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Improved Understanding of Factors Influencing the Re-emergence of Goss's Bacterial Wilt and Blight of Corn

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IMPROVED UNDERSTANDING OF FACTORS INFLUENCING THE RE-EMERGENCE OF GOSS'S BACTERIAL WILT AND BLIGHT IN CORN

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IMPROVED UNDERSTANDING OF FACTORS INFLUENCING THE RE-EMERGENCE OF GOSS'S BACTERIAL WILT AND BLIGHT IN CORN

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Goss's bacterial wilt and blight (Goss's wilt) is a serious and sometimes severe disease of corn. Goss's wilt was first identified in Dawson County Nebraska in 1969. Today Goss's wilt can be found in two countries including the U.S. and Canada, and twelve states including Nebraska, Iowa, North Dakota, South Dakota, Minnesota, Illinois, Indiana, Texas, Kansas, Colorado, Wisconsin and Wyoming. Goss's wilt was observed in Nebraska throughout the 1970's, and from the early 1980's until recently developed only sporadically. Around 2006, a re-emergence of the disease was observed in western Nebraska, northeast Colorado, and southeast Wyoming. Since then, reports of the disease have been rapidly increasing north, south, and east, and has been reported across the entire Corn Belt. A survey was implemented to determine what agronomic and environmental factors may be contributing to this development and increased incidence in corn growing regions of the U.S. The survey showed that the Goss's wilt rating assigned by seed companies, planting population density, planting date in 2011, crop rotation, and percent crop residue cover were all important factors to whether or not a field tested positive to Goss's wilt. A second study was implemented to determine if the bacterial pathogen had genetic differences based on geographical origin. The study used amplified fragment length polymorphism (AFLP) and a box polymerase chain reaction

(BOX-PCR) to separate isolates based on differences in DNA banding patterns. The study concluded that Cmn isolates were genetically similar across the entire sampling region, and isolates with similar banding patterns could be found in all states included in the survey. A third study was implemented to test if alternate hosts of the pathogen may be playing a role in the dissemination and survival of the pathogen. Results from the study indicate that three species of foxtail were alternate hosts to Cmn. These included yellow, giant, and bristly foxtail. These results will help producers better manage and potentially prevent Goss's wilt in the future.

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CHAPTER I

Literature Review

Background

Corn (*Zea mays* L.) is an important crop, not only to Nebraska agriculture, but to United States (U.S.) agriculture as a whole. Corn is native to the Americas, and was first cultivated by Native Americans in central Mexico 7,000 years ago (Priddle, 2011). Corn is believed to be derived from a grass known as teosinte. Teosinte looks different from present day corn, one reason being that its ears measure only 5.1 to 10.2 cm whereas present day hybrid corn ears measure 25.4 cm. Corn was first brought to Europe by Christopher Columbus when he discovered it in Cuba in 1492 (Gibson and Benson, 2002). In Europe, corn was first grown in gardens and small plots before it was realized that it was a valuable food crop. From Europe, corn was transported to Northern Africa, Western China, the Philippines, and into the East Indies by 1575 (Gibson and Benson, 2002).

By 1880, there were approximately 25.1 million hectares of corn grown in the U.S. (Gibson and Benson, 2002). This figure reached 40.1 million hectares in 1910 and by 1917 was over 44.5 million hectares (Gibson and Benson, 2002). In 2009, U.S. corn production fell to 35 million hectares with yields of 10,358 kg per ha^{-1} (USDA, NASS Crop Production Summary, 2009).

Currently, corn is grown in all 50 states. The top five corn producing states are Iowa, Illinois, Nebraska, Minnesota, and Indiana, respectively. Combined, these five states produce 325 billion kg of corn, or 62.8% of the total corn produced in the U.S. (USDA, NASS Crop Production Summary, 2009). Also, all five of these states have

confirmed the presence of the bacterial corn disease, Goss's bacterial wilt and blight (Goss's wilt) as of 2009 (Malvick et al., 2010, Ruhl et al., 2009, and Wysong et al., 1973).

Goss's Wilt Occurrence and Spread

Goss's wilt (*Clavibacter michiganensis* subsp. *nebraskensis*) (Vidaver and Mandel 1974) was first reported in three corn fields in Dawson County, Nebraska in 1969 (Wysong et al., 1973). By 1970, the pathogen was observed in nine counties in Nebraska: Antelope, Dawson, Fillmore, Franklin, Furnas, Gosper, Nemaha, Platte, and York. In 1971, Goss's wilt was found in Iowa, and 16 counties in Nebraska. In 1972, the number of counties with confirmed Goss's wilt in Nebraska decreased to 12, but the disease began to spread into Kansas (Wysong et al., 1973). However, by 1974 the pathogen had spread through 28 counties in Nebraska, two counties in Kansas, and one county in each of IA, CO, and SD (Schuster et al., 1975). The spread worsened by 1979 when the disease reached 53 counties in NE, and was found in Iowa, Kansas, South Dakota, Wyoming, and Colorado (Jackson et al., 2007). In the mid-to late 1980's, disease progression slowed and was only observed sporadically in cases where the plants were physically injured (Jackson et al., 2007). In 2006, re-emergence of the disease occurred in western Nebraska, southeastern Wyoming, and eastern Colorado (Jackson et al., 2007). The first reports of the disease in Indiana were confirmed in 2008 (Ruhl et al., 2009). In 2009, disease was prevalent in NE including 24 counties with confirmed samples by the UNL Plant and Pest Diagnostic Clinic, and across the Corn Belt in states including Illinois, Iowa, South Dakota, and Minnesota (Malvick et al., 2010). Also, Goss's wilt was confirmed for the first time in Texas in 2009 (Korus et al, 2010).

Pathogen Biology and Ecology

The Goss's wilt pathogen has had a variety of common names in the past which include: Goss's bacterial wilt and blight, Nebraska wilt and leaf freckles, Nebraska leaf freckles and wilt, bacterial leaf blight and wilt, and bacterial freckles and wilt. The name commonly used (Goss's wilt) was named after the late Robert M. Goss (Schuster, 1970), a professor at the University of Nebraska. The pathogen also had a scientific name change from *Corynebacterium michiganense* subsp. *nebraskense* (Vidaver and Mandel 1974) Carlson and Vidaver 1982, comb. nov. to *Clavibacter michiganensis* subsp. *nebraskensis* corrig. (Vidaver and Mandel 1974) Davis *et al*. 1984, comb. nov..

Claflin (1999) describes the pathogen as a non-motile, catalase-positive, oxidase-negative, gram-positive, wedge or club shaped rod averaging 0.5 to 2.5 µm. The organism is also classified by its orange pigmented colonies both on host tissue in the field and on nutrient broth-yeast extract agar, potato dextrose agar, and a synthetic medium supplemented with yeast extract. Colonies are usually circular, convex, glistening, and butyrous with entire margins when grown on the nutrient media mentioned above. Other distinguishing characteristics from other *Corynebacterium* include the pathogens inability to grow on 0.005% triphenyltetrazolium agar, and its specific bacteriophage sensitivity as determined by spot tests with four purified phage isolates (Vidaver and Mandel, 1974).

Vidaver and Mandel (1974) reported the optimal temperature for growth of the Goss's wilt pathogen to be between 24° C and 28° C in a controlled laboratory setting. Smidt and Vidaver (1986) reported the mean temperature for maximum bacterial growth in plants to be 27° C. The pathogen grows rapidly between 16° C and 32° C. Once mean

temperatures reach 38° C the pathogen will be unable to persist and will be dead within 24 hours. Also, small shifts in day/night air temperatures (3° C) seem to better facilitate rapid growth of the pathogen. The 27° C contrasts an earlier study conducted by Vidaver (1981) that stated the optimum temperature to be 30° C.

Gross and Vidaver (1979) state that a selective media would be useful for the quantitative recovery of the Goss's wilt pathogen from the field. In this study, researchers attempted to make a medium which was selective for *Clavibacter michiganensis* subsp. *nebraskensis* (Cmn). The CNS (*Corynebacterium nebraskense* selective medium) media is quite comparable to YDS (Yeast-extract-dextrose selective medium). However, on CNS, the growth rate is faster and orange pigmentation is more intense. Two other benefits of the CNS media are that it does not appear to influence virulence, and it is able to be stored for up to a month at 4° C without losing effectiveness. In 1986, Smidt and Vidaver discovered a problem with the underestimation of the number of bacteria when using the CNS medium. By removing lithium chloride from the medium, Cmn counts were about 100 fold higher in some instances.

Goss's Wilt Survival, Infection, and Symptoms

The Goss's wilt pathogen overwinters in infected plant residues from the previous season(s), as well as inside and on seed. Schuster (1975) reported that the pathogen can survive for up to 10 months on the soil surface in these residues, which are the primary inoculum source. A study was performed by Schuster (1975) that buried residue at various depths to test survival in the soil. Results from this study showed that on the surface the pathogen was able to survive on leaves, stems, cobs and ears. At a depth of

10.2 cm (4 in.), the pathogen only survived on stems and cobs, and at a 20.3 cm (8 in.) depth, the pathogen only survived on stems. Pure culture of the pathogen was also placed at each depth, but no viable bacteria were recovered after 10 months.

The pathogen is also able to survive in and on seed. Biddle et al. (1990) reported that the percentage of seed-borne infestation ranged from 17.1 to 30.7%, while Schuster (1975) reported 11% infested seed. In systemically infected ears Schuster (1975) reported the pathogen could be found between the scutellum and the endosperm, and near the embryo.

The rate of seed transmission was found to be less than seed-borne infestation rates. Seed transmission of the Goss's wilt pathogen ranged from 0.1-0.4% in a study conducted by Biddle et al. (1990). In a separate study, Schuster (1975) demonstrated that 1.6% of seedlings were infected when planted into sterile soil. Although the seed transmission rates are low, this should not be overlooked as a possible avenue of spread for the pathogen to new areas.

Severe Goss's wilt is observed most commonly in association with physical wounding (Claflin, 1999). Physical wounding (e.g. hail, sandblasting, heavy rain, or high winds) provides avenues for infection (Claflin, 1999, Rocheford et al., 1985). Rain and overhead irrigation are responsible for splash dispersal of the pathogen from infested residue and leaf surfaces. Since the pathogen can live epiphytically on the leaf surface and that on susceptible germ plasms of popcorn are able to support higher numbers of epiphytic Cmn bacteria (Smidt and Vidaver, 1986, Schuster et al., 1973), the source of inoculum may already be present when physical wounding occurs. Once the Goss's wilt

pathogen infects, it may persist on the vascular tissue of leaves, or move systemically through vascular bundles (Schuster, 1975).

Research reported by Schuster et al. (1973) states that Goss's wilt bacteria are able to infect through stomatal openings in corn leaves. More recently, Mallowa et al. (2012) reported that infection can occur without physical injury at a low rate of 20-30%.

Symptoms of Goss's wilt can be similar to those caused by other diseases, such as Stewart's bacterial wilt and leaf blight caused by *Erwinia stewartii* (E. F. Smith) Dye, 1963 (Schuster, 1975). Calub et al. (1974c) reports a difference in disease severity based on the age of the host plant. Plants inoculated at an age of two weeks first showed symptoms when new leaves emerged from the whorl. On these newly emerged leaves, pale streaks were visible originating at the point of injection and along the margins of the leaf. Schuster (1975) reported light greenish to yellow strips with irregular and wavy margins following the leaf veins. Other common symptoms include water soaked lesions parallel to the leaf veins, a bacterial exudate with a shiny appearance, and buggy whipping (Calub et al., 1974, Schuster, 1972). Symptoms reported by Claflin et al. (1999) include small, dark green to black, water soaked irregular areas which develop at the margins of expanding lesions known as freckles. Other characteristics of the leaf blight phase include an orange exudate and a glistening appearance on dried leaves. As leaf lesions coalesce the leaf symptoms resemble that of leaf scorching due to drought stress (Treat and Tracy, 1990). Claflin et al. (1999) stated that early infection can cause wilt, withering and even death in seedlings. In vegetatively mature diseased plants, orange vascular tissue is often seen. Husk tissue and kernels may also assume this orange

hue. Rocheford et al., (1989) also reported a wet stalk rot that can develop as a result of vascular infection.

Inoculation Techniques

Several different inoculation techniques have been assessed over the course of the last 40 years. In 1972, Schuster et al. used the cut spray method to inoculate 10-day-old inbred corn. This method involves removing the tip of the leaf, and immediately spraying the leaf with a bacterial suspension of $2.9x10^9$ cells. Reactions to the pathogen by the host were recorded after one week, and were based on the distance the bacteria moved toward the stalk from the point of inoculation. In 1974c, Calub et al. reported that the cut spray technique gave unsatisfactory and inconsistent results. Calub et al. (1974c) used two separate pin prick methods, a syringe injection method, and added 0.35% Tween 20 (Polyoxyethelene Sorbitan Monolaurate; manufactured by Sigma Chemical Co., St. Louis, Missouri) to inoculum. The pin prick method instrument first used was a device containing needles imbedded into a cork with sharp points protruding six mm. After being pricked, inoculum was applied by spray at the desired concentration. The second pin prick method added a foam sponge to the terminal end of the device which was dipped in inoculum. The syringe injection method was used at approximately the tasseling stage. This method involves injecting a bacterial suspension into the plant at the first two internodes above the ear. In treatments where 0.35% Tween was added to the suspension, the product seemingly increased the rate of infection, but also increased stunting which makes the rating process more difficult. In 1975, Schuster reported several techniques as well. Two techniques which were unsuccessful were inoculation by general atomizing and water-soaking. Contrary to the report by Calub et al. (1974c),

Schuster (1975) found the cut spray technique and syringe injection methods to be the most effective. The concentration that was reported to give the most consistent results at the V3-V5 growth stage was between $1.0x10^7$ and $1.0x10^8$ cells/ml in both susceptible and resistant plants. At a concentration of higher than $1.0x10^8$ cells/ml, two week old plants produced symptoms even in resistant lines (Calub et al., 1974b). Calub et al. (1974b) also reported that the growth stage significantly affected the ratings of resistant plants, but reported that ratings for susceptible plants were consistent across growth stages. Suparyono and Pataky (1989b) also reported a greater effect on yield loss when plants were inoculated at the V3-V5 growth stage vs. the V5-V7 growth stage.

In 1977, Vidaver used a method called the "needle-eye method" to inoculate Golden Cross Bantam sweet corn and dent corn inbred A619, two susceptible lines. The method involves thrusting the needle eye end of a number eight sewing needle into the stem of a V2-V3 leaf stage seedling 3.5 cm above the soil surface. In 1979, Carlson et al. attempted another technique which utilized a pressure injection device. The system delivered inoculum by triggering a spring-loaded piston into a chamber with a small orifice for an exit. The technique worked as well as the needle eye technique, but was about three times faster.

When producing inoculum both the use of a mixture of isolates, and the use of single known virulent strains were both attempted. Research has shown differences in virulence among different isolates collected within the same state (Schuster et al., 1972, Malvick et al., 2012). Schuster states that to ensure presence of variable pathogen population, a mixture of isolates should be used to represent this pathogen variability (Schuster, 1972). A mixture of four to six isolates was also reported in seven papers

(Gardner and Schuster, 1973 Calub et al., 1974a, Calub et al., 1974b, Calub et al., 1974c, Martin et al., 1975, Carson and Wicks, 1991 Ngong-Nassah et al., 1992).

Pathogen Variability

Virtually no variability in bacteriocin production was observed between isolates found in the first three fields in Dawson County, making it feasible to hypothesize the pathogen did originate in Dawson County, NE (Vidaver et al., 1981). Cmn showed little heterogeneity when exposed to nutritional and biochemical tests. Over the next few years, phage groups were created to classify the bacteria. In 1970 two phages were observed, and by 1972 this number increased to six phages. However when considering a bacterial pathogen, six phages is still a relatively homogeneous population. In 1987, Smidt and Vidaver conducted a study collecting isolates from a single popcorn field. Isolates were originally assigned into four groups based on colony morphology. Isolates were found to be diverse based on morphology, and bacteriopahge sensitivity. Isolates either produced CN1, CN1 and CN2, or neither. No isolate tested produced only CN2. Isolates from this study were considerably more diverse than those comprised in the 1981 study, although they all originated from one field. These results indicate increasing heterogeneity among populations of the Goss's wilt pathogen.

Virulence

Loss of virulence during storage is a problem when trying to compare isolates from different time periods. Schuster et al. (1975b) reported a loss of pathogen virulence after several months when sub-culturing Cmn on nutrient dextrose agar + thiamine (NDAT). In this study, the Goss's wilt pathogen was placed in mineral oil and distilled water, and stored at 10° C to test which would maintain pathogen virulence for

an extended period of time. The pathogen was also stored at 25° C in infested leaves and seed, which were collected from a commercial corn field. These techniques were able to maintain pathogen virulence for up to 23 months. Although all were thought to be effective storage means, the distilled water was less satisfactory due to the possibility of mutations occurring. Vidaver (1977) reported that viability and virulence could be maintained at 6° C on a nutrient broth, yeast extract medium, and on a glucose yeast-calcium carbonate medium. The three medias mentioned above were better able to maintain virulence than a semi-synthetic medium with added yeast extract. Both storage in liquid and on solid media, virulence and viability were better maintained at 6° C than at room temperature. Cultures stored at room temperature lost viability and virulence in varying amounts depending on the strain. Although these solid complex medias maintained virulence in the short-term, Vidaver (1977) still reported that lyophilization was the most satisfactory method for long-term storage with no change in virulence observed among 33% of isolates.

Schuster et al., (1975b) also attempted to restore virulence after it was lost through a variety of methods, although none proved successful. Cultures which lost virulence did not differ in colony appearance from those that were still virulent. In an attempt to restore virulence, different sources of nitrogen were added to the growing media including ammonium nitrate, ammonium sulfate, and sodium nitrate. The effect of amino acids and adenosine 3':5'-cyclic monophosphoric acid (AMP) was also studied, but neither of these seemed to influence virulence either. Another attempt was to inoculate with strains that had lost virulence, and see if the succession through a susceptible host could restore virulence. However, this attempt was also unsuccessful.

Inheritance of Resistance

Resistance to plant pathogens can be measured by a host plant's ability to slow growth and reproduction of a pathogen (Pataky et al., 1988). Although many corn inbred and hybrid lines are resistant to the Goss's wilt pathogen, and breeders have been successful in identifying tolerant lines, none are completely immune (Jackson et al., 2007). Inheritance of resistance in Goss's wilt is believed to be controlled by relatively few genes and appears to be quantitative (Carson and Wicks, 1991, Martin et al. 1975). Ngong-Nassah et al. (1992) reports that additive gene action is of primary importance in inheritance of resistance to the Goss's wilt pathogen. Martin et al. (1975) and Suparyono and Pataky (1989c) state that partial dominance for Goss's wilt can be seen by the fact that midparent Goss's wilt rating values are generally lower than F1 readings of crosses between resistant and susceptible hybrids. Ngong-Nassah et al. (1992) also states that resistant inbred lines contribute resistance to the F1's, and susceptible parents contribute to susceptibility, but the behavior of crosses among intermediate parents are less predictable. Treat et al. (1990) concluded from a study on inheritance that recurrent selection would be the most effective method when selecting for resistance to the pathogen. High correlation coefficients were observed when rating Goss's wilt plots in the same season and field, but not over time or locations due to environmental effects (Calub et al., 1974, Martin et al., 1975, Pataky, 1985, and Treat et al., 1990). Testing over multiple years and environments is also important because of significant hybrid and environment interactions (Carson and Wicks, 1991).

