related to disease management.

Like other foliar bacterial pathogens such as *Xanthomonas axonopodis* pv. *allii* (causal agent of Xanthomonas leaf blight of onion) (Schwartz & Gent, 2011) and *Xanthomonas translucens* pv. *undulosa* (causal agent of bacterial leaf streak of barley) (Adhikari et al., 2011), *Xvv* colonized the non-vascular apoplastic tissue. This information is also consistent with reports from Hartman (2018) where they could only recover the pathogen from leaves that were present at time of inoculation. The findings by Hartman (2018) and those presented here suggest that, unlike the strain of *Xvv* that infects sugarcane, *Xvv* that infects corn remains localized to the site of infection leading to a non-systemic disease response on corn and on at least sixteen other alternative hosts. The lack of systemic movement of *Xvv* could be due to the difference in morphology and physiology between corn and sugarcane (North, 1935) or it could be because *Xvv* from corn lacks effector *XopAf* or some other effector required for systemic spread that is present in *Xvv* from sugarcane. The latter statement is supported as the inoculations of corn-*Xvv* on sugarcane did not cause a systemic response.

The ability of a plant pathogen to enter their hosts is a crucial first step in the development of a foliar infection (Melotto et al., 2008). To gain access into the intercellular spaces and internal leaf tissue, many bacterial pathogens rely on intrinsic plant structures such as stomata, hydathodes, or lenticels. The stomata represent one of the most important entry routes for foliar bacterial pathogens, as they are the most abundant natural openings in the aerial part of plants that control gas exchange and water transpirations between the plant interior and the environment (Melotto et al., 2008). Although we could not capture *Xvv* entering the plant

through the stomata in real time, we hypothesized bacteria use these structures to entry the plant. This is because we observed a higher number of bacteria at this site and stomata are often kept open for a long period of time when high humidity is present in the environment. Since fields in Colorado and Nebraska are heavily watered through overhead center pivot irrigation (Turkington et al., 2004; Lichtenber, 1989) and fields in Iowa or Illinois are inherently humid, then it is possible a humid microclimate can be found inside corn fields providing the perfect environment for bacterial entrance into the corn plant.

A strong interaction was found between *Xvv* populations from North and South America, historical South African *Xvv* isolates, and the putative genome donor *Xvh* when inoculated on different corn hybrids. Bacterial leaf streak symptoms develop with all *Xvv* isolates, although this response is highly variable on the two corn varieties tested. On popcorn SAM 119 was the most aggressive isolate while CO-6 was the least aggressive. On grain corn P1151, Arg-4A and Arg-5B had highest disease progression while SAM 113 had the lowest. The results of the inoculations of SAM 119 in popcorn are similar to those found by Lang et al. (2017) who used another corn variety (DKC 61-88). In summary, South African isolates were more aggressive on popcorn while *Xvv* isolates from Argentina tend to be more aggressive on grain corn P1151. Even though popcorn and dent corn have different genetic lineages (Beadle, 1980; Kantety et al., 1995) and popcorn is more susceptible than grain corn to other corn diseases such as Stewart's bacterial wilt and blight, corn smut, northern corn leaf, and gray leaf spot (Peltier et al., 2008), the variation of *Xvv* disease response between the two corn types is interesting.

Inoculations of *Xvv* and *Xvh* on corn, sorghum, and sugarcane provided similar results to those of Lang et al. (2017). In particular, *Xvh* on corn causes water soaking symptoms with ooze but does not result in lesion expansion. *Xvh* has not recently been found on corn in the field

(Hartman, 2018; Lang et al., 2017), thus we hypothesize the amount of inoculated bacteria is higher than quantities of *Xvh* naturally found in a corn field site. Another explanation may consider that by infiltrating *Xvh* on this host the initial pathogen triggered immunity (PTI) response is bypassed. In this sense, *Xvh* may be able to colonize intercellular tissue until the plant activates the effector triggered immunity response that likely prevented greater lesion expansion. This response may also have occurred with *Xvv* inoculated on sorghum. Although there have not been reports of *Xvv* in sorghum fields, Hartman (2018) found a similar response to our inoculations on sorghum under greenhouse conditions.

There were two limitations with the infiltration experiments: 1) there was a high variability among replicates of the same *Xvv* isolate and 2) we only quantified lesion length when measuring disease response. First, the variation of the infiltration experiments predominated when one side of the infiltrated leaf showed a large symptom expansion while the other side did not. It is not known if there is a biological reaction of the plant against the bacteria or if it is just a matter of getting the bacteria inoculated in the intervenial spaces of the leaf. Spray inoculations were not performed, even though this method more closely simulates the natural infection. In preliminary experiments, it was difficult to quantitatively evaluate small differences among isolates and control the amount of bacteria inoculated onto each leaf at a given time.

Second, lesion length was the only component of disease response. All isolates produced ooze on the exterior of the leaf surfaces whether they produced lesion expansion or not. Unfortunately, ooze was not quantified, resulting in an underestimation of the importance of this phenotypic response in the isolates in which the lesion did not expand but there was ooze. Since rain and wind dispersal of bacterial ooze is likely the main method for pathogen dispersal,

quantification of exudate from infected leaves should be considered as another important component in understanding the epidemiology and aggressiveness of these isolates.

There were no significant differences in the aggressiveness of isolates of *Xvv* and *Xvh* on grain corn. This suggests that the proposed horizontal gene transfer event from *Xvh* to American *Xvv* strains (Perez-Quintero et al., 2019) did not result in an increase in aggressiveness. Establishing a link between the phenotype and genotype data is a challenging task because changes at the genome level may either be expressed or have little to no effects on the phenotypic response of a pathogen (Perfeito et al., 2007). It should be noted that phenotypic responses can also include more than increase in the leaf lesions (e.g., protein modifications or quorum sensing).

Within the context of this study, fitness is described as the ability of the bacteria to replicate and survive under a given environmental condition (Pope et al., 2010). When we evaluated growth rates of *Xvv* isolates at three temperatures, the characteristic phases of bacterial growth (lag, exponential, stationary, and death) were observed, but it was noticed that *Xvh* took longer to reach the exponential phase in all cases. At 28ºC, the temperature at which all strains grew in culture; there was no fitness advantage of *Xvv* isolates from North and South America compared to *Xvv* isolates from South Africa. However, at 22ºC, where all isolates of *Xvv* had a higher growth rate, a statistically significant difference was observed between *Xvv* from the Americas and *Xvv* from South Africa as well as contemporary *Xvh* isolates.

Furthermore, at a lower temperature, 22ºC, *Xvv* and *Xvh* isolates spend less time in lag phase and had higher growth rates than when grown at 28ºC, which was previously assumed to be optimal for bacterial multiplication in culture. Based on field observations, *Xvv*'s infection is more severe under high humidity; it is possible the humid microclimate inside the corn field had

a lower temperature than the outside environment. Additionally, it is also possible that corn has a mechanism for regulating the temperature inside the plant. For example, it has been shown that rice temperature inside the plant is lower than outside during a hot day in the summer (Liu et al., 2018). Corn may have this same thermoregulation process, although it has never been demonstrated.

Finally, we explored the role of another microbe in the bacterial leaf streak disease response. Similar to observations by Lang et al. (2017), *P. ananatis* was frequently isolated from fields infected with *Xvv*. Previous research has documented different life strategies for *P. ananatis* depending on environmental conditions (Coutinho & Venter, 2009; Goszczynska et al., 2007). For instance, *P. ananatis* was described as a pathogen of corn causing brown stalk rot in South Africa (Goszczynska et al., 2007); in other cases, *P. ananatis* has proven to be beneficial to the hosts by expressing antibacterial activity, triggering induced systemic resistance or competing for space with pathogens (Coutinho & Venter, 2009). Moreover, it is not the first time *P. ananatis* has been found in association with *Xvv*. In fact, recently both microorganisms were found infecting eucalyptus (Coutinho et al., 2015).

Based on our results, where the presence of *P. ananatis* significantly reduced symptoms of bacterial leaf streak under greenhouse conditions, and since brown stalk rot symptoms have not been reported on infected corn fields in the United States, we concluded there is not a synergistic relationship between the two organisms. Less disease may be the result of either *P. ananatis* inhibiting the growth of *Xvv* through the secretion of toxins or secondary metabolites or by competing for space or nutrients with *Xvv*. Our results support the latter as *P. ananatis* grew faster than *Xvv* in culture and failed to suppress *Xvv* colony expansion in direct competition studies. The role of *P. ananatis* raises several exciting questions about the potential use of this

organism as a biocontrol agent against bacterial leaf streak of corn. This would be beneficial since chemical control, via bactericides, are not readily available for this disease.

This research provided information on the fundamental aspects of the biology of the pathogen causing bacterial leaf streak of corn in the United States and Argentina. Through the use of molecular biology, fluorescent microscopy, and microbiological techniques we addressed questions related to the localization of *Xvv* within plant cells, behavior of *Xvv* isolates on different corn varieties, and relationship of *Xvv* with other microbes. While this research has contributed valuable information to our knowledge of *Xvv* disease ecology, further studies are needed to advance our understanding of this pathogen, including: 1) investigating the role of stomata in resistant and susceptible corn varieties to bacterial leaf streak pathogen, 2) evaluating fitness and/or virulence advantage *in planta*, 3) assessing impact on virulence capacity of both *Xvv* and *P. ananatis*, and 4) characterizing the potential epiphytic phase of *Xvv* and if so, what is the amount of bacteria necessary to change their life style and become a pathogen.

CHAPTER 3

SURVIVAL OF *XANTHOMONAS VASICOLA* PV. *VASCULORUM* IN CORN DEBRIS

3.1 INTRODUCTION

The emerging pathogen *Xanthomonas vasicola* pv. *vasculorum* (*Xvv*) causes bacterial leaf streak of corn in the United States, Argentina, and Brazil (Korus et al., 2017; Leite Jr. et al., 2018; Plazas et al., 2018). Symptoms of bacterial leaf streak on corn include: dark, water-soaked, linear lesions with wavy-edged margins that range from brown to yellow streaks. Based on field observations, it is believed *Xvv* may survive in infected residue or on alternate hosts during the winter (Hartman, 2018). As corn seedlings emerge, *Xvv* that survived in residue may be splashed by rain and irrigation water onto new leaves. The bacteria may then enter the plant through natural openings or wounds. Once inside, *Xvv* will reproduce and cause characteristic intervenial streaks on corn leaves. We have proposed that rain and wind-driven events aid in the transmission of the bacteria during the mid- to late growing season based on symptom distribution observed in the field that have experienced recent thunderstorms. After the corn is harvested, the bacteria persist in the leaves or on alternative hosts to start the cycle again in the following year.

