EPIPHYTIC SURVIVAL AND BIOFILM FORMATION OF THE GOSS'S WILT PATHOGEN *CLAVIBACTER MICHIGANENSIS* SUBSP. *NEBRASKENSIS*

By

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ABSTRACT

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Goss's wilt, a bacterial disease of corn caused by *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*), has re-emerged in the corn belt of the USA, since 2006. Corn fields in Michigan and Indiana were scouted from 2014 to 2016 for Goss's wilt, and putative *Cmn* isolates were obtained from corn leaf tissue. Known isolates were obtained from cultures or diseased leaf tissue from Indiana, Iowa, Missouri, and a culture collection at Michigan State University. All *Cmn* isolates were separated into three virulence groups and biofilm formation for each was observed in vitro. Biofilm formation of a highly virulent and a moderately virulent *Cmn* isolate was observed in vivo. All isolates produced strong biofilms on TEM grids and the slightly virulent isolate FN produced the most biofilm on glass coverslips. The highly virulent isolate GIL1 exhibited an aggregation phenotype in planta, while the moderately virulent isolate GW-20-E was observed forming biofilms within and around xylem vessels. Examination of epiphytic and endophytic was done to determine if there was any correlation to virulence. Populations of spontaneous rifampicin-resistant mutants of *Cmn* isolates that differed in virulence were tracked over time on Goss's wilt susceptible hybrids. There was no correlation between virulence and endophytic or epiphytic survival of *Cmn.*

I would like to dedicate this thesis to my incredibly supportive parents and my best friend Ryan Naughton, who helped and encouraged me through this process.

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KEY TO ABBREVIATIONS

5U/µl	5 unites per microliter
µl	Microliter
µM	Micromolar
µg/ml	Micrograms per milliliter
AFLP	Amplified fragment length polymorphism
Cm	*Clavibacter michiganensis*
Cmi	*Clavibacter michiganensisi* subsp. *insidiosus*
Cmm	*Clavibacter michiganensis* subsp. *michiganensis*
Cmn	*Clavibacter michiganensis* subsp. *nebraskensis*
Cms	*Clavibacter michiganensis* subsp. *sepedonicus*
Cmt	*Clavibacter michiganensis* subsp. *tesselarius*
CFU's/g	Colony forming units per gram
DDG's	Dried distillers grains
diH_2O	Deionized water
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
ELISA	Enzyme linked immunosorbent assay
EPS	Exopolysaccharides
fl oz/A	Fluid ounces per acre

g	Grams
gal/A	Gallons per acre
IA	Iowa
IN	Indiana
ITS	Internal transcribed spacer
J/m^2s^{-1}	Joules per meter squared times seconds
LAMP	Loop mediated amplification
LB	Lysogeny broth
lb/A	pounds per acre
mE	Millieinsteins
$MgCl_2$	Magnesium chlorite
MI	Michigan
Ml	milliliter
mm	Millimeter
mM	Millimolar
MO	Missouri
NBY	Nutrient broth yeast extract
nt	Nucleotide
OD_{600}	Optical density 600
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rcf	Relative centrifugal force

rpm	Rotations per minute
rRNA	ribosomal ribbonucleic acid
subsp.	Subspecies
UV-C	Ultraviolet-C
V3	Vegetative 3
V4	Vegetative 4

CHAPTER 1

Literature review

1.1 Corn production in Michigan

The United States produces forty percent of the world's corn, and corn is the largest crop produced in the country on a per yield basis. In 2014, a record of 14.4 billion bushels was harvested in the US. Although Michigan is not one of the major corn producing states, the crop plays a vital role in its economy. In 2014, 2.5 million acres of corn were planted by farmers in Michigan, and 360 million bushels were harvested in Michigan contributing $1.5 billion to the state's economy. Direct and indirect uses of corn provide over two billion dollars to the economy of the state. In 2015, 37% of the corn produced in Michigan was shipped to the southwest U.S. and used as feed in the southwestern US for poultry and livestock. A portion (27%) of Michigan's corn crop was used to produce ethanol from 2013-2014. Dried distiller's grains (DDGs) are a byproduct of ethanol production, and in 2013, MI produced 738,000 metric tons of DDGs. DDGs are important because they provide a high protein source for livestock. Additional uses of corn in Michigan include uses as sweeteners, corn syrup, the production of alcoholic beverages, starches, packaging peanuts, baby wipes, household cleaners, and many other biodegradable items such as cups, plastic bags and blankets (CMPM, 2015).

The production of seed corn is also vital to the economy of Michigan. The largest Monsanto seed corn plant is in Constantine Co located in southern MI. Forty percent of the US seed corn is produced by this Monsanto plant, a Pioneer Hi-Bred International plant located

nearby, and local seed corn growers in the nearby county of St. Joseph (Parker, 2010). Many local growers work for the Monsanto plant in Constantine where they harvest, sort and package seed corn (Parker, 2010). In 2010, 1.9 million bushels of seed were produced in MI almost entirely in St. Joseph County. This seed is shipped across country and to Canada and is used for the next year's crop (Parker, 2010). Remington Seeds is another seed corn company that has a plant in Sturgis MI that provides quality seed for local farmers (Remington, 2016). Potential pathogen threats to corn must be identified to ensure that damaging economic losses do not occur.

1.2 Goss's wilt disease (Background)

The bacterial disease of corn known as Goss's wilt was first discovered in 1969 in Dawson Co., Nebraska. The bacterium responsible is a gram positive, orange pigmented Corynebacterium (Vidaver and Mandel, 1974). This organism was initially named *Corynebacterium nebraskense*, and the disease it caused was called Nebraska leaf freckles and wilt. Later the disease was renamed "Goss's bacterial leaf blight and wilt" after Lincoln R. W. Goss, a former Chairman of the Department of Plant Pathology at the University of Nebraska (Wysong, et al., 1973).

The disease spread to 53 counties in Nebraska in a 10-year span (Jackson et al, 2007). In 1975 it was also confirmed in Iowa, Kansas, South Dakota, and Iowa (Treat et al., 1990). By 1979, the disease occurred in Kansas, Iowa, South Dakota and Colorado, and the causal bacterium was classified as *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*) (Agarkova et al., 2011). During the 1970s and 1980s, the disease occurred sporadically and was confined to a

few fields likely due to the use of corn varieties with partial disease resistance to Goss's wilt. (Singh et al., 2016). However, during this time period, some varieties of corn that were highly susceptible were planted, including sweet corn, dent corn hybrids and popcorn (Agarkova et al., 2011). Goss's wilt began to reemerge, and since 2006, the disease has been reported in Louisiana (Hollier, 2013), Colorado, Illinois, Iowa, Kansas, Minnesota, South Dakota, Wisconsin, Nebraska, (Singh et al., 2016) and Alberta (Howard et al., 2015).

Although Goss's wilt has yet to be confirmed in MI via completion of Koch's postulates, the disease was found only 60 miles south of MI in Indiana in the summer of 2015 (M. Botti-Marino, personal observation). From 2009 to 2013, the disease was also detected in infected corn plants by the Michigan Department of Agriculture using the enzyme linked immunosorbent assay (ELISA) kits and Agdia Immunostrip detection methods. The counties from which Goss's wilt was positively identified include Branch, Calhoun, Kalamazoo and St. Joseph (Michigan Department of Agriculture and Rural Development, personal communication). The enzyme linked immunosorbent assay tests and Agdia Immunostrips may lead to false positives because the antibodies relied on for detection may cross react with non-target bacteria (www.Agdia.com). Completion of Koch's postulates is necessary to accurately identify the pathogen in MI.

The causal agent is known to infect corn seed at a very small rate, and may be a method for the pathogen to spread to new locations and fields. For example, in 2013, Goss's wilt was also reported in a field in Louisiana in which circular patches of diseased plants formed that were 50 feet in diameter. These were only located because an aerial applicator flew over the field and spotted them. Upon further investigation, it appeared that the diseased pockets of

plants were radiating out from a central point, indicating that the initial infection may have been due to infected seed (Hollier, 2013). Identification of the pathogen can be difficult which may be preventing its identification in the state.

As mentioned previously, Goss's wilt has also been confirmed in Canada. The first report of Goss's wilt in the province of Alberta, Canada was published in August of 2013 (Howard et al., 2015). It was reported that leaves from hybrid grain and silage corn displayed symptoms typical of those observed in Goss's wilt in four counties in southern Alberta and one in central Alberta. A total of 46 fields were analyzed, and six of 11 samples tested positive for Goss's wilt. Microscopic analysis also revealed bacterial streaming that consisted of small pleomorphic gram positive rods. Further confirmation of *Cmn* was achieved using Agdia Immunostrip test kits, DNA sequencing and Koch's postulates (Howard et al., 2015).

1.3 Identification techniques for *Clavibacter michiganensis* subsp. *nebraskensis*

The genus *Clavibacter* contains only one species known as *C. michiganensis.* Within this species, there are several subspecies determined by the host that the bacterium infects as well as phenotypic and genotypic profiling. The most well-known subspecies include *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*), which causes Goss's wilt in corn; *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), which causes bacterial canker of tomato and pepper; *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), which causes potato ring rot; *Clavibacter michiganensis* subsp. *insidiosus* (*Cmi*), which causes stunting and wilting in alfalfa, and *Clavibacter michiganensis* subsp. *tessellarius* (*Cmi*), which causes bacterial mosaic in wheat (Gonzalez and Trapiello, 2014). Recently, several new subspecies have been discovered. These

include populations of *Clavibacter* isolated from tomato seed with the proposed names *Clavibacter michiganensis* subsp. *californiensis* subsp. nov. and *Clavibacter michiganensis* subsp. *chilensis* subsp. nov. (Yasuhara-Bell and Alvarez, 2015), and a novel subspecies which is pathogenic to bean with the proposed named *Clavibacter michiganensis* susp. *phaseoli* subsp. nov. (Gonzalez and Trapiello, 2014). A novel subspecies was also isolated from pepper plants (Yasuhara-Bell and Alvarez, 2015).

Bacteria in the genus *Clavibacter* belong to the family *Microbacteriaceae* and the class *Actinobacteria* (Stackebrandt et al., 2007). *Actinobacteria* refer to gram positive bacteria with a high %G+C content of their genomic DNA that also have very diverse morphologies and habitats. For example, *Streptomyces* lives in the soil, *Leifsonia* displays commensality on plants and *Bifidobacterium* displays commensality in the intestines. Additionally, it is difficult to determine which group of bacteria is phylogenetically most related to this diverse group of bacteria because it diverged from other bacteria a long time ago (Ventura et al., 2007). All of the subspecies in the genus *Clavibacter* share the following characteristics: they are aerobic, non-motile, do not form spores, and are of coryneform morphology (Lu, 2015).

Several detection methods for *Cmn* have been developed to date, but many are insufficient because they cross react with the other *Clavibacter michiganensis* subspecies. One example is a serological assay known as ELISA, as mentioned above (www.Agdia.com). Previous research has shown that use of antisera created to detect *Clavibacter michiganensis* subspecies can also generate false positives from isolates that may not be *Clavibacter* (Franken et al., 1993). Due to this, false positives may arise that require inoculation of isolates into plants to confirm positive test results with disease symptoms on host plants (Li et al., 1997).

Successful attempts have been made to detect the *Clavibacter michiganensis* subspecies via the use of Taq-man PCR and *Cm* specific primers attached to subspecies specific fluorogenic probes (Bach et al., 2003). The primer pairs for each subspecies are designed to pair with the intergenic sequences (ITS) of the rRNA operon which is found across all subspecies. The probes allow for specific detection of individual subspecies. The ITS regions for each subspecies are as follows: L43095 for *Cmt*, U09378 for *Cmi*, U09379 for *Cmm*, U09380 for *Cmm*, U90381 for *Cmn* and U09382 for *Cms* (Bach et al., 2003). These primers can amplify a DNA fragment 223 nucleotides (nt) in length. The areas that the probes target for each subspecies can be found within the 223 nt region about 81 nt to the forward primer. The researchers found that the probes used were specific to the various *Cm* subspecies, however the *Cmt* probe cross reacted slightly with the *Cms* DNA. However, the two fluorescence PCR curves that were generated are quite different. The *Cms* probe did not react with *Cmt* DNA (Bach et al., 2003).

Attempts have also been made to distinguish between *Cm* subspecies via analysis of polymorphisms in housekeeping genes. In 2011, Waleron et al (2011), utilized polymorphisms between the following genes in *Cmm, Cms, Cmi, Cmn* and *Cmt*: *recA, rpoB* and *rpoD*. Primers for PCR that targeted these genes were used, and PCR products were further analyzed via restriction fragment length polymorphism with four restriction endonucleases. The fragments generated were again subjected to PCR that was used to distinguish between the various *Cm* subspecies (Waleron et al., 2011). This was found to be an effective method for differentiation among the subspecies, especially if the endonuclease FnuDII was used (Waleron et al., 2011). Most recently, a loop-mediated amplification (LAMP) technique has been developed to detect *Cmn* specifically. This assay is able to detect the tripartite ATP-independent periplasmic (TRAP)-

type C4-dicarboxylate transport system large permease component with specific primers (Yasuhara-Bell et al.). This assay was tested with the *Cm* subspecies *Cmi*, *Cmm*, *Cmn*, *Cms*, *Cmt*, *Clavibacter michiganensis* subsp. *californiensis* subsp. nov, *Cm* isolated from pepper, *C. michiganensis* subsp. *chilensis* subsp. nov., and *C. michiganensis* subsp. *phaseoli* subsp. nov. Additional bacterial isolates used included, but were not limited to: *Microbacterium* sp., *Acidovorax avenae*, *Dickeya* sp., and *Pectobacterium* sp. All bacterial isolates were tested with both the LAMP assay, as well as the *Cmm* Immunostrip assay developed by Agdia, Inc. While several of the non-target bacterial species cross reacted with the *Cmm* Immunostrip assay, only the *Cmn* isolates used in this studied were positively identified using the LAMP assay (Yasuhara-Bell et al., 2016).

1.4 Phylogenetics/population structure of *Clavibacter michiganensis* subsp. *nebraskensis*

In the 1980s, Smidt and Vidaver characterized 50 strains of *Cmn* isolated from a popcorn field in Nebraska, based on their color, colony morphology, ability to cause disease, bacteriophage sensitivity and whether they produced a bacteriocin. They placed the strains into seven groups, which were further divided into 20 subgroups based on bacteriophage sensitivity. Strains were collected from winter to harvest from corn residue left from a crop planted that year, and were also collected from fresh plant tissue the following growing season (Smidt and Vidaver, 1987). For morphologies, the strains were divided into four groups: orange fluidal, dark orange and non-fluidal, fluidal and yellow, and colonies that appeared white when small, but later turned orange. Some of those that were orange and fluidal did not cause disease symptoms in corn, but still appeared to be *Cmn* based on morphology. For bacteriocin production *Cmm* strain 13-3 (Smidt and Vidaver, 1987) was used as an indicator strain, since the

bacteriocin that *Cmn* produces inhibits the growth of *Cmm*. *Clavibacter michiganensis* subsp. *nebraskensis* strains were found to either produce bacteriocin CN1, CN1 and CN2, or no bacteriocin. These data, along with phage sensitivity and pathogenicity testing, allowed for separation of the strains into seven groups with 20 subgroups. This study showed that populations of *Cmn* in a single field could be quite diverse (Smidt and Vidaver, 1987).

More recently, as molecular advancements have been made, studies have been done placing *Cmn* into much smaller groups based on genetic polymorphisms. These studies have also incorporated strains from varied geographic locations rather than a single field. For example, in a study conducted by Agarkova and Vidaver (2011), 131 Cmn strains collected from 1969-2009 and isolated from diverse locations geographically and temporally were placed into two major groups via amplified fragment length polymorphism (AFLP) analysis and repetitive DNA sequence-based BOX-PCR (Agarkova et al., 2011).

1.5 Disease cycle (Infection process) for Goss's wilt

Goss's wilt can become a problem in fields through infested corn residue from previous years, and from infested seed (Yasuhara-Bell et al., 2016). Plant wounds from wind-borne sand or hail, may also play a role (Eggenberger et al., 2016); it has not been determined if wounding is necessary for infection. The causal agent can infect corn at any growth stage, and symptoms appear from mid-to late season when temperatures are high (Treat and Tracy, 1990). Factors such as the environment, the planting of susceptible hybrids, rotating corn with corn and the reduced practice of tillage, may all contribute to the increase of Goss's wilt (Eggenberger et al.). Seed does become infected at low rates, as mentioned previously, but it does not readily

transmit the Goss's wilt pathogen to seedlings. Due to this, management of seed infection is not considered an important concern in areas where the disease is well established (Biddle et al., 1990). Management of corn residue is important, as several studies have shown that *Cmn* can survive for a period on host debris (Eggenberger et al., 2016).

Geographic region may also influence the survival time of the pathogen on host residue. For example, the closely related tomato pathogen (*Cmm*), survived for four months in host debris in Morocco, and up to two years in Iowa (Vega and Romero, 2015). Furthermore, in both California and Ohio, *Cmm* could be isolated from host debris for 10 months and for at least seven months in Illinois (Vega and Romero, 2015). Other factors that may influence survival of the pathogen on host residue include whether the host debris are on the surface or buried in the soil (Vega and Romero, 2015). In a study involving *Cmn*, the bacterium could not be recovered after 10 months from pure cultures mixed with soil, that were on the surface and buried 10 or 20 cm, and kept in field conditions (Biddle, 1990). The pathogen can survive on host residue, but it cannot survive well when buried in soil, especially in the absence of host residue. Eggenberger, et al. in 2012 and 2013 showed that Goss's wilt symptoms spread through fields inoculated with infested residue, as well as fields that contain wounded and inoculated plants, although the disease spread more slowly in fields inoculated with infested residue only. Furthermore, epiphytic populations of the pathogen could be recovered from asymptomatic corn plants 2.5 meters from inoculum sources in fields containing plants that were either wounded and inoculated or inoculated with infested residue. Incidence of the pathogen recovered was again higher in the wound inoculated fields (Eggenberger et al., 2016). This suggests that tillage of residue may reduce such incidences as well as rotation of a non-

host crop each year, as this would eliminate a host for the bacteria to live on, and populations would naturally decline. Additionally, certain alternative host weeds such as foxtail, shattercane, annual ryegrass, johnsongrass, large crabgrass and barnyard grass can support populations of *Cmn* (Louis, 2013, Ikley, et al., 2015). Limiting weeds in and around corn fields may help to reduce populations of *Cmn*.