Calub et al. (1974) tested the resistance of 113 corn genotypes to Goss's wilt. Results showed that the most susceptible genotype lines included N143, N59, N124, and

A632. A634 was also reported to be a very susceptible line (Ngong-Nassah et al., 1992). Schuster et al. (1972) reported lines N103, B49, R177HTA, Mo1w, Mo6, and H88 to be tolerant of the disease. Schuster et al. (1972) also reported that in a study conducted with Texas Male Sterile germ plasm, Goss's wilt did not seem to be any more or less of a problem.

Yield Loss

First reports of yield loss for the Goss's wilt pathogen in a susceptible hybrid (A619 X A632) averaged 44% when compared to a non-inoculated check of the same variety (Claflin et al., 1978). More recent reports put that number closer to 50% in severely affected fields (Claflin, 1999, Jackson et al., 2007). Jackson et al. (2007) also reported a 63% yield difference between a susceptible and a resistant hybrid in the same field. Vidaver (1977) reported a yield lag of 100 to 150 kg per ha^{-1} when using resistant hybrids vs. susceptible hybrids. Pataky et al. (1988) reported yield losses in sweet corn to be as high as 68% in susceptible varieties. In a similar study conducted by Suparyono and Pataky (1989a), four varieties of sweet corn were planted and inoculated at different leaf stages to determine impacts on yield. The four varieties were Miracle, Gold Cup, Honeycomb, and Jubilee with Goss's wilt ratings of resistant, moderately resistant, moderately susceptible, and susceptible respectively. Results showed that when susceptible plants became infected at a young age (three-to-five leaf stage), yield reductions across three years were 38%, 39% and 99%. The pathogen also reduced the ear length by as much as 50% and ear diameter by 44%.

Management

Martin et al., (1974) reported that the disease is only economically serious when host plant injury occurs concurrently with a warm and moist environment and the presence of the pathogen. The problem with this is that we have no knowledge of what the weather will be like in the coming season. Preventive management strategies are the best way to ensure population reduction of the pathogen. Preventive management strategies (e.g. planting resistant hybrids and tillage) may also be the key to slowing disease progression. Continuous corn cropping and reduced tillage may be two factors currently affecting the spread of Goss's wilt. In some areas continuous corn systems have been in place for more than 50 years. A good management strategy for anyone who has had Goss's wilt in the past would be to plant a resistant hybrid. This will ensure less yield reduction should conditions be favorable for infection. Other management strategies would be to rotate to a non-host crop or to till infested debris into the soil to reduce bacterial populations in the field (Claflin, 1999, Jackson et al., 2007).

Weed pressure may be an important consideration in the management of Goss's wilt. Goss's wilt has been isolated on two common weed species in Midwest fields, green foxtail (*Setaria viridis* L.) and shattercane (*Sorghum bicolor* L.). Schuster et al., (1975) reported that in artificial inoculations, several other grassy weeds and crops exhibited symptoms similar to those seen on corn infected with the pathogen. The species include: teosinte (*Euchlaena mexicana* Schrad.), eastern gama grass (*Tripsacum dactyloids* L.), grain sorghum (*Sorghum bicolor* L.), sudangrass (*Sorghum bicolor* L.), and sugarcane (*Saccharum officinarum*).

Once Goss's wilt infects, there is no known treatment to slow disease progression in the current growing season. Although foliar fungicides have become increasingly common, they have not been effective for management of Goss's wilt.

Summary of Literature Review

Corn is grown by farmers in every state in the U.S. The incidence of Goss's wilt has been increasing, not just in Nebraska, but across the Midwest. The Goss's wilt pathogen survives in infected debris and is able to infect plants after physical injury occurs on the plant. The pathogen is also able to survive epiphytically on leaf surfaces making it easier for Goss's wilt to infect when a severe weather event does occur. There has been research conducted on the biology of the pathogen, and resistance to it in corn. However, there has been little work done on prioritizing which agronomic factors are affecting disease progression. In addition, many questions remain about why the disease has re-emerged after so many years of low incidence, although speculations have led to questions about changes in cultural practices, resistance in current commercially available hybrids, or changes in the pathogen. The research we are proposing will help farmers as well as university and industry representatives better understand the disease which will inevitably lead to better management of the pathogen Goss's bacterial wilt and blight.

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CHAPTER I

FACTORS AFFECTING THE DEVELOPMENT AND SEVERITY OF GOSS'S BACTERIAL WILT AND BLIGHT OF *ZEA MAYS*

Abstract

Goss's bacterial wilt and blight (*Clavibacter michiganensis s*ubsp. *nebraskensis*, Cmn) has been developing in regions of North America where it has not previously been reported. Goss's wilt, a bacterial disease of *Zea mays* has been rapidly spreading across the Midwest since its reemergence in western Nebraska, northeastern Colorado, and southeastern Wyoming during the 2006 growing season. The objective of this study was to identify environmental and agronomic factors contributing to the development and severity of Cmn across the Corn Belt. To understand what factors were contributing to disease severity, a multistate survey was implemented. Of the 2,400 surveys distributed, 486 surveys were returned with leaf samples where 30% tested negative for the Cmn pathogen and 70% tested positive. Two statistical programs were used to analyze the data: Classification and Regression Tree Analysis (CART) and Random Forest. Results from both CART and Random Forest were used to help identify which agronomic and environmental factors contributed the most to the development of Cmn. A chi-square test of independence was also implemented to analyze certain portions of the dataset when responses to particular questions were < 50%. Results showed that the seed company's Goss's wilt resistance rating in the selected hybrids contributed the most to Goss's wilt development. This was followed by a high planting population (> 67,500 plants per ha^{-1}), the second most important factor for disease development. The third most influential factor for disease development was the use of a continuous corn cropping system instead of crop rotation. The fourth most important factor for disease development was a planting date prior to April 23. The fifth contributing factor to disease development was the amount of residue cover. These results were based on the environmental conditions

of the 2011 growing season. Other results contributing to Goss's wilt development included glyphosate application, foliar fungicide application and insect feeding. Results show that a number of factors could contribute to the reemergence and progression of the disease to a varying extent.

Introduction

Corn (*Zea mays* L.) is the most widely planted crop in the U.S., with 36.9 million hectares planted in 2011 alone (USDA, NASS News Release, 2011). In 2010, 53 billion kg of corn were exported, making it vital to the country's economy (USDA, NASS Agricultural Statistics, 2010). As of 2010, the presence of the disease Goss's bacterial wilt and blight (*Clavibacter michiganensis* subsp. *nebraskensis,* Cmn) impacted production in the top five corn producing states of Illinois, Indiana, Iowa, Minnesota and Nebraska. Susceptible hybrids infected with Goss's wilt have shown yield losses of up to 50% (Claflin, 1999).

Goss's wilt, a gram positive bacterium that survives and overwinters in plant residues, was first reported in three corn fields in Dawson County, Nebraska in 1969 (Vidaver and Mandel, 1974; Schuster, 1975; and Wysong et al., 1973). By 1970, Goss's wilt was also confirmed in nine counties in Nebraska: Antelope, Dawson, Fillmore, Franklin, Furnas, Gosper, Nemaha, Platte, and York. In 1971, Goss's wilt was found in Iowa and 16 counties in Nebraska. In 1972, the pathogen was confirmed in Kansas (Wysong et al., 1973). By 1974, the disease was confirmed throughout 28 counties in Nebraska, two counties in Kansas and one county in each of Iowa, Colorado and South Dakota (Schuster, 1975). The disease was further dispersed by 1979 when Goss's wilt was reported in 53 counties in Nebraska and was found in Iowa, Kansas, South Dakota,

Wyoming and Colorado (Jackson et al., 2007a). From the mid-to late 1980's through the early 2000s, disease slowed and was only observed sporadically in cases where the plants were physically injured (Jackson et al., 2007) or in very susceptible genetics, such as some popcorn fields.

Around 2006, Goss's wilt re-emerged in the regions of western Nebraska, southeastern Wyoming and northeastern Colorado (Jackson et al., 2007a). By September of 2008, the disease had been confirmed in 15 counties in Nebraska. First reports of the disease were also confirmed in Indiana in 2008 (Ruhl et al., 2009) and Texas in 2009 (Korus et al., 2010). In 2009, disease was still prevalent in Nebraska where samples submitted from 24 counties to the UNL Plant and Pest Diagnostic Clinic tested positive as well as in other states including Illinois, Iowa, South Dakota, and Minnesota (Malvick et al., 2010).

Goss's bacterial wilt and blight has two phases, the systemic wilt phase and the leaf blight phase (Claflin, 1999). The systemic wilt phase usually develops in association with an early season wounding event. This phase can kill small plants, leading to stand reduction. The wilt is caused by a buildup of bacteria in the vascular bundles, which prevents the plant from transporting water, thus causing the plant to wilt and die. The wilt phase can also occur when plants are larger, but disease is usually not as severe (Suparyono and Pataky 1989).

The second phase is the more common and less severe leaf blight phase. Foliar symptoms reported by Claflin (1999) include small, dark green to black, water soaked irregular areas of the leaf, which develop at the margins of expanding lesions known as

freckles. Other signs of the leaf blight phase include an orange bacterial exudate and a glistening appearance on the surface of leaves.

The use of Classification and Regression Tree Analysis (CART; Breiman and Friedman, 1984) has been utilized in both weed science and plant pathology as both a prediction and classification model. CART was used in both disciplines to effectively break responses into homogeneous groups (Davis et al., 2009, Gleason et al., 1994, Kim et al., 2002, Paul and Munkvold., 2004, and Williams et al., 2009). A major benefit to CART is that it takes a complex data set and breaks it into output that is easy to understand (Williams et al., 2009). A benefit to the CART analysis is that it is able to handle missing data and assign a number or categorical value, known as a surrogate variable, with relatively good accuracy based on sample similarities or differences to other surveys in the data set. CART is also able to handle data that are highly skewed, multi-modal, categorical, or either ordinal or non-ordinal in structure (Lewis, 2000).

CART analysis will take a variable, split it into two "nodes" and each "child node" can then be split into two more nodes. When a child node is split it becomes a parent node, and the two nodes that result from that are then child nodes. This type of analysis is a form of binary recursive partitioning. The most important node or the original break is known as the root node. The root node will be the first split and this variable will be split using all available data. Any node that does not break and form child nodes is referred to as a terminal node. Knowing these terms will be helpful in the interpretation of what the model has produced (Lewis, 2000). By setting the minimum split to 40 on the parent node, so no more splits were made with any less than 40 variables, the amount of output considered "noise" or variables of less importance was

reduced. This made the overall output not only easier to read, but more useful, as variables that are less important were not included.

The primary downside to the CART program is that it is relatively unstable (Hastie et al., 2001). Random Forest can be used to grow many trees making a large forest of trees, and averaging these trees will provide differences in variable importance. The main difference is that Random Forest (Breiman, 2001) will make N different trees based on the number it is programmed to and average them to give output based on a Mean Decrease Gini plot. The final output is based on the average of N number of trees, unlike in CART, where the final output is based on one tree. Another difference between CART and Random Forest is that in CART, each node is split using the best or most relevant split taken from a pool of all variables in the dataset. However, in Random Forest, the split is based on a subset that is randomly chosen at that node. Random Forest also has the ability to handle missing data. The Random Forest program is able to accurately assign a data point even when up to 80% of data is missing. Another advantage to Random Forest is its ability to avoid over fitting (Breiman, 2002). In the tree building process of CART (and Random Forest), a variable is selected to split a node based on the reduction of some impurity measure (e.g. Gini index) after the split. Naturally, the reduction in impurity is higher for splits near the root of the tree (Breiman, 2002; Breiman and Cutler 2002). Random Forest further provides a measure of importance of each variable based on the total amount of the reduction in the impurity measure when that variable is used to do splitting (Hastie et al., 2001).

Little is known about why the disease has reappeared across the Corn Belt during recent years and how current cropping practices affect disease development. The

objectives of this research were to identify both environmental and agronomic factors that have contributed to the severity of the disease across the Corn Belt and prioritize them to focus future research efforts and to improve management recommendations.

Materials and Methods

Generation of survey questions: A survey instrument was developed to gather information on agronomic and environmental factors as well as the development and severity of Goss's wilt. Responses to agronomic and environmental factors, which might affect the spread and development of Goss's wilt, including crop rotation, tillage regime, field history, irrigation, and nutrient and pesticide management. Questions were based around previously reported information about the pathogen, as well as other agronomic practices which might impact the spread and development of Goss's wilt. The survey instrument included 16 multiple choice questions that were used to aid in the understanding of the production systems being used in regions of the Corn Belt where Goss's wilt was suspected during the 2011 growing season. Information was also gathered using 24 open-ended questions about the crop production system in place, and environmental factors occurring during the 2011 growing season. A copy of the survey instrument is provided (Appendix I).

Survey distribution: The survey instrument and cover letter explaining the purpose and goals of the project, along with a gallon-size plastic bag with directions for sample submission, were distributed in a 26.25 cm x 35 cm padded, self-addressed postage-paid envelope to individuals from private industry, county extension educators and crop consultants. Contacts were asked to distribute the survey materials and trained to provide assistance to farmers and land managers to accurately report information.

Completed surveys and samples were returned in a self-addressed postage paid envelope to the Plant and Pest Diagnostic Clinic at the University of Nebraska-Lincoln, Lincoln, NE.

For the study, 2,400 survey packets were distributed. A maximum of five surveys and samples were allowed per producer. Surveys were accepted from dent corn, sweet corn and popcorn producers. Surveys were distributed from late June through much of the 2011 summer. As an incentive for submitting a survey and sample, Cmn testing was conducted at no charge on samples submitted with a completed survey a free confirmation of Cmn was provided.

Testing for C. michiganensis* subsp. *nebraskensis: Leaf samples were refrigerated upon receipt and stored until processing. When testing for Cmn, a clean pair of gloves and a sterile razor blade were used on every sample to reduce the chance of contamination. An ImmunoStrip® (ELISA) test kit (Agdia Inc. Elkhart, IN) (Korus et al., 2010) was used to test for Cmn. Manufacturer's recommended procedures for testing were used. A 2.5 cm x 1.25 cm segment of the expanding lesion was cut and macerated to extract the pathogen. A 0.5 ml aliquot was poured into a sterile 2 ml micro centrifuge tube. The ELISA strip was submersed into the liquid for 30 minutes. ELISA strip results were considered positive if two pink lines appeared and negative if one pink line appeared. Samples that did not show typical lesion development, but still tested positive were checked for bacterial streaming as a precaution against false positive test results. Samples that showed lesion development and typical symptoms, but tested negative were also checked for bacterial streaming.

Statistics: A contingency table was used to present two or more variables simultaneously by listing one variable as a row unit and the other variable as a column unit (Dowdy et al. 2004). The chi-square test of independence was used to determine whether two variables were independent. An alpha level of 0.05 was used to determine whether a test indicated significant dependence.

In the data set collected, 33% of the 40 variables were environmental and 67% were agronomic. CART was used to split nodes sequentially based on these variables. Variables that were used to split node near the root are likely to be the most important. Both environmental and agronomic variables were analyzed together to determine which variables were influencing Goss's wilt development.

When applying CART, a cost parameter value of 0.01 was used to stabilize the model. The minimum node split was set at 40. We also constructed the tree with the top 14 variables deemed to be the most important by Random Forest (we chose 14 due to a natural break between the 14 and 15 variables from the Random Forest variable importance analysis output). For this tree, a cost parameter estimate of 0.012 and a minimum node split of 50 were used.

For open ended questions, responses were grouped for analysis. For a sample size of 500, approximately 10 groups per variable were used. Variables that were amended in this manner included GPS coordinates, planting population and planting date. Either GPS coordinates or Township/Range/Section numbers were reported in 58% of the 486 responses. The GPS coordinates of the remaining 42% of responses were estimated based on a city in the center of the county in which the field was located. Township/Range/Section numbers and city estimates were made from the following site:

http://citylatitudelongitude.com. Latitude and Longitude coordinates were split into five groups. Longitude ranged from 86.15 to 108.47 degrees with groups from 86.00 to 90.49 degrees, 90.50 to 94.99 degrees, 95.00 to 99.49 degrees, 99.50 to 103.99 degrees, and 104.00 to 108.49 degrees. Latitudes ranged from 37.38 to 46.98 degrees with groups from 37.00 to 38.99 degrees, 39.00 to 40.99 degrees, 41.00 to 42.99 degrees, 43.00 to 44.99 degrees, and 45.00 to 46.99 degrees.

Planting populations ranged from 50,000 plants per ha^{-1} to 100,000 plants per ha^{-1} and were grouped into ten categories: 50,000 to 54,999, 55,000 to 59,999, 60,000 to 64,999, 65,000 to 69,999, 70,000 to 74,999, 75,000 to 79,999, 80,000 to 84,999, 85,000 to 89,999, 90,000 to 94,999, 95,000 to 100,000 plants per ha^{-1}. Planting dates ranged from April 11th to June 4th, comprising 55 days. Five groups of 11 days each were broken down from this: April 11th to April 21st, April 22nd to May 1st, May 2nd to May 12th, May 13th to May 23rd, and May 24th to June 4th.

Hybrid Cmn ratings were also amended. There were hybrids from 20 different seed companies represented in survey responses, and 16 had a rating scale of either one to nine, where nine is susceptible, or one to nine where one is susceptible. Since nine of the 16 companies represented in the survey responses used the one to nine scale, with one being susceptible, this scale was used, and the other seven company's ratings were inverted. Two of the four remaining companies did not have a rating system for Cmn resistance. The other two companies had ratings on a scale of one to five, and one to ten. The companies on a five number rating scale were doubled, so the numbers were 2,4,6,8,10 instead of 1,2,3,4,5. Then these numbers were transferred onto a scale of nine

by rounding, where 0.9=1, 1.8=2, 2.7=3, 3.6=4, 4.5=5, 5.4=5, 6.3=6, 7.2=7, 8.1=8 and 9.0=9.

Chi Square Analysis: A chi-square test of independence was conducted on 26 variables versus Cmn successfully. Questions where less than 50% (243) of the 486 returned surveys were completed were not used in the CART and Random Forest analyses. A chi-square test of independence was implemented on the responses for these factors. Four questions from the survey about grass weed pressure during the current growing season, grass weed pressure from the previous growing season, insect pressure for the current season, and where these insects were feeding during the current season, did not contain a sufficient number of responses to be included into the Random Forest and CART analysis.