Survival of plant pathogens is a key factor that ensures the development of primary inoculum and persistence of a disease in a determined area. In annual crops for example, pathogens may survive the winter in infected plant debris, soil, seeds, resting structures, alternative hosts, and even in insect vectors (Schuster & Coyne, 1974). *Xanthomonas* species have been shown to be

highly adaptable to different hosts and environments (Jacques et al., 2016; Leyns et al., 1984). For instance, *X. oryzae* pv. *oryzae* (Sakthivel et al., 2001), *X. campestris* pv. *carotae* (Kuan et al., 1985), and *X. campestris* pv. *campestris* (Randhawa & Schaad, 1894) survive on seeds of their respective hosts (rice, carrot, and crucifers). *Xanthomonas* may also survive in the soil such as *X. vasicola* pv. *musacearum* (Mwebaze et al., 2006), or in crop debris such as *X. axonopodis* pv. *allii* (Gent et al., 2005) and *X. campestris* pv. *armoraciae* (Zhao et al., 2002).

Research on the persistence of *Xvv* has focused on finding potential alternative hosts in or near corn fields (Hartman, 2018) and in evaluating the seed transmission rate of this pathogen (Arias et al., 2017). Evidence on the host range showed that big bluestem and bristly foxtail are two grasses determined to be hosts of *Xvv* in both greenhouse and field conditions (Hartman, 2018). So far, survival in naturally-contaminated corn seeds can only be detected at very low levels (0.1 to 0.5% in pooled seedling samples); therefore, this mode of persistence is unlikely (Arias et al., 2017). Furthermore, survival of *Xvv* on crop debris has not yet been explored. Considering that the incidence of bacterial leaf streak of corn is greatest in northeastern Colorado, western Nebraska, and western Kansas, where continuous corn production is a common practice (USDA-NASS, 2018b), and that corn residue is often left in the field to serve as a protective layer in sandy soils and to graze cattle (personal communication, Brett Adler, Colorado Corn Grower), it is likely that the amount of residue left on the soil may represent a significant reservoir for initial inoculum of the bacterial pathogen.

The goal of this study was to evaluate the potential of corn residue as primary inoculum for the development of the bacterial leaf streak of corn. A three-year study was conducted by leaving fine mesh bags with infected residue either on the surface or buried 10 cm below ground and collecting them after winter had passed. Several techniques were evaluated to isolate and

quantify the pathogen as well as to correlate these results with changing environmental conditions on the studied locations. This information will be valuable to design agronomic practices designed to reduce and/or eliminate the amount of inoculum and prevent further spread of the bacterial leaf streak pathogen.

3.2 MATERIALS AND METHODS

3.2.1 Field studies

Survival of *Xvv* in plant residue was evaluated through a three-year study in continuous corn fields during the winters of 2016, 2017, and 2018. Different locations across Colorado, Nebraska, and Iowa ensured a longitudinal gradient from dry to humid climates and from sandy to silty clay soils (Table 3.1). During the first year, three fields were located in eastern Colorado. For the second year study, we collected samples in four fields in Colorado, six locations across Nebraska, and two sites in Iowa (Figure 3.1). All of the fields had significant disease incidence during the three years. In October of each year, ten corn leaves, naturally infected with *Xvv*, were collected and placed inside fine nylon mesh bags. A total of ten bags were placed at each field, five bags were left on the soil surface and five bags were buried 10 cm below ground. Bags on the surface were covered with 1 inch plastic garden fence to protect them from being blown away. Soil samples were collected from the top 10 cm of soil and analyzed for texture class at the Water and Plant Testing Laboratory at Colorado State University (Table 3.1). Symptomatic corn leaves were confirmed to be positive for infection with the bacterial leaf streak pathogen through isolation and PCR strategies at the laboratory (see below). Sample bags were recovered in April of the following year, and processed for further analyses.

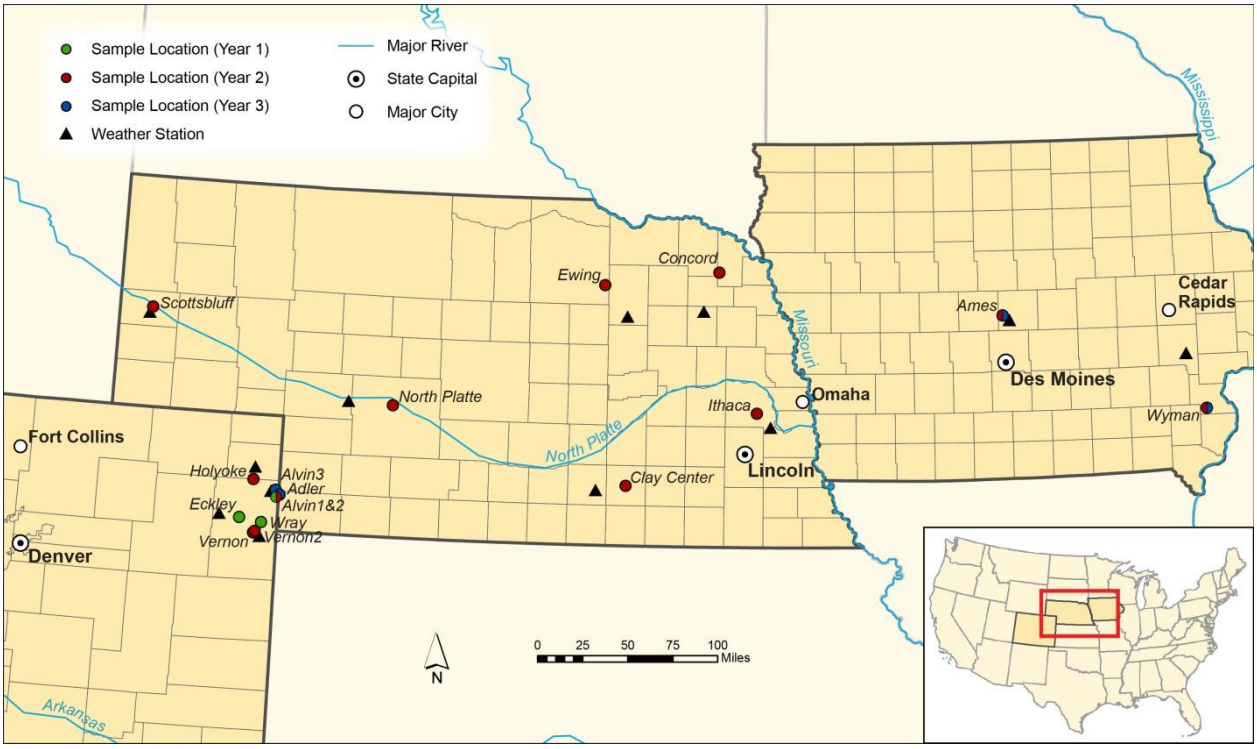

Figure 3.1: ***Description of field locations in Colorado, Nebraska, and Iowa during three-year study****. The map shows the coordinates of the locations for each year and the closest weather stations to the sites.*

Table 3.1: Description of locations in Colorado, Nebraska, and Iowa for three-year survival study on corn debris

Year	State	Location	County	Irrigated	Soil texture class
Year 1 2016-2017	Colorado	Alvin	Yuma	Yes	sandy loam
	Colorado	Eckely	Yuma	Yes	sandy loam
	Colorado	Wray	Yuma	Yes	sandy loam
Year 2 2017-2018	Colorado	Vernon	Yuma	No	sandy clay loam
	Colorado	Vernon 2	Yuma	Yes	sandy clay loam
	Colorado	Alvin 2	Yuma	Yes	sandy loam
	Colorado	Holyoke	Phillips	Yes	sandy clay loam
	Nebraska	Ithaca	Saunders	No	silty clay loam
	Nebraska	Clay Center	Clay	Yes	silt loam
	Nebraska	Concord	Dixon	Yes	silty clay loam
	Nebraska	Ewing	Holt	No	sandy loam
	Nebraska	North Platte	Lincoln	Yes	loam fine sand
	Nebraska	Scottsbluff	Scotts Bluff	Yes	loam fine sand
	Iowa	Ames	Story	No	silty clay loam
	Iowa	Wyman	Washington	No	silty clay laom

Year 3 2018-2019	Colorado	Alvin 3	Yuma	Yes	sand
	Colorado	Adler-18	Yuma	Yes	sand
	Iowa	Ames	Story	No	silty clay loam
	Iowa	Wyman	Washington	No	Silty clay laom

3.2.2 Isolation and molecular detection of suspected *Xvv* strains from corn residue

Samples recovered from the fields at the beginning of each experimental year (October) were isolated according to Lang et al. (2017). At the end of each experimental year (April), bacterial samples were isolated by first cutting tissue in smaller pieces with sterile scissors and measuring 1 g of residue. Each sample was then soaked in either 9 mL of 100 mM $MgSO_4$ or 9 mL of 10 mM PBS for 15 min at room temperature. Samples were shaken by inversion for 2 min and serial dilutions were prepared from 10^0, corresponding to original sample, until 10^{-5}. Next, 20 µL from each solution was plated onto nutrient agar (NA) and yeast dextrose calcium carbonate (YDC) plates. Suspected colonies were further purified until obtaining pure cultures. A second methodology for isolation included the incubation of samples for 1 and 24 h at 28 ºC in a 10 mL 10 mM PBS solution. Serial dilutions and plating were performed as described previously.

Molecular detection was done through colony PCR of the 16S rRNA gene. DNA template was prepared by boiling 1 colony in 10 µL of sterile water at 95ºC for 5 min. PCR reactions (50 µL) contained 2 µL of boiled DNA template, 0.2 µM of each primer (515-F (GTGCCAGCMGCCGCGGT), 806-R (GGACTACHVGGGTWTCTAAT)), 1X GoTaq reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 0.25 unit/µL GoTaq DNA polymerase enzyme (Promega). The cycling conditions were as follows: initial denaturation at 94ºC for 3 min, following 35 cycles of 94ºC for 45 s, 50ºC for 1 min and 72ºC for 1:30 min, and the final extension period at 72ºC for 10 min. PCR fragments were separated in a 1.5% agarose gel for 45 min at 90 V, and fragments were extracted and purified using the DNA clean & concentrator kit

(ZYMO Research). Sequencing was performed with 5 ng/µL of each PCR product at Quintara Biosciences (Fort Collins, CO) and analyzed using Geneious software (version 10.0.7). Sequence identities were determined using Blastn from the NCBI database.

3.2.3 Inoculation of residue corn samples into healthy corn plants

To test pathogenicity of corn residue from the 2016 locations, three inoculation methodologies were used. For the first two procedures (infiltration and stab on the stem), 0.5 g of residue sample from surface and from below ground were measured from each location and then added to 20 mL of 10 mM PBS pH 7.4. Solutions were incubated at 28ºC for 24 h at 200 rpm. Inoculations were done with 3-week-old popcorn plants under maintained greenhouse conditions (30 ± 1ºC, 16 h day length, and 80% relative humidity) by either infiltrating into the intercellular spaces of leaves on either side of the abaxial vein or by stabbing the lower stem with 50 µL of each solution. Symptom development was monitored during seven and fourteen days post inoculation.