Other factors that influence the development of Goss's wilt are the growth stage of hybrids, environmental factors such as plant stress, internal bacterial populations, and age of inoculation. For example, it was shown that early maturing hybrids from Midwestern germplasm, tend to be more susceptible to the disease than intermediate and late maturing hybrids (Rocheford et al., 1989). Furthermore, it was shown in a study by Suparyono and Pataky (1989) that disease symptoms were more severe when a Goss's wilt susceptible sweet corn hybrid was inoculated at the three to five leaf stage in comparison to hybrids inoculated at later growth stages (five-to seven-leaf or seven-to nine-leaf). It should be noted that disease ratings were still high for the susceptible hybrid when inoculated at the three growth stages. Hybrids with moderate resistance did not exhibit severe symptoms when inoculated at later growth stages. In this study, it was also found that yield for more resistant hybrids that were inoculated at later growth stages was the highest (Pataky and Suparyono, 1989). Carson and Wicks (Carson and Wicks, 1991) examined the relationship between the Goss's wilt symptoms of leaf freckles and wilting, and yield losses in related maize hybrids. Hybrids that differed in resistance to Goss's wilt were inoculated at the four-to six- and eight-to 10-leaf stages via a pin prick method. Ten random plants per plot were then rated for disease severity at the midtassel growth stage on a scale from one-nine, with nine referring to highly symptomatic plants.

Although yield loss could be significantly associated with disease severity ratings, only about 40% of the yield loss observed could be associated with disease severity ratings. Some of the hybrids used had significant reductions in yield when compared to hybrids with disease ratings that were not significantly different (Carson and Wicks, 1991).

Temperature is also an important factor influencing disease development for plant pathogenic bacteria. Several studies conducted by Sharabani et al., demonstrated how temperature influences the ability of the closely related subspecies *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) to infect its host, tomatoes, and develop cankers on the fruit (Sharabani et al., 2014). When tomato seedlings were inoculated, they wilted and died more quickly when temperatures ranged from 21-24^{o}C and when temperatures ranged from 18-23^{o}C, compared to temperatures that ranged from 15-18^{o}C or temperatures that ranged from 28-31^{o}C. Plants died most quickly when the average temperature was 26^{o}C. The expression of virulence factors of *Cmm*, population growth, and canker development on tomato seedlings were affected by temperature (Sharabani et al., 2014). Additionally, the rate of disease symptoms of *P. solanacearum* on tomato increase at temperatures between 30^{o}-35^{o}C, and even resistant tomato cultivars may exhibit disease symptoms at these temperatures (Hayward, 1991). This shows that temperature is a very important factor for the growth of phytopathogenic bacteria and the development of disease. It is most likely the same case with the development of disease and population growth of *Cmn*. It is known that the bacterium favors a temperature between 24^{o}C and 28^{o}C (Langemeier, 2012), so a temperature around this would probably best favor disease development.

1.6 Methods of disease management of Goss's wilt

Agricultural practices such as crop rotation and tillage can reduce inoculum levels of the Goss's wilt pathogen. Partially resistant Goss's wilt hybrids may be used, but may not always be available to growers. Due to this, chemical controls may still be necessary to help growers retain yields. In 2012 and 2013, Mehl et al., studied the effects of copper hydroxide and citric acid on Goss's wilt severity and corn yield. In this study, varieties of corn that were susceptible to Goss's wilt, were grown at the University of Illinois Crop Sciences Research and Education Center (CSREC). In their experiments, they included a non-treated control and applied the two previously mentioned chemicals to plants 24 hrs before inoculating them with Cmn, as well as 25 hrs after and five-seven days after. There was no significant difference in yield between the plants treated with copper hydroxide and citric acid and the non-treated controls. (Mehl et al., 2015).

In 2012, Oser et al. conducted leaf treatment trials to manage the disease in Nebraska. In this study the researchers used the corn hybrid DKC 37-38, which was given a rating of seven out of nine for Goss's wilt rating, which equated to a "poor". The plants were inoculated two times and simultaneously injured in such a way to mimic hail damage. Inoculum was made of five *Cmn* strains in tryptic soy broth and water. The treatments were the following: a non-treated control, Kocide 3000 (46.1% copper hydroxide) (1.5 lb/A), Procidic (citric and organic acids) (6.0 fl oz/A), Oxidate (27.1% hydrogen dioxide, 2.0% peroxyacetic acid) (1.0 gal/A), and Headline AMP (metconazole and pyraclostrobin) (10 fl oz/A). Treatments were applied before inoculation, after inoculation and after lesions had formed. There were no significant

differences in disease severity or yield between the treated and non-treated controls (Oser et al., 2013).

Studies with *Clavibacter michiganensis michiganensis* (*Cmm*), have shown that applications of bactericides such as copper hydroxide and streptomycin can reduce *Cmm* populations and spread (Hausbeck et al., 2000). In this study, tomato seedlings that were inoculated with *Cmm* were treated with either mancozeb, copper hydroxide, mancozeb mixed with copper hydroxide, streptomycin, streptomycin mixed with copper hydroxide, and mancozeb mixed with copper hydroxide 12 hrs before spraying. All treatments except mancozeb, reduced both the spread of and population size of the bacteria on tomato plants in the greenhouse to less than $5.0x10^5$ CFU/g of tissue. Additionally, all greenhouse treatments except mancozeb, increased plant survival in the field and tomato yields when compared to the untreated inoculated control. Results found suggest that bactericide sprays may be used to control bacterial populations of *Cmn* when applied early and prior to the buildup of high bacterial populations.

1.7 Cmn epiphytic and endophytic populations studies

Phytopathogenic bacteria can survive epiphytically on plant hosts, build populations, and enter host tissues through natural openings, such as stomata and hydathodes. These bacterial populations can survive UV radiation and significant temperature changes that occur on plant surfaces (Wilson et al., 1999). The increase of endophytic populations is crucial for disease development. The pathogenic bacterial strain *P. syringae* pv. *syringae* B728a-GFP, builds up epiphytic and endophytic populations on bean leaves, and at 95% RH the bacteria

move into stomata after only three days. After six days, bacterial streaming could be observed (Sabaratnam and Beattie, 2003). This suggests that both the buildup of endophytic and epiphytic populations of phytopathogenic bacteria may be important for initiation of disease symptoms in hosts.

Little research has been conducted concerning the epiphytic and endophytic survival of *Cmn*, but populations of the pathogen have been recovered from the surfaces of maize leaves in the field (Ahmad et al., 2015). Populations of some of the *Clavibacter* subspecies have the ability to survive on non-host plants and farm equipment (Bach et al., 2003). Goss's wilt leaf blight symptoms may appear at the tips of apparently undamaged maize leaves in fields and have typically been observed around the time of tasseling (Mallowa et al., 2016). *Cmn* can enter through wounds in maize plants caused by hail and sand to insight disease (Agarkova et al., 2011). Mallowa et al. (2016), determined that Goss's wilt susceptible maize hybrids that were grown to the V4-V5 leaf stage and spray inoculated without detectable wounding showed Goss's wilt symptoms seven days post inoculation (dpi), after being exposed to high levels of humidity and ambient humidity. Leaves that were only spray inoculated were viewed via an SEM to observe external and internal populations. Just 12 hrs after spray inoculation, *Cmn* cells could be observed on stomata, around the bases of trichomes and around guard cells. Aggregates of *Cmn* cells were also found in substomatal chambers just 72 hrs after inoculation (Mallowa et al., 2016). This suggests that populations of *Cmn* most likely build external populations and move inward through natural openings in the plant over time, possibly leading to disease symptoms.

1.8 Biofilm formation of gram positive bacteria

A significant part of bacterial associations with plants is the formation of biofilms, which are aggregations of cells, extracellular polymeric substances (EPS), proteins and DNA. Biofilms can range from flat films to complex structures and allow bacteria to survive harsh environments. For some pathogenic bacteria that inhabit the xylem or phloem of plant tissues, such as *Xanthomonas campestris* pv. *campestris* (*Xcc*), biofilm formation is necessary for virulence. Once the pathogen enters the vascular tissue of its host through wounds, it not only forms biofilms, which allow it to disperse and spread throughout the plant, but it also produces exoenzymes and exopolysaccharides, regulated by proteins and a DSF signal synthase. The exoenzymes allow the pathogen to break down host plant tissues, while the exopolysaccharide, known as xanthan gum, allows the pathogen to form biofilms (Ramey et al., 2004). The fire blight pathogen of apple trees, *Erwinia amylovora*, requires production of the EPS amylovoran to form biofilms in its host and efficiently colonize and infect the vascular tissues (Koczan et al., 2009).

In a study conducted in 2011, a (GFP)-labeled strain of *Cmm* was tracked in planta to determine where the pathogen preferred to form biofilms within the vascular tissues and how it moved throughout them to successfully cause systemic infection and disease. Certain plasmid encoded genes were also determined to be necessary for movement of the pathogen into the vascular tissue to incite disease, by tracking a mutant lacking these virulence genes (Chalupowicz et al., 2012). In this same study, biofilm formation by *Cmm* in tomato xylem sap, M9 minimal medium and LB medium was observed. It was shown that *Cmm* produced more

biofilm in xylem sap, suggesting that there may be a chemical(s) in the sap that triggers biofilm formation by the pathogen (Chalupowicz et al., 2012).

Little is known about the biofilm formation of *Cmn* in corn. In a recent study conducted by Mbofung et al. (2016), colonization of a rifampicin resistant strain of *Cmn*, 91-R, was viewed in corn leaf tissue. Leaf fractures were taken from lesions of infected maize plants and viewed with a scanning electron microscope. Cells of the *Cmn* isolate were shown to mainly be colonizing the xylem and were also found in the mesophyll. Bacterial cells were densely aggregated in both areas. Interestingly, *Cmn* cells were mostly observed at the spiral and annular rings of the xylem, and on the inner walls of the xylem vessels (Mbofung et al., 2016). Additionally, when samples were observed at time points soon after inoculation, *Cmn* cells were found mostly in the metaxylem. This is not what is observed with the closely related subspecies *Cmm*, which was mostly found to inhabit the protoxylem in its tomato host (Mbofung et al., 2016). This study also suggested that initial colonization of the metaxylem, especially of the annular and spiral rings, may allow the bacteria to further colonize the xylem lumen, by aiding in initial attachment of bacterial cells (Mbofung et al., 2016). Interestingly, this type of bacterial biofilm development to colonize the xylem lumen was also observed for *Pantoea stewartii* subspecies *stewartii* (Koutsoudis et al., 2006). This bacterium causes bacterial wilt and leaf blight of maize, but is gram negative, unlike *Cmn*. Although *Pantoea stewartii* is transmitted by the corn flea beetle, it is similar to *Cmn* in that it inhabits the xylem of corn plants, leading to disease symptoms (Merighi et al., 2003). Another important aspect in the formation of biofilms by gram positive bacteria is the formation of pili. Understanding of how this works in gram positive bacteria was bolstered by a study of *Actinomyces naeslundii* by

Yeung et al. (Mandlik et al., 2007). Past studies as well as recent work have shown that pili mediate bacterial adhesion to host cells, invasion and eventual biofilm formation, leading to disease symptoms (Mandlik et al., 2007). Pili formation in the *Clavibacter michiganensis* subspecies has yet to be explored.

1.9 Objectives

The objectives for this research were: (1.) To examine the relationship between virulence and biofilm formation; (2.) To investigate the epiphytic and endophytic survival of *Clavibacter michiganensis* subsp. *nebraskensis* on Goss's wilt susceptible corn hybrids; and (3.) To confirm the presence of Goss's wilt disease in Michigan through scouting, bacterial isolation, and identification techniques.

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CHAPTER 2

The role of biofilm formation in virulence of the Goss's wilt pathogen *Clavibacter michiganensis* subspecies *nebraskensis*.

2.1 ABSTRACT

Goss's wilt is a bacterial disease of corn that has reemerged in the corn belt of the US since 2006. The causal agent is *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*). For many xylem-dwelling plant pathogenic bacteria, such as *Cmn*, biofilm formation within host tissues is important for colonization of and survival within host tissue, and is often associated with virulence. It was hypothesized that *Cmn* isolates produce biofilms. It was also hypothesized that there would be an association with the amount of biofilm produced by an isolate and its level of virulence. In order to test these hypotheses, 19 *Cmn* isolates that varied in virulence levels were observed for biofilm formation in vivo and in vitro. In vivo methods involved observing inoculated corn xylem and plant leaf surfaces with a scanning electron microscope for biofilm formation. In vitro methods involved a glass coverslip method to qualify biofilm formation, and the observation of biofilm formation on gold mesh transmission electron microscopy grids with a scanning electron microscope to qualify biofilm formation. It was determined that *Cmn* does produce biofilms in vivo and in vitro, but there was no clear correlation to virulence.

2.2 INTRODUCTION

Bacterial cells form complex assemblages known as biofilms, as well as smaller groupings of cells (aggregates), on plant tissue surfaces (Danhorn, 2007). Biofilms are often formed by cells connected in a network of extracellular polysaccharides (EPS) and additionally proteins and DNA, and are initiated by cells attaching to a surface. Exopolysaccharides are a major component of bacterial biofilms, and often promote colonization of phytopathogenic bacteria of their hosts (Danhorn, 2007). Biofilms also allow bacterial cells to survive exposure to harsh environmental conditions such as exposure to UV radiation, bactericides, and desiccation. For example, *Pseudomonas syringae* cells survived better in larger aggregates when subjected to desiccation, while those in smaller groupings or solitary cells tended to die (Morris and Monier 2003). These biofilms can be compared to communities which harbor various cell types exchanging nutrients and DNA, are often complex and go through several steps to develop (Watnick and Kolter 2000).

Many xylem-dwelling plant pathogenic bacteria produce biofilms and this has been correlated to virulence. For example, *Erwinia amylovora*, the causal agent of fireblight of apple and pear, forms biofilms within the xylem of host tissue, which contributes to the wilting observed during *E. amylovora* infection (Koczan et al., 2009). Several other wilt pathogens form biofilms within xylem vessels in host leaves and stems, including *Pantoea stewartii* subsp. *stewartii,* causal agent of Stewart's wilt of corn (Roper, 2011), *Ralstonia solanacearum*, causal agent of bacterial wilt (Alvarez et al., 2010), *Xanthomonas campestris* pathovar *campestris*, which causes black rot disease of crucifers (Crossman and Dow, 2004), and *Xylella fastidiosa*, causal agent of Pierce's disease of grape and other hosts (Newman et al., 2003). *Clavibacter*

michiganensis subsp. *michiganensis,* causal agent of bacterial canker of tomato, also produces biofilms in host xylem. With both scanning electron and confocal laser-scanning microscopy, it was observed that this bacterium forms large biofilm-like aggregates in host xylem (Chalupowicz et al., 2012).

Bacteria in biofilms are often more protected than planktonic cells from environmental stress factors and plant host defenses. *Clavibacter michiganensis* subsp. *sepedonicus* cells in biofilms were shown to be more resistant to treatments with sodium hypochlorite, quaternary ammonium, and hydrogen peroxide, than were planktonic cells (Howard, et al., 2015). Phytopathogenic bacteria are also protected from plant host defenses when in biofilms. For example, pathogenic bacteria in biofilms in the rhizosphere produce compounds that can protect them against antimicrobials produced by root exudates (Rudrappa et al., 2008). Additionally, cells of the plant pathogen *Psedomonas aeruginosa* were found to be resistant to an antimicrobial known as rosmarinic acid when they were in a biofilm. This antimicrobial could, however, kill planktonic cells (Walker et al., 2004). Knowledge of the biofilm formation of plant pathogenic bacteria may lead to better understanding of pathogen-host interactions, from which effective management strategies can be devised.

The bacterial phytopathogen *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*) belongs to a group of gram-positive bacteria that consists of one species, *C. michiganensis*, and is taxonomically divided into subspecies based on host specificity (Eichenlaub et al., 2006). *Cmn* is responsible for a disease of corn known as Goss's wilt, which consists of a systemic wilt phase, where the bacterium colonizes the xylem of its host, and a leaf blight phase, which is more common. During the leaf blight phase, wavy lesions are observed that often have dark,

water-soaked spots at their margins, which are referred to as freckles (Jackson et al., 2007). *Cmn* is a vascular pathogen known to inhabit the xylem of its host, but it is also known to survive epiphytically on its host before entering tissue through wounds and possibly naturally openings, leading to infection and disease symptoms (Eggenberger et al., 2016). To date, there is no published research regarding the biofilm formation of *Cmn* within and on its host, and how this may be related to virulence.

Since bacterial biofilms serve to protect phytopathogenic bacteria from harsh conditions and aid in host colonization, we hypothesized that *Cmn* isolates with high virulence would produce stronger biofilms, while strains that were less virulent would produce weaker biofilms. To test this theory, *Cmn* isolates which differed in virulence were studied using qualitative and quantitative biofilm assays. Biofilm formation by *Cmn* in planta was also observed microscopically, both within leaves and on leaf surfaces.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and growth conditions.

The 19 bacterial isolates (Table 2.1) were used to quantify and qualify biofilm formation. Two isolates (GW-20-E and GIL1) were used to visualize biofilm formation within and on corn leaf tissues.

2.3.2 Virulence rating of *Cmn* isolates

The first method used to evaluate virulence of all 19 *Cmn* isolates (Table 2.1) was a cut inoculation method. Plants of a Goss's wilt susceptible corn hybrid that were grown to the vegetative 3 to the vegetative 4 (V3-V4) leaf stage, were cut inoculated across three major veins with a sterile scalpel (Mbofung, et al., 2016), 11cm from the leaf sheath, between the leaf

edge and midrib. A total of 400µl of bacterial suspension was sprayed over each wound with an airbrush (Master Airbrush, model TC-60, TCPGlobal, San Diego, CA.). Negative controls were wounded, but not inoculated. Plants were incubated on a greenhouse bench for eight days. Next, vegetative 3 leaves of plants were imaged with a NIKON D5200 and evaluated for percent of leaf area infected using APS Assess 2.0 (2002-2008, Manitoba Canada). There were four biological replicates per treatment, and the averages of the percent of infected leaf tissue were recorded for each treatment. Data was assessed using the multivariate method hierarchical clustering in the statistical program JMP to rank the isolates into virulence groups (Figure 2.1).