Variables for herbicide active ingredients, and other diseases present were used in the Random Forest analysis, and a chi-square test of independence was conducted to determine if there was a correlation between a factor and a sample testing positive for Cmn.

If a chi-square test did not have at least 10 responses in each of the four possible outcomes the results were not included. These variables were reported in the results section below.

Results

Survey respondents submitted leaf samples from 509 locations with 486 accompanied by at least partially completed surveys that were used for the CART and Random Forest analyses. Surveys were returned from late June through late October 2011. Samples were requested from fields with and without the disease. Returned

surveys included samples where 30% tested negative and 70% tested positive for Cmn. Dent corn comprised 97% of samples returned and the remaining 3% were received from popcorn fields. Completed surveys represented eight states, including Nebraska, Iowa, South Dakota, Indiana, Colorado, Kansas, Minnesota, and Missouri. Positive samples were received from every state except Missouri.

Chi-square results: Questions containing 243 or more responses (50%), were used in the final analysis. Due to certain questions having < 243 responses, not all factors were included in the CART and Random Forest analysis. Alternative crops planted, inches of irrigation water applied, past disease severity, estimates of insect damage, estimates of weed density from both the current and past growing seasons, the rate of insecticide, rate of herbicides, and rate and timing of foliar fungicide application and the amount of any nutrients applied were unable to be included incorporated into the data analysis due to inadequate responses. There were three questions on the history of disease severity in the field, severity of Goss's wilt on leaf area caused by lesions in the 2011 season, and the estimated yield loss caused by lesion development that were removed from the data set due to the subjective nature of the responses.

Five of the variables tested from the survey results were correlated with the presence or absence of disease in the field.

A chi-square test was used to determine if other reported diseases in the field were correlated with the development of Goss's wilt. The four most commonly reported diseases in the survey were gray leaf spot (*Cercospora zeae-maydis*), common rust (*Puccinia sorghi*), eyespot (*Aureobasidium zeae*) and northern corn leaf blight (*Exserohilum turcicum*). No correlations were found between the occurrence of the four

most commonly reported diseases and Goss's wilt (Table 1). Likewise, there was no correlation between the lack of other diseases and the presence of Goss's wilt. However, in Figure 1, common rust is shown to influence a sample testing positive for Cmn when the common rust pathogen is present.

The four plant parts reported as feeding sites by insects were roots, ears, stalks, and leaves. These four parts were analyzed for a correlation with Goss's wilt presence. Insects feeding on the plant roots, and leaves were correlated with the presence of Goss's wilt (Table 2). From these responses, there was insufficient data to determine if stalk feeding insects were causing an increase in Goss's wilt development.

The five most commonly reported insects in the surveys were corn rootworm (*Diabrotica* spp.), corn borer (*Ostrinia nubilalis* Hubner), aphids (Order Hemiptera; Family Aphididae), corn earworm (*Helicoverpa zea* (Boddie), and grasshoppers (Order Orthoptera; Family Acrididae), yet corn earworm and grasshoppers did not contain sufficient data to successfully run a chi-square test. The only correlation found was between corn rootworm feeding both as juveniles and adults on the roots and silks or leaves, respectively (Table 3).

For the past growing season, the five most commonly reported weed species were foxtail spp. (*Setaria* spp.), giant foxtail (*Setaria faberi* Herrm.), green foxtail (*Setaria viridis* (L.) Beauv.), woolly cupgrass (*Eriochloa villosa* (Thunb.) Kunth), and crabgrass (*Digitaria* spp.). The following weed species were reported as alternate hosts to Goss's wilt and were present in the survey results: green foxtail (*Setaria viridis* (L.) Beauv.), volunteer corn (*Zea mays* L.), barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) and shattercane (*Sorghum bicolor* (L.) Moench ssp. *arundinaceum* (Desv.) (Schuster et al.,

1975, Wysong, 1981). The alternate hosts that were reported did contain sufficient data to conduct a chi-square test. During the current growing season, the five most commonly reported weed species were foxtail spp., including giant foxtail, green foxtail, woolly cupgrass and sandbur (*Cenchrus longispinus* (Hack.) Fern.), The known alternate host group did include sufficient data to conduct a chi-square test. No correlations were observed between the presence of known alternate host weeds in a field during the previous season or the current growing season on Goss's wilt development and Cmn survival (Table 4).

A correlation between the presence of Goss's wilt and the use of a foliar fungicide was observed (Table 5). A chi-square test was conducted on the use of foliar fungicides, a test of the triazole versus the strobilurin fungicide class and a test of the three different strobilurin active ingredients, including azoxystrobin, pyraclostrobin, and trifloxistrobin,. The only chi-square test containing sufficient data was on the use of a foliar fungicide. No correlation was observed with the use of an insecticide.

The four most commonly applied herbicide active ingredients applied during the current growing season were glyphosate, atrazine, metolachlor and acetochlor. The only chemical active ingredient with a correlation to the presence of Goss's wilt was glyphosate (Table 6).

A chi-square test was conducted on different application methods and/or timings of nitrogen, phosphorus and potassium and the presence of Goss's wilt. The four different methods and timings for each of the three nutrients were pre-plant application, at plant application, side-dress application, and fertigation application. Due to insufficient data, a chi-square test on side-dress phosphorus and potassium and the

fertigation of all three nutrients were unable to be conducted. No correlations were observed between the application of, and/or timings of nutrient application and the presence of Cmn (Table 7). Nitrogen application was however found to be one of the top 14 most important variables for the development of Cmn resulting from the Random Forest analysis (Figure 3). Nitrogen application at planting was also illustrated on Figure 1.

CART analysis: Results from the CART analysis show that at a planting population of < 67,500 seeds per ha^{-1}, 73% of samples tested negative for the presence of Cmn, while 27% of samples tested positive (Figure 1). When planting populations were > 67,500 seeds per ha^{-1}, 25% of samples tested negative for Cmn, while 75% of samples tested positive. Using the CART analysis, planting population was the most important variable for the presence of Cmn. The second most important variable was the Goss's wilt disease rating for the hybrid assigned by the seed company. At a rating of > 5.2, where the higher the rating the more resistant the hybrid, 30% of samples were negative, and 70% of samples were positive for Cmn. When a hybrid had a rating of < 5.2, 12% of samples tested negative to Cmn, while 88% tested positive. The third most important variable, according to the CART analysis, was longitude. At a longitude of < 95.73, 35% of samples tested negative, and 65% tested positive for Cmn. At a longitude of > 95.73, 6% of samples tested negative for Cmn, and 94% tested positive. If a producer planted on or before April 23, 44% of the fields located at a longitude of < 95.73 tested negative for Goss's wilt. If a producer planted after April 23, 24% of the fields located at a longitude of < 95.73 reported tested negative for Goss's wilt. When all 40 variables are observed, the nine most influential agronomic and environmental variables were planting

population, Goss's wilt disease rating, longitude, planting date, crop rotation, yield history, common rust, growth stage, and nitrogen application method (Figure 1).

Random Forest: The final analysis used was Random Forest. Random Forest uses the decreases in Gini index attributed to each variable to rank their importance (Figure 3). A difference is observed in that Random Forest found the assigned Goss's wilt rating for hybrids to be the most important variable for a sample to test positive for Cmn, while CART found a planting population of > 67,500 plants per ha^{-1} to be the most influential variable. Between the two outputs, several variables were at a different level of importance, but overall, the same variables had the greatest effect on a samples testing positive for Cmn.

Based on the Random Forest output, a natural break was observed between the 14^{th} and 15^{th} variable. Based on this break, the top 14 variables were tested to determine which variables were the most influential on the presence or absence of Goss's wilt. Again, planting population, followed by assigned Goss's wilt hybrid rating, longitude, and planting date were the four most important variables (Figure 2). Figure 1 reports more variables, including growth stage, application of nitrogen at the time of planting and common rust than Figure 2. The only variable observed in Figure 2 that is not observed in Figure 1 is irrigation type. When inputting the top 14 variables from the Random Forest output in CART, longitude is the one environmental variable observed and the remaining six variables observed are agronomic and include planting population, Goss's wilt hybrid rating, planting date, irrigation type, rotation, and nitrogen application method.

Discussion

Results from the survey were collected subjectively. Samples received were sent in based on the belief that the sample would test positive for Cmn. The project did yield 70% of samples that were positive and 30% of samples that tested negative. Based on these numbers we felt confident that we would be able to see differences in production practices for the producers who did, or did not have the Goss's wilt pathogen in their field.

Results of the Random Forest analysis indicated that the Goss's wilt hybrid rating assigned by each company was the most important variable for a sample to test positive for Cmn. Jackson et.al (2007) reported that prior to 2006, only 25% of seed companies in Nebraska were publicizing their hybrids' ratings for Goss's wilt. From results of the survey, 90% of seed companies had published Goss's wilt hybrid ratings. By publishing these ratings and producers taking them into consideration, more fields might be planted with resistant genetics reducing disease severity and progression. The assigned Goss's wilt hybrid rating may be observed three and two times, respectively in Figure 1 and Figure 2. In all instances there was a higher probability of Cmn when planting a more susceptible hybrid. Based on the survey selective breeding is the most influential way to protect a field from Goss's wilt.

For the development of disease, the next most important factor was planting population. As shown in Figure 1 and Figure 2, high planting populations were observed in association with Cmn. This observation is taken from the root node in the CART analysis where planting populations above 67,500 seeds per ha^{-1} tend to have more fields with confirmed Cmn. At a planting population of > 79,500 seeds per ha^{-1}, the same trend

of more Cmn infected fields is seen within these higher planting populations. This has been shown to be the case in the fungal species, white mold (*Sclerotinia sclerotium*), where closer row spacing, varieties with dense canopies, and higher amounts of irrigation were all contributing factors to higher disease incidence. The most open plant canopy was warmer, drier and had a lower incidence of white mold (Blad et al., 1978). The suspected origin of this bacteria was in a semi-arid environment, so the bacterium should thrive in a warmer, drier canopy, and not in a cooler, wetter canopy, as does the previously described pathogen (Wysong et al., 1973). This is contradictory to Figure 2, which shows when using center pivot or gravity/flood irrigation, 13% of the samples planted after April 23 tested negative for Cmn. When using subsurface irrigation or no irrigation, 37% of the fields planted after April 23 tested negative for Cmn. This would indicate that the pathogen may be adapting to a more humid environment, since the use of above ground irrigation creates a more humid environment.

The survey indicates that the third most important factor for the presence of Goss's wilt is crop rotation. Crop rotation that incorporates non-host plants is a key factor in reducing population densities of plant pathogens, weeds, and insect pests (Liebman and Dyck, 1993; Wright, 1984; and Peters et al., 2003). Crop rotation can improve soil structure and increase yields (Bullock, 1992). Based on the surveys that were submitted, producers planting continuous corn and planting before April 23 had a 70% chance for their sample testing negative for Cmn. This is compared to producers who were using a crop rotation, and whether it is corn-soybean or corn-corn-soybean rotation, they had a 45% chance that their field that was planted before April 23 that their sample would test negative for Cmn.

A later planting date might also make a field more likely to be infected with Cmn. At a longitude of > 95.3, 6% of samples with an assigned Cmn rating of > 5.2 tested negative. At this higher longitude planting dates will be later because of cooler night temperatures causing soil temperatures to stay cooler longer.

The amount of residue cover on a field is a variable of importance from the Random Forest analysis for a sample testing positive to Cmn. Since this pathogen is residue born, the pathogen will occur more commonly in no-till or reduced-till fields, where more residue is left on the soil surface (Schuster, 1975).

An application of glyphosate was correlated to a sample testing positive to Cmn (Table 6). It is unknown if this is caused by the incorporation of reduced tillage when using a glyphosate product as a primary means of weed control, or if there is another possible reason for this correlation. However, glyphosate can reduce a plant's resistance to certain pathogens in a plant, including *Phytophthora megasperma* f. sp. *Glycinea* (Keen et al., 1982). According to Bently (1990), EPSPS inhibition affects the biosynthesis of proteins, auxins, pathogen defense compounds, phytoalexins, folic acid, precursors of lignins, flavonoids, plastoquinone and other alkaloid and phenolic compounds. Several of these compounds are essential for the defense response to plant pathogens and is responsible for increased susceptibility to certain plant pathogens (Pline-Srnic, 2005). No correlations were observed between the other three most commonly used herbicide active ingredients reported in the survey.

A correlation is noted between both root feeding insects and leaf feeding insects and a sample testing positive to Cmn (Table 2). The correlation was observed in fields where root feeding insect pressure were reported in 48% of the samples tested positive

for Cmn, while fields that reported having root feeding insects, 5% tested negative to Cmn. This could indicate that there is the potential for the pathogen to enter through wounds in the root system or natural openings in the plant (Mallowa et al., 2012). No correlation was observed for ear feeding insects and a sample testing positive to Cmn. Samples that reported having leaf feeding insects present had 18% of samples tested positive to Cmn and 82% tested negative, while fields that reported no insect feeding on the leaves had 63% of samples test positive. Since the pathogen is known to occur after a physical wounding event, such as hail, high winds, hard rainfall, or sandblasting (Claflin, 1999) the wound from an insect feeding sight should be enough to allow the pathogen to enter the plant.

The three most commonly reported insects in the survey were corn rootworm, corn borer, and aphids. One of these three commonly reported insects correlated to the presence of Goss's wilt and that insect was corn rootworm. The feeding of this insect could have either been on the roots as juveniles, or on the silks or leaves as adults. This relates to our information because as a plant is wounded, the pathogen will then have an avenue by which to enter the plant.

The application of a foliar fungicide was correlated to a sample testing positive to Cmn. Several of the factors previously listed would indicate that a sample testing positive to Cmn may be related to potential stress issues on the plant. When observing the results of CART, Random Forest and the chi-square tests the following factors may have contribute to this stress. High and low yields, field conditions for lower yielding fields tend to be more stressful (i.e. less productive ground, dry land situations, higher weed, insect, and plant pathogen pressure). When observing the variable of higher yields,

planting populations are higher, so more inter-plant competition arises, causing stress on the individual plants. The application of glyphosate would also put stress on the plant as previously discussed. Insect feeding causes stress on the plant from the wounds caused by the insect. However, the application of a foliar fungicide will not cause plant stress. The use of a foliar fungicide for increased plant health and harvest-ability has increased dramatically with higher corn prices and changes in weather patterns (Wise and Mueller, 2011). In 46% of trials conducted over the last ten years, the application of a foliar fungicide has shown positive yield differences from an untreated control that were statistically significant. The question which arises is: why is there a correlation between the application of a foliar fungicide and the presence of Cmn. Further research will need to be conducted to determine if this is a coincidence or if there is an adjuvant or active ingredient in the foliar fungicide application causing this correlation.

Another trend depicted in Figure 1, also shown to be important on Figure 3, was growth stage. This may be due to the fact that only 10% of the surveys were received before the silking (R1) growth stage, or by the time producers noticed a problem, they may have just assumed that is was the plant senescing. Another reason that more surveys were received at this time may be due to the extra stress put on the plant around silking and pollination causing the plants to be more susceptible to the pathogen at these stages. A final factor may have been that producers were out checking fields more around this time.

In conclusion, several factors have been identified including the use of susceptible hybrids, high planting populations, early planting dates, amount of residue cover, and the use of continuous corn that may help explain why this pathogen has been so successful in

disseminating over the past six years. Future research will need to be done in the areas of plant breeding, studies on planting population density, the use of crop rotation, planting date and residue cover may all be useful in identifying the best ways to combat this pathogen. Based on the survey planting resistant hybrids, planting later, using a crop rotation, reducing the amount of inoculum by incorporating residue, and lowering plant population may all be ways to combat the spread, development, and severity of Goss's wilt.

The writers would like to acknowledge all of the public and private industry collaborators who helped make this survey a success. We would like to acknowledge the North Central Integrated Pest Management Center for funding to help make this project possible. We would like to thank the UNL Plant and Pest Diagnostic Clinic for the use of their space. I would especially like to thank Amy Timmerman, Debbie Pederson, and all others whose technical assistance helped make this project a success.

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Table 1. Chi-square correlations of other diseases of corn present in the field where Goss's wilt is suspected and *Clavibacter michiganensis* subsp. *nebraskensis* test results.

Other Diseases Present in Field				
		C. michiganensis subsp. *nebraskensis* test		
Disease		Positive	Negative	P-value
		——— % ———		
Gray Leaf Spot	yes	61	19	0.3392
	no	14	6	
Northern Corn Leaf Blight	yes	17	6	0.9315
	no	57	20	
Common Rust	yes	10	3	0.8762
	no	65	22	
Eye Spot	yes	14	4	0.5702
	no	61	21	
No other disease	yes	9	4	0.4848
	no	66	21	
n		327		

Table 2. Chi-square correlations of insect feeding habits and *Clavibacter michiganensis* subsp. *nebraskensis* test results.

Insect Feeding Habits				
		C. michiganensis subsp. *nebraskensis* test		
Insects Feeding		Positive	Negative	P-value
		——— % ———		
Root Feeding Insects	yes	48	5	0.0005
	no	33	14	
Ear Feeding Insects	yes	43	9	0.6494
	no	38	10	
Leaf Feeding Insects	yes	18	9	0.0005
	no	63	10	
n		200		

Table 3. Chi-square correlations of corn feeding insects and *Clavibacter michiganensis* subsp. *nebraskensis* test results.

Corn Feeding Insects				
		C. michiganensis subsp. *nebraskensis* test		
Insect		Positive	Negative	P-value
		%		
Corn Rootworm	yes	59	10	0.0258
	no	22	9	
Corn Borer	yes	15	4	0.9673
	no	66	15	
Aphid	yes	11	4	0.4226
	no	70	15	
n		200		

Table 4. Chi-square correlations of reported *Clavibacter michiganensis subsp. nebraskensis* alternate host species and *Clavibacter michiganensis* subsp. *nebraskensis* test results.

Reported Alternate Cmn Host Species				
		C. michiganensis subsp. *nebraskensis* test		
Hosts Present		Positive	Negative	P-value
		%		
Current Season (2011)	yes	21	6	0.8126
	no	54	19	
n		221		
Past Season (2010)	yes	22	6	0.8999
	no	54	18	
n		170		

Table 5. Chi-square correlations the application of a foliar fungicide and/or a soil or foliar insecticide and *Clavibacter michiganensis* subsp. *nebraskensis* test results.