The third method of inoculation of the residue samples consisted on grinding 1 g of residue with a sterile mortar and pestle, and dissolving the extract into 100 mL of sterile water. Samples were incubated for 1 and 24 h at 28 ºC, 220 rpm. All the leaves in three popcorn plants were sprayed with 50 mL of solution after each time point and placed in a mist bench with misting every hour for 2 min during 12 h. For all inoculations, *Xvv* CO-5 was used as positive control and sterile, distilled water was used as negative control.

3.2.4 Minimum amount of inoculum to cause disease in the greenhouse

Corn hybrid (Pioneer P1151) was grown in a 1:1 mix of Promix-BX Biofungicide + Mycorrhizae (Quakertown, PA) under greenhouse conditions (30 ± 1 ºC, 16 h day length, and 80% relative humidity). Bacterial isolate *Xvv* CO-5 was cultured in peptone sucrose agar (PSA) for 24 h at 28ºC and suspended to 10^8 CFU/mL in sterile, distilled water. Serial dilutions were prepared from 10^8 CFU/mL until 10^1 CFU/mL. Three weeks after planting, plants were either infiltrated or sprayed with each dilution. Infiltration was done as described by Lang et al. (2017) and spray inoculations were performed with ~15 mL of bacterial suspension. After inoculation, plants were placed under high humidity (>90% relative humidity) for 48 h in growth chamber and then they were transferred to standard greenhouse conditions. Sterile, distilled water was used as a negative control in all inoculations. Three independent replicates were done per each dilution and treatment. Quantification of the lesion length in the infiltrated plants was done by measuring the expansion distance beyond the infiltration site. Severity after spray was scored by counting the number of leaves infected over the total number of leaves on a plant (Hartman, 2018). Symptom development was assessed at 7 days post inoculation for each treatment.

3.2.5 Quantification of *Xvv* from corn debris using real time PCR and high- throughput assay

DNA extractions using a Kingfisher kit (Thermo Scientific) were performed with 50 mg of each sample from year 1 and following manufacturer recommendations. DNA concentrations were measured in Nanodrop One (Thermo Scientific) and dilutions of all samples to 20 ng/µL were made. Real-time PCR was performed as described in Stulberg et al. (2019). Predicted concentrations in ng/µL were obtained using standard curve of the positive *Xvv* CO-5 strain.

Three random samples from each bag at all the locations (10 bags per location) were sent to the National Agricultural Genotyping Center (NAGC) in Fargo, ND. These samples were tested against the high-throughput protocol for quantification of *Xvv* (NAGC, 2019). Quantity of bacteria was expressed in colony forming units per gram of residue (CFU/g). One independent sample consisted of the average of the three technical replicates per bag. Average values of independent replicates were obtained depending on their location and status within the field (surface or buried). Quantification was done at the end of year 1 study and at the beginning and end of year 2 and 3 of the survival study. Based on r-student residual outlier test, the Clay Center field in Nebraska for year 2 was an outlier. Therefore, this site was discarded for further analysis.

For statistical analysis, a one-way ANOVA using log (Quantity) transformation was performed in R (version 3.5.2) with the lm and anova functions. Log transformation was done to satisfy ANOVA assumptions. All pairwise comparisons were done using emmeans function from the emmeans package with Tukey adjustment test (HSD, honestly significant difference) and $\alpha = 0.05$.

3.2.6 Weather data

Historical certified weather data was downloaded from the National Center for Environmental Information (NCEI) (www.ncdc.noaa.gov). This data described the global summary of rain and snow in each of the months the bags were left on the fields from 2017 until 2019. Inches of rain and snow were correlated with quantities of *Xvv* for the second and third year across the different states. Sample correlation coefficients were obtained using JMP software (JMP 13.0.0).

3.2.7 Percentage degradation of quantities of *Xvv*

Percentage degradation in each location for the second and third year considered the average initial quantity of *Xvv* DNA when bags were left on the field and average of surface and buried samples when bags were collected after six months. Percentage degradation was calculated using the following formula:

$$\%\ degradation = \frac{Average\ Initial\ Quantity - Average\ Final\ Quantity}{Average\ Initial\ Quantity} * 100$$

Statistical differences on the means of percentage degradation of Xvv in surface and buried sample were evaluated through the student's t-test in R (version 3.5.2) with t.test function and $\alpha = 0.05$.

3.2.8 Natural infection of *Xvv* onto emerging corn seedlings in greenhouse conditions

Three greenhouse survival experiments were performed to simulate the natural infection of *Xvv* from crop debris to emerging corn seedlings. Four amounts of residue (5, 7, 9, 18 g) were placed on the surface of the soil in pots with three independent replicates per each amount for the first and second experiments. The third experiment had different amounts of residue (5, 10, 15 g). Each amount was placed in triplicate on the surface of pots and also mixed with the soil. All experiments were done under two environments: "wet environment" inside mist bench (misting every 40 min for 30 s during 12 h) and a "dry environment" under greenhouse conditions (30 ± 1ºC, 16 h day length). High relative humidity was present in wet environment. Independently of the environment plants were watered once every other day. Four seeds of P1151 corn hybrid were allowed to grow in each pot for six weeks and observations of bacterial leaf streak symptoms were made twice per week.

3.3 RESULTS

Potential survival of *Xvv* on corn litter in the first year study was evaluated through two conventional methods: isolation of suspected bacteria from corn residue and pathogenicity tests. We tried three isolation methodologies involving dilution plating of PBS, $MgSO_4$, and H_2O solutions. From here, 50 suspected bright yellow colonies were isolated and proceed to molecular identification using the 16S rRNA gene. Unfortunately, none of these colonies belonged to the genus *Xanthomonas* (Table 3.2). The majority of the isolated bacteria, 34%, corresponded to *Curtobacterium* spp. This genus has the same morphology as *Xanthomonas* spp. in culture but is a gram-positive organism and a general soil inhabitant (Silva Júnior et al., 2012). Out of all the isolated bacteria, the closest phylogenetically to the *Xanthomonas* genus was *Stenotrophomonas* spp. This genus was first grouped with *Xanthomonas* until 1993, when its taxonomy was revised and it was placed its own genus (Palleroni & Bradbury, 1993). In contrast to *Xanthomonas* spp., no *Stenotrophomonas* species are known to be phytopathogenic (Ryan et al., 2009). The other isolated bacteria are common soil inhabitants such as *Frigoribacterium* spp. (Dastager et al., 2008) and *Massilia* spp. (Ofek et al., 2012) and potential opportunistic animal pathogens like *Serratia* spp. (Grimont & Grimont, 2006).

Table 3.2: Different genera of bacteria recovered from litter studies

Isolated Bacteria	Total	%
Curtobacterium spp.	17	34
Stenotrophomonas spp.	9	18
Massilia spp.	5	10
Chryseobacterium spp.	4	8
Frigoribacterium spp.	5	10
Uncultured bacterium	3	6
Salinibacterium spp.	2	4
Pseudomonas lini	2	4
Serratia spp.	1	2

Microbacterium spp.	1	2
Pedobacter spp.	1	2
Total	50	100

Since it was not possible to isolate *Xvv* from any of the residue samples, we then focused on evaluating the pathogenicity potential of the crop debris. To this end, we inoculated extracts of corn residue, which were previously incubated on 20 mL of PBS for 24 hours at 28ºC, into healthy corn plants. None of the implemented inoculation strategies (infiltration, stab on the stem, and spray) resulted in the characteristic symptoms of bacterial leaf streak after seven days post inoculation under greenhouse conditions (Figure 3.2).

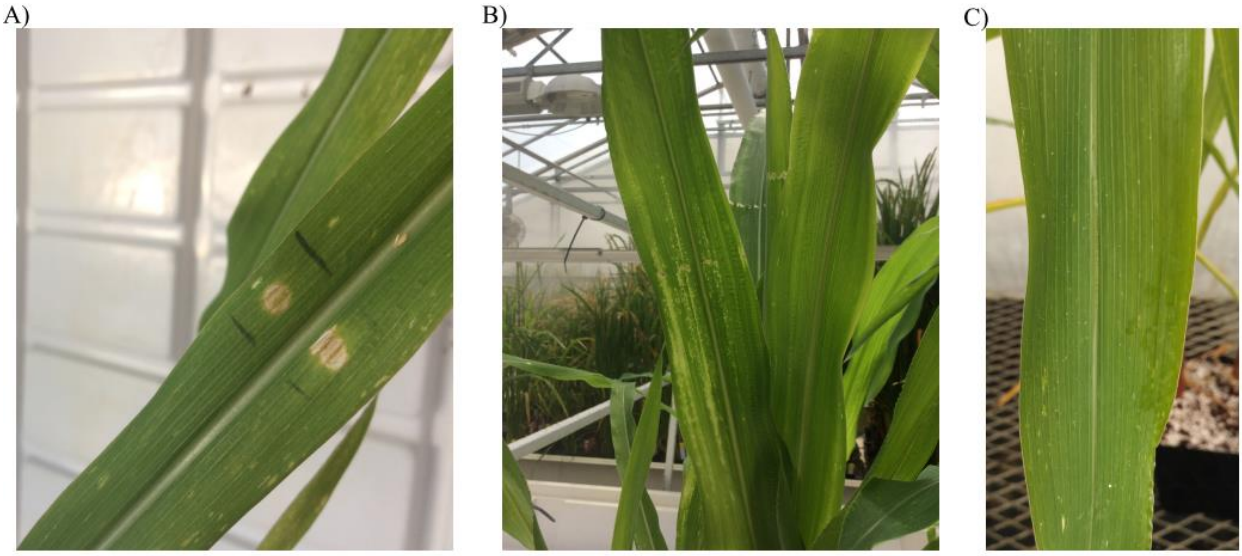

Figure 3.2: ***Three inoculation strategies using extract from corn debris.*** *A) Infiltration on the back side of the plant. Black lines delimitated the infiltration site. B) Stab on the lower stem lead to holes in the middle of the newest leaf. C) Spray of corn debris extract across the entire plant did not show any symptoms.*

Through greenhouse inoculation experiments, we found that the minimum amount of inoculum to cause disease is 10^5 CFU/mL of bacterial suspension. Therefore, it is possible that the amount of *Xvv* present in the corn residue extracts was lower than the one used in the inoculations. At this point, it was unknown if the bacteria were even present in any of the studied

samples. To address this concern, molecular detection methods were carried out first in the lab using a real time PCR protocol (Stulberg et al., 2019) and second through a high-throughput quantification method developed at the National Agricultural Genotyping Center (NAGC). For the identification protocol, samples were classified depending on their type (surface or buried) in each location of eastern Colorado.