A stab inoculation method was also utilized. Corn plants were inoculated by making two wounds per plant at right angles to each other 4cm above the soil line with a 1ml syringe, to the center of the stem (Vidaver, 1977), after which 0.25ml of inoculum, adjusted to an OD_{600} of 0.2, was injected into each wound. This was done for each of the 19 *Cmn* isolates, and there were four biological replicates per isolate. Plants inoculated with *Clavibacter michiganensis* subsp. *michiganensis* isolate 1010 or half strength potassium phosphate buffer (PBS) were also used as negative controls. After ten days, plants were evaluated for disease symptoms on a scale of 0-5 (0 = a healthy plant; 1 = a plant with minor wilting, no stunting, and lesions on at least two leaves. Bacterial ooze may or may not be present on leaf tips; 2 = a plant with wilting, stunting lesions on three or more leaves and possible bacterial ooze on leaf tips; 3 = a plant with wilting, stunting lesions on all leaves and possible bacterial ooze on leaf tips; 4 = a plant with severe wilting, severe stunting, lesions on all leaves and possible bacterial ooze on leaf tips; and 5 = a plant that was completely wilted, stunted and dead). *Clavibacter michiganensis* susp. *nebraskensis* isolates were assigned virulence ratings based on these numbers (Table 2.1).

2.3.3 Quantification of biofilm of *Cmn* isolates

All 19 isolates were grown for two days in 5ml of half strength LB broth in a rotary shaker at 200 rpm, set to 28^{o}C. Cultures were adjusted to the same concentration, after which 200µl were placed into wells of a 24-well plate containing 2ml of 0.5x LB and a glass coverslip set at a 45-degree angle. Six replications were completed per isolate, and 0.5x LB broth served as a negative control, while *Erwinia amylovora* strain Ea110 served as a positive control. A Breathe-Easy® membrane (Sigma-Aldrich Co. LLC.) was placed over the wells and the plate was incubated for three days at 28^{o}C.

Next, the supernatant was removed from each well and glass coverslips were stained for one hour with 10% crystal violet to stain biofilm that had formed on the coverslips. After one hour, coverslips were rinsed in a tray of diH_20 for several minutes. Finally, the remaining crystal violet stain was resolublized with 200µl of a solution of 40% methanol and 10% acetic acid, for each coverslip. The mixture from each coverslip was then placed into individual wells of a 96 well plate so that an OD_{600} reading could be taken. Data was analyzed using Tukey's HSD test in the statistical program JMP.

2.3.4 Qualification of biofilm formation of *Cmn* isolates

The following assay was adapted from Zeng et al., 2013. One hundred µl of 0.5x LB were placed into individual wells of a 96 well plate. Next, one G300-Au mesh transmission electron microscopy grid (Electron Microscopy Sciences, Hatfield, PA.) was placed into the bottom of each well. Fifty microliters of all 19 *Cmn* isolates, that were adjusted to the same concentration, were placed into individual wells. There were three replicates per isolate, and

0.5x LB broth served as a negative control, while *Erwinia amylovora* strain Ea1189 served as positive control. A Breathe-Easy® membrane (Sigma-Aldrich Co. LLC.) was placed over the 96 well plate, after which the plate was incubated at 28^{o}C for three days.

After three days, 100µl of paraformaldehyde-glutaraldehyde (2.5% of each compound in 0.1 M sodium cacodylate buffer; Electron Microscopy Sciences, Hatfield, PA) was added to each well and left on the bench at room temperature for one hour. Next, all liquid was carefully removed from the wells, and 100µl of 25%, 50%, 75% and 90% ethanol were placed in series into the wells for 30 minutes each to dehydrate the mesh discs. Next, the gold grids were flooded with 100% ethanol three times for 15 minutes each. Discs were then dried with a critical point dryer (EMCPD300, Leica Microsystems). Grids were then mounted on aluminum stubs on which carbon tabs had been placed. Stubs were placed into a petri dish and put in a container under vacuum until they were visualized with a Scanning Electron Microscope (JEOL 7500F SEM) for biofilm formation.

2.3.5 SEM visualization of *Cmn* isolates in planta

Plants of a Goss's wilt susceptible hybrid were grown up to the V4-V5 leaf stage in the greenhouse at 28^{o}C. Plants were planted in SUREMIX (Michigan Growers Products, Inc.^{tm}) soil in square, 1135.62cm^{3} pots, after which they were fertilized with approximately a teaspoon of Osmocote® Smart-Release® 14-14-14 plant food (ScottsMiracle-grow) upon emergence. The V3 leaves of plants were wounded by cutting across three major leaf veins, 8cm from the leaf sheath, between the midrib and the leaf edge. Corn plants were inoculated with either of the bacterial isolates GW-20-E or GIL1, which had been adjusted to an OD_{600} of 0.2, or mock inoculated to serve as negative controls. Approximately 400µl of inoculum was applied to each

wound with an airbrush (Master Airbrush, model TC-60, TCPGlobal, San Diego, CA.). Plants were sampled 6 and 8 dpi from lesions that formed from the point of inoculation. Leaves were processed for imaging with an SEM scope, as described above, except they were dehydrated in 95% ethanol rather than 90% ethanol, as the final ethanol treatment. Cross sections of samples were visualized for biofilm formation in the leaf xylem.

2.3.6 SEM visualization of *Cmn* biofilms on leaf surfaces

Clavibacter michganensis subsp. *nebraskensis* isolates GW-20-E and GIL1 were grown for two days in NBY broth, spun down, resuspended in 0.5x PBS and adjusted to the same concentration. The Vegetative 3 leaves of corn plants were inoculated with either of the bacterial isolates or non-inoculated to serve as negative controls. Approximately 1ml of suspension of inoculum was applied to entire V3 leaves with an airbrush. Plants were sampled 2, 6, and 8 dpi by cutting approximately 1mm squares 4cm from the V3 leaf tip and 4cm from the V3 leaf sheath. Leaves were processed for imaging with an SEM scope, as mentioned previously, and visualized for biofilm formation on the surface of the leaf.

2.4 RESULTS

2.4.1 Virulence rating of *Cmn* isolates

Tukey's HSD test in the statistical program JMP was utilized to separate the 19 *Cmn* isolates used in this study into three groups based on lesion length/virulence (figure 2.1). This test was unable to separate the isolates into distinct groups. The isolates were next separated into three distinct virulence groups via hierarchical clustering (figure 2.2), including highly virulent (red), moderately virulent (green), and slightly virulent (blue) isolates. There was a lot

of variation between experiments for each isolate in the highly virulent and the moderately virulent groups, as indicated by the blue squares to the right of the isolates in the figure.

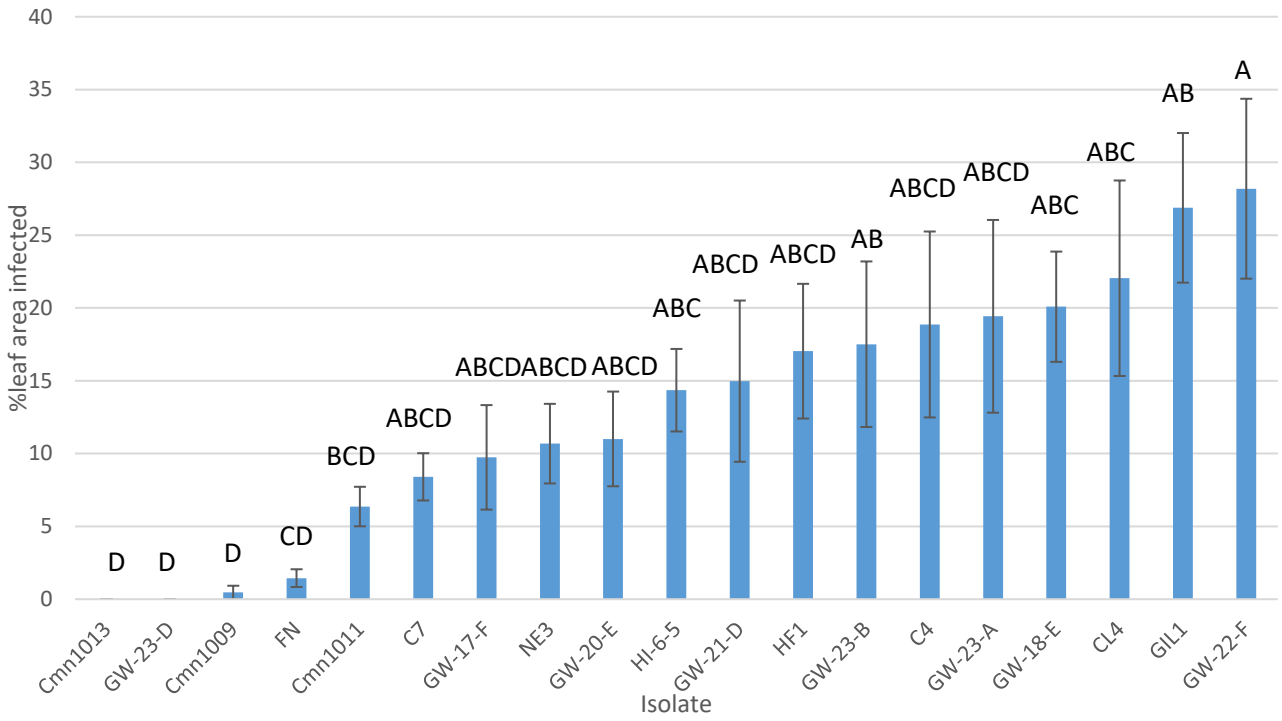


Figure 2.1. Percent area of corn leaves infected with each of the 19 *Clavibacter michiganensis* subsp. *nebraskensis* isolates. V3 leaves of Plants were wounded then inoculated with suspensions of either of the 19 *Cmn* isolates. After 8 days, the percent of leaf area infected was measured. Results above are of the means of two independent experiments. Bars with letters that differ from those of other bars denote significant differences, as found with Tukey's HSD test.

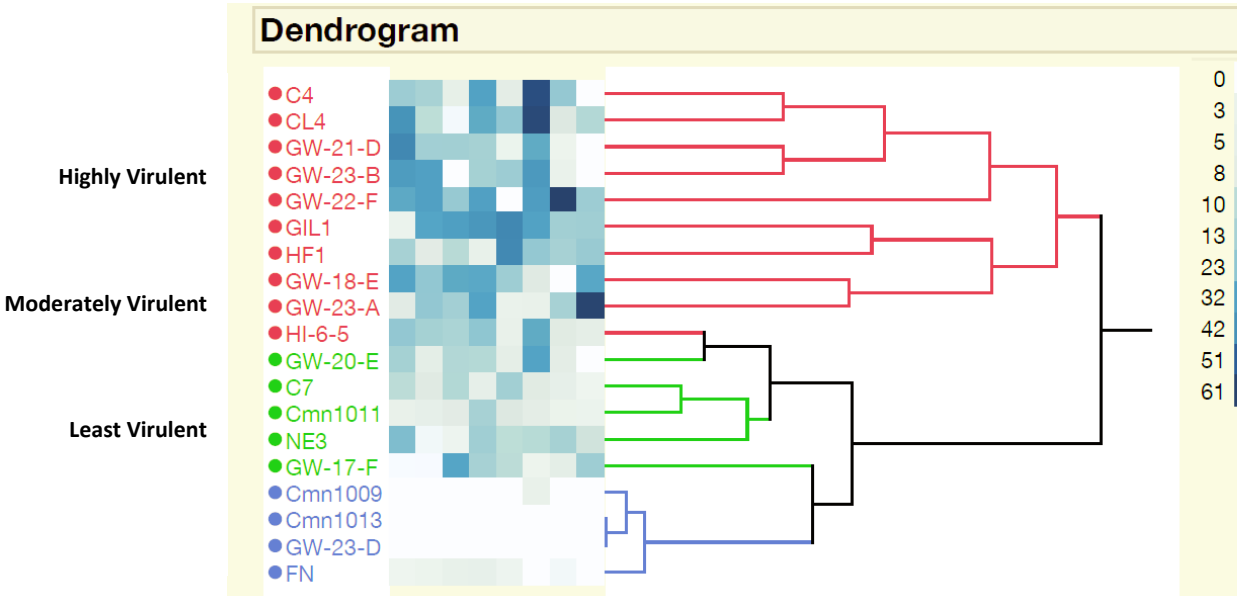


Figure 2.2. Dendogram separating all 19 *Clavibacter michiganensis* subsp. *nebraskensis* isolates into three virulence groups based on hierarchical clustering and includes highly virulent isolates with the greatest percentage leaf area diseased (red), moderately virulent isolates with moderate percentage leaf area diseased (green) and slightly virulent isolates with the least percentage leaf area diseased (blue). Blue squares indicate percent leaf area infected for eight biological replicates per isolate divided into two experiments.

2.4.2 Quantifaction of biofilm formation of *Cmn* isolates

The *Cmn* isolate FN, which was one of the least virulent isolates (Figure 2.3) produced the most biofilm. Isolate GW-20-E, which is moderately virulent, produced a significant amount of biofilm. The most virulent isolates did not produce high amounts of biofilm (Figure 2.3), but produced similar amounts of biofilm compared to the positive control *Erwinia amylovora*.

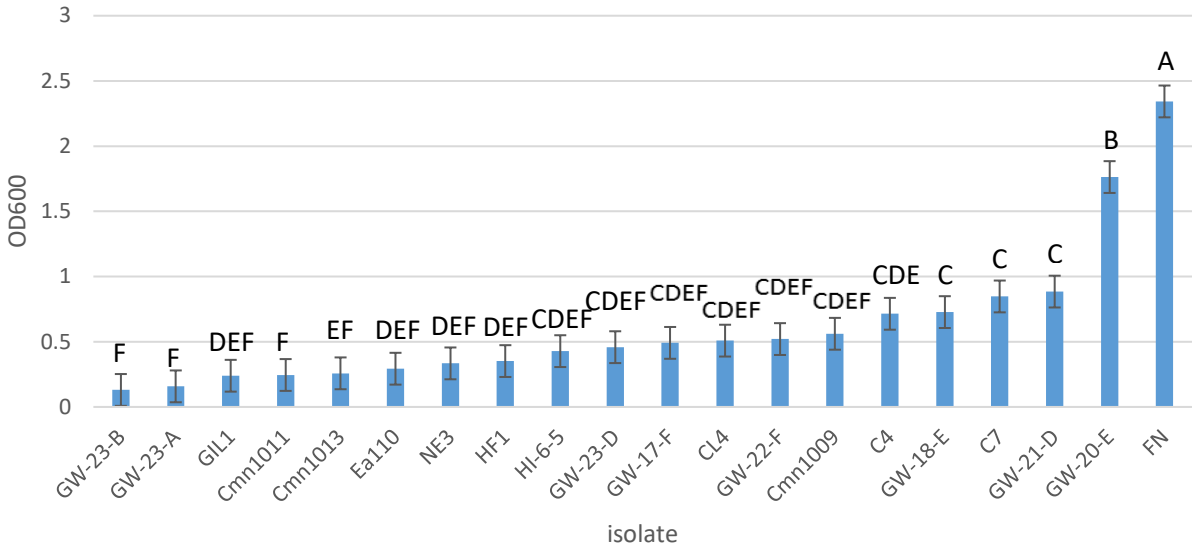


Figure 2.3. Biofilm formation on glass coverslips of *Cmn* isolates. Results above are of the means of two independent experiments. Bars with letters that differ from those of other bars denote significant differences, as found with Tukey's HSD test. Overlapping error bars indicate no significant difference between isolates.

2.4.3 Qualification of biofilm formation of *Cmn* isolates

Each of the 19 *Cmn* isolates, were visualized forming biofilms on transmission electron microscopy grids via a scanning electron microscope (JEOL 7500F). Representatives of these isolates can be seen in Figure 2.4 below.

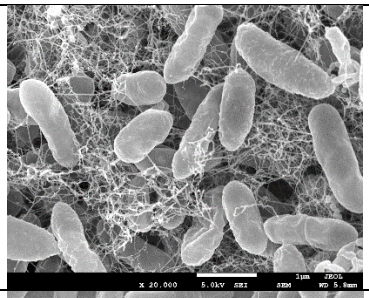
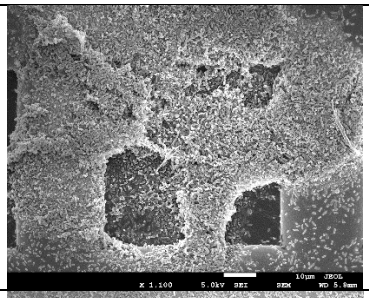
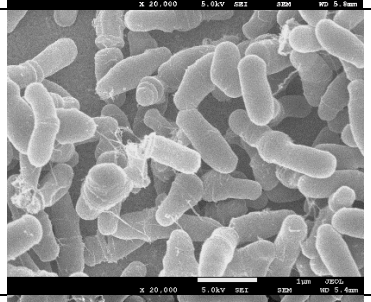
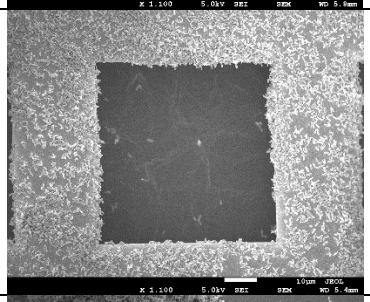
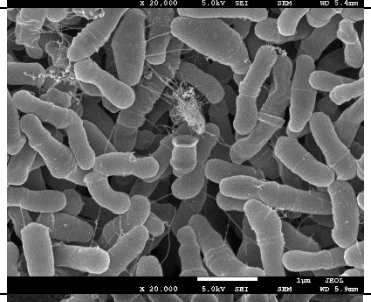
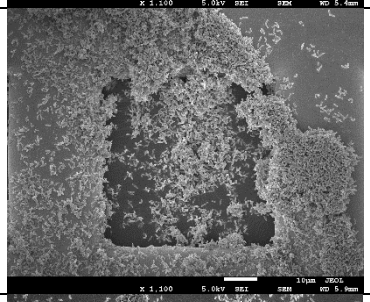
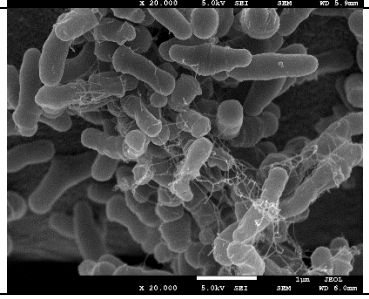
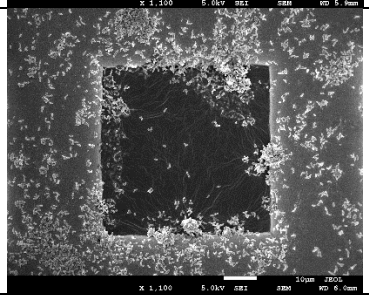
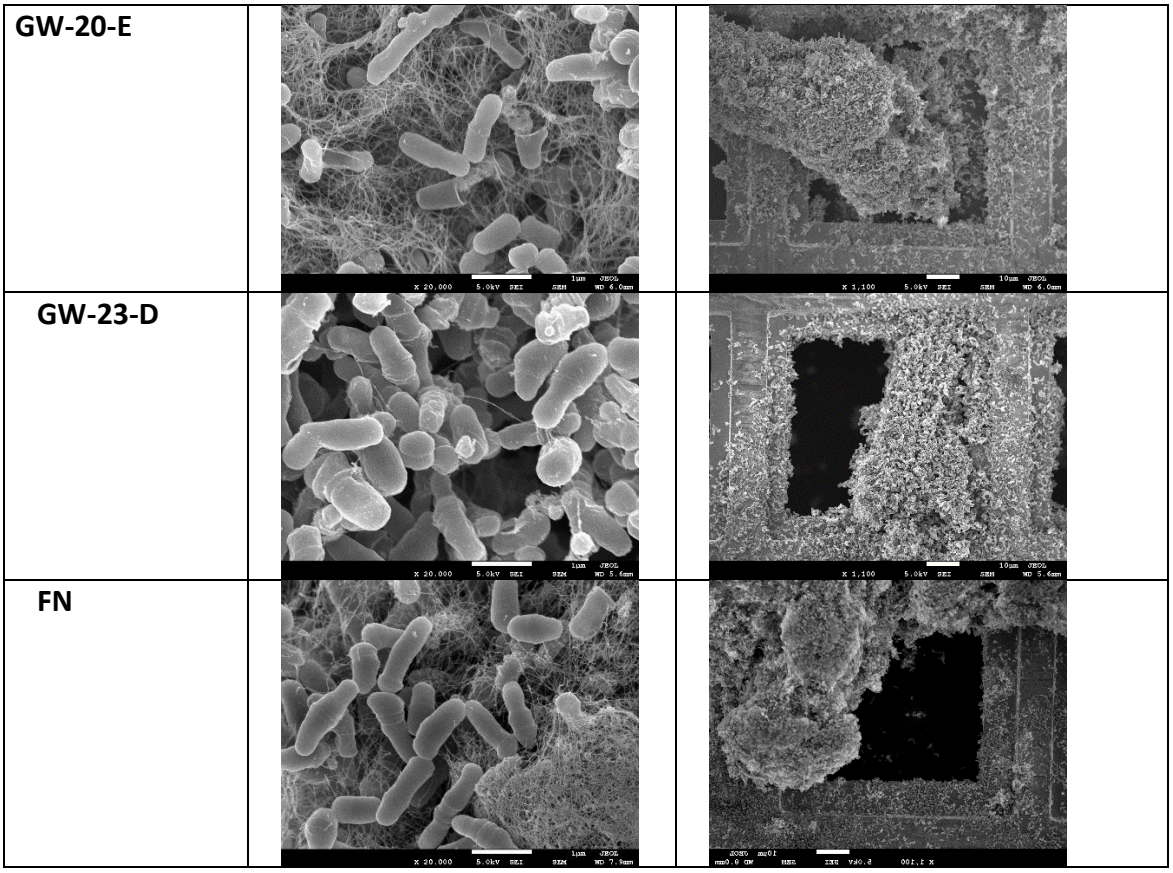
Isolate	20,000x	1,100x
Ea1189		
GW-22-F		
GIL1		
C7		