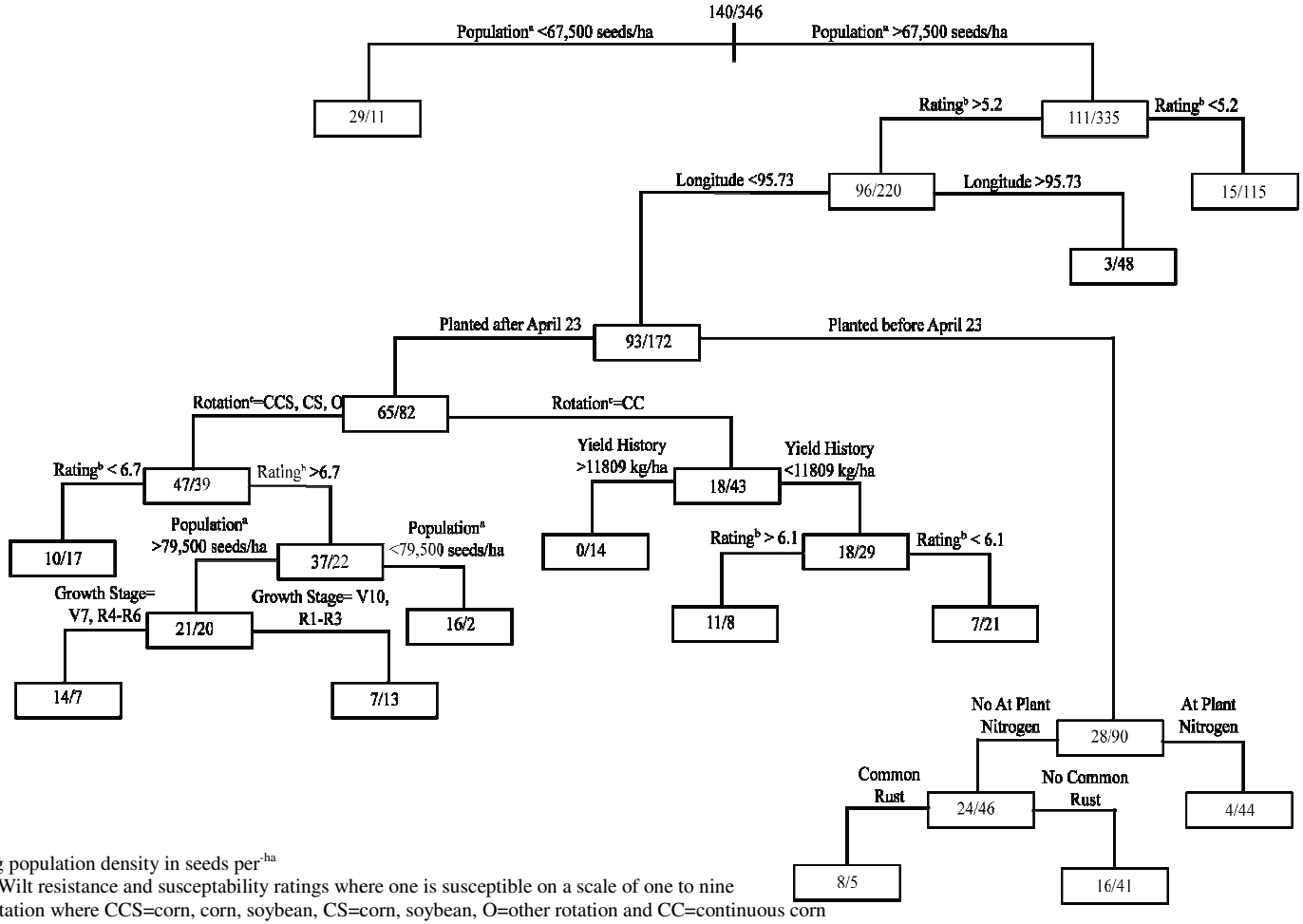
Insecticide or Fungicide Application				
		C. michiganensis subsp. *nebraskensis* test		
Application		Positive	Negative	P-value
		%		
Foliar Fungicide	yes	26	6	0.0010
	no	45	23	
Insecticide	yes	15	6	0.6433
	no	56	23	
n		486		

Table 6. Chi-square correlations of the application of a herbicide active ingredient and *Clavibacter michiganensis* subsp. *nebraskensis* test results.

Herbicide Active Ingredients		*C. michiganensis* subsp. *nebraskensis* test		
Active Ingredient		Positive	Negative	P-value
		%		
Glyphosate	yes	54	16	0.0101
	no	19	11	
Atrazine	yes	41	14	0.8037
	no	33	12	
Metolachlor	yes	26	9	0.9334
	no	48	17	
Acetochlor	yes	26	9	0.9834
	no	48	17	
n		397		

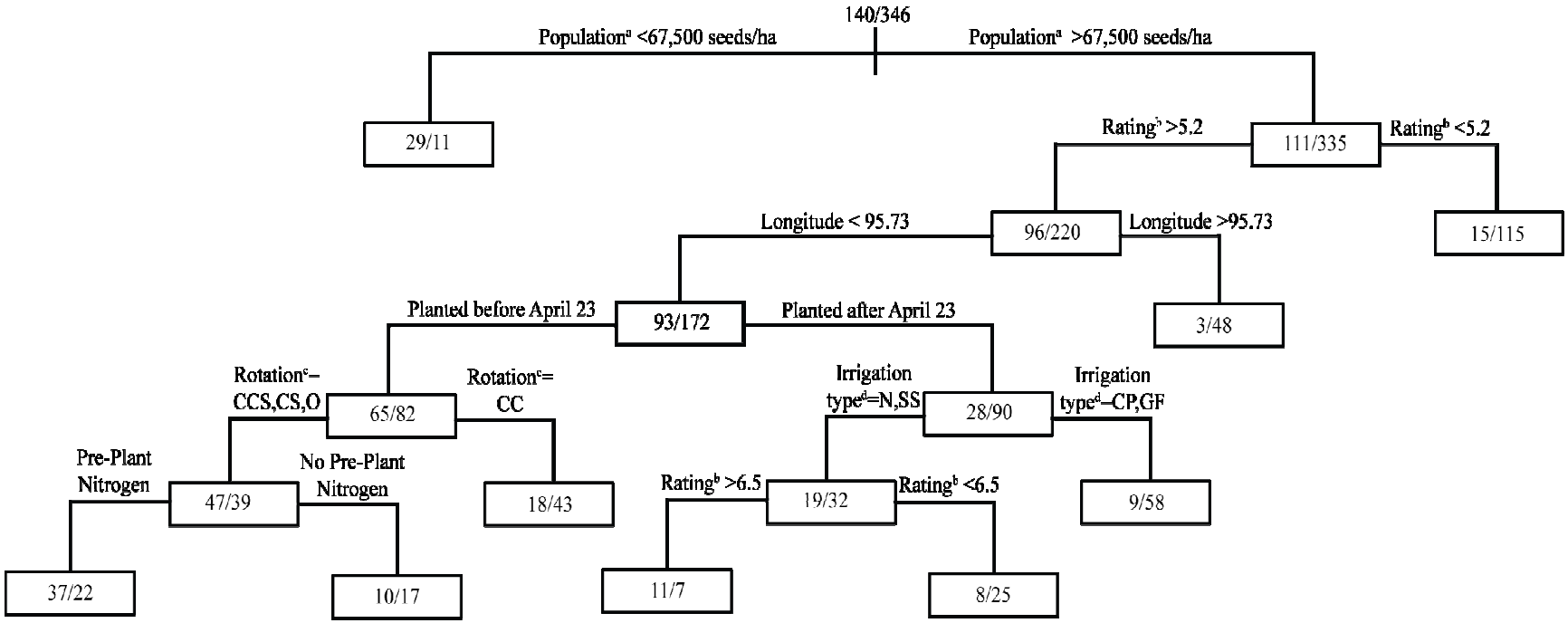
Table 7. Chi-square correlations of nitrogen, phosphorus, and potassium applications timing and *Clavibacter michiganensis* subsp. *nebraskensis* test results.

Nutrient Application Methods				
		C. michiganensis subsp. *nebraskensis* test		
Method		Positive	Negative	P-value
		%		
Pre-plant nitrogen	yes	55	21	0.0862
	no	19	5	
At plant nitrogen	yes	29	8	0.1549
	no	45	18	
Side dress nitrogen	yes	19	7	0.9162
	no	55	19	
n		390		
Pre-plant phosphorus	yes	50	19	0.2627
	no	25	6	
At plant phosphorus	yes	31	10	0.6216
	no	43	16	
n		292		
Pre-plant potassium	yes	59	22	0.8964
	no	14	5	
At plant potassium	yes	19	8	0.6803
	no	54	19	
n		212		



[a]Planting population density in seeds per[-ha]
[b]Goss's Wilt resistance and susceptability ratings where one is susceptible on a scale of one to nine
[c]Crop rotation where CCS=corn, corn, soybean, CS=corn, soybean, O=other rotation and CC=continuous corn

Figure 1. CART analysis of all 40 variables



[a]Planting population density in seeds per[-ha]
[b]Goss's Wilt resistance and susceptability ratings where one is susceptible on a scale of one to nine
[c]Crop rotation where CCS=corn, corn, soybean, CS=corn, soybean, O=other rotation and CC=continuous corn
[d]Type of irrigation used where N=none, SS=sub-surface irrigation, CP=center pivot and GF=gravity/flood

Figure 2. CART analysis of the top 14 factors as produced by the Random Forest output.

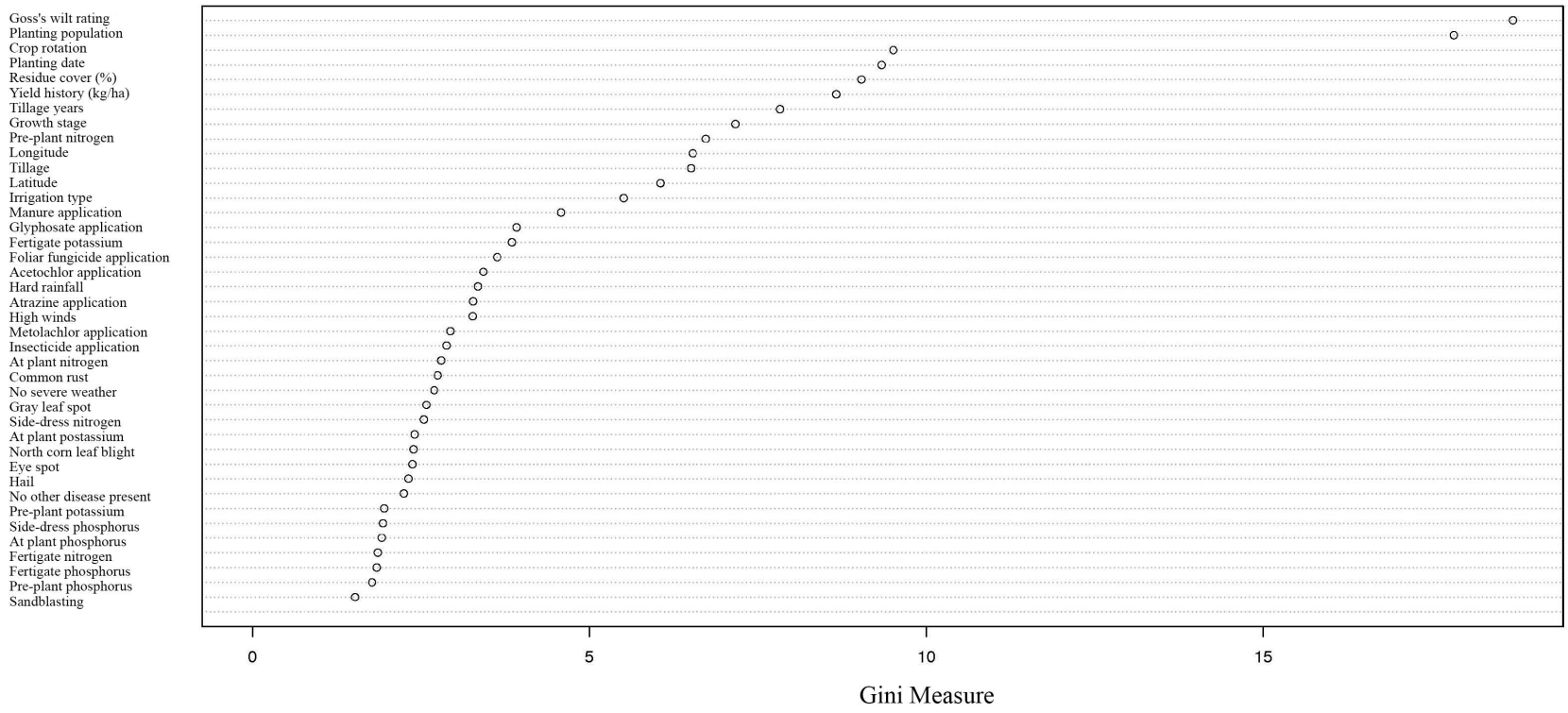


Figure 3. Random Forest analysis of all 40 variables listed in order of importance.

CHAPTER II

A GEOGRAPHICAL BASED ASSESMENT OF GENETIC DIVERSITY WITHIN CONTEMPORARY *CLAVIBACTER MICHIGANENSIS* SUBSP. *NEBRASKENSIS* USING BOX-PCR AND AFLP FINGERPRINTING TECHNIQUES

Abstract

Clavibacter michiganensis subsp. *nebraskensis* (Cmn) is a gram-positive bacteria and is the causal agent of Goss's bacterial wilt and blight. The disease was first discovered in 1969 in Dawson County Nebraska. The pathogen has since disseminated to 12 states in the U.S. The objective of this research was to determine if a change in the genetics of the Cmn pathogen is responsible for its increased incidence in many corn growing regions of the U.S. To better understand the genetics of contemporary Cmn isolates, amplified fragment length polymorphism (AFLP) and rep-polymerase chain reaction (rep-PCR) DNA fingerprinting techniques were implemented. A large sample size (n=466) was used to determine if isolates from eight different states had any geographical based differences in DNA composition. Cluster analysis was performed using the unweighted pair group method using arithmetic averages algorithm (UPMGA), and results presented in a dendrogram and a map of the distribution of contemporary isolates. Results showed that three main groups of isolates exist. This consists of a large group A, small group B, and a heterogeneous group C consisting of several subgroups for both DNA fingerprinting techniques. The mean similarity cutoff used for rep-PCR was 92.5%, and 87% for AFLP. Rep-PCR resulted in large group A consisting of 82% of isolates tested, group B contained 16% and seven sub-groups in group C contained the following 2%. AFLP resulted in 80% of isolates in the large group A, 10% of isolates in group B, and 22 sub-groups in group C containing the last 10% of isolates. Variability in DNA composition was 11.5% using rep-PCR, and 21% using the AFLP techniques. Isolates were then based on the county in which they originated. No geographical basis for groupings was noted using the DNA fingerprinting techniques used.

Introduction

Goss's bacterial wilt and blight is a bacterial disease of corn (*Zea mays* L.) caused by *Clavibacter michiganensis* subsp. *nebraskensis* (Cmn) (Vidaver and Mandel, 1974). Cmn was first observed in Dawson County Nebraska in 1969 in three fields (Vidaver and Mandel, 1974). Cmn spread to nine counties in Nebraska by 1970. By 1971 the pathogen was found in 16 counties in Nebraska, and one county in Iowa. The pathogen was well dispersed being found in 53 counties in Nebraska, and also found in Colorado, Iowa, Kansas, South Dakota, and Wyoming. Disease incidence was reduced after 1980 about and was only observed sporadically until its re-emergence in 2006 in western Nebraska, northeast Colorado, and southeast Wyoming (Jackson et al., 2007). Cmn has also been confirmed in Indiana, Minnesota and Texas (Korus et al., 2011, Malvick et al., 2010, and Ruhl et al., 2009).

Cmn has been described as non-motile, catalase positive, oxidase negative, gram-positive, wedge or club shaped rod averaging 0.5 to 2.5 µm (Claflin, 1999). Cmn is also classified by its orange pigmented colonies both on host tissue and broth-yeast extract agar. Colonies are usually circular, convex, glistening, and butyrous with entire margins when grown on the media mentioned above (Vidaver and Mandel, 1974). The optimal temperature for the pathogen is between 24 C and 28 C in a laboratory setting, and 27 C in a field setting (Smidt and Vidaver, 1986b).

Severe Goss's wilt is observed most commonly in association with physical wounding (Claflin, 1999). Physical wounding (e.g. hail, sandblasting, heavy rain, or high winds) provides avenues for infection (Claflin, 1999, Rocheford et al., 1985). Rain and

overhead irrigation are responsible for splash dispersal of the pathogen from infected residue and infested leaf surfaces. Since the pathogen can live epiphytically on the leaf surface and that on susceptible germ plasms of popcorn, higher numbers of epiphytic Cmn bacteria were documented (Smidt and Vidaver, 1986b, Schuster et al., 1973), the source of inoculum may already be present when physical wounding occurs. Once the Goss's wilt pathogen infects, it may persist on the vascular tissue of leaves, or move systemically through vascular bundles (Schuster, 1975).

Goss's bacterial wilt and blight has two phases, the systemic wilt phase and the leaf blight phase (Claflin, 1999). The systemic wilt phase usually develops in association with an early season wounding event. This phase can kill small plants, leading to stand reduction. The wilt is caused by a buildup of bacteria in the vascular bundles, which prevents the plant from transporting water, thus causing the plant to wilt and die. The wilt phase can also occur when plants are larger, but disease is usually not as severe (Suparyono and Pataky 1989).

The second phase is the more common and usually less severe leaf blight phase. Foliar symptoms reported by Claflin (1999) include small, dark green to black, water soaked irregular areas of the leaf, which develop at the margins of expanding lesions known as freckles. Other signs of the leaf blight phase include an orange bacterial exudate and a glistening appearance on the surface of leaves.

The Goss's wilt pathogen overwinters in infected plant residues from the previous season(s), as well as inside and on seed. Schuster (1975) reported that the pathogen can survive for up to 10 months on the soil surface in these residues, which are the primary inoculum source. The pathogen is also able to survive in and on seed. Biddle et al.

(1990) reported that the percentage of seed-borne infestation ranged from 17.1 to 30.7%, while Schuster et al. (1975) reported 11% infested seed. In systemically infected ears Schuster et al. (1975) reported the pathogen could be found between the scutellum and the endosperm, and near the embryo.

The rate of seed transmission was found to be less than seed-borne infestation rates. Seed transmission of the Goss's wilt pathogen ranged from 0.1-0.4% in a study conducted by Biddle et al. (1990). In a separate study, Schuster et al. (1975) demonstrated that 1.6% of seedlings were infected when planted into sterile soil. Although the seed transmission rates are low, this should not be overlooked as a possible avenue of spread for the pathogen to new areas.

Little is known about why the disease has reappeared across the Corn Belt during recent years. The objective of this research was to determine if a change in the biology of the Cmn pathogen is responsible for its increased incidence in many corn growing regions of the U.S.