First, the detection method in our lab predicted low levels of *Xvv* DNA in the corn residue. Average detected DNA concentrations ranged from 0.0017 to 0.0037 ng/µL and only 5 samples out of the 32 tested had a concentration above 0.02 ng/µL, which was the accurate detection and quantifiable level for the established standard curve (Table 3.3). Second, quantification of the amount of bacteria at the NAGC allowed the analysis of a high number of samples in a short period of time. Average values are represented in CFU/g of residue (Figure 3.3). Statistical analyses showed that quantity of *Xvv* inside buried bags were significantly higher than quantities of *Xvv* inside bags left on the surface of the fields. Even though different standard curves were used in the detection methods, quantities of *Xvv* DNA were similar in both laboratories.

Table 3.3: Molecular identification of *Xvv* from corn residue using real-time PCR protocol

Location	**Type**	**Number of samples**	**Average concentration (ng/µL)**
Eckley	Surface	5	0.0368
	Buried	4	0.0105
Wray	Surface	3	0.0047
	Buried	3	0.0017
Alvin	Surface	3	0.0425
	Buried	3	0.0061

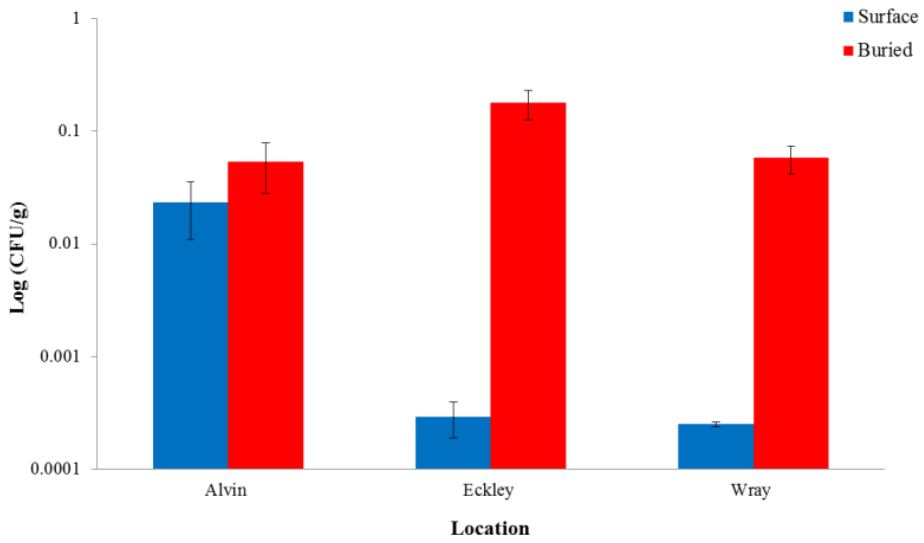

Figure 3.3: ***Quantities of*** **Xvv** ***DNA were higher in the buried samples than in the surface samples.*** *The graph represents the amount of* Xvv *for year 1 in Log (CFU/g) (y-axis) per each location (x-axis) in Colorado. Blue bars denote surface residue and red bars represent buried residue 10 cm below ground.*

In contrast to the results of year 1, quantification of *Xvv* from corn residue for year 2 (2017-2018) and 3 (2018-2019) per type showed that the amount of *Xvv* found on the surface was significantly higher than the quantity of *Xvv* found beneath the soil per each location (p-value < 0.0001 for both years) (Figure 3.4A and 3.4B). Additionally, results for both years showed a strong interaction of type (buried vs. surface) and location (p-value < 0.0001).

To complement these findings, we also evaluated the relationship between precipitation and quantity of *Xvv* in corn residue. Precipitation was divided in inches of rain and inches of snow fallen during the time the bags were left on the field (six months). As expected, for year 2, rain in Colorado was significantly lower than in Nebraska and Iowa (Figure 3.4A) (NCEI, 2018). In addition, there were no significantly differences in the quantity of snow in the three states. For the third year study, there was more rain and snow in Iowa than in Colorado, although no significant differences were observed (p-value = 0.17) (Figure 3.4B).

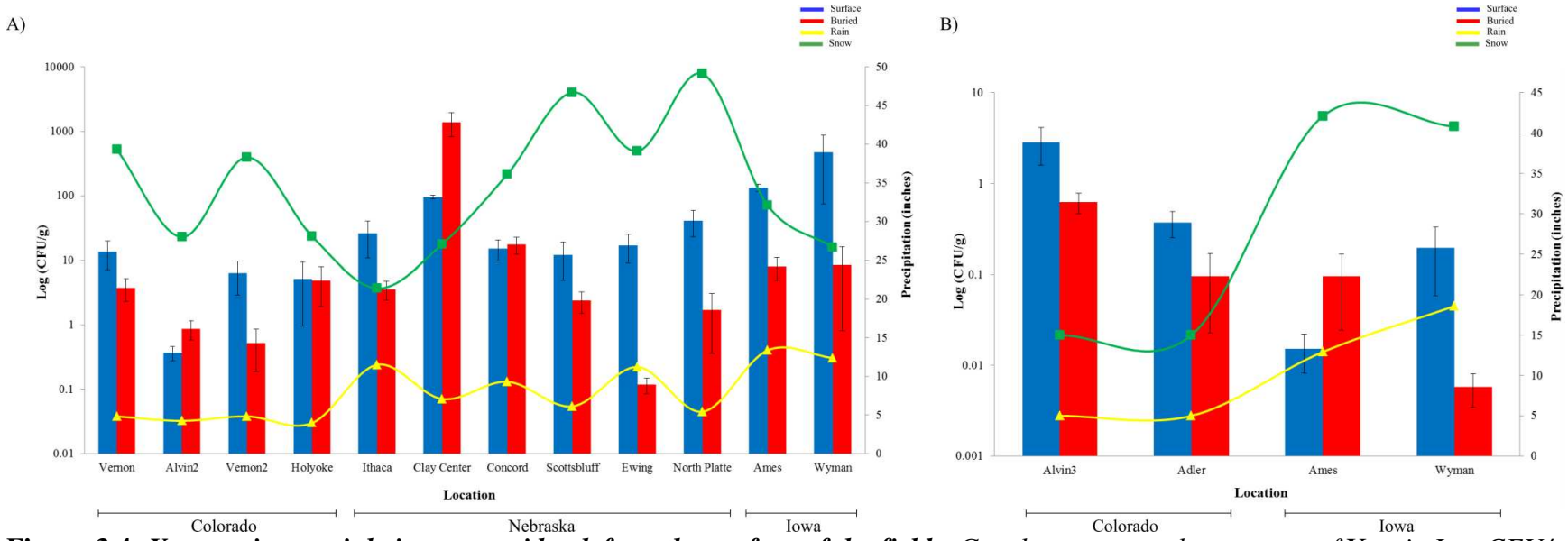

Figure 3.4: ***Xvv survives mainly in corn residue left on the surface of the fields.*** *Graphs represent the amounts of* Xvv *in Log CFU/g (y-axis) per each location (x-axis) for year 2 (A) and year 3 (B), respectively. Blue bars denote surface residue, red bars represent buried residue, yellow lines show the total inches of rain and green lines show the total inches snow from October to April for year 2 and 3 of the survival study. Results are shown in the log scale of amount of Xvv.*

The relationship between the environmental factors (rain and snow) and the quantities of *Xvv* per type (surface and buried) of residue showed a positive correlation between rain and amount of *Xvv* for both surface and buried samples during the second year study (Figure 3.5A and 3.5B). In contrast, there was a negative correlation between snow and quantities of *Xvv* in surface and below ground residue for the same year (Figure 3.5C and 3.5D). Although the sample correlation coefficients did not denote a strong linear relationship between the pairs, there was 95% coverage in all the comparisons. We observed there was a stronger correlation of rain vs surface *Xvv* quantities (r = 0.549) than rain vs buried *Xvv* quantities (r = 0.400). The lowest correlation coefficient was between the snow and buried *Xvv* quantities (r = -0.208).

For year 3, there were only four studied locations; therefore the correlation coefficients were not robust with this sample size (data not shown). In general, the same trend of an inverse relationship between snow and quantities of *Xvv* was observed: more snow may be related to fewer *Xvv*. It is worth noting that the inches of snow in Colorado were underestimated because there was missing data for three out of the six months the bags were left on the field (NCEI, 2018), hence it was not possible to infer a relationship.

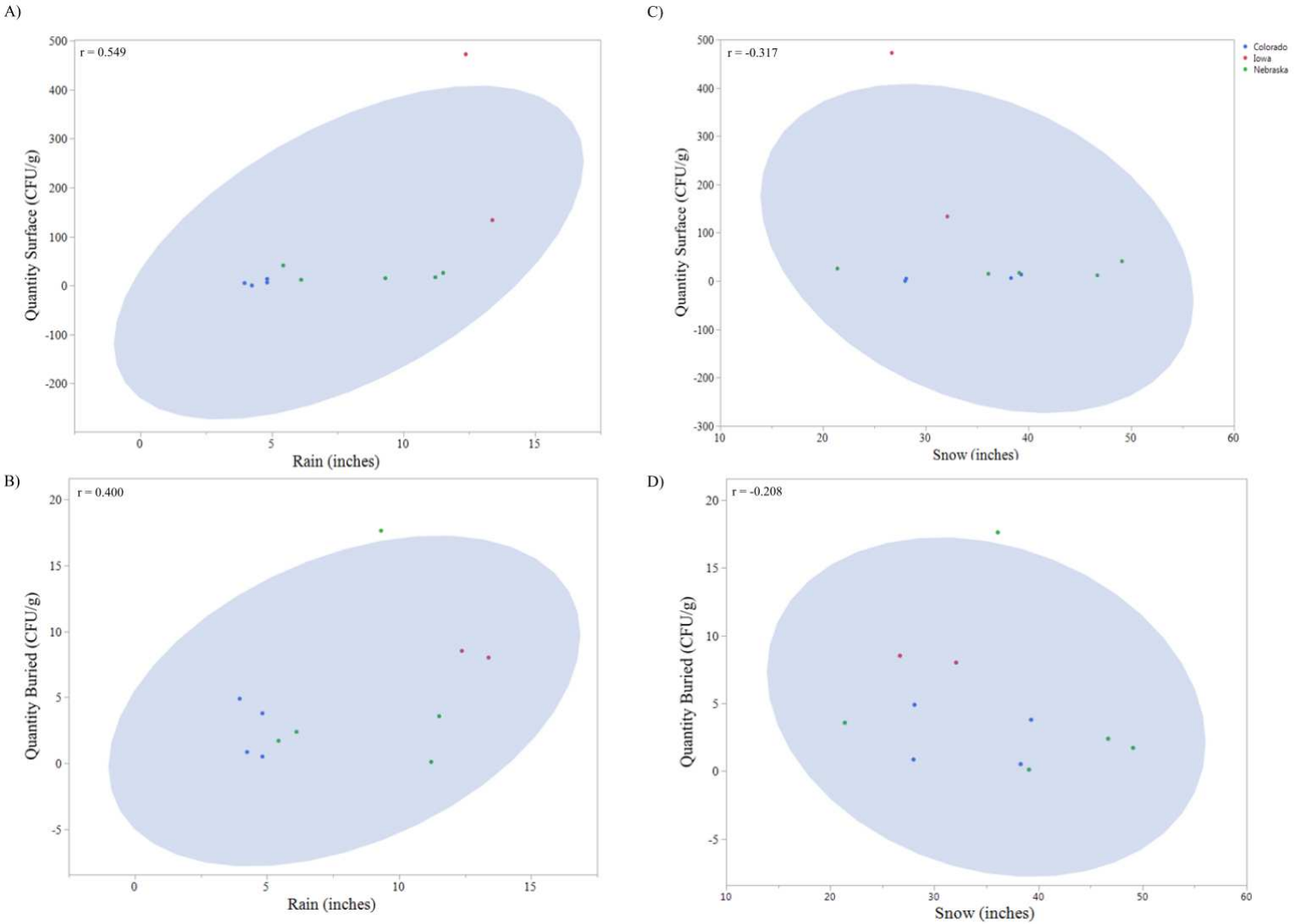

Figure 3.5: ***Correlation of quantities of Xvv and precipitation in the form of rain and snow for year 2.*** *A) and B) show correlation of quantity of* Xvv *in surface and buried (y-axis) versus inches of rain (x-axis) for Colorado, Nebraska, and Iowa. C) and D) show correlation of quantity of* Xvv *in surface and buried (y-axis) versus inches of snow for Colorado, Nebraska, and Iowa. The values in the left top corner in each graph denote the sample correlation coefficient for each relationship correlation.*