Figure 2.4. Visualization of *Cmn* isolates from different virulence groups forming biofilms on TEM grids via a scanning electron microscope (JEOL 7500F) at 20,000x and 1,100x. The images are in descending order with the top row being the positive control (Ea1189), the next two rows showing the highly virulent isolates, the middle two rows representing moderately virulent isolates and finally the bottom two rows representing least virulent isolates

Figure 2.4 (cont'd)



2.4.4 SEM visualization of *Cmn* isolates in planta

Both isolates GIL1 and GW-20-E were observed aggregating or forming biofilms in the V3 leaves of a Goss's wilt susceptible corn hybrid six and eight days after inoculation (figure 2.5).

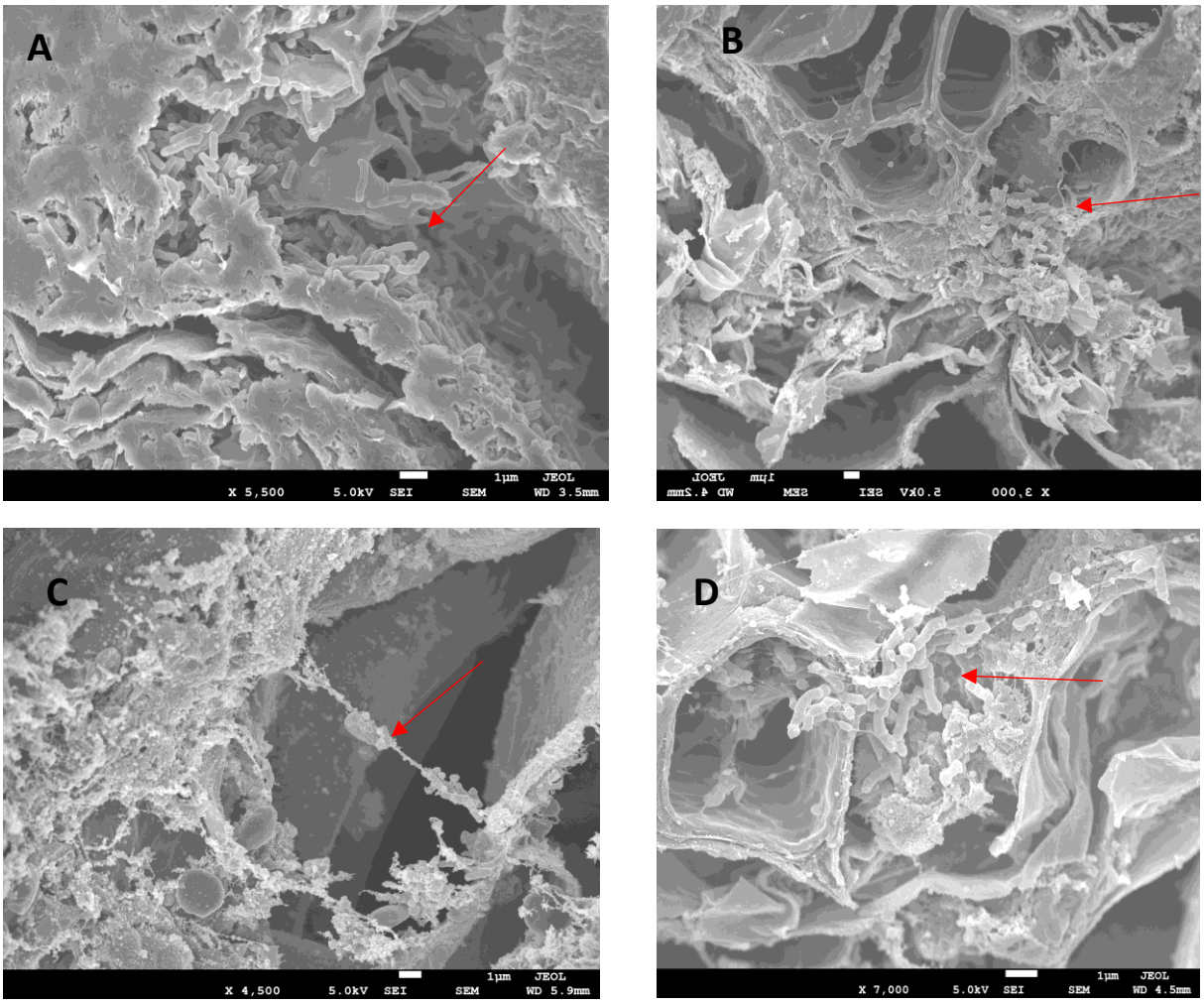


Figure 2.5. Images of *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*) isolates forming biofilm in the V3 leaves of Goss's wilt susceptible corn hybrid. The red arrow shows cells of *Cmn* isolate GIL1 aggregating in a V3 corn leaf 8dpi (A). The red arrow shows cells of *Cmn* isolate GIL1 forming a biofilm near a xylem vessel 6 dpi (B). Cells of *Cmn* isolate GW-20-E forming a biofilm across a xylem vessel 6 dpi (C). The red arrow points to a grouping of cells. Isolate GW-20-E forming a biofilm in a V3 corn leaf 8 dpi, as indicated by the red arrow (D).

2.4.6 SEM visualization of *Cmn* isolates on leaf surfaces

Both *Cmn* isolates GIL1 and GW-20-E were observed on the V3 leaves of a Goss's wilt susceptible corn hybrid, 6 dpi. Cells of GIL1 and GW-20-E seemed to be spread randomly throughout the leaf surface. Additionally, some cells of GW-20-E were found around stomata (Figure 2.6).

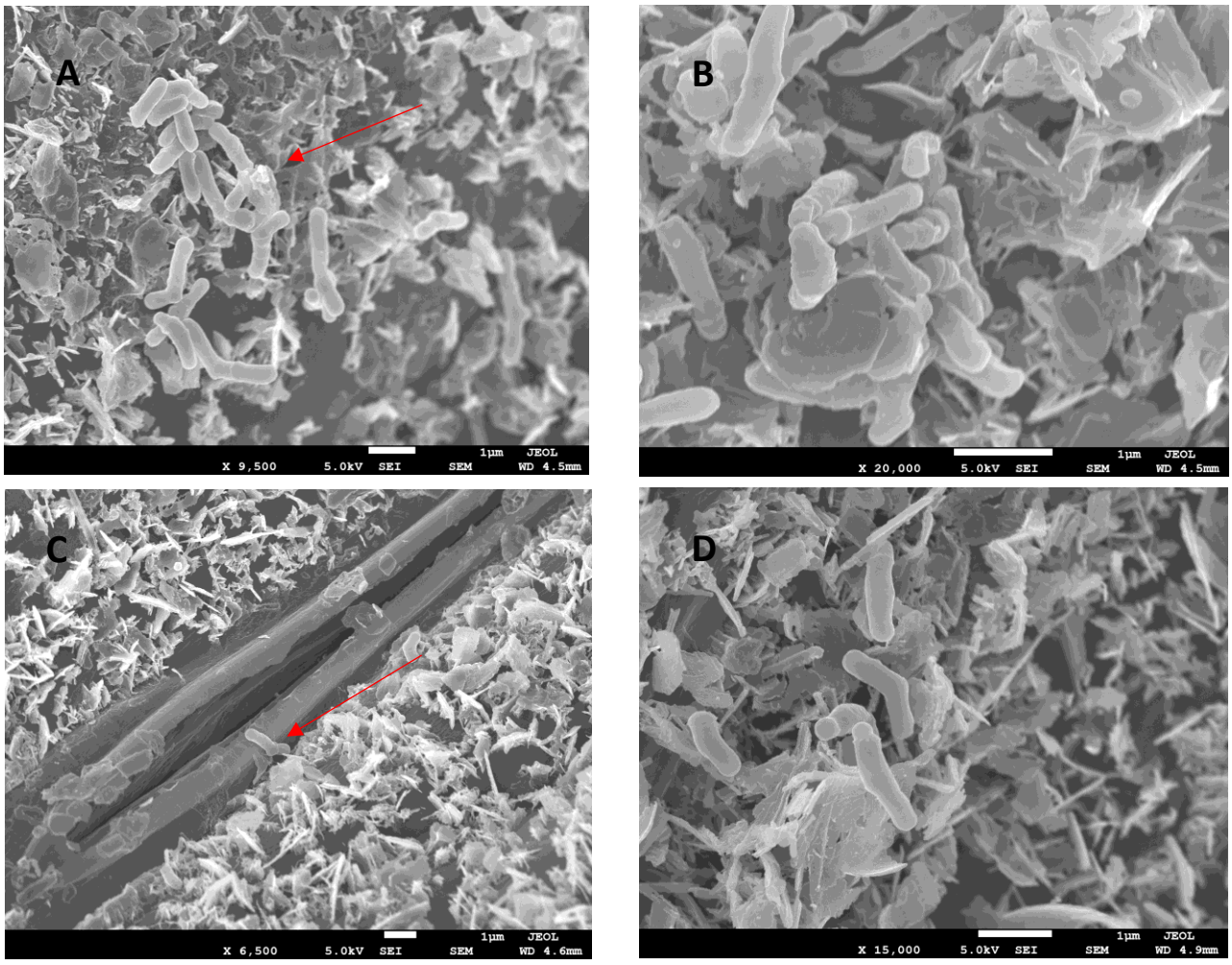


Figure 2.6. Images of *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*) isolates aggregating on corn leaves 8 dpi. The red arrow points to cells of *Cmn* isolate GW-20-E aggregating 4cm from the leaf sheath of V3 corn leaf (A). Cells of *Cmn* isolate GW-20-E aggregating 4 cm from the leaf tip of a V3 corn leaf (B). A single cell of *Cmn* isolate GW-20-E near a stomate on a V3 corn leaf, as indicated by the red arrow (C). Cells of *Cmn* isolate GIL1 4 cm from the leaf sheath of a V3 corn leaf (D).

Table 2.1. The table below lists the isolates used in these studies. Note that isolates did not originate from Michigan, but were in cultures at the university. Disease ratings are based on a stab inoculation method after which disease severity was rated on a scale from 0-5 (0 = a healthy plant; 1 = a plant with minor wilting, no stunting, and lesions on at least two leaves. Bacterial ooze may or may not be present on leaf tips; 2 = a plant with wilting, stunting lesions on three or more leaves and possible bacterial ooze on leaf tips; 3 = a plant with wilting, stunting lesions on all leaves and possible bacterial ooze on leaf tips; 4 = a plant with severe wilting, severe stunting, lesions on all leaves and possible bacterial ooze on leaf tips; and 5 = a plant that was completely wilted, stunted and dead). Numbers are the averages for eight biological replicates divided into two separate experiments.

Isolate	Origin	Disease rating (0-5)
GW-17-F	Indiana (Kiersten Wise)	4.25
GW-18-E	Indiana (Kiersten Wise)	4
GIL1	Iowa (Alison Robertson)	4.75
C4	Iowa (Alison Robertson)	3.25
HF1	Iowa (Alison Robertson)	3.25
NE3	Iowa (Alison Robertson)	3
FN	Iowa (Alison Robertson)	3
CL4	Iowa (Alison Robertson)	3.25
HI-6-5	Iowa (Alison Robertson)	3.25
C7	Iowa (Alison Robertson)	3
GW-20-E	Missouri (Patti Wallace)	2.75
GW-21-D	Missouri (Patti Wallace)	3.75
GW-22-F	Missouri (Patti Wallace)	4
GW-23-A	Missouri (Patti Wallace)	3.25
GW-23-B	Missouri (Patti Wallace)	2.25
GW-23-D	Missouri (Patti Wallace)	0.75
Cmn1009	Michigan State University	1.0
Cmn1013	Michigan State University	0.25
Cmn1011	Michigan State University	3.5

2.5 DISCUSSION

By using hierarchical clustering and a stab inoculation method, the 19 *Cmn* isolates used in this study were separated into three virulence groups. For the cut inoculation method, there was a lot of variation observed among the highly virulent and the moderately virulent isolates. This may be due to the method of wounding and application of inoculum. It is possible that not all V3 leaves were cut similarly along three leaf veins or that inoculum was applied quite evenly over wounds. In vitro it was observed that these isolates could form biofilms, but there was no

clear correlation between virulence and biofilm formation. For example, FN was one of the strongest biofilm formers in the glass coverslip assay, however it was one of the least virulent. The scanning electron micrograph of this bacteria (Figure 2.4), however revealed that this isolate produces a great deal of fibrous material in its biofilm. GW-20-E produced a similar biofilm to FN (Table 2.2), but it was moderately virulent. This fibrous material may be what was observed for these two isolates in the glass coverslip assay, leading to the higher OD_{600} readings observed for these two isolates (Figure 2.3). Most of the highly virulent isolates, for example GIL1, seemed to produce strong biofilms when their scanning electron micrographs were observed, but did not produce strong biofilms in the glass coverslip assay. It was observed that they produce less fibrous material in their biofilms, unlike FN and GW-20-E. It is interesting that these two isolates produced more fibrous material than other isolates. Such fibrous materials are often associated with biofilms and help bacterial cells to link to each other and attach to plant surfaces. One example is *Agrobacterium tumefaciens*. When stimulated by host roots, cells of this bacterium produce cellulose fibrils that help them to attach to plant surfaces and to each other to create large aggregations. Mutants deficient in cellulose production have a reduced capability to attach to plant surfaces (Matthysse et al., 2005). Perhaps GW-20-E and FN overproduce fibrous materials that may be beneficial in biofilm formation and attachment to plant surfaces.

It is possible that other virulence factors, besides biofilm formation, may be important for virulence of the Goss's wilt pathogen. For example, the causal agent of bacterial wilt and canker of tomato *Clavibacter michiganensis* subsp. *michiganensis,* requires genes encoding specific serine proteases, as well as additional plasmid borne virulence factors. Mutants of the

wildtype lacking the ability to produce these virulence factors were found to only colonize inoculation sites, while the wildtype colonized more of the plant (Chalupowicz et al., 2011). Perhaps GW-20-E is lacking in certain virulence factors, such as serine proteases, that may be necessary to break down host tissue so that the bacteria can better travel through and colonize its host. As mentioned above, GW-20-E and FN were also seen to produce more fibrous materials in in vitro biofilms. These dense masses could hinder these two isolates from translocating through their host, leading to reduced colonization and a reduction in disease symptoms. One example of a plant pathogen where movement through the host and its relation to virulence has been well documented is the causal agent of Pierce's disease of grape, *Xylella fastidiosa*. It was found that mutants deficient in hemagglutinin proteins were more virulent than the wildtype because they tended to aggregate less, could move more freely through host xylem, and could better colonize xylem vessels (Guilhabert and Kirkpatrick, 2005).

Two representative *Cmn* isolates that differed in virulence were observed for biofilm formation on and within a Goss's wilt susceptible corn hybrid. This was done to see if biofilm formation was necessary for colonization of its host. For this assay, both GW-20-E and GIL1 were seen forming biofilms within their hosts 6 and 8 dpi. GIL1 was seen aggregating in host tissue, but it was unclear if the bacteria was colonizing only xylem vessels, as the tissue observed was greatly diseased leading to collapsed vascular bundles. Bacterial cells appeared to inhabit the mesophyll because of this. GW-20-E was seen forming biofilms around xylem lumen and across xylem vessels. This seems logical, since GW-20-E produced more fibrous material in in vitro biofilms. Perhaps this isolate is better at forming biofilms internally when compared to GIL1. GIL1 may translocate more easily than GW-20-E because of a reduction in

fiber production, leading to a greater breakdown of tissue and an increased expression of disease symptoms.