Materials and Methods

Source of isolates: Isolates were collected during the 2011 growing season as part a multistate survey on the progression of Cmn in the Corn Belt (Langemeier et al. 2012). A total of 466 isolates from eight states were included in the analysis. A set of 22 isolates from a previous paper published on the diversity of Cmn were also included for comparison. These isolates were from 1970-2009 and were from two different states including Nebraska and Iowa (Agarkova et al., 2011).

Samples were received into the UNL Plant and Pest Diagnostic Clinic and refrigerated upon arrival. Samples were tested for the presence of Cmn with an

ImmunoStrip® (ELISA) test kit (Agdia Inc. Elkhart, IN) designed for *Clavibacter michiganensis* subsp. *michiganensis* (Korus et al., 2010). Manufacturer's recommended procedures for testing were used. Each sample submitted contained four to five leaves. For each sample that tested positive for Cmn, four samples of expanding lesion, each from a separate lesion on a separate leaf, were macerated in 100 µl of water. The suspension containing the bacteria was then transferred to a petri dish of *Corynebacterium nebraskense* selective medium (CNS) minus LiCl, a selective agar medium developed for isolating the Cmn pathogen, (Gross and Vidaver, 1979, Smidt and Vidaver, 1986). The liquid was streaked using a bacterial loop and flamed before and after each transfer. Plates were grown for four days at 25 C. After four days, a single colony was transferred to a new petri dish to form a pure culture. Pure cultures were tested with the Agdia ImmunoStrip® to ensure the culture was Cmn. After a positive confirmation from the ImmunoStrip®, a single colony was placed in long term storage in a Microbank™ vial (Pro-Lab Diagnostics Inc. Toronto Canada). Directions for long term storage were followed as provided by the manufacturer.

DNA extraction: Isolates were removed from the -80 C freezer and plated on nutrient broth yeast extract (NBY) agar (Vidaver 1967). After three days of growth a single colony was placed in a 125 ml Erlenmeyer flask containing 20 ml of NBY broth. Cultures were grown for 24 to 48 hours on a rotary shaker. Genomic DNA was isolated using a Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin). Manufacturer recommended procedures were followed. Vials of DNA were stored at -20 C. DNA concentrations were determined and recorded with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware).

Pathogenicity assay: Isolates were tested for virulence in replicates of three. From each Erlenmeyer flask, 1.0 ml of bacterial suspension was plated onto a petri dish of NBY medium. These cultures were transferred to new plates monthly. Golden Cross Bantam (GCB) sweet corn, a known susceptible host for Cmn was planted in steam pasteurized soil in 15.2 cm pots at a seeding rate of six seeds per pot (Vidaver, 1977, Smidt and Vidaver, 1987). GCB was planted at a depth of 3.75 cm. One week after emergence pots were thinned to three plants per pot. The greenhouse temperature was set at a day/night fluctuation of 28/22 C. Supplemental artificial light was used to produce 16 hours of light to eight hours of darkness per day.

The inoculation process was done in sets of 103 isolates per time due to limitations in greenhouse space. The space was used successively from February 1st until July 10th. Cultures were grown on NBY for four days at 25 C. Bacterial colonies were then suspended in 10 mM PO_4 buffer at a pH of 7.1 (Carlson et al., 1979). Inoculum densities were adjusted to a concentration of $1.0x10^7$ (Schuster, 1975, Pataky 1985) with a Spectronic 20 set at 620 nm. Inoculum concentrations were verified in 10% of samples by plating serial dilutions of inoculum aliquots on NBY agar. Inoculum was kept on ice while being transported to the greenhouse facilities and during the inoculation process. The syringe injection method was used to create two successive wounds perpendicular to each other made 2.54 cm above the soil line into plant stems at approximately 18 days in age (V2 toV3) (Schuster et al., 1975, Agarkova et al., 2011). The process involved a 26-gauge needle attached to the syringe filled with inoculum. The inoculum was deposited in the stem by pushing the needle through the stem, forming a five µl drop of bacterial suspension on the bevel of the needle, and withdrawing the needle back through the stem.

Inoculation with sterile phosphate buffer in 10% of pots was done as a control. Pots were evaluated for visual symptoms, and percent of leaf area affected by lesion development seven days after inoculation. Asymptomatic pots were kept for an additional week to monitor for latent infection. Re-isolation onto NBY was performed on 10% of pots with typical symptoms and cultures were checked using the ImmunoStrip® test to ensure the lesions were caused by Cmn.

BOX-PCR: BOX-PCR was carried out with the primer sequence that corresponds to BOX A1 element (Louws et al., 1994, 1998, Agarkova et al., 2011). The sequence was 5' –CTA CGG CAA GCC GAC GCT GAC G custom DNA Oligos, synthesized by Life Technologies (Grand Island, New York). The PCR reaction and amplification were followed as described by Smith et al., (2001), and Agarkova et al., (2011) with slight modifications. The following procedure was calibrated for 10 PCR reactions at 20.0 µl per tube. The ingredients used in the master mix were 40.0 µl of 5X Crimson LongAmp® *Taq* DNA Polymerase PCR Buffer with 15 mM $MgCl_2$ (New England BioLabs Inc. Beverly, Massachusetts), 16.0 µl of 2.5 mM Deoxynucleotide Solution mix (dNTP's) (New England BioLabs Inc. Beverly, Massachusetts), 2.0 µl of 5U/µl Crimson LongAmp® Taq DNA Polymerase, 40.0 µl at 5 µM of the BOX-A1 primer, and 112.0 µl of distilled water. Aliquots of 19.0 µl of master mix, and 1.0 µl of template DNA (5 ng/µl) were dispensed into a 200 µl PCR tube. All products used in the master mix were kept at – 20 C until the reactions were run including the extracted DNA. The BOX-PCR reaction runs at an initial denaturation at 94 C for 5 minutes, 35 cycles of 94 C for 1 min, 50 C for 1 min, and 72 C for 2 min with a final elongation of 15 min at 72 C.

After the PCR reaction was completed 1.5 ul of PCR product was loaded into a 1.5% agarose gel (Fischer Scientific). The gel was submersed in 0.5X TBE buffer and stained using Ethidium Bromide at a working concentration of 0.5 mg/L. The DNA marker used was the 2-Log DNA Ladder (0.1-10.0 kb) (New England BioLabs Inc., Beverly, Massachusetts). The DNA ladder was loaded in every six to seven lanes at positions 1,7,14,21,28,35, and 40. The gels were run for 1.5 hrs. at 200V in a 35 cm gel box. Digital images were captured with the ChemiDoc EQ System (Bio-Rad, Hercules, California). Images were converted into 8-bit TIFF files for subsequent analysis of the fingerprint using GelCompare II (version 5.1) software (Applied Maths, Kortrijk, Belgium). TIFF files were normalized and similarity of banding patterns were calculated by applying the different bands product-moment correlation coefficient. Cluster analysis was performed using the unweighted pair group method using arithmetic averages algorithm (UPMGA), and results presented in a dendrogram and a map of the distribution of contemporary isolates (Smith et al., 2001).

AFLP: AFLP was carried out using processes described previously (Vos et al., 1995) with slight modifications (Agarkova et al., 2006, Agakova et al., 2011). Previously Agarkova et al. (2011) tested several enzyme-primer combinations and reported *Hpa*II+0 and *Eco*R1+0 provided the best resolution for Cmn. The sequence for *Hpa*II+0 and *Eco*R1+0 are (5' –CGA TGA GTC CTC ACC GGA) and (5' –GAC TGC GTA CCA ATT C). The *Eco*R1+0 primer was equipped with an infrared fluorescent dye, IRDye 700 (LI-COR Inc., Lincoln, Nebraska).

Six steps were followed to get the DNA from pure DNA to the desired product. The steps were to prepare restriction reactions, anneal adapter pairs, prepare ligation

reaction, making AFLP core mix, selective amplification, and electrophoresis of AFLP products.

Preparing restriction reactions: For ten reactions, a mixture of 11.0 µl 10X Ligase buffer with 10mM ATP (New England BioLabs Inc. Beverly, Massachusetts), 11.0 µl of Sodium Chloride (NaCl, concentration if 0.5 M), 5.5 µl Bovine serum albumin (BSA, concentration of 1 mg/ml), 0.5 µl HpaII 4 base pair (bp) cutter (concentration of 10 U) (New England BioLabs Inc. Beverly, Massachusetts), 0.5 µl EcoR1 6 bp cutter (concentration of 50 U) (New England BioLabs Inc. Beverly, Massachusetts), 6.5 µl sterile DDW. Aliquot 3.5 µl of master mix into each 200 µl PCR tube. Template DNA diluted in 5.5 µl DDW was added at a concentration of approximately 500 ng/ml to each tube of master mix for a final volume of 9.0 µl per PCR tube. Tubes were gently mixed and incubated for 2 hours at 37 C followed by 15 minutes at 70 C to inactivate restriction enzymes in a G-Storm GS1 Thermocycler (Gene Technologies Ltd. Fitzroy Vic, Australia).

Annealing Adapter Pairs: EcoR1 and HpaII adapter pairs synthesized by Life Technologies (Grand Island, New York) were heated to boiling in a distilled water bath for five minutes. Adapters were then diluted to a working concentration of 5 uM and 50 uM for EcoR1 and HpaII, respectively. After dilutions were completed adapter pairs were stored at – 20 C.

Preparing Ligation Reaction: For ten reactions, a mixture of 3.0 µl 10X Ligase buffer with 10mM ATP (New England BioLabs Inc. Beverly, Massachusetts), 1.0 µl of Sodium Chloride (NaCl, concentration if 0.5 M), 0.5 µl Bovine serum albumin (BSA,

concentration of 1 mg/ml), 1.65 µl of I Weiss unit T4DNA Ligase (New England BioLabs Inc. Beverly, Massachusetts), 0.5 µl EcoR1 adapter pair (concentration of 5 uM), 0.5 µl HpaII adapter pair (concentration of 50 uM), and 3.85 µl DDW. Aliquot 3.0 µl to each restriction reaction tube for a final volume of 12.0 µl per PCR tube. Tubes were gently mixed and incubated for 2 hours at 20 C in a G-Storm GS1 Thermocycler (Gene Technologies Ltd. Fitzroy Vic, Australia). A dilution of 1:10 of samples was performed with 0.1X Tris EDTA (TE) buffer and product was stored at – 20 C.

AFLP Core Mix: For ten reactions, a mixture of 10.0 µl 10X ThermoPol™ reaction buffer 15 mM $MgCl_2$ (New England BioLabs Inc. Beverly, Massachusetts), 3.2 µl of 1.25 mM Deoxynucleotide Solution mix (dNTP's) (New England BioLabs Inc. Beverly, Massachusetts), 0.625 µl of 5U/µl ThermoPol™ Taq DNA Polymerase, 2.0 µl 5 uM AFLP *Hpa*II selective primer synthesized by Life Technologies (Grand Island, New York), 2.0 µl 5 uM AFLP *Eco*R1 selective primer synthesized by LI-COR Inc. (Lincoln, Nebraska), and 72.175 µl DDW.

Selective Amplification: Aliquot 9.0 µl of AFLP core mix and 1.0 µl or product from the ligation reaction into a 200 µl PCR tube. The AFLP reaction runs at an initial denaturation at 94 C for 2 minutes, 10 cycles of 94 C for 30 sec, a step down 66 C -1 C for 30 sec, and 72 C for 1 min, 20 cycles of 94 C for 30 sec, 56 C for 30 sec, and 72 C for 1 min with a final elongation of 5 min at 72 C in a G-Storm GS1 Thermocycler (Gene Technologies Ltd. Fitzroy Vic, Australia). Amplified product was mixed with 5 µl of Blue Stop Solution. Product was heated to 92 C for 3 minutes, and immediately placed on ice.

Electrophoresis of PCR products: Product was loaded (0.8 µl) into a 6.5% polyacrylamide gel on a LI-COR Long ReadIR™ DNA Sequencer (model 4200, Lincoln, Nebraska). Plates were 25 cm and spacers were 25 mm thick. The DNA marker used was the 50-700 BP Sizing Standard (LI-COR, Lincoln, Nebraska). The DNA ladder was loaded in every five to eight lanes at 1.0 µl per lane at positions 1,9,17,25,33,41, and 47. Electrophoresis data was automatically collected and simultaneously recorded during the run. Images were converted into 8-bit TIFF files for subsequent analysis of the fingerprint using GelCompare II (version 5.1) software (Applied Maths, Kortrijk, Belgium). TIFF files were normalized and similarity of banding patterns were calculated by applying the different bands product-moment correlation coefficient. Cluster analysis was performed using the unweighted pair group method using arithmetic averages algorithm (UPMGA), and results presented in a dendogram and a map of the distribution of contemporary isolates (Smith et al., 2001). All isolates were run twice starting with the AFLP amplification and electrophoresis steps and compared visually to ensure similar banding patterns.

Results

Pathogenicity: The pathogenicity test resulted in a total of 10% of isolates being avirulent, 4% were not tested due to contamination, and 86% being virulent. Virulent isolates showed typical symptoms of Cmn including freckling, exudate, stunting and buggy whipping. Serial dilutions resulted in viable bacterial counts ranging from $3.5x10^6$ to $6.5x10^7$. Re-isolation also resulted in 100% of samples tested being confirmed for Cmn. Differences in virulence were noted in greenhouse trials, but were not reported due

to variations based on proximity to vents and doors. No differences in groupings based on an isolate being virulent or avirulent were observed from the results (Table 1, Figure 3). Isolates in group A, B, and C were found to be both virulent and avirulent in the BOX-PCR and AFLP analyses. The 10% of isolates found to be avirulent were from 25 of the 85 (29%) counties represented in the analysis.

BOX-PCR analysis of Cmn isolates: The reproducibility of the BOX-PCR procedure was assessed visually to ensure banding patterns were similar, and each isolate was run twice starting from the PCR reaction. Cluster analysis of banding patterns resulted in three groups using a similarity cutoff of 92.5%. The first grouping (A) consisted of 82% (n=384) of isolates. Group A contained isolates from 81 counties in eight states. Group A also contained 17 isolates from the previous study conducted by Agarkova et al., (2011). Group B contained 16% (n=74) of isolates and consisted of isolates from 40 counties in six states. There were also three isolates from the previous study observed in this group. Group C was a very heterogeneous mix of isolates and by the 92.5% similarity value, actually contains 7 subgroups (Table 1). Group C1, C2, C4, C5, C6, and C7 each contained 0.22% (n=1) of isolates. Group C3 contained 0.44% (n=2). These isolates were grouped into one group (C) containing 2% (n=8) of all isolated tested. These isolates originated in seven counties in two states (Figure 1).

AFLP analysis of Cmn isolates: The reproducibility of the AFLP procedure used described previously (Agarkova et al., 2011). Cluster banding analysis again resulted in one main group, a smaller group, and a very heterogeneous group of isolates. A similarity value of 87% was used. The largest group again being group A contained 80% (n=376) of isolates representing 84 counties in eight states. This group also

contained 15 of the isolates from the previous study. Group B contained 10% (n=45). Group B consisted of isolates from 38 counties in four states. Three isolates from the previous study were found in this group. Group C was again very heterogeneous and using the similarity cutoff value of 87% was broken down into 22 subgroups. Group C4, C5, C8, C10, C12, C14, C17, C18, C19, C20, C21, and C22 each contained 0.22% (n=1) of isolates. Groups C1, C6, and C13 each contained 0.44% (n=2). Groups C3, C7, C11, C15, and C16 each contained 0.66% (n=3). Groups C9 contained 0.88% (n=4). Group C2 contained 1.76% (n=8). These isolates were grouped into one group (C) containing 10% (n=45) of all isolated tested. These isolates originated in 31 counties in six states (Figure 2). Four isolates from the previous study were observed in group C.

Distribution of combined isolates: All isolates from Dawson County, NE were in group A and B for both old isolates from the previous study, and contemporary isolates (Table 1). Isolates were put in the composite data set based on a combination of the BOX-PCR and AFLP analyses. A map of the current distribution of isolates in the Corn Belt shows no geographical basis for the separation of isolates collected during the 2011 growing season (Figure 3). Five of the eight states represented in the analysis had isolates that were in group A, B, and C. In the remaining three states, only one sample was received and may be the reason that there is not more diversity observed in these states. In counties with group A only isolates, there were between one and four isolates per county with an average of 1.9 isolates per county. In counties with group A and B isolates there were between one and 15 isolates with an average of 4.5 isolates per county. In counties with group A and C isolates there were between one and eight isolates with an average of 3.3 isolates per county. In counties with group A, B, and C

isolates there were between three and 34 isolates with an average of 10.4 isolates per county. The more samples received from a particular county, the more diverse the counties isolates were.

Discussion

Previous research on the virulence of Cmn is limited, but results from a few studies have been reported (Malvick et al., 2012, Schuster, 1975, Schuster et al., 1975b, Vidaver, 1977). Isolates were tested for virulence in the greenhouse over a six month period. During this time, isolates were maintained on NBY due to limitations in greenhouse space to test for virulence. Reduction or complete loss of virulence often occurs when sub-culturing on nutrient agar media with bacterial pathogens (Schuster et al., 1975b). Vidaver (1977) reports that during early studies it became clear that Cmn readily loses virulence. Vidaver (1977) also reported that virulence was lost more rapidly that viability when storing isolates at room temperature. Schuster et al., (1975b) was unable to restore virulence with five successive passages through a susceptible corn line. This loss of virulence may be partially to blame for the number of isolates that were shown to be avirulent in the pathogenicity study. Differences in virulence have also been reported for Cmn (Schuster, 1975, Malvick et al., 2012). These differences in virulence were noted, but due to differences in symptoms based on location in the greenhouse, these results are not reported in this paper. Studies on pathogen population diversity have also been reported, but in limited numbers again (Smidt and Vidaver, 1987, Vidaver, 1977).

Virtually no variability in bacteriocin production was observed between isolates found in the first three fields in Dawson County (Vidaver et al., 1981). Cmn showed little heterogeneity when exposed to nutritional and biochemical tests. Over the next few years, phage groups were created to classify the bacteria. In 1970 two phages were observed, and by 1972 this number increased to six phages. However when considering a bacterial pathogen, six phages is still a relatively homogeneous population. In 1987, Smidt and Vidaver conducted a study collecting isolates from a single popcorn field. Isolates were originally assigned into four groups based on colony morphology. Isolates were found to be diverse based on morphology, and bacteriophage sensitivity. Isolates either produced CN1, CN1 and CN2, or neither. No isolate tested produced only CN2. Isolates from the study conducted in 1987 were considerably more diverse than those comprised in the 1981 study, although they all originated from one field. These results indicate increasing heterogeneity among populations of Cmn. Agarkova et al. (2011) also found some heterogeneity among isolates collected over a 40 years period using AFLP and rep-PCR techniques.