The percentage degradation for each location in Colorado and Nebraska for year 2 and Colorado and Iowa for year 3 were calculated based on the quantities of *Xvv* at the beginning and end of each experimental year (Table 3.4). There is a high percentage of degradation of *Xvv* DNA in the corn litter, above 58%, from the fall to the spring of each year. In the second year, the highest degradation occurred at location Vernon 2 in northeastern Colorado with a 99.9% degradation of detectable *Xvv* in buried corn residue. The lowest degradation of *Xvv* occurred for surface samples at the North Platte, Nebraska location with a 59.5% reduction. For the third year, the highest degradation corresponded to 98.85% in the location Alvin 3, Colorado and the lowest degradation of *Xvv* corresponded to 58.28% in Ames, Iowa.

Comparison of the percentage degradation of surface and buried samples showed that there was a higher degradation in the below ground samples than in the surface residue for all the locations (Table 3.4). However, the mean of the percentage degradation of *Xvv* in surface versus buried were not significantly different from each other in both years (year 2 p-value = 0.074, year 3 p-value = 0.17 when $\alpha = 0.05$).

Table 3.4: Percentage degradation of *Xvv* from October to April of two consecutive years in Colorado, Nebraska, and Iowa locations

State	**Percentage Degradation Year 2**			**State**	**Percentage Degradation Year 3**		
	Location	Surface	Buried		Location	Surface	Buried
Colorado	Vernon	86.9%	96.4%	**Colorado**	Alvin 3	94.8%	98.9%
	Alvin 2	96.5%	91.8%		Adler	92.5%	98.1%
	Vernon 2	98.9%	99.9%	**Iowa**	Ames	58.3%	91.3%
	Holyoke	69.4%	70.9%		Wyman	62.5%	84.2%
Nebraska	Ithaca	74.4%	96.5%				
	Concord	85.1%	82.6%				
	Scottsbluff	88.2%	97.6%				
	Ewing	83.2%	99.9%				
	North Platte	59.5%	98.3%				

It has been reported that in a field with 100-150 bushels of grain per acre, there are 62.5 g of corn residue per square foot left on the surface after harvest (62.5 g/ ft^2 (KSU-Extension, 1989; USDA-NRCS, 2018; USDA-NASS, 2018b). Based on this information, we then estimated the amount of *Xvv* on the surface (CFU) per square foot and per hectare (ha) in year 2 (Table 3.5). From here, we found that there is an average of $5.9*10^3$ CFU/ft^2, $2.9*10^{10}$ CFU/ha. Although the values of CFU per hectare are low, it is important to consider that this number will vary from different collection points in the field and it is likely *Xvv* has a patchy distribution in the residue across each location. This may be due to a variety of factors including quantity of residue, soil moisture, snow cover, etc.

Table 3.5: Estimation of the amount of *Xvv* DNA per square foot and per hectare for year 2 of survival study

State	Location	Average (CFU/g)	CFU/square foot	CFU/ha
Colorado	Vernon	13.62	$8.51*10^2$	$5.72*10^9$
	Alvin 2	0.369	$2.30*10^1$	$1.55*10^8$
	Vernon 2	6.33	$3.96*10^2$	$2.66*10^9$
	Holyoke	5.15	$3.22*10^2$	$2.16*10^9$
Nebraska	Ames	133.74	$8.36*10^3$	$5.62*10^{10}$
	Clay Center	95.81	$5.99*10^3$	$1.98*10^{11}$
	Concord	15.13	$9.45*10^2$	$1.09*10^{10}$
	Scottsbluff	11.98	$7.49*10^2$	$4.03*10^{10}$
	Ewing	17.04	$1.06*10^3$	$6.36*10^9$
	North Platte	41.09	$2.57*10^3$	$5.03*10^9$
Iowa	Wyman	472.33	$2.95*10^4$	$7.16*10^9$
	Ithaca	25.94	$1.62*10^3$	$1.73*10^{10}$

1 ft^2= 62.5 g of corn residue
1 ha= 107639 ft^2

Finally, to determine if infected residue is able to transfer bacteria to emerging seedlings in the greenhouse, we set up different experiments under two environmental conditions. The first condition consisted of a wet environment where plants where placed in a mist bench with constant mist for 12 h. The second condition simulated a dry environment where plants were

placed in greenhouse conditions with low humidity and watered from the top only once per day. These experiments were repeated three times with different amount of corn residue that was close to the real amount that is left on the field. In the first and second replicate, corn debris was left on the surface of the pots and water was splashed by the mist and through the nozzle in both environments. In the third replicate, corn residue was also mixed with the soil to simulate the buried samples. None of the emerged plants presented characteristic symptoms of bacterial leaf streak. Therefore, transmission of *Xvv* from leaf debris was not observed in a greenhouse scale experiment. This experiment also supported our previous analyses where the amount of *Xvv* is low and it may not be evenly distributed in the residue.

3.4 DISCUSSION

Plant pathogens in temperate climates must survive the winter when their host plants are dormant or absent (Schuster & Coyne, 1974). Survival is a key factor for the development of disease because it ensures the permanence of the pathogen in an environment and allows it to be, in most cases, the primary inoculum for the next growing season. In this research, we investigated the role of corn residue as potential primary inoculum source for the development of the disease bacterial leaf streak of corn. This is important because many fields in eastern Colorado and western Nebraska, where the disease has a high incidence, have continuous corn production and therefore, corn debris may present a greater risk for increasing disease severity and yield loss.

Similar to our research, other studies have evaluated the role of crop debris left on the surface and buried for several foliar bacterial pathogens (Gent et al., 2005; Jones et al., 1985; Thapa,

2014; Vega & Romero, 2016; Zhao et al., 2002). Examples include diseases caused by *X. campestris* pv. *armoraciae* on brassica (Zhao et al., 2002), *X. axonopodis* pv. *allii* on onion (Gent et al., 2005), and *Clavibacter michiganensis* subsp. *michiganensis* on tomato (Vega & Romero, 2016). The difference of this research with the aforementioned studies is that our samples were naturally infected while those studies were artificially inoculated with a known amount of the respective bacterial pathogen. Natural inoculation has a great value because we ensured our results are due to the interaction of hosts and pathogen without any human intervention and it also has a realistic bacterial concentration and physiology.

We first tried to recover *Xvv* from corn residue following conventional isolation and culture methods as described by Schuster and Coyne (1974). Unfortunately, because isolation techniques were not successful, a molecular detection method was used to identify the pathogen exposed to the surface or below ground corn fields. This was similar to Zhao et al. (2002) which also developed a PCR protocol to study survival of *P. syringae* on tomato.

Our results from year 2 and 3 suggest that quantities of *Xvv* are mainly found on corn residue left on the surface of the field. These results are consistent with reports of pathogen survival on host debris on other foliar diseases such as gray leaf spot (Kinyua, 2003; Ward & Nowell, 1998) and Goss's wilt (Wise et al., 2010). The main difference among the three-year study is that for the first year bacteria were found in higher proportions in samples below ground. A possible explanation for this discrepancy is that the sample size for year 1 was small compared to the other years. We could not control the sample size at this point because the fields were used to graze cattle and we did not account for the animals eating and destroying the demarcation zone. In fact, out of 10 expected replicates per each type at a location, we could only recover at most

three of them. To prevent this bias, five replicates per each type ensured a statistical power of 90% for year 2 and 3 across several locations in Colorado, Nebraska, and Iowa.

The high percentage of degradation of *Xvv* from fall to the spring provided an explanation why we could not isolate the pathogen in the lab. In this sense, the isolation of the bacteria would be dependent upon the rate of decay of infested plant tissues. Since we could only isolate the pathogen at the beginning of the study for year 2 and 3 but not at the end of the winter, we conclude the amount of *Xvv* in the corn residue was below the threshold of isolation and culture for this specific pathogen. The higher degradation of *Xvv* in the buried residue samples was likely the result of a greater decomposition of the corn residue itself when it is located in the soil than when it is on the soil surface.

Variation in abiotic conditions could also explain observed differences in the survival of *Xvv* in host debris. Specifically, in year 2 precipitation in the form of rain was positively correlated with *Xvv* survival quantities in both surface and below ground residue samples. Sites in Colorado had less rain and significantly lower quantities of *Xvv*, contrary to sites in Iowa where more rain was correlated to a higher amount of *Xvv* survival. Hence, *Xvv* had a higher chance to survive depending on the amount of water each field received.

Based on field observations (personal communication, Kirk Broders & Tamra Jackson-Ziems), *Xvv* is transferred from corn debris to new seedlings through water splashing early in the growing season. This water may come from rain or irrigation. Although Colorado sites had lower amounts of rain, these fields were heavily irrigated, and this may be the reason there were still high levels of bacterial leaf streak in the next growing season. These findings are consistent with those found by Hartman (2018) that demonstrated the importance of irrigation and continuous corn production as factors that increase disease development.

One limitation of these studies is that we could only quantity the amount of *Xvv* DNA present in each sample but we could not quantify the number of living cells. While *Xvv* quantities in the residue may be low, the high amount of residue across a field increases the opportunity for survival. Several factors may influence the survival of *Xvv* in corn residue, for example, infection levels during growing season, uneven distribution of residue due to machine clumping, and localization of bacteria within the plant. In any case, only a single infection is required to result in colonization and exudation of bacterial cells of *Xvv*. Since the secondary spread from plant to plant through splashed ooze has proven to be very efficient at moving the disease throughout a field, even lower numbers of living bacteria will have the potential to perpetuate the disease for the next growing season.