Both isolates were also observed on corn leaf surfaces 6 dpi. Cells of GW-20-E were seen aggregating more than those of GIL1, and some cells were observed clustering near stomata. No significant aggregations were observed for either of the isolates. Biofilm formation or aggregation may still be important for the survival of *Cmn* externally. For example, in a study focusing on *Xanthomonas axonopodis* pv. *citri*, biofilm formation of this bacterium was observed on lemon leaf surfaces. A mutant deficient in producing an important component of biofilms of this bacterium, xanthan, could not produce biofilms as strong as that of the wildtype and exhibited reduced survival and growth (Rigano et al., 2007). Since *Cmn* is known to survive epiphytically, it is likely that it still produces aggregations and biofilms on corn leaf surfaces. This would help the bacterium to survive longer epiphytically and to withstand environmental stressors. This experiment should be carried out for a longer period and perhaps with a higher inoculum concentration to determine if the bacterium does indeed aggregate on corn leaf surfaces. Since the bacterium also survives on host residue, experiments should be conducted observing possible aggregation or biofilm formation on senesced corn leaf surfaces, as this could help the bacteria to overwinter.

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CHAPTER 3

Exploration of the epiphytic and endophytic survival of *Clavibacter michiganensis* subsp. *nebraskensis* isolates on a Goss's wilt susceptible corn hybrid and a corn hybrid partially resistant to Goss's wilt.

3.1 ABSTRACT

The gram-positive bacterium *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*), is the causal agent of Goss's wilt in corn. Research into the epidemiology and management of this disease has increased since its re-emergence in the corn belt in 2006. The causal agent is known to have an epiphytic phase, and it is thought that this may be important for the spread, survival, and virulence of the pathogen. In this study, the relationship between virulence and epiphytic and endophytic survival was examined, as well as the relationship between survival of the pathogen and hybrid susceptibility to Goss's wilt. To achieve this, populations of two spontaneous rifampicin-resistant *Cmn* mutants, which differed in virulence, were enumerated over time, on a susceptible Goss's wilt hybrid, as well as a partially resistant hybrid. To track epiphytic populations, corn leaf samples were sonicated in 0.5x phosphate buffered saline (PBS), after which 10-fold dilutions were drop plated onto nutrient broth yeast extract (NBY) agar containing rifampicin at 100µg/ml, to obtain colony forming units per gram of tissue (CFU's/g). To track endophytic populations, corn leaf samples were subjected to ultraviolet-C radiation to kill epiphytic populations. These leaves were ground in 0.5x PBS, and 10-fold dilutions were drop plated onto NBY agar containing rifampicin at 100µg/ml to obtain CFU's/g. No significant differences were found between epiphytic and endophytic populations for either of the spontaneous rifampicin-resistant mutants on the highly susceptible hybrid or the partially resistant hybrid 10 dpi. Additionally, no significant differences were seen among the

rifampicin-resistant mutants 8 and 12 dpi on the highly susceptible Goss's wilt hybrid. This suggests that virulence may not be directly related to the ability of the pathogen to build up populations.

3.2 INTRODUCTION

Plant leaf surfaces provide a habitat for many microorganisms, including yeast, fungi and bacteria (Lindow and Brandl, 2003). Populations of bacteria that inhabit leaf surfaces are said to inhabit the phylloplane, and interest in such microbial populations arose due to crop losses caused by bacterial epiphytes, such as *Pseudomonas syringae*, that are also phytopathogenic (Hirano and Upper 2000). Many species of bacteria live as endophytes and epiphytes either within or on plant tissues, respectively. Endophytic populations consist of microorganisms that can be isolated from plants, but do not cause detectable injury to the plants (Araujo et al. 2002). Endophytic populations can enter host tissue through wounds, natural openings or by penetrating host tissue through the action of cellulase and pectinase enzymes. These endophytic populations establish themselves from epiphytic populations that may inhabit either the phylloplane or the rhizoplane (Hallmann et al. 1997). Since the survival of epiphytic plant pathogens can lead to build up and spread of the pathogen, knowledge of the epiphytic and endophytic survival of phytopathogenic bacteria is important to develop effective control strategies.

As mentioned previously, endophytic bacteria can be isolated from internal plant parts, but do not cause noticeable disease. This definition also extends to bacterial pathogens that may later incite disease when conditions are appropriate (Lodewyckx, et al., 2002). Certain plant pathogens have an epiphytic phase where they buildup populations on host tissue and

this is an important aspect of their spread and ability to incite disease (Miller and Schroth, 1972; Romantschuck, 1992; McGuire et al., 1991). To incite disease, bacteria must be able to penetrate host tissue via some mechanism (Melotto, M. et al., 2006). For example, phytopathogenic bacteria may alter leaf surfaces or enter plants through natural openings to achieve this goal. This includes bacterial pathovars of *P*seudomonas *syringae* and *X*anthomonas *campestris*, and *Erwinia* spp. (Renick et al., 2008; Chan and Goodwin, 1999; Salmond, 1994), which have been known to survive epiphytically on their hosts before causing disease. As an example, some strains of *P*. *syringae* exhibit ice nucleation activity which can damage host tissue via frost injury. For these strains, bacterial population size is directly correlated to the amount of frost damage (Hirano and Upper, 2000). When such damage occurs, the bacteria is better able to colonize its host internally (Hirano and Upper, 2000). The same result was found for the phytopathogen *Erwinia herbicola*, where the amount of frost damage was correlated to the population of bacterial cells on corn leaves (Lindow, et al., 1978). This is evidence that both the ability to enter host tissue through wounds or natural openings, and the ability to build up large populations, are necessary for colonization and infection of host tissue. Epiphytic populations of *Cmn* have also been recovered from host tissues in the field (Ahmad et al., 2015). Although *Cmn* is known to enter its host through wounds caused by sand and hail, it has been speculated that severe wounding may not be necessary for infection. This is because diseased corn plants with no apparent wounds have been discovered in corn fields with disease symptoms beginning at the tips of upper leaves (Mallowa et al., 2016). This suggest that wounds may not be necessary for the pathogen to enter its host, and that epiphytic populations may enter through natural openings as well, such as stomata and hydathodes.

Many factors influence the ability of a bacterial species to inhabit leaf surfaces as an epiphyte. One major component is the availability of nutrients that bacterial cells can utilize. For example, a carbon source, a nitrogen source, and inorganic compounds must be available for proliferation and growth of bacterial cells. These may be provided by the leaf itself through exudates or through insect deposition (Mercier and Lindow, 1999). Bacterial cells must also be able to survive such harsh conditions as ultraviolet radiation and desiccation. For example, species of *P. syringae* survived better during drying conditions than bacterial species that were not commonly found as epiphytes (O'Brien and Lindow, 1989), suggesting that common epiphytes must adapt strategies to colonize plant leaf surfaces and survive harsh conditions. Bacteria will often inhabit "protected sites" to survive these harsh conditions. Examples include the base of trichomes, stomata, and at epidermal cell wall junctions (Beattie and Lindow, 1999). Thus, to survive epiphytically, bacterial cells must be able to gain access to required nutrients, as well as to exploit these "protected sites." The behavior of colonization of protected sites can be seen by the common bacterial epiphyte and plant pathogen *P. syringae.* For example, a strain of this bacteria that was pathogenic on bean was found to preferentially aggregate around trichomes and veins on the leaves of bean plants grown in the greenhouse (Monier and Lindow, 2004).

Furthermore, bacterial species will survive differently based on plant host and plant physiology. A great example of this is in a study conducted in 1989 that examined the survival of *Pseudomonas syringae* species under several environmental conditions. The epiphytic growth of these species was also compared to that of bacterial species that are not typically found as epiphytes. It was determined that plants with a waxier cuticle like pea, oat, and corn

retained less initial bacterial populations than those that possessed more trichomes, such as bean, tomato and cucumber (O'Brien and Lindow, 1989). Furthermore, in a study examining colonization of *Pantoea agglomerans* and *Clavibacter michiganensis* subsp. *nebraskensis* on corn hybrids that varied in cuticular wax biosynthesis, it was determined that a mutant hybrid deficient in cuticular wax production supported higher bacterial populations on its leaves than did the wildtype hybrid (Marcell and Beattie, 2002). Susceptibility of the host may also play a role in how well plant pathogens survive epiphytically. For example, greater populations of pathogenic *P. syringae* were found on susceptible bean lines in the field when compared to more resistant lines (Daub and Hagedorn, 1981). Additionally, it was determined that three pathogenic races of *P. glycinea* grew 1,000-fold from 1 to 2 weeks on more susceptible soybean cultivars, while their populations remained unchanged or greatly declined on more resistant cultivars (Mew and Kennedy, 1971).

We hypothesized that epiphytic populations of *Cmn* may build up on host leaf surfaces prior to entry of the bacterium through wounds and/or natural openings, leading to disease. It was also hypothesized that corn hybrids that varied in susceptibility to Goss's wilt would support differing populations of the pathogen. Furthermore, it was speculated that more virulent *Cmn* isolates may survive longer on their host and build up greater populations epiphytically and endophytically. To test this theory, several corn hybrids were screened for susceptibility to Goss's wilt and a Goss's wilt susceptible hybrid and a hybrid partially resistant to Goss's wilt were chosen. The survival of two spontaneous rifampicin resistant *Cmn* mutants, that differed in virulence, was tracked epiphytically and endophytically over time on these hybrids. First, each isolate was tracked separately on both hybrids to see if there were

differences in epiphytic and endophytic populations on the two hybrids. Next, both isolates were tracked at the same time on the chosen susceptible hybrid to see if virulence was correlated to survival of *Cmn*.

3.3 MATERIALS AND METHODS

3.3.1 Preparation of bacterial isolates for virulence screening

Isolates of *Cmn* were obtained from Alison Robertson at Iowa State University, Goss's wilt infected leaf tissue from Missouri and Indiana, sent from Patricia K. Wallace and Kiersten Wise respectively, and from a bacterial culture collection at Michigan State University (Table 3.1). Bacterial isolates were grown in 5ml of nutrient broth yeast extract (NBY) in a rotary shaker set at 200rmp and 28^{o}C, for 48 hours. Next, isolates were spun down in a centrifuge at 541 RCF for 10 minutes, after which they were resuspended in half strength phosphate buffered saline (PBS). Cultures of isolates were adjusted to an OD_{600} of 0.2 ($5.0x10^8$ CFU/mL) and placed on ice until they were ready for use.

3.3.2 Virulence screening of isolates

Plants of a Goss's wilt susceptible corn hybrid was grown in the greenhouse to the V3-V4 leaf stage. Seeds were sown in individual square, 1135.62cm^3 pots in SUREMIX (Michigan Growers Products, Inc.^{tm}) and fertilized with a teaspoon of osmocote® Smart-Release® 14-14-14 (ScottsMiracle-Grow) upon emergence. Next, corn plants were inoculated by making two wounds per plant at right angles to each other 4cm above the soil line with a ml syringe (Vidaver, 1977), after which 0.25ml of inoculum was injected into each wound. This was done for all 19 *Cmn* isolates (Table 3.1), and there were four biological replicates per isolate, each of

which consisted of one plant per pot. Plants inoculated with *Clavibacter michiganensis* subsp. *michiganensis* isolate 1010 or half strength PBS were also used as negative controls. After ten days, plants were evaluated for disease symptoms on a scale of 0-5 (0 = a healthy plant; 1 = a plant with minor wilting, no stunting, and lesions on at least two leaves. Bacterial ooze may or may not be present on leaf tips; 2 = a plant with wilting, stunting lesions on three or more leaves and possible bacterial ooze on leaf tips; 3 = a plant with wilting, stunting lesions on all leaves and possible bacterial ooze on leaf tips; 4 = a plant with severe wilting, severe stunting, lesions on all leaves and possible bacterial ooze on leaf tips; and 5 = a plant that was completely wilted, stunted and dead). *Clavibacter michiganensis* subsp. *nebraskensis* isolates were given virulence ratings based on these numbers (Table 3.1).

The second method used to evaluate virulence of all 19 *Cmn* isolates (Table 3.1) was a cut inoculation method. Plants of a Goss's wilt susceptible corn hybrid that were grown to the V3-V4 leaf stage, were cut inoculated across three major veins with a sterile scalpel (Mbofung, et al., 2016), 11cm from the leaf sheath, between the leaf edge and midrib. A total of 400 µl of bacterial suspension was sprayed over each wound with an airbrush (Master Airbrush, model TC-60, TCPGlobal, San Diego, CA.). Negative controls were wounded, but not inoculated. Plants were incubated on a greenhouse bench for 8 days. Next, V3 leaves of plants were imaged with a NIKON D5200 and evaluated for percent of leaf area infected using APS Assess 2.0 (2002-2008, Manitoba Canada). There were four biological replicates per treatment and the averages of the percentage of infected leaf tissue were recorded for each treatment. Data were assessed using the multivariate method hierarchical clustering in the statistical program JMP to rank the isolates into virulence groups (Figure 3.1).

3.3.3 Selection of spontaneous rifampicin resistant-mutants

Spontaneous rifampicin resistant-mutants were selected for two *Cmn* isolates that differed in virulence so that their populations could be tracked over time. The isolates chosen were GIL1, highly virulent and isolate GW-20-E, moderately virulent. Spontaneous rifampicin-resistant mutants were obtained by plating liquid cultures of *Cmn* isolates GIL1 and GW-20-E on NBY agar plates containing rifampicin at 100µg/ml. Plates were incubated for five days at 28^{o}C, after which surviving colonies were picked and mass streaked. Mass streaked plates were incubated at 28^{o}C, after which bacterial growth was swabbed and stored at -80^{o}C.

Both rifampicin-resistant mutants were tested to ensure that they maintained the same virulence level as the wild type, highly virulent and moderately virulent (data not shown). Additionally, growth curves of the wildtype and the rifampicin-mutants used in this study were generated (Figures 3.2-3.3). The mutants were named GIL1-RIF4 and GW-20-E-RIF.

3.3.4 Screening for susceptibility of corn hybrids to Goss's wilt

Two day old cultures of the spontaneous rifampicin-resistant mutant GIL1-RIF4 were adjusted to an OD_{600} of 0.12, washed and resuspended in 0.5x PBS. The V4 leaves of corn hybrids were inoculated by dipping sterile scissors in inoculum and cutting the leaves from the edge to the midrib 18cm from the leaf tip. There were four replicate plants per hybrid. After ten days, hybrids were rated for susceptibility to Goss's wilt in two ways. First, lesion lengths were measured with a ruler from the point of inoculation (Figure 3.4). Second, inoculated V4 leaves were cut into 1cm pieces with sterile scissors, placed into 20ml of 0.5x PBS and ground with a Polytron® (Model PT 10-35 GT, Kinematica). Tenfold dilutions from 0-10^{-8} were drop

plated out on NBY agar containing rifampicin at 100µg/ml. This was done to obtain CFU/g to calculate *Cmn* populations for each corn hybrid (Figure 3.5). Data was analyzed using Tukey's HSD test in the statistical software program JMP. From these experiments, a Goss's wilt susceptible and a partially resistant hybrid were chosen for tracking epiphytic and endophytic populations of *Cmn*.

3.3.5 Generation of an ultraviolet-C (UV-C) radiation kill curve

An ultraviolet-C (UV-C) radiation kill curve (Figure 3.6) was generated to determine the amount of UV-C radiation needed to kill external populations of *Cmn*. Cultures of GIL1 and GW-20-E were grown for two days in a rotary shaker set at 200 rmp and 28^{o}C, washed, resuspended in 0.5x PBS and transferred to sterile glass petri dishes. The cultures were next exposed to a UV-C germicidal light (Sankyo Denki, G15T8) with an output of 1.4 J/m^{2}s^{-1} in a dark room. Cultures were subjected to 100, 200, 300 and 400 J/m^{2}s^{-1} successively. Initially and after each exposure, cultures were dilution plated onto NBY agar plates to obtain colony forming units (CFU's). Additionally, cultures were agitated in between exposures to ensure that all cells were exposed to UV-C radiation and were not shaded by other cells.

3.3.6 Inoculation of corn hybrids with isolates and sample processing

To look at the survival of *Cmn* isolates on corn hybrids that differed in susceptibility to Goss's wilt, fifty milliliters of either GW-20-E RIF or GIL1-RIF4 were grown on a rotary shaker set to 200 rpm and 28^{o}C, in 200 mL flasks for three days. Cultures were spun down, washed, resuspended in 0.5x PBS and adjusted to an OD_{600} of 0.2. Cultures were kept on ice until they were ready for use. The V3 leaves of the chosen Goss's wilt susceptible hybrid and the chosen

partially resistant hybrid were spray inoculated with an airbrush (Master Airbrush, model TC-60, TCPGlobal, SanDiego, CA.) with approximately 1ml of bacterial suspension evenly on both sides. Non-inoculated controls were included. Plants were left on the bench at room temperature to dry, after which they were randomly placed in a humidity chamber set to mist the plants for 1 minute every two hours. Non-inoculated controls were maintained on the greenhouse bench to prevent contamination.

The V3 leaves of both corn hybrids were sampled 0, 1, 2, 6 and 10 dpi and there were eight samples per hybrid. Half of the samples were subjected to 400J/m^2s^{-1} of UV-C radiation on both abaxial and adaxial surfaces to kill external bacterial populations. The irradiated leaves were then cut and placed in 20ml of 0.5x PBS in Agdia® sample bags (ACC 00925, Agdia Inc., Elkhart, IN.) and ground with an Agdia® tissue homogenizer (ACC 00900, Agdia, Inc., Elkhart, IN.). Leaf prints were taken of treated leaves onto NBY agar to ensure that external bacterial cells had been killed. Tenfold dilutions were then drop plated on NBY agar plates containing rifampicin at 100µg/mL to obtain internal populations. The other half of samples were cut into 1cm pieces and placed into glass tubes containing 20ml of 0.5x PBS. These were sonicated for seven minutes at an operating frequency of 35 khz in a sonicator (VWR International LLC, Radnor PA, model 97043-972), after which tenfold dilutions were drop plated onto NBY agar plates containing rifampicin at 100µg/ml to obtain external populations. Only one spontaneous rifampicin-resistant mutant was utilized at a time for each experiment. Each experiment was run twice for a total of four experiments. All data was analyzed using Tukey's HSD test in the statistical program JMP (Figures 3.7-3.10).

To test if virulence was correlated to survival of *Cmn*, an additional experiment was carried out. It followed the same method as above, however, both spontaneous rifampicin-resistant mutants were sprayed on the V3 leaves of the chosen Goss's wilt susceptible hybrid only. Plants that were sprayed with either of the mutants were placed randomly in the humidity chamber and sampled 0, 8 and 12 dpi. Samples were processed, plated out, and CFU's/g were enumerated, as mentioned above. This was done so that comparisons could be made among the mutants that could not be made in the previous experiment, since both mutants were not included at the same time per experiment. All data was analyzed using Tukey's HSD test in the statistical program JMP (Figures 3.11-3.12).