AFLP is a fingerprinting technique that can be used reliably to characterize plant pathogenic bacteria (Vos et al., 1995). The technique has proven successful when attempting to subtype and identify C. michiganensis subspecies (Agarkova et al., 2011, de Leon et al., 2009). The technique is also able to discriminate at the subspecies level. Another benefit to AFLP is that is has a much higher level of genetic resolution due to the large number of fragments available for analysis (Agarkova et al., 2011). A disadvantage of the AFLP procedure is that is requires expensive equipment and that

large portions of DNA may be excluded from the banding profile if they do not contain the proper restriction sites for the enzymes used.

Repetitive sequence-based polymerase chain reaction (rep-PCR) has also proven useful in subtyping subspecies in the *Clavibacter* genus. Louws et al. (1998) successfully used rep-PCR to differentiate between all five subspecies contained within the *Clavibacter michiganensis* genus and species. PCR techniques have also proven useful in showing differences in subspecies of *C. michiganensis* (Kleitman et al., 2008, Louws et al., 1998, Smith et al., 2001).

Both AFLP and BOX-PCR have shown useful in subtyping of plant pathogens at the *Clavibacter michiganensis* specie and sub-specie levels. These techniques have proven successful in several subspecies in the *Clavibacter* genus including *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), and *Clavibacter michiganensis* subsp. *nebraskensis* (Cmn). Both techniques use similar approaches in creating DNA fragments. Both techniques utilize the entire genome to generate polymorphic DNA banding patterns.

Similarly to the study conducted by Agarkova et al., (2011) three major groups were found among isolates tested during this study. Isolates from the previous study were also included in this study as a check. Isolates 20038 and 20047 were found to be in group C, or the heterogeneous group using AFLP. These were also found to be heterogeneous by Agarkova et al. (2011). Isolate 20079-B was also found to be different from (group A3 in old study, group B in new study) other isolates. This isolate was also in a different group than the majority of isolates tested (Table 1). BOX-PCR techniques

also showed isolates 20038, and 20047 to be in the very heterogeneous group C. This confirms what Agarkova et al., (2011) found (Table 1).

The current study also found many differences from the study done in 2011. Overall, isolates from the 2011 survey were found to be very similar in DNA composition based on the two fingerprinting techniques used. Using similarity values of 87% for AFLP and 92.5% for rep-PCR we were able to categorize almost all isolates into three homogeneous groups. The research showed one diverse group at the bottom of the dendrogram that contained only 2% of isolates using rep-PCR and 10% of isolates using AFLP (Figure 1 and 3). These data shows that Cmn is still very homogeneous in nature, but based on the number of isolates in group C there is limited heterogeneity in this bacterial population. Differences were found from the 2011 study in that this research showed that BOX-PCR only found 2% of isolates to be in the diverse group while the 2011 study showed 6% of isolates to be in the diverse group. Implementing the AFLP fingerprinting technique our research showed 10% of isolate fell into group C, while the 2011 study showed 7% of isolates were in this diverse group. This shows that since 1969 when the pathogen was first identified, little mutation in the bacterial DNA has been observed based on the two fingerprinting techniques described. Based on a large sample pool of contemporary isolates (n=443) we were able to show that the diversity of Cmn that exists does not seem to have a geographical basis based on the two fingerprinting techniques used.

From a management standpoint knowing that this diversity is widespread may be helpful in combating this disease. If a seed company wants to test for Cmn in one state, but market seed in other states, knowing that this diversity exits naturally may help them

in their Goss's wilt screening program. Using inoculum that contains isolates from each of these three groups should help to screen out susceptible hybrids and inbreds. This way if they were to sell seed to other regions, they would know that a hybrid that was tolerant in their region, should be tolerant to Cmn in other regions.

In conclusion this research supports the research conducted by Agarkova et al., (2011) in that Cmn isolates can be divided into one large homogeneous group, and two smaller groups. This research also shows that although there is some heterogeneity in the Cmn population, but overall it is a very homogeneous. This research also shows no geographical basis for re-emergence, indicating that either a change in the DNA makeup of the bacterial has not occurred, or that the change could not be observed using the two fingerprinting techniques implemented in this project.

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Table 1. Isolates of *Clavibacter michiganensis* subsp. *nebraskensis* used in this study.

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
20033	NE	Kearney	Yes	2003	C3	B	BC
20038	NE	Lincoln	Yes	2003	C3	C6	C
20047	NE		Yes	2004	C3	C3	C
31341	NE	Hall	Yes	2004	A	A	A
31491	NE	Dawson	Yes	2004	A	B	AB
2009000479	NE	Antelope	NA	2009	B	A	AB
001A	NE	Chase	Yes	2011	A	A	A
001B	NE	Chase	No	2011	C15	B	BC
001C	NE	Chase	No	2011	C22	A	AC
003A	NE		Yes	2011	A	A	A
003B	NE		Yes	2011	A	A	A
004A	NE	York	No	2011	A	A	A
004B	NE	York	No	2011	A	A	A
004C	NE	York	No	2011	A	A	A
004D	NE	York	No	2011	A	A	A
006A	NE	Box Butte	Yes	2011	A	A	A
010A	NE	Box Butte	No	2011	A	A	A
010B	NE	Box Butte	Yes	2011	A	A	A
011A	NE		Yes	2011	A	A	A
014A	NE		Yes	2011	A	A	A
015A	NE	Saline	No	2011	C20	A	AC
016A	NE	Scotts Bluff	Yes	2011	A	B	AB
016B	NE	Scotts Bluff	Yes	2011	A	A	A
020A	NE	Kimball	Yes	2011	B	A	AB
020B	NE	Kimball	Yes	2011	A	A	A
029B	IA	Sac	Yes	2011	C4	A	AC
032A	IA	Poweshiek	Yes	2011	A	A	A
032B	IA	Sac	No	2011	A	A	A
037A	IA	Sac	Yes	2011	A	A	A
037B	IA	Sac	Yes	2011	A	A	A
038A	IA	Sac	No	2011	A	C2	AC
039A	IA	Sac	Yes	2011	C5	A	AC
043A	NE	Banner	Yes	2011	A	A	A
043B	NE	Banner	Yes	2011	A	A	A
044B	NE	Scotts Bluff	Yes	2011	A	B	AB
045A	IA	Story	Yes	2011	B	A	AB
045B	IA	Story	Yes	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
046A	NE	Antelope	Yes	2011	A	B	AB
048A	NE	Lincoln	Yes	2011	A	A	A
049A	NE	Lincoln	Yes	2011	A	A	A
049B	NE	Lincoln	Yes	2011	A	B	AB
051A	NE	Lincoln	Yes	2011	B	B	B
051B	NE	Lincoln	Yes	2011	A	A	A
054A	NE	Holt	Yes	2011	A	A	A
054C	NE	Holt	Yes	2011	A	A	A
056A	NE	Cheyenne	No	2011	A	A	A
056B	NE	Cheyenne	No	2011	B	A	AB
060A	NE	Butler	Yes	2011	A	B	AB
060B	NE	Butler	Yes	2011	A	A	A
060C	NE	Butler	Yes	2011	A	B	AB
064A	NE	Furnas	Yes	2011	A	A	A
064B	NE	Furnas	Yes	2011	A	A	A
064D	NE	Furnas	Yes	2011	A	A	A
065A	NE	Dawson	Yes	2011	A	A	A
065B	NE	Dawson	Yes	2011	A	A	A
066B	NE	Colfax	Yes	2011	A	A	A
067C	IA	Iowa	Yes	2011	A	B	AB
068B	IA	Buena Vista	Yes	2011	A	A	A
069A	NE	Holt	Yes	2011	A	A	A
069B	NE	Holt	Yes	2011	B	C1	BC
071B	IA	Plymouth	Yes	2011	C1	A	AC
072A	IA	Cherokee	Yes	2011	B	B	B
073C	IA	Plymouth	No	2011	A	A	A
074A	NE	Platte	Yes	2011	B	B	B
074B	NE	Platte	Yes	2011	C6	B	BC
075B	NE	Platte	No	2011	C11	C7	C
076B	IA	Plymouth	Yes	2011	A	A	A
076C	IA	Plymouth	Yes	2011	B	A	AB
078A	NE	York	Yes	2011	C14	A	AC
078B	NE	York	Yes	2011	A	B	AB
084A	IA	Buena Vista	No	2011	A	B	AB
084B	IA	Buena Vista	No	2011	A	A	A
085A	IA	Buena Vista	No	2011	A	A	A
085C	IA	Buena Vista	Yes	2011	A	B	AB
088A	NE	Madison	Yes	2011	A	A	A
088B	NE	Madison	Yes	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
088C	NE	Madison	Yes	2011	A	A	A
090A	NE	Fillmore	Yes	2011	A	A	A
090B	NE	Fillmore	Yes	2011	A	A	A
092A	NE	Holt	Yes	2011	B	A	AB
092B	NE	Holt	Yes	2011	A	A	A
094A	IA	Manona	Yes	2011	B	A	AB
094B	IA	Manona	Yes	2011	A	A	A
098A	IA	Manona	Yes	2011	A	A	A
098B	IA	Manona	Yes	2011	A	A	A
098C	IA	Manona	Yes	2011	A	B	AB
100A	IA	Sac	Yes	2011	A	A	A
100B	IA	Sac	Yes	2011	A	A	A
102A	NE	Dawson	Yes	2011	B	A	AB
102D	NE	Dawson	Yes	2011	A	A	A
103A	IA	Pottawattamie	Yes	2011	A	A	A
105A	MN	Nicollet	Yes	2011	A	A	A
105B	MN	Nicollet	Yes	2011	A	A	A
105D	MN	Nicollet	Yes	2011	A	B	AB
106A	IA	Cerro Gordo	Yes	2011	A	B	AB
106B	IA	Cerro Gordo	Yes	2011	A	A	A
106D	IA	Cerro Gordo	Yes	2011	A	B	AB
109B	NE	Colfax	Yes	2011	A	A	A
110A	IA	Jasper	Yes	2011	A	A	A
110C	IA	Jasper	Yes	2011	C10	A	AC
112A	NE	Cedar	Yes	2011	B	A	AB
112B	NE	Cedar	Yes	2011	A	A	A
113A	NE	Dodge	Yes	2011	B	A	AB
116A	IA	Plymouth	Yes	2011	A	A	A
117A	NE	Colfax	Yes	2011	B	A	AB
117B	NE	Colfax	Yes	2011	A	A	A
117C	NE	Colfax	Yes	2011	A	A	A
120A	NE		Yes	2011	A	A	A
120B	NE		Yes	2011	A	A	A
120D	NE		Yes	2011	A	B	AB
121A	NE	Hall	Yes	2011	A	A	A
121B	NE	Hall	Yes	2011	A	A	A
124A	NE	platte	Yes	2011	A	A	A
124B	NE	Platte	Yes	2011	A	A	A
124D	NE	Platte	No	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
126D	IA	Dubuque	No	2011	A	B	AB
127A	IA	Dubuque	Yes	2011	A	A	A
127B	IA	Dubuque	Yes	2011	A	A	A
127C	IA	Dubuque	Yes	2011	A	A	A
128A	IA	Delaware	Yes	2011	A	A	A
128B	IA	Delaware	Yes	2011	A	A	A
128C	IA	Delaware	Yes	2011	A	A	A
128D	IA	Delaware	Yes	2011	A	B	AB
145A	IA	Butler	Yes	2011	B	B	B
145B	IA	Butler	Yes	2011	A	A	A
146B	IA	Butler	Yes	2011	C13	B	BC
146C	IA	Butler	Yes	2011	A	A	A
151B	IA	Jones	Yes	2011	A	A	A
151CA	NE	Madison	Yes	2011	A	A	A
151CB	NE	Madison	Yes	2011	A	A	A
151CC	NE	Madison	Yes	2011	A	A	A
151CD	NE	Madison	Yes	2011	A	A	A
158B	NE	Perkins	Yes	2011	A	A	A
161CA	NE	Hall	Yes	2011	A	A	A
161CB	NE	Hall	Yes	2011	A	A	A
162CA	NE	Hall	Yes	2011	A	A	A
162CB	NE	Hall	Yes	2011	A	A	A
163CA	NE	Hall	Yes	2011	A	B	AB
163CB	NE	Hall	Yes	2011	A	A	A
163CC	NE	Hall	Yes	2011	A	A	A
166A	IA	Clinton	Yes	2011	A	B	AB
166B	IA	Clinton	Yes	2011	A	A	A
166CA	NE	Holt	No	2011	A	A	A
166CB	NE	Holt	Yes	2011	A	A	A
167A	IA	Clinton	Yes	2011	B	A	BA
167B	IA	Clinton	Yes	2011	C8	A	CA
167C	IA	Clinton	Yes	2011	A	B	AB
167D	IA	Clinton	Yes	2011	A	A	A
168B	IA	Clinton	Yes	2011	A	A	A
168C	IA	Clinton	No	2011	A	B	AB
171B	NE	Cuming	Yes	2011	A	B	AB
171C	NE	Cuming	Yes	2011	B	A	BA
173A	NE	Dodge	Yes	2011	C11	A	CA
173B	NE	Dodge	Yes	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
175A	IA	Delaware	Yes	2011	B	A	AB
175B	IA	Delaware	Yes	2011	A	A	A
178A	IA	Black Hawk	Yes	2011	C2	A	AC
178B	IA	Black Hawk	Yes	2011	A	A	A
180B	IA	Dubuque	Yes	2011	A	B	AB
181B	IA	Hardin	Yes	2011	B	A	AB
181C	IA	Hardin	Yes	2011	A	B	AB
181D	IA	Hardin	Yes	2011	A	A	A
182A	IA	Marion	Yes	2011	C2	A	AC
182B	IA	Marion	Yes	2011	A	A	A
182D	IA	Marion	Yes	2011	A	A	A
185B	IA	Mitchell	Yes	2011	A	A	A
189A	IA	Benton	Yes	2011	A	A	A
189B	IA	Benton	Yes	2011	B	A	AB
189C	IA	Benton	Yes	2011	A	A	A
190A	IA	Benton	NA	2011	A	A	A
190B	IA	Benton	Yes	2011	A	A	A
191A	IA	Delaware	Yes	2011	A	B	AB
191B	IA	Delaware	Yes	2011	A	A	A
191C	IA	Delaware	Yes	2011	A	A	A
193D	NE	Lincoln	Yes	2011	A	B	AB
194A	NE	Dodge	Yes	2011	B	A	AB
194B	NE	Dodge	Yes	2011	B	A	AB
194C	NE	Dodge	NA	2011	A	A	A
195B	IA	Tama	No	2011	A	B	AB
195C	IA	Tama	Yes	2011	A	A	A
196A	NE	Valley	Yes	2011	C21	A	AC
196B	NE	Valley	Yes	2011	A	B	AB
196C	NE	Valley	Yes	2011	A	A	A
197A	NE	Jefferson	Yes	2011	A	A	A
197B	NE	Jefferson	No	2011	A	A	A
198D	NE	Delaware	Yes	2011	A	B	AB
1994NC+	NE	Adams	Yes	1994	A	A	A
2006 Scotts Bluff	NE	Scotts Bluff	Yes	2006	A	A	A
2007-24C	NE	Chase	Yes	2007	B	A	AB
20079-B	NE	Chase	Yes	2007	C15	A	AC
206A	NE	Antelope	Yes	2011	A	B	AB
206B	NE	Antelope	Yes	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
208A	IA	Muscatine	Yes	2011	A	A	A
208B	IA	Muscatine	Yes	2011	A	A	A
209A	IA	Cedar	Yes	2011	A	A	A
209B	IA	Cedar	Yes	2011	A	A	A
209C	IA	Cedar	Yes	2011	C2	A	AC
211A	NE	Saline	Yes	2011	A	A	A
211B	NE	Saline	Yes	2011	A	A	A
212B	IA	Carroll	Yes	2011	A	A	A
212C	IA	Carroll	NA	2011	A	B	AB
213A	IA	Carroll	Yes	2011	A	B	AB
221A	CO	Yuma	Yes	2011	A	A	A
221B	CO	Yuma	NA	2011	A	A	A
222CA	NE	Box Butte	Yes	2011	A	A	A
224B	NE	Saline	Yes	2011	B	A	AB
224C	NE	Saline	Yes	2011	A	A	A
225A	NE	Hall	Yes	2011	A	A	A
225C	NE	Hall	Yes	2011	A	A	A
225CA	NE	Washington	Yes	2011	B	A	AB
225CB	NE	Washington	Yes	2011	A	A	A
225CC	NE	Washington	Yes	2011	A	A	A
227A	CO	Yuma	Yes	2011	A	A	A
228A	CO	Yuma	Yes	2011	A	A	A
228B	CO	Yuma	Yes	2011	A	A	A
228C	CO	Yuma	Yes	2011	A	A	A
228D	CO	Yuma	Yes	2011	A	B	AB
229A	CO	Yuma	Yes	2011	A	A	A
229C	CO	Yuma	Yes	2011	A	A	A
230CA	NE	Washington	Yes	2011	A	A	A
230CB	NE	Washington	Yes	2011	A	A	A
230CC	NE	Washington	Yes	2011	A	A	A
231A	CO	Yuma	Yes	2011	A	B	AB
231B	CO	Yuma	Yes	2011	A	A	A
235A	NE	Platte	Yes	2011	A	A	A
235B	NE	Platte	Yes	2011	A	A	A
235C	NE	Platte	Yes	2011	C2	A	AC
236A	NE	Platte	Yes	2011	A	A	A
236B	NE	Platte	Yes	2011	A	A	A
238A	NE	Platte	Yes	2011	A	A	A
238B	NE	Platte	Yes	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
238C	NE	Platte	Yes	2011	A	A	A
241A	IA	Cedar	Yes	2011	C15	A	AC
241B	IA	Cedar	Yes	2011	B	A	AB
243CA	NE	Buffalo	Yes	2011	A	A	A
243CC	NE	Buffalo	Yes	2011	A	A	A
247A	IA	Delaware	Yes	2011	B	A	AB
247B	IA	Delaware	Yes	2011	A	A	A
247C	IA	Delaware	No	2011	C2	A	AC
256A	IA	Dubuque	Yes	2011	A	A	A
256B	IA	Dubuque	Yes	2011	A	A	A
256C	IA	Dubuque	Yes	2011	A	B	AB
257A	IA	Dubuque	Yes	2011	A	A	A
257B	IA	Dubuque	NA	2011	A	A	A
258A	IA	Dubuque	Yes	2011	A	A	A
258B	IA	Dubuque	No	2011	A	A	A
258C	IA	Dubuque	No	2011	A	A	A
258D	IA	Dubuque	Yes	2011	A	A	A
259A	IA	Dubuque	Yes	2011	A	A	A
259B	IA	Dubuque	Yes	2011	C16	A	AC
260A	IA	Dubuque	Yes	2011	A	A	A
260B	IA	Dubuque	Yes	2011	A	A	A
261A	IA	Benton	Yes	2011	A	A	A
261B	IA	Benton	No	2011	A	A	A
264A	IA	Butler	Yes	2011	A	A	A
264B	IA	Butler	Yes	2011	A	A	A
268A	NE	Custer	Yes	2011	A	A	A
268B	NE	Custer	Yes	2011	A	A	A
271A	NE	Nance	Yes	2011	A	A	A
271B	NE	Nance	NA	2011	A	A	A
272A	NE	Nance	Yes	2011	A	B	AB
273A	IN	Pulaski	Yes	2011	A	A	A
273B	IN	Pulaski	Yes	2011	A	A	A
274A	NE	Custer	Yes	2011	C2	A	AC
274B	NE	Custer	NA	2011	C11	A	AC
274CA	NE	Platte	Yes	2011	C1	A	AC
274CB	NE	Platte	Yes	2011	A	A	A
274CC	NE	Platte	Yes	2011	A	B	AB
275CA	NE	Platte	No	2011	A	A	A
275CB	NE	Platte	Yes	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
275CC	NE	Platte	No	2011	A	A	A
275CD	NE	Platte	Yes	2011	A	A	A
276B	IN	Pulaski	Yes	2011	A	B	AB
277A	CO	Yuma	Yes	2011	A	B	AB
277B	CO	Yuma	Yes	2011	A	A	A
277D	CO	Yuma	Yes	2011	A	B	AB
278A	IN	Pulaski	Yes	2011	A	A	A
278B	IN	Pulaski	Yes	2011	A	B	AB
278C	IN	Pulaski	Yes	2011	A	A	A
279A	IN	Pulaski	Yes	2011	A	A	A
280B	IN	Pulaski	Yes	2011	A	A	A
281A	IA	Delaware	No	2011	A	B	AB
282A	IA	Delaware	Yes	2011	A	A	A
282B	IA	Delaware	Yes	2011	C16	A	AC
282CA	NE	Antelope	Yes	2011	C2	A	AC
283A	IA	Delaware	Yes	2011	A	A	A
283B	IA	Delaware	Yes	2011	A	A	A
286A	IA	Cherokee	Yes	2011	A	A	A
286B	IA	Cherokee	Yes	2011	A	A	A
286C	IA	Cherokee	No	2011	A	A	A
295A	IN	Pulaski	Yes	2011	A	A	A
295B	IN	Pulaski	Yes	2011	A	A	A
295C	IN	Pulaski	NA	2011	A	A	A
301A	IA	Osceola	No	2011	A	A	A
301B	IA	Osceola	Yes	2011	A	A	A
311CA	NE	Antelope	Yes	2011	A	A	A
311CB	NE	Antelope	Yes	2011	A	A	A
311CC	NE	Antelope	Yes	2011	A	A	A
312A	SD	Clay	Yes	2011	A	A	A
312B	SD	Clay	Yes	2011	A	A	A
312C	SD	Clay	Yes	2011	A	A	A
312CB			Yes	2011	A	A	A
313CA	IA		Yes	2011	A	A	A
313CC	IA		Yes	2011	A	B	AB
315CA	IA		No	2011	A	A	A
317A	IA	Calhoun	Yes	2011	A	A	A
317B	IA	Calhoun	Yes	2011	A	A	A
318A	NE	Greeley	Yes	2011	A	A	A
318B	NE	Greeley	Yes	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
319B	NE	Howard	Yes	2011	C16	A	AC
324A	NE	Hall	Yes	2011	A	A	A
324B	NE	Hall	No	2011	A	A	A
326A	NE	Hall	Yes	2011	C9	A	AC
326C	NE	Hall	NA	2011	A	A	A
327A	NE	Hall	Yes	2011	A	A	A
327B	NE	Hall	Yes	2011	A	A	A
330B	NE	Perkins	Yes	2011	A	A	A
331A	IA	Fayette	Yes	2011	A	A	A
331B	IA	Fayette	Yes	2011	C9	A	AC
332A	IA	Fayette	No	2011	A	A	A
332B	IA	Fayette	No	2011	A	A	A
334A	IA	Butler	Yes	2011	A	A	A
334B	IA	Butler	Yes	2011	A	A	A
336C	NE	Boone	NA	2011	A	A	A
337A	NE	Butler	Yes	2011	B	A	AB
337B	NE	Butler	NA	2011	A	A	A
338B	NE	Dixon	No	2011	A	A	A
339CA	NE	Platte	Yes	2011	A	A	A
339CB	NE	Platte	Yes	2011	A	A	A
339CD	NE	Platte	Yes	2011	B	A	AB
344A	IA	Mahaska	Yes	2011	A	A	A
349A	NE	Keith	Yes	2011	A	A	A
349D	NE	Keith	Yes	2011	A	B	AB
364A	NE	Colfax	Yes	2011	A	A	A
364B	NE	Colfax	No	2011	A	A	A
364C	NE	Colfax	NA	2011	A	A	A
365A	CO	Yuma	Yes	2011	A	A	A
366A	NE	Perkins	No	2011	C6	A	CA
366C	NE	Perkins	No	2011	A	B	AB
370A	IA	Buchanan	Yes	2011	A	C4	AC
370B	IA	Buchanan	Yes	2011	A	A	A
371A	IA	Black Hawk	Yes	2011	A	A	A
371B	IA	Black Hawk	Yes	2011	A	A	A
371C	IA	Black Hawk	Yes	2011	B	B	B
374B	SD	McPherson	Yes	2011	A	A	A
374C	SD	McPherson	Yes	2011	A	B	AB
374D	SD	McPherson	Yes	2011	A	A	A
375A			Yes	2011	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
375C			No	2011	A	B	AB
376A	NE	Cedar	NA	2011	A	B	AB
376B	NE	Cedar	NA	2011	A	A	A
377A	NE	Cedar	Yes	2011	A	A	A
377B	NE	Cedar	Yes	2011	A	B	AB
377C	NE	Cedar	Yes	2011	A	A	A
379A	NE	Cedar	Yes	2011	A	A	A
379C	NE	Cedar	Yes	2011	A	A	A
388A	NE	Rock	Yes	2011	A	A	A
388B	NE	Rock	Yes	2011	A	A	A
388CA	NE	Holt	Yes	2011	A	A	A
388CB	NE	Holt	Yes	2011	A	A	A
388CC	NE	Holt	No	2011	A	B	AB
390B	SD	Union	Yes	2011	A	A	A
390C	SD	Union	Yes	2011	C7	A	AC
393B	NE	Perkins	Yes	2011	A	A	A
394A	IA	Carroll	No	2011	C19	A	AC
396A	IA	Benton	Yes	2011	A	A	A
396B	IA	Benton	Yes	2011	A	B	AB
396D	IA	Benton	NA	2011	A	B	AB
400A	NE	Perkins	Yes	2011	B	A	AB
400B	NE	Perkins	Yes	2011	C7	A	AC
417A	NE	Hall	Yes	2011	B	A	AB
417B	NE	Hall	Yes	2011	A	A	A
417C	NE	Hall	Yes	2011	A	A	A
418A	NE	Hall	Yes	2011	A	A	A
418B	NE	Hall	Yes	2011	A	A	A
418C	NE	Hall	Yes	2011	A	A	A
418D	NE	Hall	Yes	2011	A	A	A
419A	NE	Hall	Yes	2011	A	B	AB
419B	NE	Hall	Yes	2011	C7	B	BC
420A	NE	Hall	Yes	2011	A	A	A
420B	NE	Hall	Yes	2011	A	B	AB
420C	NE	Hall	Yes	2011	A	A	A
421A	NE	Hall	Yes	2011	A	A	A
421B	NE	Hall	Yes	2011	A	A	A
421C	NE	Hall	Yes	2011	A	A	A
422B	NE	Hall	No	2011	A	A	A
422C	NE	Hall	No	2011	A	A	A