In conclusion, the present study has shown that corn debris left on the surface of the field is likely an important source of inoculum for the development of bacterial leaf streak in continuous corn production systems. Even though, isolation of *Xvv* was not possible, this is the first report that shows considerable amounts of *Xvv* found on corn residue after an overwintering season. Other research has evaluated the role of alternative hosts as potential inoculum reservoirs (Hartman, 2018) and the amount of *Xvv* in seeds (Arias et al., 2017), but both studies only showed detection of the pathogen at low incidence levels under a specific time point and not overtime in naturally infected corn fields.

Since *Xvv* degrades quickly in residue over just a 3-month period, a single season crop rotation to a non-host could be the most effective management strategy for this disease. Another strategy to decrease disease pressure, if planting corn in the next season, could be to remove crop debris from the field or perform deep tillage. Additional research is needed to determine the rate of survival of *Xvv* and the exact moment at which the first infection occurs. To this end, field

evaluations with detailed sampling every day after planting will be crucial to observe initial symptoms of bacterial leaf streak of corn in the field.

CHAPTER 4:

CONCLUSIONS AND OPPORTUNITIES FOR DISEASE MANAGEMENT

This research focused on understanding aspects of the disease ecology of the emerging bacterial pathogen *Xanthomonas vasicola* pv. *vasculorum*. Similar to other foliar pathogens, *Xvv* likely enters through the stomata or wounds and remains restricted to the leaf tissue, acquiring nutrients in the apoplast of the plant cells. From mid- to late season, bacteria continue to spread by the formation of exudates which are splashed to other parts of the same plant, as well as adjacent plants and in between fields. This ensures the persistence of the pathogen during the corn growing season.

We also found that several *Xvv* isolates vary in aggressiveness and that popcorn is more susceptible to the pathogen than grain corn P1151. *Xvv* can infect and cause large lesions on corn. On sorghum, *Xvv* has the ability to form exudates, but there was no significant lesion expansion under greenhouse conditions. In contrast, *Xvh* causes large streaks on sorghum but not on corn. Both *Xvv* and *Xvh* had a similar response on sugarcane with formation of exudates but no lesion expansion. Although *Xvv* and *Xvh* have not been found in the field on sorghum and corn respectively, the interaction between these two pathogens is important to the evolution of *Xvv* infection on corn. The relationship between *Xvv* and the endophyte *P. ananatis* is significant because this endophyte has the potential to be used as a biocontrol agent for *Xvv*. However, further research is required to explore this possibility.

Furthermore, we showed that *Xvv* quantities were higher in infected residue left on the surface from the previous growing season. In this sense, continuous corn production and no-

till/reduced till practices may increase the risk of disease development because inoculum will remain on the residue. Although quantities of *Xvv* were low in the corn debris, only one infection event is necessary to perpetuate the disease for the next growing season.

Based on the previous information, there are several opportunities for disease management. Rotation to a non-host crop and tillage will decrease the chance of survival of the pathogen by removing the bacteria from its primary host and promoting degradation of the corn residue. If rotation is not an option, management of grasses and volunteer corn should be done as they can potentially serve as reservoirs for *Xvv* during the winter. Even though tillage is recommended, growers should consider soil erosion as a negative impact of this practice. Preventing movement of residue between fields may decrease disease incidence as well. Sanitation practices should also be implemented, especially by cleaning debris from machinery when used in multiple fields and harvesting heavily-infected fields last.

Finally, proper identification of the disease is crucial since chemical control methods are not available like those used with other foliar diseases of corn. Currently, there are no commercially available corn hybrids with resistance to *Xvv*. Therefore, identification of pathogen effectors involved in virulence as well as quantitative trait loci (QTL) for disease resistance in corn will aid in the design of resistant hybrids which will likely be the most effective and practical means of disease control.

REFERENCES

Adhikari TB, Gurung S, Hansen JM, Bonman JM, 2011. Pathogenic and genetic diversity of *Xanthomonas translucens* pv. *undulosa* in North Dakota. *Phytopathology* 102, 390-402

Ahmad AA, Askora A, Kawasaki T, Fujie M, Yamada T, 2014. The filamentous phage xacf1 causes loss of virulence in *Xanthomonas axonopodis* pv. *citri*, the causative agent of citrus canker disease. *Frontiers Microbiology* 5.

Arias S, Block CC, Mayfield DA, Jackson-Ziems TA, Broders KD, Munkvold GP, 2017. Occurrence of *Xanthomonas vasicola* on maize seeds in the United States San Antonio, TX.

Astua-Monge G, Minsavage GV, Stall RE, Davis MJ, Bonas U, Jones JB, 2000. Resistance of tomato and pepper to t3 strains of *Xanthomonas campestris* pv. *vesicatoria* is specified by a plant-inducible avirulence gene. *Molecular Plant Microbe Interactions* 13, 911–921.

Bart R, Cohn M, Kassen A, Mccallum EJ, Shybut M, Petriello A, Krasileva K, Dahlbeck D, Medina C, Alicai T, Kumar L, Moreira LM, Rodrigues Neto J, Verdier V, Santana MA, Kositcharoenkul N, Vanderschuren H, Gruissem W, Bernal A, Staskawicz BJ, 2012. High-throughput genomic sequencing of cassava bacterial blight strains identifies conserved effectors to target for durable resistance. *Proceedings of the National Academy of Sciences* 109, 1972-1979.

Bartetzko V, Hammes UZ, Sonnewald S, Börnke F, Vogel F, Hartner K, Stadler R, 2009. The *Xanthomonas campestris* pv. *vesicatoria* type III effector protein XopJ inhibits protein secretion: Evidence for interference with cell wall–associated defense responses. *Molecular Plant Microbe Interactions* 22, 655–664.

Beadle GW, 1980. The ancestry of corn. *Scientific American* 242, 112-119.

Beattie GA, Lindow SE, 1995. The secret life of foliar bacterial pathogens on leaves. *Annual Review Phytopathology* 33, 145–172.

Bennett AF, Dao KM, Lenski RE, 1990. Rapid evolution in response to high-temperature selection. *Nature* 346, 79–81.

Bossi L, Fuentes JA, Mora G, Figueroa-Bossi N, 2003. Prophage contribution to bacterial population dynamics. *Journal of Bacteriology* 185 6467–6471.

Bradbury JF, 1986. Guide to plant pathogenic bacteria. *CAB International*.

Brady C, Cleenwerck I, Venter S, Vancanneyt M, Swings J, Coutinho T, 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). *Systematic and Applied Microbiology* 31, 447-460.

Breiman L, 2001. Random forests. *Machine Learning Journal* 45, 5–32.

Breiman L, Friedman JH, Olshen RA, Stone CJ, 1984.Classification and regression trees. In. *Wadsworth International Group.* Belmont, California, 358.

Broders K. Status of bacterial leaf streak of corn in the United States. *Proceedings of the Proceedings of the Integrated Crop Management Conference*, *2017*, 111-115.

Brüssow H, Canchaya C, Hardt WD, 2004. Phages and the evolution of bacterial pathogens: From genomic rearrangements to lysogenic conversion. . *Microbiology Molecular Biology Review* 68, 560–602.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC, 1994. Green fluorescent protein as a marker for gene expression. *Science* 263, 802-805.

Chicaybam L, Barcelos C, Peixoto B, Carneiro M, Gomez-Limia C, Redondo P, Lira, Carla,, Paraguassú-Braga F, Farias Meira De Vasconcelos Z, Barros L, Bonamino MH, 2017. An efficient electroporation protocol for the genetic modification of mammalian cells. *Frontiers in Bioengineering and Biotechnology* 4, 1-13

Choi K-H, Gaynor JB, White KG, Lopez C, Bosio M, Karkhoff-Schweizer RR, Schweizer HP, 2005. Tn7-based broad-range bacterial cloning and expression system. *Nature Methods* 2, 443–448.

Coakley SM, Scherm H, Chakraborty S, 1999. Climate change and plant disease management. *Annual Review of Phytopathology* 37, 399-426.

Coutinho T, 1987. Bacterial streak disease of maize: Specificity and cultivar susceptibility: University of Natal, Master of Science.

Coutinho TA, Van Der Westhuizen L, Roux J, Mcfarlane SA, Venter SN, 2015. Significant host jump of *Xanthomonas vasicola* from sugarcane to a *Eucalyptus grandis* clone in South Africa. *Plant Pathology* 64, 576-581.

Coutinho TA, Venter SN, 2009. *Pantoea ananatis*: An unconventional plant pathogen. *Molecular Plant Pathology* 10, 325-335.

Coutinho TA, Wallis FM, 1991. Bacterial streak disease of maize (*Zea mays* L.) in South Africa. *Journal of Phytopathology* 133, 112–113.

Curland RD, Gao L, Bull CT, Vinatzer BA, Dill-Macky R, Van Eck L, Ishimaru CA, 2018. Genetic diversity and virulence of wheat and barley strains of *Xanthomonas translucens* from the upper midwestern United States. *Phytopathology* 108, 443-453.

Damicone J, Cevallos F, Olson J, 2018. First report of bacterial leaf streak of corn caused by *Xanthomonas vasicola* pv. *vasculorum* in Oklahoma. *Plant Disease* 102, 437-437.

Darrasse A, Carrère S, Barbe V, Boureau T, Arrieta-Ortiz ML, Bonneau S, Briand M, Brin C, Cociancich S, Durand K, Fouteau S, Gagnevin L, Guérin F, Guy E, Indiana A, Koebnik R, Lauber E, Munoz A, D Noël L, Pieretti I, Poussier S, Pruvost O, Robène-Soustrade I, Rott P, Royer M, Serres-Giardi L, Szurek B, Van Sluys M-A, Verdier V, Vernière C, Arlat M, Manceau C, Jacques M-A, 2013. Genome sequence of *Xanthomonas fuscans* subsp. *fuscans* strain 4834-r reveals that flagellar motility is not a general feature of Xanthomonads. *BMC Genomics* 14, 1471-2164.

Darsonval A, Darrasse A, Durand K, Bureau C, Cesbron S, Jacques MA, 2008a. Adhesion and fitness in the bean phyllosphere and transmission to seed of *Xanthomonas fuscans* subsp. *fuscans*. *Molecular Plant Microbe Interactions* 22, 747–757.

Darsonval A, Darrasse A, Meyer D, Demarty M, Durand K, Bureau C, Manceau C, Jacques MA, 2008b. The type III secretion system of *Xanthomonas fuscans* subsp. *fuscans* is involved in the phyllosphere colonization process and in transmission to seeds of susceptible beans. *Applied Environmental Microbiology* 74, 2669-2678.

Dastager SG, Lee JC, Ju YJ, Park DJ, Kim CJ, 2008. *Frigoribacterium mesophilum* sp. nov., a mesophilic actinobacterium isolated from Bigeum Island, Korea. *International Journal of Systematic and Evolutionary Microbiology* 58, 1869-1872.