3.3.7 Recovery of CFU/g of *Cmn* isolates

Plants of a Goss's wilt susceptible hybrid were grown up to the V3-V4 leaf stage in square, 1135.62cm^3 pots containing SUREMIX (Michigan Growers Products, Inc.^{tm}). Plants were fertilized with one teaspoon of Osmocote® Smart-Release® 14-14-14 plant food (ScottsMiracle-grow) upon emergence. Next, 6 *Cmn* isolates, which had been previously grouped into three rankings of virulence (figure 3.2) were grown for 24 hours in NBY broth on a rotary shaker, spun down, resuspended in 0.5x PBS and adjusted to an OD_{600} of 0.2. The vegetative 3 leaves of the corn plants were wounded by cutting across three major veins between the midrib and edge of the leaf, 8cm from the sheath, with a sterile scalpel. Next, suspensions of the bacterial isolates were sprayed over the wounds with an airbrush sprayer (Master Airbrush, model TC-60, TCPGlobal, San Diego, CA.). Approximately 400µl was sprayed on each wound. Corn plants were also mock inoculated to serve as a negative control. The inoculated V3 leaves were sampled 0, 1, 2, 4 and 6 days dpi, weighed, placed into 20ml of 0.5x PBS, and ground with a

polytron® (Model PT 10-35 GT, Kinematica). Tenfold dilutions were made of the liquid formed and drop plated onto NBY agar containing cyclohexamide at 100µg/mL, to obtain CFU's/g of tissue. There were four replicates per bacterial isolate per sampling day. Data was analyzed using Tukey's HSD test in JMP (Figure 3.13).

3.4 RESULTS

3.4.1 Virulence screening of isolates

For the stab inoculation method, Isolate GIL1 was the most virulent, with a rating of 4.75 out of 5 (0 = a healthy plant; 1 = a plant with minor wilting, no stunting, and lesions on at least two leaves. Bacterial ooze may or may not be present on leaf tips; 2 = a plant with wilting, stunting lesions on three or more leaves and possible bacterial ooze on leaf tips; 3 = a plant with wilting, stunting lesions on all leaves and possible bacterial ooze on leaf tips; 4 = a plant with severe wilting, severe stunting, lesions on all leaves and possible bacterial ooze on leaf tips; and 5 = a plant that was completely wilted, stunted and dead). This was followed by GW-17-F, which had a rating of 4.25 and GW-22-F and GW-18-E, which both had ratings of 4. For this method, these four isolates were ranked as highly virulent. Isolates identified as moderately virulent were Cmn1011, GW-23-A, GW-21-D, C7, HI-6-5, CL4, FN, NE3, HF1 and C4 with scores ranging from 3 to 3.75. Finally, the isolates GW-20-E, GW-23-B, GW-23-D, Cmn1009 and Cmn1013 were determined to be the least virulent with scores ranging from 2.75 to 0.75.

For the cut inoculation method, the 19 *Cmn* isolates were separated into three virulence groups (Figure 3.1). The groups were highly virulent isolates (red), moderately virulent isolates (green) and the least virulent isolates (blue). Blue squares in the figure indicate the percentage of leaf area infected for eight biological replicates per isolate, divided into two experiments.

Differences in shades of blue for squares for specific isolates showed that there was a lot of variation in % leaf area infected for the highly virulent and the moderately virulent isolates, among the two experiments. The least virulent isolates showed the least amount of variation among the two experiments. Furthermore, only 10 of the isolates grouped similarly to how they did in the stab inoculation method. Some exceptions include CL4, which grouped as highly virulent in this method and FN, which grouped as one of the least virulent isolates in this method.

Table 3.1. Goss's wilt disease ratings of *Clavibacter michiganensis* subsp. *nebraskensis* isolates inoculated to corn via a stab inoculation method and a cut inoculation method. Stab inoculation disease ratings are based on a scale from 0-5 (0 = a healthy plant; 1 = a plant with minor wilting, no stunting, and lesions on at least two leaves. Bacterial ooze may or may not be present on leaf tips; 2 = a plant with wilting, stunting lesions on three or more leaves and possible bacterial ooze on leaf tips; 3 = a plant with wilting, stunting lesions on all leaves and possible bacterial ooze on leaf tips; 4 = a plant with severe wilting, severe stunting, lesions on all leaves and possible bacterial ooze on leaf tips; and 5 = a plant that was completely wilted, stunted and dead). Percent Area of leaf tissue infected for each isolate is shown for the cut inoculation method.

Isolate	Source	Disease rating (0-5)	% Area leaf infection (cm^2)
GW-17-F	Indiana, Kiersten Wise	4.25	9.74
GW-18-E	Indiana, Kiersten Wise	4	20.09
GIL1	Iowa, Alison Robertson	4.75	26.88
C4	Iowa, Alison Robertson	3.25	18.87
HF1	Iowa, Alison Robertson	3.25	17.03
NE3	Iowa, Alison Robertson	3	10.68
FN	Iowa, Alison Robertson	3	1.45
CL4	Iowa, Alison Robertson	3.25	22.05
HI-6-5	Iowa, Alison Robertson	3.25	14.36
C7	Iowa, Alison Robertson	3	8.4
GW-20-E	Missouri, Patti Wallace	2.75	11.01
GW-21-D	Missouri, Patti Wallace	3.75	14.97
GW-22-F	Missouri, Patti Wallace	4	28.19
GW-23-A	Missouri, Patti Wallace	3.25	19.43
GW-23-B	Missouri, Patti Wallace	2.25	17.51
GW-23-D	Missouri, Patti Wallace	0.75	0
Cmn1009	Michigan State University	1.0	0.46
Cmn1013	Michigan State University	0.25	0
Cmn1011	Michigan State University	3.5	6.37

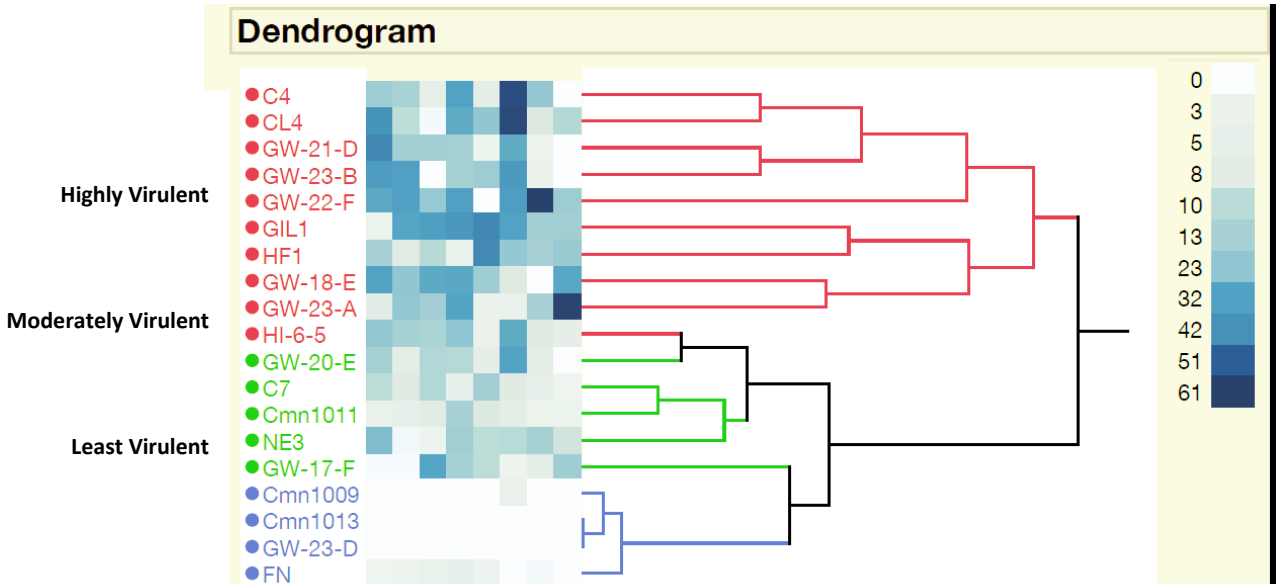

Figure 3.1. Separation of all 19 *Clavibacter michiganensis* subsp. *nebraskensis* isolates into three virulence groups based on hierarchical clustering and includes highly virulent isolates with the greatest percentage leaf area diseased (red), moderately virulent isolates with moderate percentage leaf area diseased (green) and slightly virulent isolates with the least percentage leaf area diseased (blue). Blue squares indicate percent leaf area infected for 8 biological replicates per isolate, divided into two experiments.

3.4.2 Selection of rifampicin-resistant mutants

The mutants used in this study were found to be as virulent as the wildtypes (data not shown). However, they were found to grow slower than the wildtypes (Figures 3.2-3.3). The rifampicin-resistant mutants were still able to reach the same concentration as the wildtype in liquid broth, which was to an OD_{600} of approximately 1.8 for GIL1 and an OD_{600} of approximately 1.6 for GW-20-E.

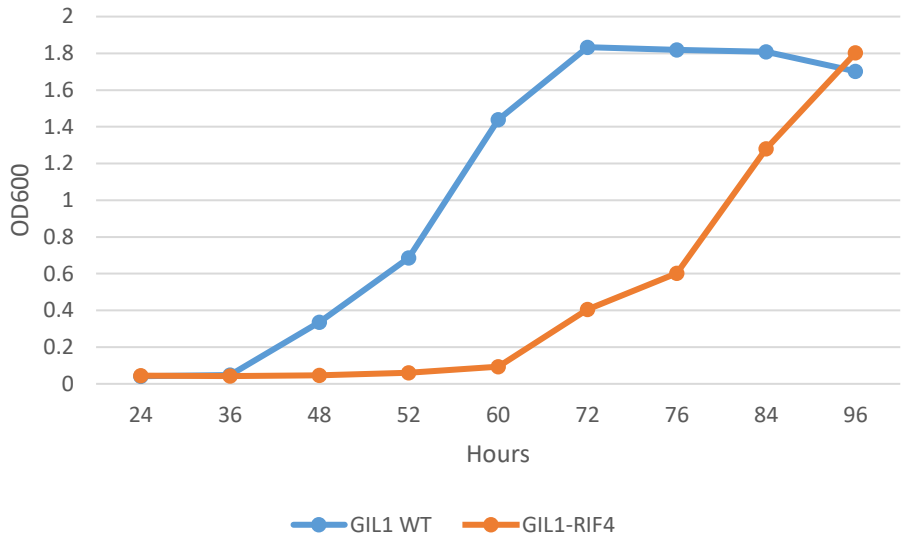

Figure 3.2. Growth Curve for *Cmn* isolates GIL1 and the rifampicin resistant-mutant GIL1-RIF4. The isolates were grown in 50mL of NBY broth on a rotary shaker at 28°C. The y-axis shows the OD_{600}, while the x-axis refers to the time in hours. GIL1-RIF4 grows slower than the wildtype.

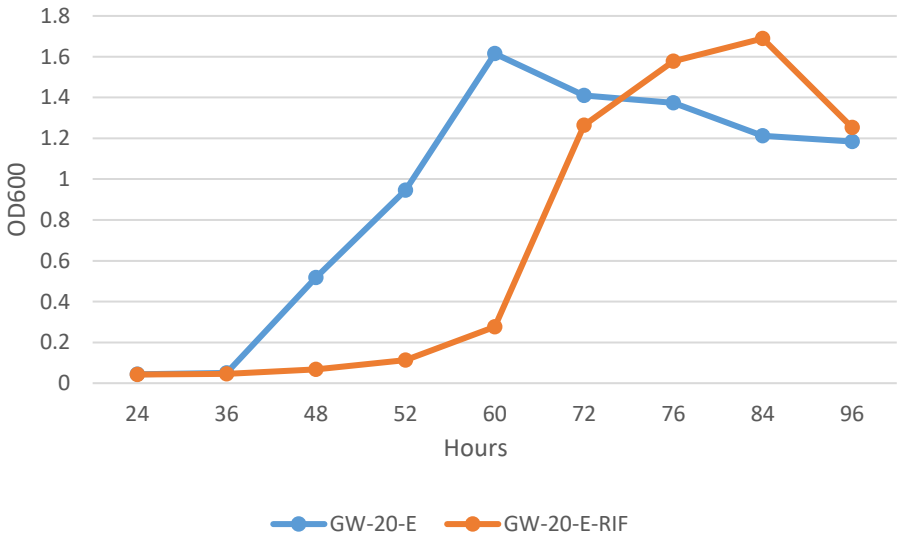

Figure 3.3. Growth curve for the *Cmn* isolate GW-20-E and the rifampicin-resistant mutant GW-20-E RIF. The isolates were grown in 50mL of NBY broth on a rotary shaker at 28°C. The y-axis shows the OD_{600}, while the x-axis refers to the time in hours. GW-20-E-RIF grows slower than the wildtype.

3.4.3 Screening of susceptibility of corn hybrids to Goss's wilt

Five corn hybrids were screened for their susceptibility to Goss's wilt so that a susceptible hybrid and a partially resistant hybrid could be used in these experiments. It was found that hybrid two was the most susceptible while hybrid one appeared to be partially resistant. Hybrid two had one of the longest lesion lengths on the V4 leaf 10 dpi with GIL1-RIF4, along with hybrid five (Figure 3.4), while hybrid one had the shortest. Additionally, the most CFU's/g were recovered from hybrid two, again along with hybrid five (Figure 3.5), while the least were recovered from hybrid one. Due to this, hybrid two was chosen as the susceptible Goss's wilt hybrid to be used in these studies, while hybrid one was chosen as the partially resistant hybrid to be used in these studies.

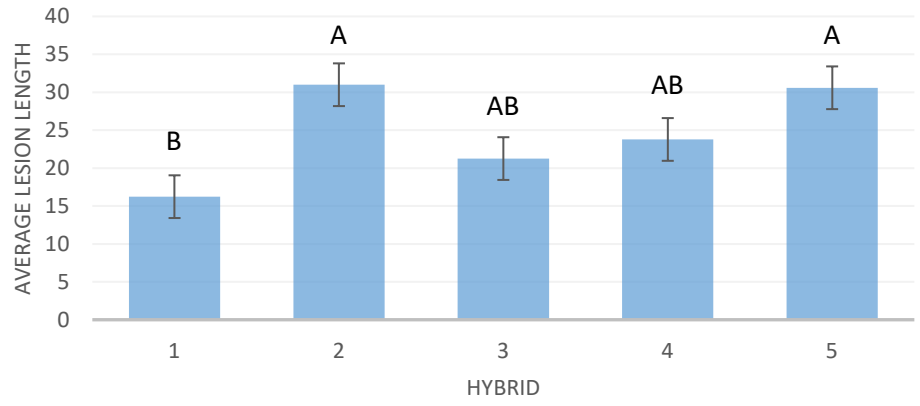

Figure 3.4. Average lesion length on the V4 leaves of four different corn hybrids 10 days after inoculation with *Cmn* isolate GIL1-RIF4.

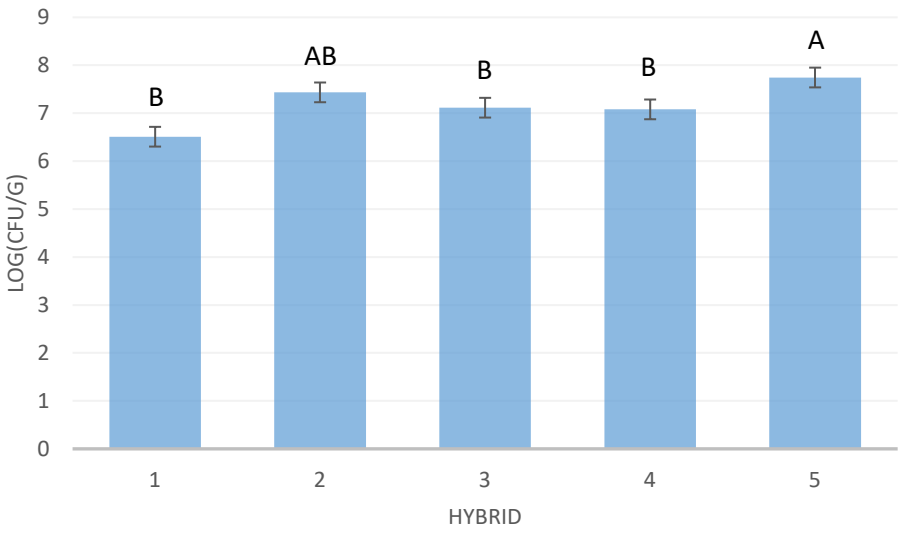

Figure 3.5. Bacterial populations (log CFU's/g) recovered from Goss's wilt susceptible corn hybrids 10 days post inoculation with GIL1-RIF4.

3.4.4 Generation of a UVC radiation kill curve

A UV-C radiation kill curve was generated to determine how much UV-C light should be applied to V3 corn leaves to kill external populations, so that internal populations of bacterial cells could be obtained. It was determined that 400J/m^2s^{-1} was sufficient to reduce cell numbers by 10^4-fold in liquid culture. This amount of UV-C radiation did not kill all of the bacterial cells, but it was observed that subjecting leaves to a longer exposure would wilt the leaves, which could result in killing of internal populations.

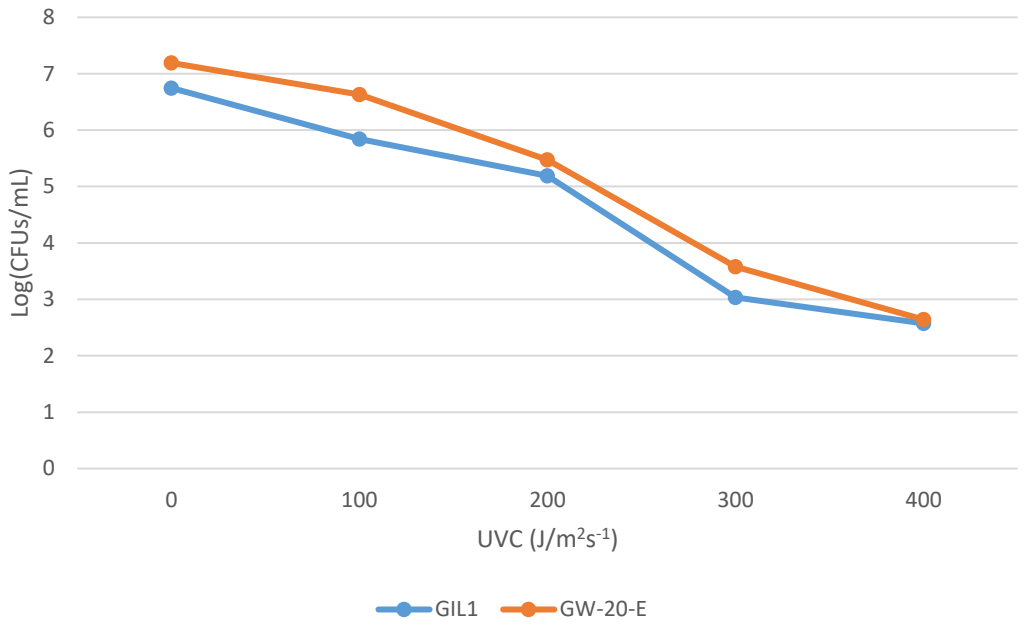

Figure 3.6. UV-C radiation kill curve for *Cmn* isolates GIL1 and GW-20-E. Liquid cultures of both isolates were subjected to 0, 100, 200, 300 and 400 J/m^2s^{-1} of UVC light successively, and plated onto NBY to obtain CFU's.