Table 1 continued

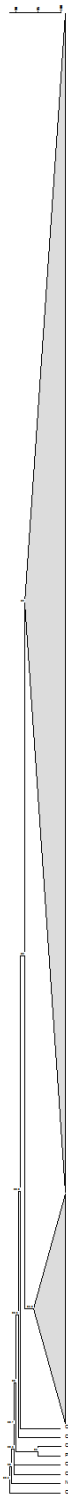
Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
428B	IA	Johnson	Yes	2011	A	A	A
429A	IA	Johnson	Yes	2011	A	A	A
429B	IA	Johnson	Yes	2011	A	A	A
429C	IA	Johnson	Yes	2011	A	A	A
430B	IA	Johnson	Yes	2011	A	B	AB
431A	IA	Johnson	Yes	2011	B	A	AB
431B	IA	Johnson	Yes	2011	A	A	A
431C	IA	Johnson	Yes	2011	A	A	A
434A	IA	Cedar	Yes	2011	C18	A	AC
435A	IA	Cedar	Yes	2011	A	B	AB
435B	IA	Cedar	Yes	2011	A	A	A
435D	IA	Cedar	Yes	2011	A	A	A
438A	IA	Dubuque	Yes	2011	A	B	AB
438B	IA	Dubuque	Yes	2011	A	A	A
438D	IA	Dubuque	Yes	2011	A	A	A
442CB	NE	Polk	No	2011	A	A	A
444A	IA	Hamilton	Yes	2011	A	A	A
444B	IA	Hamilton	Yes	2011	B	A	AB
445A	IN	Pulaski	Yes	2011	A	B	AB
445B	IN	Pulaski	Yes	2011	B	A	AB
445C	IN	Pulaski	NA	2011	A	B	AB
446A	IN	Newton	Yes	2011	A	A	A
446B	IN	Newton	Yes	2011	A	A	A
446C	IN	Newton	Yes	2011	B	A	AB
447A	IN	Jasper	Yes	2011	B	A	AB
447B	IN	Jasper	Yes	2011	C9	A	AC
447C	IN	Jasper	Yes	2011	A	A	A
449A	IA	Boone	Yes	2011	A	B	AB
449B	IA	Boone	Yes	2011	A	A	A
449C	IA	Boone	Yes	2011	A	C3	AC
449D	IA	Boone	Yes	2011	B	A	AB
452A	IA	Boone	Yes	2011	A	A	A
452B	IA	Boone	Yes	2011	C13	A	AC
453A	IA	Boone	Yes	2011	A	A	A
454A	IA	Pottawattamie	Yes	2011	B	A	AB
458B			Yes	2011	A	A	A
472A	SD	Bon Homme	Yes	2011	A	A	A
472B	SD	Bon Homme	Yes	2011	A	A	A
478A	NE	Dixon	Yes	2011	A	A	A

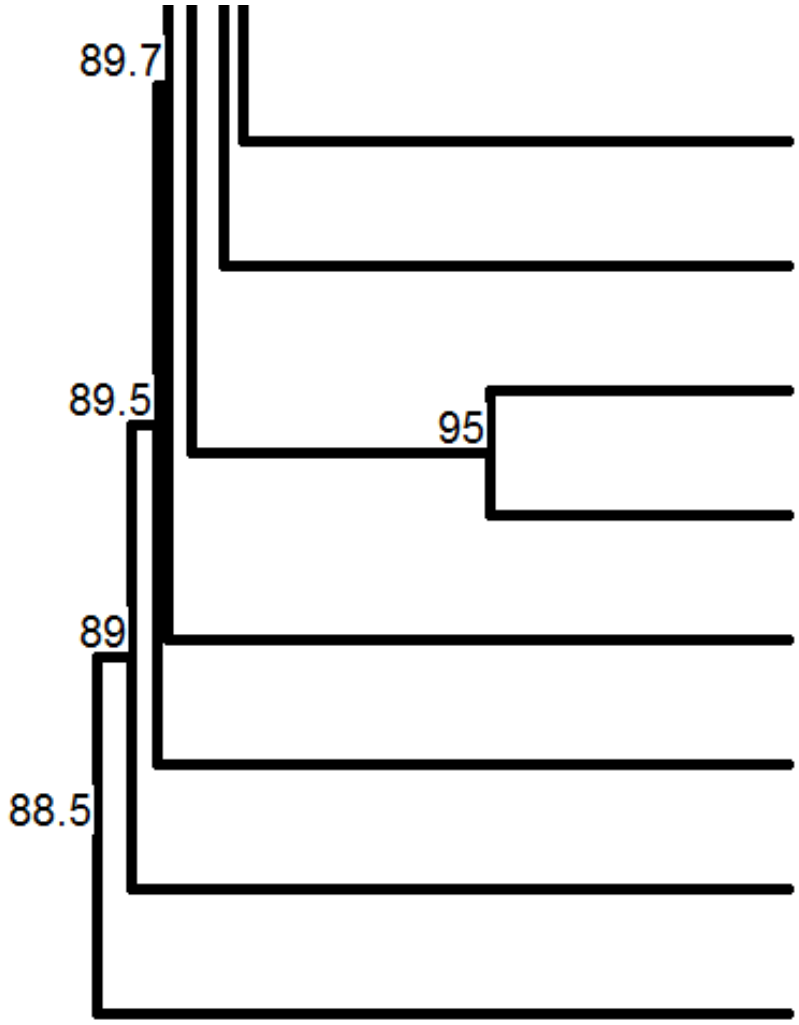
Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
478B	NE	Dixon	Yes	2011	B	A	AB
484A	NE	Nance	Yes	2011	A	A	A
485NDA	MN	Wilkin	Yes	2011	A	A	A
485NDB	MN	Wilkin	Yes	2011	A	A	A
486NDA	MN	Wilkin	Yes	2011	C17	A	AC
486NDB	MN	Wilkin	Yes	2011	A	A	A
488A			Yes	2011	A	A	A
488B			Yes	2011	A	B	AB
489B	NE		Yes	2011	A	B	AB
489D	NE		Yes	2011	B	A	AB
490B	NE	Madison	Yes	2011	A	A	A
495CA	NE	Holt	Yes	2011	A	A	A
495CB	NE	Holt	Yes	2011	C12	A	AC
495CC	NE	Holt	Yes	2011	A	A	A
495CD	NE	Holt	Yes	2011	B	A	AB
496CA	NE	Holt	Yes	2011	A	A	A
496CB	NE	Holt	Yes	2011	A	A	A
496CC	NE	Holt	NA	2011	C2	A	AC
499CA	NE	Knox	Yes	2011	A	A	A
499CB	NE	Knox	Yes	2011	A	A	A
504NDA	ND	LaMoure	Yes	2011	B	A	AB
504NDB	ND	LaMoure	Yes	2011	A	A	A
521CA	MN	Traverse	No	2011	A	A	A
572CA	KS	Wallace	Yes	2011	C9	A	AC
572CB	KS	Wallace	Yes	2011	A	A	A
572CC	KS	Wallace	Yes	2011	A	A	A
CN18-1	NE	Dawson	Yes	1969	A	B	AB
CN18-4	NE	Dawson	Yes	1970	A	A	A
CN38-1	NE	Furnas	Yes	1970	A	A	A
CN48-1	NE	Hitchcock	Yes	1972	A	A	A
CN68-1	NE	Keith	Yes	1975	A	A	A
CN72-1	NE	Chase	Yes	1974	A	A	A
CN72-25A	NE	Chase	Yes	1982	A	A	A
CN72-40	NE	Chase	Yes	1982	A	A	A
F32	NE	Morrill	Yes	2009	B	C5	BC
F78	NE	Scotts Bluff	Yes	2009	A	A	A
G2	NE	Scotts Bluff	Yes	2008	A	A	A
HANSONA	NE	Saunders	Yes	2011	A	A	A
Indiana	IN	Pulaski	Yes	2009	A	A	A

Table 1 continued

Isolate	State	County	Pathog-enicity	Year Isolated	AFLP Group	BOX-PCR Group	Overall Group
WELTZB	NE	Saunders	Yes	2011	A	A	A

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95
89
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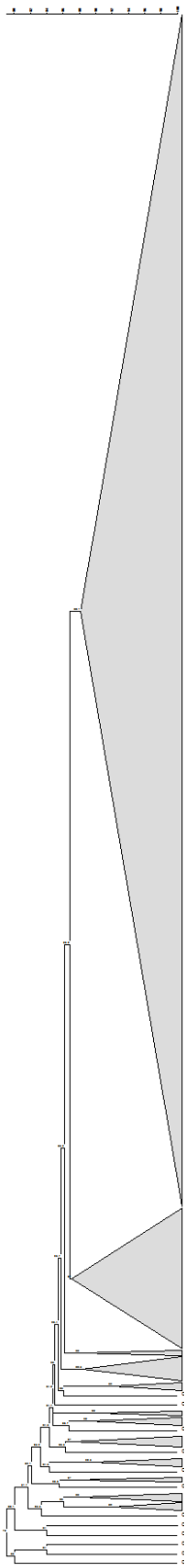