De Nazareno NRX, Lipps PE, Madden LV, 1992. Survival of *Cercospora zeae- maydis* in corn residue in Ohio. *Plant Disease* 76, 560-563.

Dyer RA, 1949.Botanical surveys and control of plant disease. . In. *Annual Report Department of Agriculture, South Africa.* 119-121. (275.)

Earth-Data, 2018.Earth observing system data and information system (EOSDIS). In. *Scientific Data.* https://earthdata.nasa.gov/about. Accessed July 2018

Esgalhado ME, Roseiro JC, Amaral Collago MT, 1995. Interactive effects of pH and temperature on cell growth and polymer production by *Xanthomonas campestris*. *Process Biochemistry* 50, 667-671.

Fargier E, Fischer-Le Saux M, Manceau C, 2011. A multilocus sequence analysis of *Xanthomonas campestris* reveals a complex structure within crucifer-attacking pathovars of this species. *Systematic and Applied Microbiology* 34, 156-165.

Figueroa-Bossi N, Uzzau S, Maloriol D, Bossi L, 2001. Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in salmonella. *Molecular Microbiology* 39 260-271.

Fletcher J, Luster D, Bostock R, Burans J, Cardwell K, Gottwald T, Mcdaniel L, Royer M, Smith K, 2010. *Emerging infectious plant diseases*. Washington: ASM Press.

Garrett KA, Jumpponen A, Gomez-Montano L, 2010. Emerging plant diseases: What are our best strategies for management? *In: Kleinman, EL, JA Delborne, KA Cloud-Hansen and J. Handelsman (eds.). Controversies in Science and Technology: From Evolution to Energy* 3, 152-160.

Gent DH, Lang JM, Bartolo ME, Schwartz HF, 2005. Inoculum sources and survival of *Xanthomonas axonopodis* pv. *allii* in Colorado. *Plant Disease* 89, 507-514.

Gilbertson RL, Rand RE, Hagedorn DJ, 1990. Survival of *Xanthomonas campestris* pv. *phaseoli* and pectolytic strains of *X. campestris* in bean debris. *Plant Disease* 74, 322-327.

Goszczynska T, Venter SN, Coutinho TA, 2007. Isolation and identification of the causal agent of brown stalk rot, a new disease of corn in South Africa. *Plant Disease* 91, 711-718.

Grimont F, Grimont PaD, 2006. The genus *Serratia*. In. *The prokaryotes.* 219-244.

Gupta AK, Solanki, I.S., , Bashyal, B.M., , Singh Y, Srivastava K, 2015. Bakanae of rice - an emerging disease in Asia. *The Journal of Animal & Plant Sciences* 25, 1499-1514.

Hall BG, Acar H, Nandipati A, Barlow M, 2014. Growth rates made easy. *Molecular Biology and Evolution* 31, 232-238.

Hartman TM, 2018. Investigation of alternative hosts and agronomic factors affecting *Xanthomonas vasicola* pv. *vasculorum,* causal agent of bacterial leaf streak of corn. DigitalCommons@University of Nebraska - Lincoln: University of Nebraska - Lincoln Master of Science.

Hayward AC, 1974. Latent infections by bacteria. *Annual Review Phytopathology* 12, 87-97.

Hirano SS, Upper CD, 1983. Ecology and epidemiology of foliar bacterial plant pathogens. *Annual Review Phytopathology* 21, 243-269.

Hyre RA, 1943. New records and unusual occurrence of plant diseases. *Plant Disease* 27, 553-554.

Iimura K, Hosono A, 1996. Biochemical characteristics of *Enterobacteri agglomerans* and related strains found in buckwheat seeds. *International Journal of Food Microbiology* 30, 243-253.

Jacques MA, Arlat M, Boulanger A, Boureau T, Carrere S, Cesbron S, Chen NW, Cociancich S, Darrasse A, Denance N, Fischer-Le Saux M, Gagnevin L, Koebnik R, Lauber E, Noel LD, Pieretti I, Portier P, Pruvost O, Rieux A, Robene I, Royer M, Szurek B, Verdier V, Verniere C, 2016. Using ecology, physiology, and genomics to understand host specificity in *Xanthomonas*. *Annual Review Phytopathology* 54, 163-187.

Jain R, Rivera MC, Moore JE, Lake JA, 2003. Horizontal gene transfer accelerates genome innovation and evolution. *Molecular Biology and Evolution* 20, 1598-1602.

Jamann TM, Plewa D, Mideros SX, Bissonnette S, 2019. First report of bacterial leaf streak of corn caused by *Xanthomonas vasicola* pv. *vasculorum* in Illinois. *Plant Disease* 103, 1018-1018.

Jones JB, Phoronezny KL, Stall RE, Jones JP, 1985. Survival of *Xanthomonas campsteris* pv. *vesicatoria* in Florida on tomato crop residue, weeds, seeds, and volunteer tomato plants. *Phytopathology* 76, 430-434.

Kang SH, Cho HS, Cheong H, Ryu CM, Kim JF, Park SH, 2007. Two bacterial endophytes elicitng both growth promotion and plant defence on pepper (*Capsicum annum* L.). *Journal of Microbiology Biotechnology* 17, 96-103.

Kantety RV, Zeng X, Bennetzen JL, Zehr BE, 1995. Assessment of genetic diversity in dent and popcorn (*Zea mays* L.) inbred lines using inter simple sequence repeat (ISSR) amplification. *Molecular Breeding*, 365-373.

Karamura G, Smith J, Studholme D, Kubiriba J, Karamura E, 2015. Comparative pathogenicity studies of the *Xanthomonas vasicola* species on maize, sugarcane and banana. *African Journal of Plant Science* 9, 385-400.

Kauffman HE, Reddy APK, 1974. Seed transmission studies of *Xanthomonas oryzae* in rice. *Phytopathology* 65, 663-666.

Kim KS, Taylor SE, Gleason ML, Koebler KJ, 2002. Model to enhance site-specific estimation of leaf wetness duration. *Plant Disease* 86, 179-185.

Kinyua ZM, 2003. Genetic structure, virulence chracteristics and survival of'*Cercospora*'populations causing maize grey leaf spot in Kenya: Royal Holloway, University of London, PhD.

Korus KA, Lang JM, Adesemoye AO, Block CC, Pal N, Leach JE, Jackson-Ziems TA, 2017. First report of *Xanthomonas vasicola* causing bacterial leaf streak on corn in the United States. *Plant Disease* 101.

Kranz WL, Irmak S, Van Donk SJ, Yonts CD, Martin DL, 2008.Irrigation management for corn. In: Extension UONL, eds. *Institute of Agriculture and Natural Resources.* http://extension.unl.edu/publications.

KSU-Extension, 1989.Estimating corn residue. In: Kansas State University, ed. Cooperative Extension Service 589-592.

Kuan TL, Minsavage GV, Gabrielson RL, 1985. Detection of *Xanthomonas campestris* pv. *carotae* in carrot seed. *Plant Disease* 69, 758-760.

Lang JM, Ducharme E, Ibarra Caballero J, Luna E, Hartman T, Ortiz-Castro M, Korus K, Rascoe J, Jackson-Ziems TA, Broders K, Leach JE, 2017. Detection and characterization of

Xanthomonas vasicola pv. *vasculorum* (Cobb 1894) comb. nov. causing bacterial leaf streak of corn in the United States. *Phytopathology* 107, 1312-1321.

Langemeier CB, Robertson AE, Wang D, Jackson-Ziems TA, Kruger GR, 2017. Factors affecting the development and severity of Goss's bacterial wilt and blight of corn, caused by *Clavibacter michiganensis* subsp. *nebraskensis*. *Plant Disease* 101, 54-61.

Leite Jr. RP, Custódio AaP, Madalosso T, Robaina RR, Duin IM, Sugahara VH, 2018. First report of the occurrence of bacterial leaf streak of corn caused by *Xanthomonas vasicola* pv. *vasculorum* in brazil. *Plant Disease* 103.

Lenski RE, Simpson S, Nguyen TT, 1994. Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. *Bacteriology* 176, 3140–3147.

Lewis Ivey ML, Tusiime G, Miller SA, 2010. A polymerase chain reaction assay for the detection of *Xanthomonas campestris* pv. *musacearum* in banana. *Plant Disease* 94, 109-114.

Leyns F, De Cleene M, Swings JG, De Ley J, 1984. The host range of the genus *Xanthomonas*. *The Botanical Review* 50, 308-356.

Lichtenber E, 1989. Land quality, irrigation development, and cropping patterns in the Northern High Plains. *American Journal of Agricultural Economics* 71, 187-194.

Lin H, Gudmestad N, 2012. Aspects of pathogen genomics, diversity, epidemiology, vector dynamics, and disease management for a newly emerged disease of potato: Zebra chip. *Phytopathology* 103, 524-537.

Lindow SE, Brandl MT, 2003. Microbiology of the phyllosphere. *Applied Environmental Microbiology* 69, 1875–1883.

Liu T, Li R, Zhong X, Jiang M, Jin X, Zhou P, Liu S, Sun C, Guo W, 2018. Estimates of rice lodging using indices derived from UAV visible and thermal infrared images. *Agricultural and Forest Meteorology* 252, 144-154.

Mcfarlane S, Coutinho TA, 2010.Gummosis of sugarcane. In: Coutinho Ta GT, Lennox Cl, Venter Sn, ed. *Bacterial Diseases of Plants in South Africa.* Briza Publications, 114-115.

Melotto M, Underwood W, He SY, 2008. Role of stomata in plant innate immunity and foliar bacterial diseases. *Annual Review Phytopathology* 46, 101-122.

Mew TW, Alvarez AM, Leach JE, Swings J, 1993. Focus on bacterial blight of rice. *Plant Disease* 77, 5-12.

Moffett ML, 1983. Bacterial plant pathogens recorded in Australia.

Morris CE, Barny MA, Berge O, Kinkel LL, Lacroix C, 2017. Frontiers for research on the ecology of plant-pathogenic bacteria: Fundamentals for sustainability: Challenges in bacterial molecular plant pathology. *Molecular Plant Pathology* 18, 308-319.

Mwebaze JM, Tusiime G, Tushemereirwe WK, Kubiriba J, 2006. The survival of *Xanthomonas campestris* pv. *musacearum* in soil and plant debris. *African Crop Science Journal* 14, 121 - 127.

NAGC, 2019.Bacterial leaf streak. National Agricultural Genotyping Center. ed. *North Dakota Farmers Union.* In: https://www.genotypingcenter.com/services/testing/. Accessed March 2019.

Navi SS, Bandyopadhyay R, Thirumala Devi K, Reddy DVR, 2002. Bacterial leaf streak of sorghum: A new report from India. *ISMN* 43, 61-53.

NCEI, 2018.Global summary of the month. In: https://www.ncei.noaa.gov/. Accessed April 2019

Nguyen TN, Phan QG, Duong LP, Bertrand KP, Lenski RE, 1989. Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12. *Molecular Biology and Evolution* 6, 213–225.