3.4.5 Inoculation of corn hybrids with isolates and sample processing

For the initial experiment, there were no significant differences observed in endophytic or epiphytic populations between GIL1-RIF4 (highly virulent) on the susceptible hybrid or the partially resistant hybrid (Figures 3.7-3.8), 2, 6, and 10 dpi. The same could be seen for GW-20-E RIF (moderately virulent), except for a significant difference between external and internal populations, 6 dpi on the more susceptible hybrid (Figures 3.9-3.10).

For the second experiment, there were no significant differences for endophytic or epiphytic populations observed among GIL1-RIF4 or GW-20-E-RIF on the Goss's wilt susceptible hybrid (Figures 3.11-3.12).

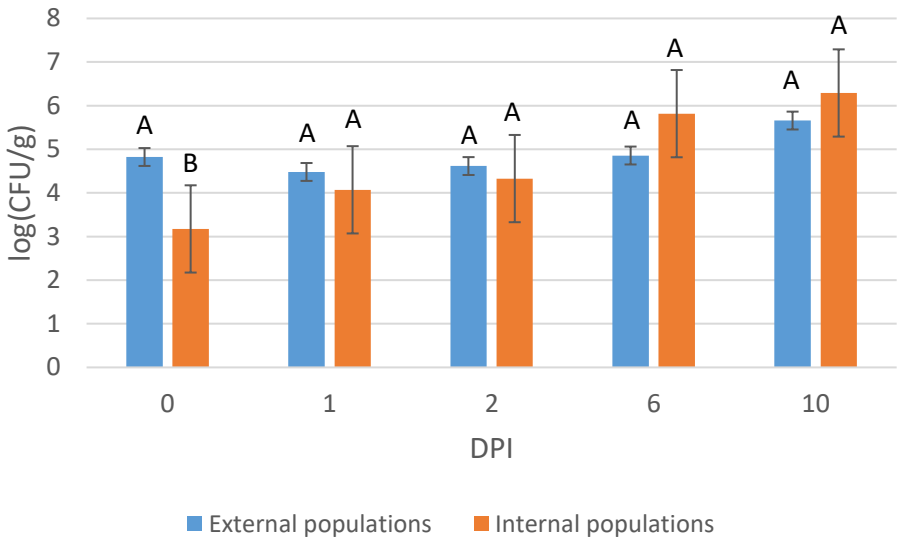

Figure 3.7. External vs internal populations of GIL1-RIF4 on a Goss's wilt susceptible corn hybrid. No statistical differences were seen among isolates, except at 0 dpi, as determined with Tukey's HSD.

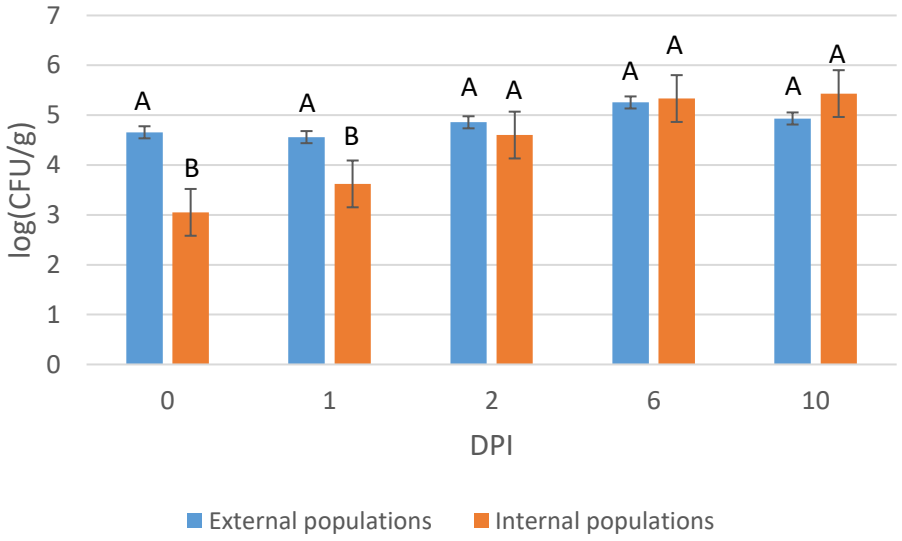

Figure 3.8. External vs internal populations of GIL1-RIF4 on a corn hybrid partially resistant to Goss's wilt. No statistical differences were seen among isolates, except at 0 and 1 dpi, as determined with Tukey's HSD.

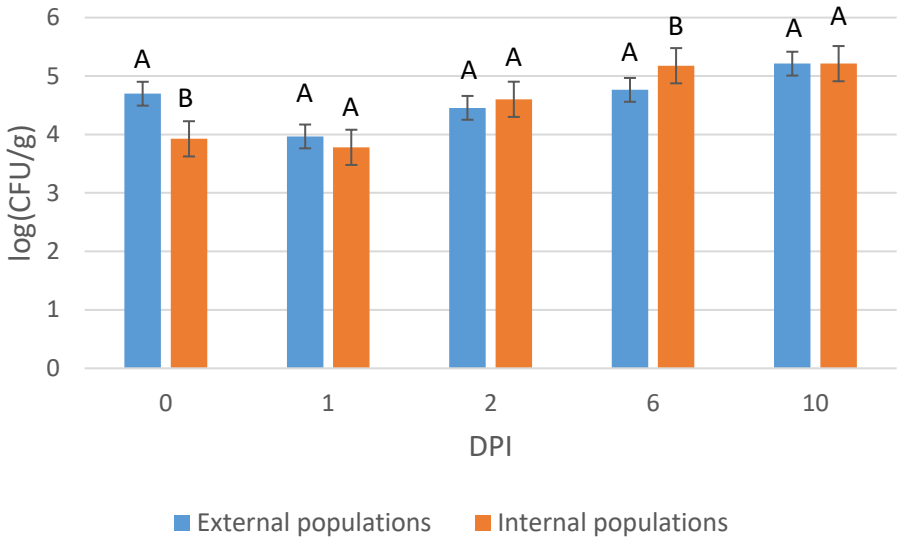

Figure 3.9. External vs internal populations of GW-20-E RIF on a Goss's wilt susceptible corn hybrid. No statistical differences were seen among isolates, except at 0 dpi, as determined with Tukey's HSD.

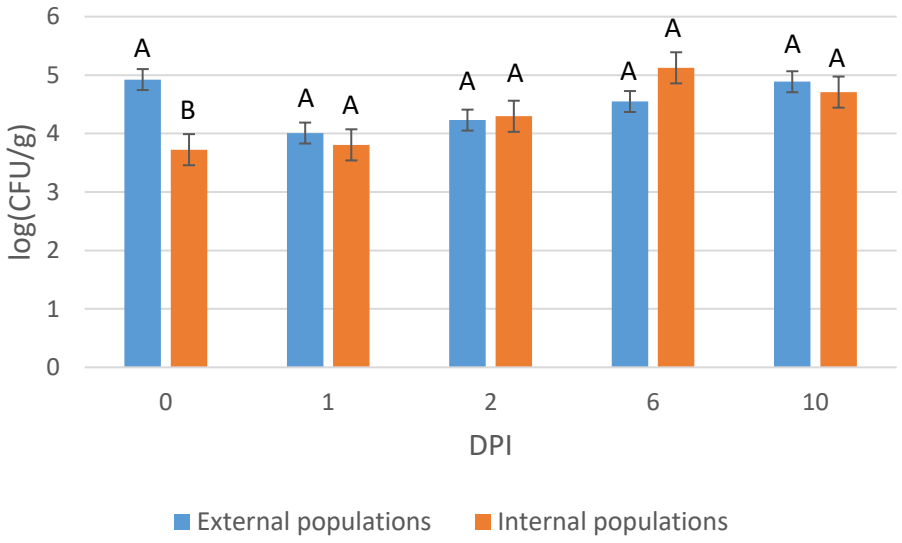

Figure 3.10. External vs internal populations of GW-20-E RIF on a corn hybrid with partial resistance to Goss's wilt. No statistical differences were seen among isolates, except at 0 dpi, as determined with Tukey's HSD.

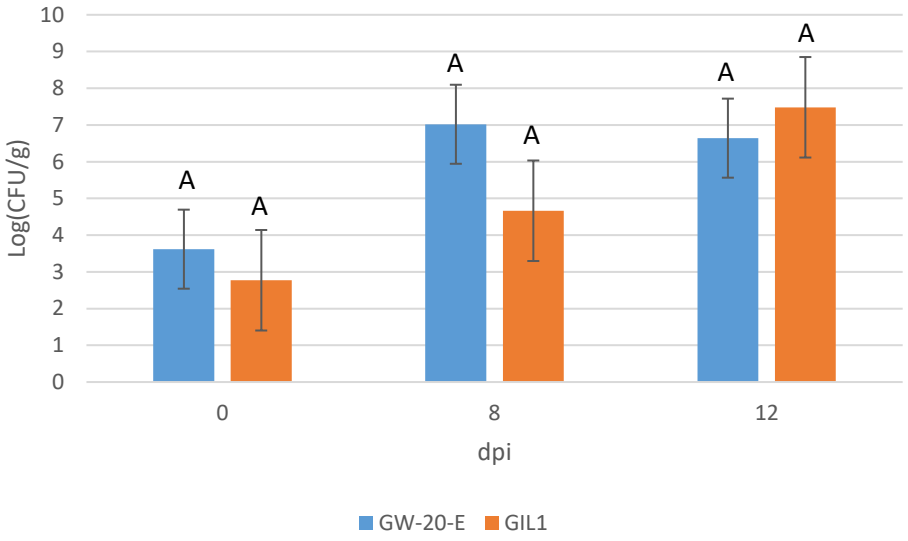

Figure 3.11. Bar graph showing internal populations of *Cmn* isolates over time. The Y-axis shows the Log of CFU's/g recovered, while the Y-axis shows the dpi. There was no significant difference between the populations of both isolates 0, 8 and 12 dpi. Data was analyzed using Tukey's HSD test in the statistical program JMP.

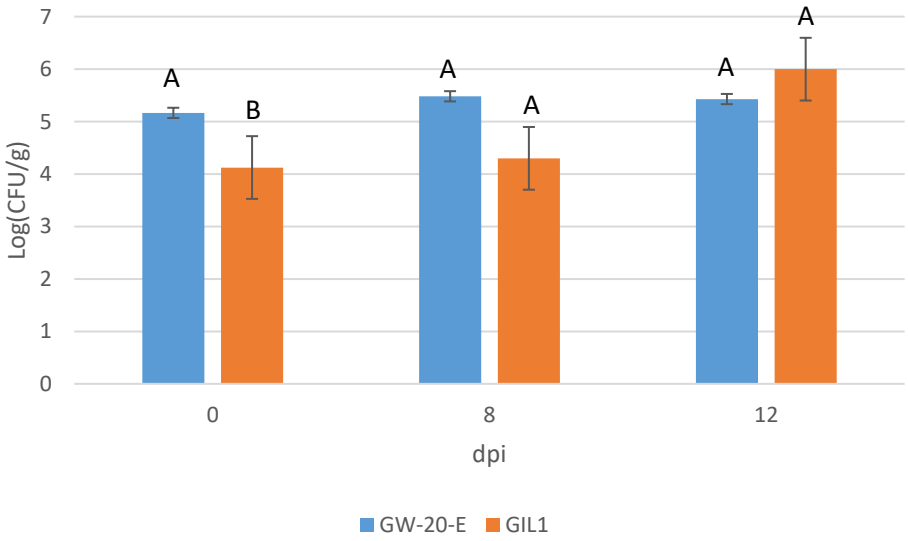

Figure 3.12. External populations of *Cmn* isolates on corn leaves over time. Data was analyzed using Tukey's HSD test in the statistical program JMP.

3.4.6 Recovery of CFU/g of *Cmn* isolates

None of the isolates that differed in virulence differed significantly in populations recovered except for populations of Cmn1009. This is interesting because Cmn1009 is grouped with the least virulent isolates (figure 3.7). GW-23-D, which is also one of the least virulent isolates had population levels that dropped 1 and 2 dpi, but by day six had risen to the levels of all the isolates besides Cmn1009.

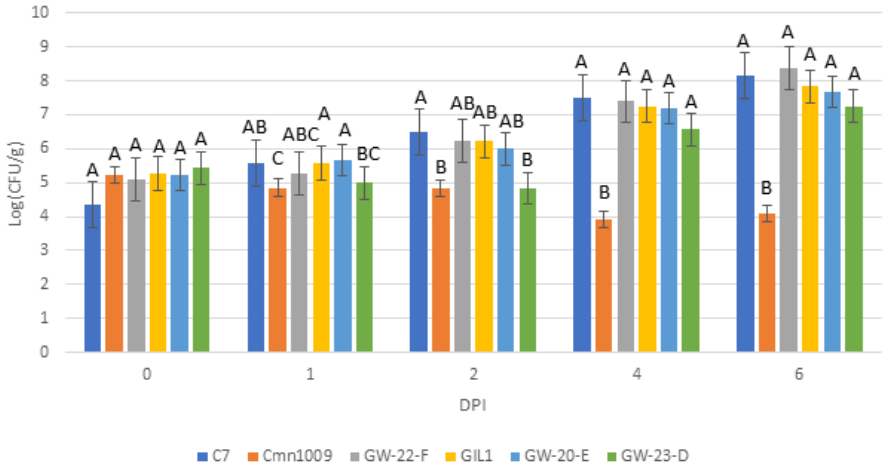

Figure 3.13. Bargraph showing *Cmn* isolates recovered over time, that represent different virulence groups. The Y-axis shows the log of CFU's recovered per gram of tissue while the X-axis shows the dpi. None of the populations of the isolates were significantly different 6 dpi, except for Cmn1009, which was one of the least virulent isolates.

3.5 DISCUSSION

In this study, we utilized two different inoculation methods to separate 19 *Cmn* isolates into three different virulence groups. From this, we could choose a highly virulent isolate, GIL1, and a moderately virulent isolate GW-20-E, for our epiphytic and endophytic population studies. There was a great deal of variation among the highly virulent and the

moderately virulent strains for the cut inoculation method, while there was little to no variation among the least virulent strains. Hierarchical clustering was able to separate the 19 *Cmn* isolates into three virulence groups, regardless. The variation observed may be due to the method of which the leaves were cut and inoculum applied. Perhaps not all leaves were cut across three major veins consistently or perhaps inoculum was not sprayed consistently over wounds.

By using UV-C radiation we could kill 4-fold bacterial cells of our chosen *Cmn* isolates in order to separate epiphytic and endophytic populations of our spontaneous rifampicin-resistant mutants. While this method did not kill all the bacterial cells, it was determined to be an effective method to separate the majority of epiphytic bacteria from endophytic bacteria. It was determined that *Cmn* isolates build up epiphytic and endophytic populations over time, however, there were no significant differences between epiphytic and endophytic populations for either GIL1-RIF4, highly virulent, or GW-20-E RIF, moderately virulent, on corn hybrids that differed in susceptibility to Goss's wilt. Furthermore, there were no significant differences between epiphytic and endophytic populations among GIL1-RIF4 and GW-20-E RIF on a Goss's wilt susceptible hybrid. This agrees with findings that there were no significant differences associated with virulence among epiphytic populations of *Cmn* or those recovered from diseased tissue on a Goss's wilt susceptible corn hybrid by Ahmad et al. (2015). This suggests that the ability of *Cmn* to survive epiphytically may not be associated with disease, but may be a way for the pathogen to spread. It is also possible that both isolates used in this study grew well because they were both under conditions of high humidity and moisture. For example, it was found that population sizes for pathogenic and nonpathogenic *P. syringae* strains were

similar under high relative humidity (Wilson et al., 1999). Pathogenic strains, however, survived better when subjected to drying conditions than did nonpathogenic strains. In this study, bacterial cells were not observed for survival under dry conditions, but perhaps a noticeable difference would have been seen among isolates if they had been subjected to desiccation.

Another factor that may have influenced these results is that only one *Cmn* isolate was run at a time on both a highly susceptible hybrid and a partially resistant hybrid, but were only run together on the more susceptible hybrid. It is possible that both isolates were able to colonize this hybrid well because it was highly susceptible, even though the isolates differed in virulence. This study should be repeated looking at the survival of these two isolates at the same time on a resistant as well as a susceptible hybrid. It was found, for example, that higher populations of *P. morsprunorum* could be found on a more susceptible cultivar of cherry when compared to a more resistant one (Crosse, 1963).

As a final experiment to test whether the survival of *Cmn* was linked to virulence, isolates that differed in virulence were sprayed onto the V3 leaves of the chosen Goss's wilt susceptible hybrid and CFU's/g were recovered to see if isolates that were more virulent could survive better than those that were less virulent, epiphytically. For the *Cmn* isolates evaluated, none differed significantly in populations 6 dpi, except for one of the least virulent isolates Cmn1009, which was one of the least virulent isolates. Although, this isolate could not survive well, there was no clear correlation between virulence and the ability to survive externally, as all other isolates reached populations that were not significantly different, 6 dpi. This again may be because all isolates were subjected to ideal conditions, allowing all bacterial isolates to

proliferate. Perhaps if this experiment would have been conducted under drying conditions, then noticeable differences in populations among isolates would be observed.

Finally, several phytopathogenic bacterial species are known to have the ability to infect alternative hosts, with or without causing disease symptoms. Some of these species include *Xylella fastidiosa, Cmn,* and *Xanthomonas axonopodis* pv. *vignicola* (Chatelet et al., 2011; van der Wolf et al., 2005; Sikirou and Wydra, 2004). This knowledge is important to understand the epidemiology of these pathogens and to devise effective methods of control. It is also known that *Cmn* can survive on certain weed hosts and cause disease symptoms. For example, it was found that *Cmn* can cause disease on annual ryegrass, Johnson grass, and large crabgrass (Ikley et al., 2015). Other alternative hosts of *Cmn* include 4 *Setaria* species that are often found in corn fields (Langmeier et al., 2014). In all of these studies, knowledge of how the various pathogenic bacteria survive on their alternative hosts is important because populations of the bacteria have the potential to build up and serve as inoculum sources for their main hosts. Future work should also examine the ability of *Cmn* to survive epiphytically and endophytically on weed hosts, and how this may impact spread of Goss's wilt.

This research shows that *Cmn* can survive epiphytically and enophytically and that control of epiphytic populations is important because it could potentially be a way for the bacteria to build up populations and spread. This is supported by a study where epiphytic populations of *Cmn* could be found 15 days on symptomless corn leaves that were 2.5 meters away from a source of inoculum (Eggenberger, et al., 2016). This research also shows that epiphytic and endophytic populations of *Cmn* may able to survive regardless of virulence, if conditions are optimal. It remains to be seen how isolates that differ in virulence can survive

desiccation. Since the Goss's wilt pathogen survives on host debris, it is possible that different isolates could better tolerate drying conditions.

APPENDIX

APPENDIX

Scouting for Goss's wilt in Michigan

A.1. MATERIALS AND METHODS

A.1.1 Collection of bacterial isolates

Known and unknown bacterial strains were collected during the summer and fall of 2014 (table A.1), the summer of 2015 (table A.2) and the summer of 2016 (table A.2). Isolates obtained from leaf samples were processed by cutting up diseased tissue after surface sterilization in 5% bleach, and pouring sterile 85% saline over the tissue to bring out bacterial cells. Dilutions of 10^{-1} and 10^{-2} were made and 100µl were plated onto the semi-selective CNS media, except for in 2016 when dilutions were plated onto NBY agar. Two replicates of each dilution were performed. Once bacterial colonies had grown, colonies of orange color and varying morphology were separately streaked out onto NBY agar. Loopfulls of bacteria from these streaks were streaked out onto NBY agar to obtain single colonies. Single colonies were then mass streaked onto new NBY plates to obtain a bacterial lawn, which was taken up with a sterile cotton swab and put into cryotubes containing 20% or 15% glycerol for storage at -80°C. In 2014, 42 isolates were obtained from five counties in MI, 18 isolates from four counties in IN, and 18 isolates from four counties in MO. Additionally, eight known *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn)* isolates were obtained from Iowa and two from Michigan State University. In 2015, 60 isolates were obtained, 14 of which were from two counties in Indiana and 46 that came from five counties in MI. An additional 79 isolates were isolated from a field in IN that was badly infected with Goss's wilt. The field where samples were collected from in IN was confirmed to have Goss's wilt by researchers at Purdue University in this year. In the

summer of 2016, 12 isolates were obtained from four counties in MI from leaves suspected to be infected with *Cmn*.

A.1.2 Identification of *Clavibacter michiganensis* subsp. *nebraskensis* via a Hypersensitive Response in Four-o'clocks (2014)

To prepare inoculum, 3µl of each bacterial isolate were taken from freezer stocks and added to 3ml of NBY broth in 14ml falcon tubes. Tubes were placed into a rotary shaker set at 28°C for 48 hours. Cells were next centrifuged at 2,000g for 15 minutes. After discarding the supernatant, the bacterial pellet was re-suspended in sterile diH_2O (Gitaitis, 1990). *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*) cells grown up this way were previously determined to contain approximately 10^8 cells/ml, via dilution plate counts. Inoculum was maintained on ice until plants were inoculated.

Clavibacter michiganensis subsp. *michiganensis* (*Cmm)* strain *Cmm*1010, *Pseudomonas syringae* strain PF5 and sterile diH_2O were used as negative controls during this experiment. Plants were grown in a growth chamber maintained at 24°C with a 400 mE light level and a 14 hour light period. Plants were routinely given nutrient water after sprouting. Once fully expanded leaves had formed, they were infiltrated with inoculum in ten different spots per isolate via a blunt syringe method. Plants were checked for a hypersensitive response 36-48 hours after inoculation and up to two weeks for additional symptoms and results were recorded (Table A.1). An image of the HR response seen can be observed in image A.1.

A.1.3 Identification of putative *Cmn* isolates with PCR

In both 2014 and 2015, all putative *Cmn* isolates were grown in 5ml of NBY broth for two days after which their DNA was extracted using DNeasy Blood & Tissue Kits for gram-positive bacteria. About 1.5mL of bacterial culture were transferred to 1.5ml centrifuge tubes and tubes were centrifuged for 10 minutes at 7,500 rpm. The supernatant was discarded and the bacterial pellet was resuspended in 180uL of enzymatic lysis buffer. The mixture was incubated for 30 minutes, after which 25µl of protinase K and 200µl of Buffer AL were added. The mixture was vortexed and incubated at 56^{o}C for 30 minutes. Next, 200µl of ethanol was added to the mixture and vortexed, after which the mixture was pipetted into a DNeasy Mini spin column set in a 2ml collection tube. The liquid in the collection tube was discarded after centrifuging the mixture at 8000 rpm for one minute. Next, the DNeasy spin column was set in a new 2ml collection tube, 500µl of AW1 wash buffer was added and he mixture was centrifuged for 1 minute at 8000 rpm. The liquid in the collection tube was again discarded and the DNeasy mini spin column was set in another 2ml collection tube. 500µl of AW2 was buffer was added and the mixture was centrifuged for three minutes at 14,000 rpm. The liquid in the collection tube was again discarded, the spin column was placed back into the same collection tube, and centrifuged again for one minute at 14,000 rpm. Finally, the DNeasy Mini spin column was put into a 1.5ml centrifuge tube, 200µl of AE elution buffer was placed onto the DNeasy membrane and inubated at room temperature for one minute. This was then centrifuged for one minute at 8,000 rpm to elute.

DNA that had been extracted by the above method was checked with a nanodrop for purity and used in a PCR method adapted for the various *Cm* subspecies (H-J Bach et al. 2003), in 2014 and 2015 only. In 2016, colony PCR was utilized. For the method adapted from H-J

Bach et. al, 25µl reactions were carried out. Each reaction consisted of 2.5µl of 10x PCR buffer, 16.18µl of sterile water, 2.0µl of 50mM $MgCl_2$, 1.0µl of 10µM forward primer, 2.0µl of 10µM revers primer, 0.2µl of 25mM deoxynucleotide triphosphates, 0.125µl of Taq DNA polymeraze (invitrogen) at 5U/µl and 1µl of DNA template.

The PCR protocol consisted of one hold at 95^{o}C for 10 minutes to denature DNA, 35 cycles at 95^{o}C for 20s and 66^{o}C for 60s. PCR product was visualized on an agarose gel and compared to a known *Cmn* isolate that was used as a positive control. Water was used as a negative control. Each PCR reaction was replicated twice and positive or negative results were recorded (table A.1-A2). In 2016, the same PCR protocol was used, as mentioned previously, but colony PCR was utilized rather than extraction of DNA. For this procedure, a single bacterial colony was placed into 13µl of sterile distilled water and boiled at 95^{o}C for five minutes. One microliter of this mixture was placed into each reaction tube.

A.1.4 Identification of putative *Cmn* isolates with inoculation of corn hybrids

Inoculum was prepared by adding 3µl of isolate stock to 5ml of NBY broth in a 14ml falcon tube. Tubes were placed in a rotary shaker set to 28°C and allowed to grow for 48 hours. Inoculum was then put into 50ml conical tubes and centrifuged at 2000 rpm for 10 minutes. After discarding the supernatant, bacterial pellets were re-suspended in 0.01M potassium phosphate buffer, pH 7.2 (Vidaver, 1977). Potassium phosphate buffer and a known isolate of *Cmn* were used as negative and positive controls, respectively. Inoculum was maintained on ice until used.

Two Goss's wilt susceptible corn lines were grown in the greenhouse until they reached the V3-V4 leaf stage. Inoculum was then injected into the stems of the plants 4 cm from the soil line in two wounds made at 90 degree angles from each other with a 25 gauge needle attached to a 1ml syringe. Each wound received half a ml of bacterial suspension and each inoculation was replicated twice. Corn plants were observed for disease symptoms 10 days after inoculation (table A.1-A2).

A.2 RESULTS

A.2.1 Isolates collected in 2014

No isolates from Michigan were positive for *Cmn* via either of the three testing methods used, PCR for *Cm*, an HR response in 4-o'clocks, and disease symptoms in corn. Eight known isolates of *Cmn* were obtained from Iowa and two from Michigan State University, which all tested positive for the testing methods used. Additionally, six *Cmn* isolates were isolated from Missouri and two isolates were isolated from Indiana.

Table A.1. Table listing all *Cmn* isolates collected in 2014, their origin, how they were isolated, and *Cmn* identification test results. Test results shown are for PCR identification, an HR response in 4-o'clocks, and disease symptoms in Goss's wilt susceptible corn hybrids.

Isolate	Year Isolated	obtained from	Isolated from	PCR + or -	HR response	disease in corn + or
Cmn1013	2014	MI	culture	+	No HR	+
Cmm1010	2014	MI	culture	+	HR response	-
Cmn1009	2014	MI	culture	+	No HR	+
GIL1	2014	IA	culture	+	chloroic w/ drk cntr	+
C4	2014	IA	culture	+	chloroic w/ drk cntr	+
HF1	2014	IA	culture	+	chloroic w/ drk cntr	+

Table A.1 (cont'd)

NE3	2014	IA	culture	+	chloroic w/ drk cntr	+
FN	2014	IA	culture	+	chloroic w/ drk cntr	+
CL4	2014	IA	culture	+	chloroic w/ drk cntr	+
HI-6-5	2014	IA	culture	+	chloroic w/ drk cntr	+
C7	2014	IA	culture	+	chloroic w/ drk cntr	+
CmmG11	2014	MI	culture	-	No HR	-
GW-1-A	2014	MI	leaf	-	No HR	-
GW-1-B	2014	MI	Leaf	-	No HR	-
GW-1-C	2014	MI	Leaf	-	No HR	-
GW-1-D	2014	MI	Leaf	-	No HR	-
GW-2-A	2014	MI	Leaf	-	No HR	-
GW-2-B	2014	MI	Leaf	-	No HR	-
GW-3-A	2014	MI	Leaf	-	No HR	-
GW-3-B	2014	MI	Leaf	-	No HR	-
GW-3-C	2014	MI	Leaf	-	No HR	-
GW-3-D	2014	MI	Leaf	+/-	No HR	-
GW-4-A	2014	MI	Leaf	-	No HR	-
GW-4-B	2014	MI	Leaf	-	No HR	-
GW-4-C	2014	MI	Leaf	-	No HR	-
GW-5-A	2014	MI	Leaf	+/-	No HR	-
GW-6-A	2014	MI	Leaf	-	No HR	-
GW-6-B	2014	MI	Leaf	-	No HR	-
GW-6-C	2014	MI	Leaf	-	No HR	-
GW-7-A	2014	MI	Leaf	+/-	No HR	-
GW-8-A	2014	MI	Leaf	-	No HR	-
GW-8-B	2014	MI	Leaf	-	No HR	-
GW-8-C	2014	MI	Leaf	-	No HR	-
GW-9-A	2014	MI	Culture	-	No HR	-
GW-9-B	2014	MI	Culture	-	No HR	-
GW-9-C	2014	MI	Culture	-	No HR	-
GW-9-D	2014	MI	Culture	-	No HR	-
GW-9-E	2014	MI	Culture	-	Faint chlorosis	-
GW-9-F	2014	MI	Culture	-	No HR	-
GW-9-G	2014	MI	Culture	-	No HR	-
GW-10-A	2014	MI	Leaf	-	No HR	-
GW-11-A	2014	MI	Leaf	-	No HR	-

Table A.1 (cont'd)

GW-11-B	2014	MI	Leaf	-	No HR	-
GW-12-A	2014	MI	Leaf	-	No HR	-
GW-12-B	2014	MI	Leaf	-	No HR	-
GW-12-C	2014	MI	Leaf	-	No HR	-
GW-13-A	2014	MI	Leaf	-	No HR	-
GW-13-B	2014	MI	Leaf	-	No HR	-
GW-13-C	2014	MI	Leaf	-	No HR	-
GW-13-D	2014	MI	Leaf	-	No HR	-
GW-14-A	2014	MI	Leaf	-	No HR	-
GW-14-B	2014	MI	Leaf	-	No HR	-
GW-14-C	2014	MI	Leaf	-	No HR	-
GW-14-D	2014	MI	Leaf	-	No HR	-
GW-15-A	2014	IN	leaf	-	No HR	-
GW-16-A	2014	IN	leaf	+/-	No HR	-
GW-16-B	2014	IN	leaf	+/-	No HR	-
GW-16-C	2014	IN	leaf	-	chlorotic spots	-
GW-17-A	2014	IN	leaf	-	No HR	-
GW-17-B	2014	IN	leaf	-	No HR	-
GW-17-C	2014	IN	leaf	+	No HR	-
GW-17-D	2014	IN	leaf	+	chlorotic spots	-
GW-17-E	2014	IN	leaf	-	No HR	-
GW-17-F	2014	IN	leaf	+	chlorotic w/ drk cntr	+
GW-18A	2014	IN	leaf	+	Faint chlorosis	-
GW-18-B	2014	IN	leaf	-	No HR	-
GW-18-C	2014	IN	leaf	+	No HR	-
GW-18-D	2014	IN	leaf	+	Faint chlorosis	-
GW-18-E	2014	IN	leaf	+	chlorotic no drk 9dpi	+
GW-19-A	2014	IN	leaf	+	No HR	-
GW-19-B	2014	IN	leaf	+	No HR	-
GW-19-C	2014	IN	leaf	+	No HR	-
GW-19-D	2014	IN	leaf	-	No HR	-
GW-20-A	2014	MO	leaf	-	Faint chlorosis	-
GW-20-B	2014	MO	leaf	-	No HR	-
GW-20-C	2014	MO	leaf	-	No HR	-
GW-20-D	2014	MO	leaf	-	No HR	-
GW-20-E	2014	MO	leaf	+	chlorotic w/ drk cntr	+
GW-21-A	2014	MO	leaf	-	No HR	-

Table A.1 (cont'd)

GW-21-B	2014	MO	leaf	-	No HR	-
GW-21-C	2014	MO	leaf	-	Faint chlorosis	-
GW-21-D	2014	MO	Leaf	+	chlorotic w/ drk cntr	+
GW-22-A	2014	MO	Leaf	-	Faint chlorosis	-
GW-22-B	2014	MO	Leaf	-	Faint chlorosis	-
GW-22-C	2014	MO	Leaf	-	Faint chlorosis	-
GW-22-D	2014	MO	Leaf	-	No HR	-
GW-22-E	2014	MO	Leaf	-	No HR	-
GW-22-F	2014	MO	Leaf	+	chlorotic w/ drk cntr	+
GW-23-A	2014	MO	culture	+	chlorotic w/ drk cntr	+
GW-23-B	2014	MO	Culture	+	chlorotic w/ drk cntr	+
GW-23-C	2014	MO	Culture	-	No HR	-
GW-23-D	2014	MO	Culture	+	chlorotic w/ drk cntr	+

Although no *Cmn* isolates were recovered from MI in 2014, a unique hypersensitive response was discovered for the pathogen, that differed from the closely related subspecies *Cmm*, as can be seen in figure A.1. below. On the left is shown the HR response in a 4-o'clock inoculated with *Cmn* isolate CL4, as compared to a 4-o'clock inoculated with *Cmm* isolate Cmm1010, on the right. While the *Cmm* infected plant exhibits a typical HR response with the whole point of inoculation dying and becoming brown, the *Cmn* inoculated plant shows a much different response. While the whole point of inoculation becomes sunken and chlorotic, only the center dies and becomes brown.

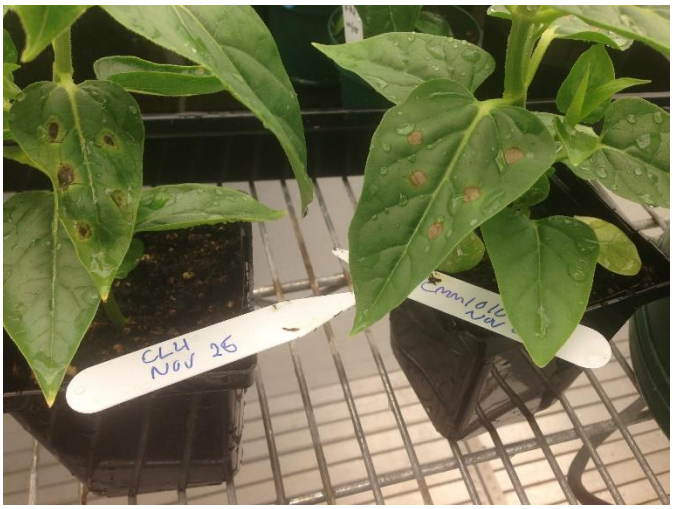

Figure A.1. This image shows the HR response of 4-o'clocks infected with *Cmn* isolates (left) vs *Cmm* isolates (right).

The image below shows a Goss's wilt susceptible corn hybrid 10 dpi with a highly virulent *Cmn* isolate. Symptoms include, freckling, wilting, stunting and bacterial ooze.

Figure A.2. This image shows Goss's wilt susceptible corn hybrids 10 dpi with a highly virulent *Cmn* isolate

A.2.2 Isolates collected 2015-2016

The table below shows all the isolates collected from 2015-2016. Isolates were only tested via PCR in these years. No isolates tested positive via PCR.

Table A.2. Table showing all isolates collected from 2015-2016. None of these isolates tested positive for *Cmm* via PCR.

# of isolates	Year isolated	Origin	PCR +
94	2015	Indiana	0
46	2015	Michigan	0
12	2016	Michigan	0

A.3 DISCUSSION

Michigan and Indiana were scouted for Goss's wilt during the Summer of 2014 and 2015, but only Michigan in the Summer of 2016. During this time, a bacterial culture collection was created, which included isolates from MO and IA. No isolates from Michigan were found to be positive for Goss's wilt, however, *Cmn* isolates were isolated from *Clavibacter michiganensis* subsp. *nebraskensis* infected tissue received from both MO and IN. Known *Cmn* isolates were obtained from Iowa. In addition, a unique HR response was discovered for *Cmn* isolates used in this study. This HR response is not like a typical HR response, but appears to be more like a disease symptom. Symptoms do not spread beyond the point of inoculation, much like a typical HR response, however. It is speculated that Goss's wilt will soon be in MI if it is not in the state already, as it was found as close as an hour away from the MI border in IN in 2015. Scouting for Goss's wilt should continue yearly, especially near the border of IN as a precaution and to prevent unwanted spread of the disease into MI. Resistant corn hybrids should be planted as a precaution and rotated out with a non-host crop, like soybeans, every other year.

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