North DS, 1935. The gumming disease of sugarcane, its dissemination and control.

Ofek M, Hadar Y, Minz D, 2012. Ecology of root colonizing *Massilia* (oxalobacteraceae). *PLoS One* 7, e40117.

Palleroni N, Bradbury J, 1993. *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) swings et al. 1983. *International Journal of Systematic Bacteriology* 43, 606-609.

Paul PA, Munkvold GP, 2004. A model-based approach to preplanting risk assessment for gray leaf spot of maize. *Phytopathology* 94, 1350-1357.

Peltier AJ, Esker PD, Bradley CA, Robertson A, Paul PA, 2008.Corn foliar diseases identification and management field guide. In: Extension TOSU-, ed.

Perez-Quintero AL, Ortiz-Castro M, Wu G, Lang JM, Liu S, Chapman TA, Chang C, Ziegle J, Peng Z, White FF, Plazas MC, Leach JE, Broders K, 2019. Genomic acquisitions in emerging populations of *X. vasicola* pv. *vasculorum* infecting corn in the U.S. and Argentina. *Phytopathology* In review.

Perfeito L, Fernandes L, Mota C, Gordo I, 2007. Adaptive mutations in bacteria: High rate and small effects. *Science* 317, 813-815.

Péros JP, Girard JC, Lombard H, Janse JD, Berthier Y, 1994. Variability of *Xanthomonas campestris* pv. *vasculorum* from sugarcane and other gramineae in Reunion Island. Characterization of a different Xanthomonad. *Journal of Phytopathology* 142, 177-188.

Plazas MC, De Rossi RL, Brücher E, Guerra FA, Vilaró M, Guerra GD, Wu G, Ortiz-Castro MC, Broders K, 2018. First report of *Xanthomonas vasicola* pv. *vasculorum* causing bacteria leaf streak of maize (*Zea mays*) in Argentina. *Phytopathology* 102, 1026.

Pope CF, Mchugh TD, Gillespie SH, 2010. Methods to determine fitness in bacteria. *Methods Molecular Biology* 642, 113-121.

Qhobela M, Claflin LE, Nowell DC, 1990. Evidence that *Xanthomonas campestris* pv. *zeae* can be distinguished from other pathovars capable of infecting maize by Restriction Fragment Length Polymorphism of genomic DNA. *Canadian Journal of Plant Pathology* 12, 183-186.

Qiu Y, Kaiser C, Schmidt C, Robertson A, Jamann TM, 2019.Identification of Quantitative trait loci effective against bacterial leaf streak of maize.Unpublished

Randhawa PS, Schaad NW, 1894. Selective isolation of *X. campestris* pv. *campestris* from crucifers seeds. *Phytopathology* 74, 258-272.

Reddy APK, Mackenzie DR, Rouse DI, Rao AV, 1979. Relationship of leaf-blight severity to grain-yield of rice. *Phytopathology* 69, 967-969.

Ricaud C, Autrey LJC, 1989. *Gumming disease*. Elsevier.

Rothrock CS, Woodward JE, Kemerait RC, 2015. Cotton diseases: Seed and seedling, bacterial blight, and vascular wilt. *Crops and Soils* 48, 32-41.

Ryan RP, Monchy S, Cardinale M, Taghavi S, Crossman L, Avison MB, Berg G, Van Der Lelie D, Dow JM, 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nature Review Microbiology* 7, 514-525.

Sakthivel N, Mortensen CN, Mathur SB, 2001. Detection of *Xanthomonas oryzae* pv. *oryzae* in artificially inoculated and naturally infected rice seeds and plants by molecular techniques. *Applied Microbiology and Biotechnology* 56, 435-441.

Sanko TJ, Kraemer AS, Niemann N, Gupta AK, Flett BC, Mienie C, Bezuidenhout CC, 2018. Draft genome assemblages of 10 *Xanthomonas vasicola* pv. *zeae* strains, pathogens causing leaf streak disease of maize in South Africa. *Genome Announcements* 6.

Sapkota S, Zhang Q, Chittem K, Mergoum M, Xu SS, Liu Z, 2018. Evaluation of triticale accessions for resistance to wheat bacterial leaf streak caused by *Xanthomonas translucens* pv. *undulosa*. *Plant Pathology* 67, 595-602.

Schulze-Lefert P, Panstruga R, 2011. A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. *Trends in Plant Science* 16, 117-125.

Schuster ML, Coyne DP, 1974. Survival mehcanims of phytopathogenic bacteria. *Annual Review Phytopathology* 2, 199-221.

Schwartz H, Gent DH, 2011.Xanthomonas leaf blight of onion. In: Colorado State Unviersity, ed. Colorado State Unviersity- Extension.

Schwartz HF, Otto KL, Gent DH, 2003. Relation of temperature and rainfall to development of Xanthomonas and Pantoea leaf blights of onion in Colorado. *Plant Disease*, 11-14.

Shiotani H, Uematsu H, Tsukamoto T, Shimizu Y, Ueda K, Mizuno A, Sato S, 2009. Survival and dispersal of *Xanthomonas citri* pv. *citri* from infected satsuma mandarin fruit. *Crop Protection* 28, 19-23.

Shrestha RK, Rosenberg T, Makarovsky D, Eckshtain-Levi N, Zelinger E, Kopelowitz J, Sikorski J, Burdman S, 2013. Phenotypic variation in the plant pathogenic bacterium *Acidovorax citrulli*. *PLoS One* 8, e73189.

Silva Júnior TaF, Negrão DR, Itako AT, Soman JM, Maringoni AC, 2012. Survival of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in soil and bean crop debris. *Journal of Plant Pathology* 94 331-337.

Singh R, Sunder S, 2012. Foot rot and bakanae of rice: An overview. *Review Plant Pathology* 5, 565-604.

Singh RP, Hodson DP, Jin Y, Lagudah ES, Ayliffe MA, Bhavani S, Rouse MN, Pretorius ZA, Szabo LJ, Huerta-Espino J, Basnet BR, Lan C, Hovmoller MS, 2015. Emergence and spread of new races of wheat stem rust fungus: Continued threat to food security and prospects of genetic control. *Phytopathology* 105, 872-884.

Smith DL, Groves C, Lueloff S, 2018.Bacterial leaf streak of corn confirmed for the first time in Wisconsin. In. *Wisconsin Crop Manager Newsletter.*

Studholme DJ, Wicker E, Abrare SM, Aspin A, Bogdanove A, Broders K, Dubrow Z, Grant M, Jones JB, Karamura G, Lang J, Leach J, Mahuku G, Nakato GV, Coutinho T, Smith J, Bull CT, 2019. Transfer of *Xanthomonas campestris* pv. *arecae* and *Xanthomonas campestris* pv. *musacearum* to *Xanthomonas vasicola* (Vauterin) as *Xanthomonas vasicola* pv. *arecae* comb. nov., and *Xanthomonas vasicola* pv. *musacearum* comb. nov. and description of *Xanthomonas vasicola* pv. *vasculorum* pv. nov. *Phytopathology* In review.

Stulberg MJ, Santillana G, Studholme DJ, Kasiborski1 B, Ortiz Castro M, Broders K, Arias S, Block C, Munkvold G, Rascoe J, 2019. Genomics-informed molecular detection of *Xanthomonas vasicola* pv. *vasculorum* strains causing severe bacterial leaf streak of corn. *Phytopathology* Accepted May 2019.

Thapa S, 2014. Fiel survival of *Xanthomonas cucurbitae*, the causal agent of bacterial spot of pumpkin, and efficacy of selected chemicals and biocontrol agents for control of the diseases: University of Illinois at Urbana-Champaign,, Master of Science.

Turkington TK, Kuzyk A, Dunn R, Mclaren D, Irvine B, Clearn RM, Patrick SK, 2004. Irrigation and plant disease management. *Plant Disease* 81, 49-52.

Tushemereirwe W, Kangire A, Ssekiwoko F, Offord LC, Crozier J, Boa E, Rutherford M, Smith JJ, 2004. First report of *Xanthomonas campestris* pv. *musacearum* on banana in Uganda. *Plant Pathology* 53, 802-802.

USDA-NASS, 2018a.Corn statistics. In: United States Department of Agriculture USDA, ed. National Agricultural Statistics Service. http://www.nass.usda.gov. Accessed August 2018

USDA-NASS, 2018b.Crop production summary. In: United States Department of Agriculture USDA, ed. National Agricultural Statistics Service. http://www.nass.usda.gov. Accessed July 2018

USDA-NRCS, 2018.Corn stover. In: United States Department of Agriculture USDA, ed.Natural Resources Conservation Service. https://www.nrcs.usda.gov/wps/portal/nrcs/site/national/home/. Accessed March 2019.

Vauterin L, Hoste B, Kersters K, Swings J, 1995. Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology,* 45, 472-489.

Vega D, Romero AM, 2016. Survival of *Clavibacter michiganensis* subsp. *michiganensis* in tomato debris under greenhouse conditions. *Plant Pathology* 65, 545-550.

Wang XQ, Allen TW, Wang H, Peterson DG, Nichols RL, Liu A, Li XD, Deng P, Jia D, Lu SE, 2019. Development of a qPCR protocol to detect the cotton bacterial blight pathogen, *Xanthomonas citri* pv. *malvacearum*, from cotton leaves and seeds. *Plant Disease* 103, 422-429.

Ward JMJ, Nowell DC, 1998. Integrated management practices for the control of maize grey leaf spot. *Integrated Pest Management Reviews* 3, 177-188.

Ward JMJ, Stromberg EL, Nowell DC, Nutter FW, 1999. Gray leaf spot: A disease of global importance in maize production. *Plant Disease* 83, 884-895.

Wasukira A, Coulter M, Al-Sowayeh N, Thwaites R, Paszkiewicz K, Kubiriba J, Smith J, Grant M, Studholme DJ, 2014. Genome sequencing of *Xanthomonas vasicola* pathovar *vasculorum* reveals variation in plasmids and genes encoding lipopolysaccharide synthesis, type-IV pilus and type-III secretion effectors. *Pathogens* 3, 211-237.

Wise K, Ruhl G, Creswell T, 2010.Goss's bacterial wilt and leaf blight. In: Purdue University, ed. *Purdue extension.*

Young JM, Bradbury JF, Davis RE, Dickey RS, Ercolani GL, Hayward AC, Vidaver AK, 1991. Nomenclatural revisions of plant pathogenic bacteria and list of names 1980-1988. *Review of Plant Pathology* 70, 211-221.

Zhao YF, Damicone JP, Bender CL, 2002. Detection, survival, and sources of inoculum for bacterial diseases of leafy crucifers in Oklahoma. *Plant Disease* 86, 883-888.

Zwietering MH, Jongenburger I, Rombouts FM, Van't Riet K, 1990. Modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, 1875-1881.