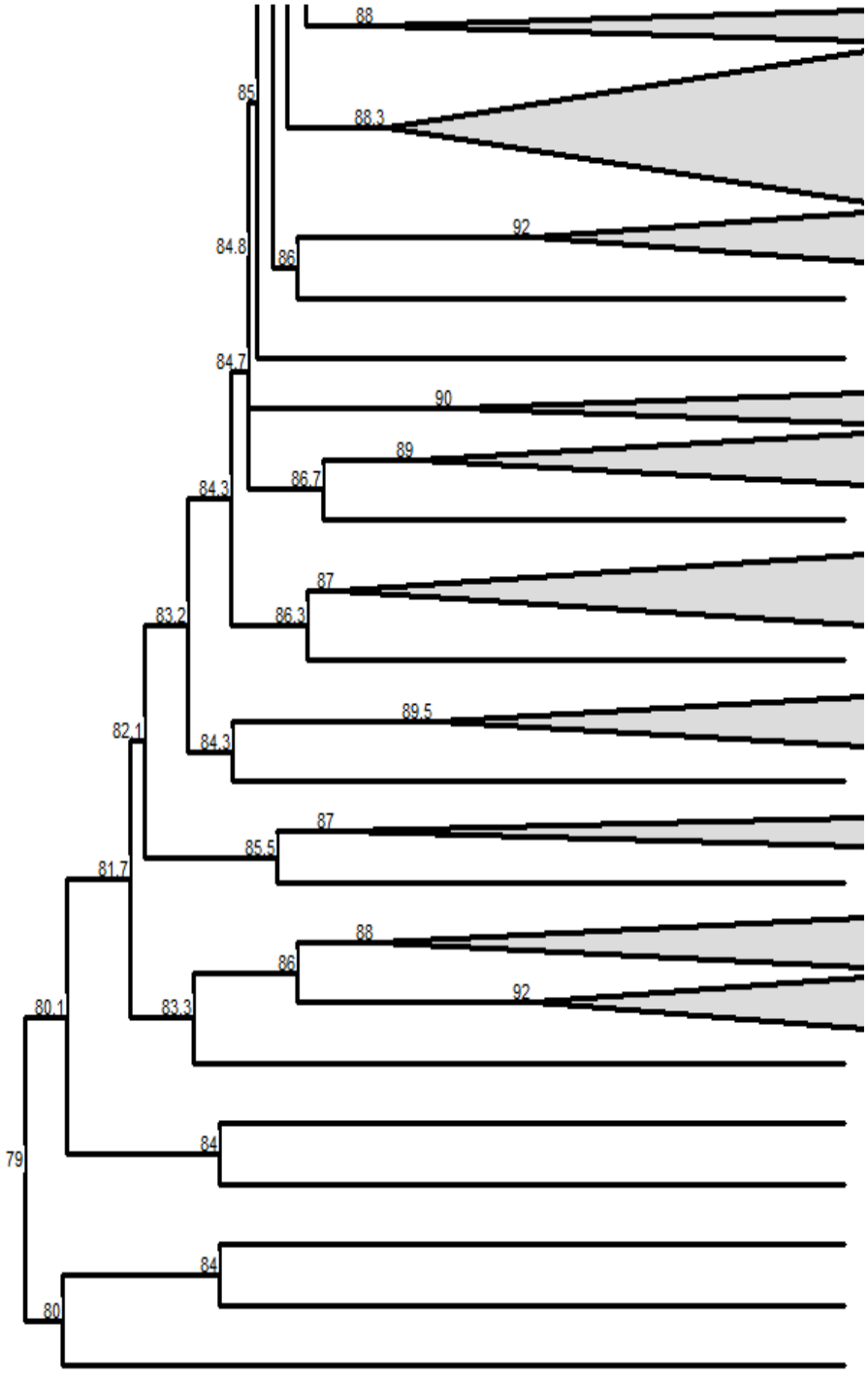
88
85
88.3
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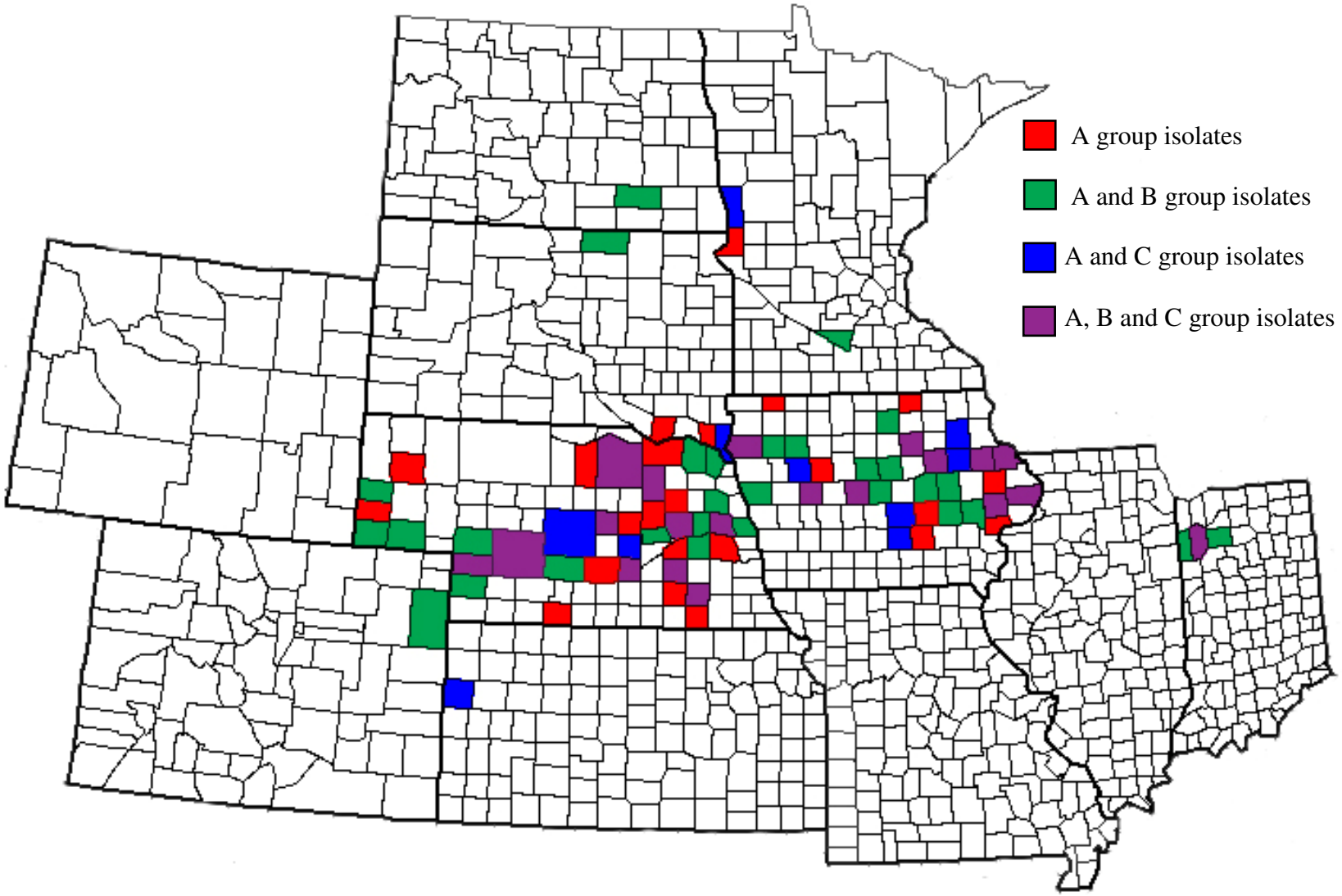

Figure 5. Distribution of isolates collected during the 2011 survey. Group A isolates had an average of 1.9 isolates per county represented on the map. Group A and B had an average of 4.5 isolates per county represented on this map. Group A and C had an average of 3.3 isolates per county on this map. Group A, B, and C had an average of 10.4 isolates per county represented on this map.

CHAPTER III

FOUR COMMON SETARIA SPECIES ARE ALTERNATE HOSTS FOR *CLAVIBACTER MICHIGANENSIS* SUBSP. *NEBRASKENSIS*, CAUSAL AGENT OF GOSS'S BACTERIAL WILT AND BLIGHT OF *ZEA MAYS*

Abstract

Goss's bacterial wilt and blight, caused by *Clavibacter michiganensis* subsp. *nebraskensis* (Cmn) has re-emerged as an important disease of *Zea mays* (corn) in the Midwest. Results from a 2011 multi-state survey indicated that *Setaria* spp. (foxtail) were commonly present in corn fields with a history of Cmn. The objective of this research was to determine if *Setaria* spp. that are common in the Midwest are susceptible to Cmn. In the greenhouse, seedlings of four *Setaria* spp. including *S. viridis* (green foxtail), *S. faberi* (giant foxtail), *S. verticillata* (bristly foxtail), *S. pumila* (yellow foxtail), and *Zea mays* (Golden Cross Bantam sweet corn) were inoculated with a suspension of 1.0×10^7 bacteria cells. A mixture of four Cmn isolates were used for inoculation whose virulence was demonstrated during previous testing. The trial was arranged in a randomized complete block design and repeated one additional time. Percent of symptomatic leaf area affected was visually estimated eight days after inoculation. Bacterial streaming was confirmed microscopically and Cmn was re-isolated from the four *Setaria* species. *S. faberi* was the most susceptible in both trials with 22% and 18% leaf area affected in trials 1 and 2, respectively. *S. viridis* was the next most susceptible having 12% and 10% leaf area affected, respectively. *S. verticillata* was also susceptible having 8% and 5% leaf area affected followed by *S. pumila*, which was the least susceptible with 5% and <1% leaf area affected, respectively. Results indicate that these four *Setaria* spp. are susceptible to Cmn and could serve as an alternate host and inoculum source potentially reducing the effectiveness of crop rotation to manage for Cmn.

Introduction

Goss's bacterial wilt and blight (*Clavibacter michiganensis* subsp. *nebraskensis*, Cmn) (Vidaver and Mandel, 1974) was first reported in three *Zea mays* L. (corn) fields in Dawson County, Nebraska in 1969 (Wysong et al., 1973). Cmn has since been confirmed in ten states including Nebraska, Colorado, Kansas, Iowa, Wyoming, South Dakota, Minnesota, Illinois, Indiana, and Texas (Schuster, 1975, Jackson et al., 2007, Ruhl et al., 2009, Korus et al., 2010, Malvick et al., 2010,). Cmn overwinters in infected plant residue from the previous season(s), as well as inside and on seed (Schuster, 1975).

Studies on host range were conducted by Schuster (1975) to determine what other species might serve as alternate hosts to Cmn. Schuster (1975) reported that teosinte (*Euchlaena mexicana* Schrad.), eastern gama grass (*Tripsacum dactyloides* L.), green foxtail (*Setaria viridis* (L.) Beauv.), grain sorghum (*Sorghum vulgare* L.), sudangrass (*Sorghum bicolor* (L.) Moench ssp. drummondii (Nees ex Steud.) de Wet & Harlan), sugarcane (*Saccharum officinarum*), and shattercane (*Sorghum bicolor* (L.) Moench ssp. arundinaceum (Desv.) de Wet & Harlan) are documented susceptible hosts of Cmn. Wysong et al., (1981) also reported that barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) was an alternate host, although this species was not confirmed in trials by Schuster (1975). Naturally occurring infection has been reported in *Sorghum bicolor* (L.) Moench ssp. arundinaceum (Desv.) de Wet & Harlan, *S. viridis* (L.) Beauv., and *Echinochloa crus-galli* (L.) Beauv. (Wysong et al., 1981). All other mentioned species' susceptibility were confirmed following artificial inoculation in greenhouse trials.

Setaria spp. are commonly observed growing in row crop fields, along terraces, waterways, field borders and roadsides. As Cmn becomes more prevalent, management

of alternate hosts may play an important role in protecting a corn crop from this particular pathogen. *S. viridis* (L.) Beauv. has been reported as an alternate host previously, and in earlier studies performed by Schuster (1975), although *S. pumila* (Poir.) Roemer & J.A. Schultes was not a confirmed host for Cmn. Since only two species were previously tested, and *Setaria* spp. continue to be a problem in row crop production, an experiment was designed to test four commonly occurring *Setaria* spp. in Nebraska for their susceptibility to Cmn following mechanical inoculation. These species included giant foxtail (*Setaria faberi* Herrm.), green foxtail (*Setaria viridis* (L.) Beauv.), bristly foxtail (*Setaria verticillata* (L.) Beauv.), and yellow foxtail (*Setaria pumila* (Poir.) Roemer & J.A. Schultes) to determine which species might serve as alternate hosts to the Cmn. The objective of this research was to determine if *Setaria* spp. that are common in the Midwest are susceptible to Cmn, and if so, to what degree (as determined via visual ratings).

Materials and Methods

Bacterial isolates and inoculum preparation. More than 600 Cmn isolates were collected from infected, symptomatic corn leaves submitted to the UNL Plant and Pest Diagnostic Clinic as part of a multi-state survey conducted during the 2011 growing season (Langemeier et al. 2012). Four isolates were randomly selected with diverse geographic origins in Nebraska and with confirmed virulence following inoculation of Golden Cross Bantam sweet corn (GCB) in the greenhouse that produced typical symptoms (*data not shown*). The isolates included in the study originated from Dodge, Cheyenne, Hall, and Butler counties in Nebraska.

Inoculum was made from a mixture of all four isolates, consisting of 25% from each isolate (Schuster, 1972). Isolates were grown on *Corynebacterium nebraskense* selective medium (CNS), a selective agar medium developed for isolating the Cmn pathogen, (Gross and Vidaver, 1979) for four days at 25 C. Bacterial colonies were then suspended in 10 mM PO_4 buffer at a pH of 7.1 (Carlson et al., 1979). Inoculum concentrations were adjusted to a concentration of 1.0 $x10^7$ (Schuster, 1975) with a Spectrophotometer (Spectronic 20D+ Thermo Scientific Waltham, MA), set at 620 nm.

Grass seed. The *S. viridis* (L.) Beauv., *S. faberi* Herrm., *S. verticillata* (L.) Beauv., and *S. pumila* (Poir.) Roemer & J.A. Schultes seed were provided by the University of Nebraska-Lincoln Department of Agronomy and Horticulture. The seed were stored in a freezer in the greenhouses at -15 C prior to experimental setup.

Greenhouse conditions and experimental design. The experimental design was a split-plot arranged in a randomized complete block design, where plants inoculated with bacteria were maintained separate from those inoculated with sterile phosphate buffer to prevent cross-contamination. The experiment was repeated in time, and conducted in the University of Nebraska-Lincoln Plant Pathology greenhouse. The greenhouse day/night temperature was 28/22 C. Supplemental artificial light was used to produce 16 hours of light to eight hours of darkness per 24 hr period.

Setaria spp. seeds were planted into steam pasteurized soil in 15.2 cm. pots at a seeding rate of 30 seed per pot, and thinned to five plants per pot one week after emergence. *Setaria* spp. seed were planted at a depth of 1.5 to 2.5 cm, to optimize germination rates (Buhler and Mester, 1991; Dawson and Bruns, 1962). GCB was

planted a depth of 3.75 cm. GCB was included in the study as a positive control to insure that the inoculum was virulent and produced typical symptoms (Vidaver, 1977, Smidt and Vidaver, 1987). GCB was planted at five seeds per pot and thinned to three plants per pot. Inoculation took place at 30 days and 24 days after planting for trial 1 and trial 2, respectively. This is when plants were large enough to effectively use the inoculation device (Table 1). Inoculation was conducted via a modified multiple needle inoculation method as described by Andrus (1948). Each pot was mechanically inoculated by squeezing the inoculation device in four different places with the center of the device located at the center of the whorl of three to five stems per squeeze, ensuring that each of the five *Setaria* spp. and three GCB plants had one or more leaves that were wounded and inoculated (Figure 1).

Koch's postulates were completed with symptomatic leaf tissue. Bacteria were isolated from symptomatic leaf tissue and cultured onto CNS medium. Agdia ImmunoStrips® (ELISA) test kit (Agdia Inc. Elkhart, IN) designed for *Clavibacter michiganensis* subsp. *michiganensis* were used to test both symptomatic leaf tissue and cultures. Manufacturer's recommended procedures for testing were used (Korus et al., 2010). Symptomatic leaf tissue was also examined microscopically (20X) for bacterial streaming. Finally disease symptoms were visually estimated eight days after inoculation and percentage of leaf area affected by lesion development was estimated visually (0-100%) (Suparyono and Pataky, 1989.).

Statistics. An ANOVA was conducted on both trials with SAS 9.2, (GLM Procedure, Version 9.2, SAS Institute Inc., Cary, NC) using an F-test.

Results

Typical symptoms of Cmn were observed and documented on GCB indicating that the inoculum was viable (Figure 2). In both studies, the five replications of all the *Setaria* spp. and GCB showed at least one symptomatic leaf per pot in the pots inoculated with Cmn. Leaves were tested with the ImmunoStrips® and all tested positive (*data not shown*). Bacterial streaming was also confirmed in all five replications that were inoculated with Cmn (Figure 4). Koch's postulates were performed and bacterial colonies typical (apricot-orange in color, circular, convex, glistening, and butyrous delimited from a darker center zone) Cmn were observed on CNS (Vidaver and Mandel, 1974)

Atypical leaves (yellowing, or necrotic tissue) in pots inoculated only with phosphate buffer were also tested with the ImmunoStrips®, and all were examined microscopically for bacterial streaming, but none of the leaves tested positive with the ImmunoStrips®, and no bacterial streaming was observed.

Disease severity. Symptoms developed on inoculated leaves in all four species of *Setaria* spp. Percent of leaf area affected by lesion development for *S. faberi* Herrm. was 22% in trial 1 and 18% in trial 2 (Figure 3). The next most susceptible species was *S. viridis* (L.) Beauv. In trials 1 and 2, 12% and 10% of leaf area was affected (Table 1). *S. verticillata* (L.) Beauv. had 8% and 5% leaf area affected in trials 1 and 2 respectively. *S. pumila* (Poir.) Roemer & J.A. Schultes was the least susceptible in these trials with 5% and <1% leaf area affected for the two trials.

A significant difference between trial 1 and trial 2 was observed when using a P-value of .05, so trials were not combined for analysis.

Discussion

Plants of the four *Setaria* spp. were found to be susceptible to Cmn, but at varying severity. *S. viridis* (L.) Beauv., a known alternate host for Cmn, was reported to be susceptible to Cmn in both greenhouse trials, as well as naturally occurring in field settings (Schuster et al., 1975, and Wysong et al., 1981). Contrary to previous research by Schuster, (1975) this research suggests that *S. pumila* (Poir.) Roemer & J.A. Schultes is an alternate host for Cmn. This research also confirmed that both *S. verticillata* (L.) Beauv. and *S. faberi* Herrm. are hosts of Cmn. This may be the result of a change in the biology of the pathogen over time, genetic differences in the population tested for each *Setaria* spp., or a change in the biology of the host plant.

These possibilities are the reason for using a mixture of isolates. Documented differences in colony color, colony margin development, and bacteriocin production have been reported with Cmn (Vidaver and Mandel, 1974, and Smidt and Vidaver, 1987). More recently, differences in the DNA makeup of Cmn have been reported (Agarkova et al., 2011). Research also shows differences in virulence among different isolates collected within the same state (Malvick et al., 2012). If these differences exist, there is the possibility that one isolate may be virulent on *Zea mays*, but not on *Setaria* spp. and vice versa. Therefore a representative mixture of Cmn was randomly selected from different *Zea mays* growing areas of the state where *Zea mays* and *Setaria* spp. may be growing in the same field.

This research should help producers better manage for Cmn by knowing that control of these four *Setaria* spp. could reduce inoculum. One effective way to reduce inoculum load is to rotate with non-host crops (Schuster, 1975). Since Cmn is a residue-borne pathogen, it is possible for Cmn to over winter in infected corn residue, infect a *Setaria* species growing in a field of soybean, wheat, or any other non-host crop, and survive in the *Setaria* residues until the following growing season. During subsequent seasons, the Cmn surviving in the *Setaria* residue could infect the corn crop, thus reducing the effectiveness of rotation by not eliminating the inoculum source.

In summary, this research confirmed that *S. pumila* (Poir.) Roemer & J.A. Schultes, *S. verticillata* (L.) Beauv., and *S. faberi* Herrm. are alternate hosts for Cmn. It also confirmed *S. viridis* (L.) Beauv. as an alternate host as reported by Schuster (1975), and Wysong et al. (1981). Additional research is necessary to test if other species not previously tested could be potential alternate hosts for this particular pathogen.

Acknowledgement

The authors thank Lowell Sandell, University of Nebraska-Lincoln for providing the *Setaria* seed used in this experiment. I would also like to thank Dr. Thomas Powers and Pat Lambrecht for their technical assistance.

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Table 1. Average plant height taken to the center of the whorl at inoculation, days until first symptoms appeared (freckling), and percent leaf area affected for greenhouse inoculated *Setaria* species.

Species common names	Average height at inoculation[z]	Days until symptoms appeared	Percent leaf area affected by disease
	cm	days	%
Setaria faberri giant foxtail	21,20[y]	4,5	22,18
Setaria viridis green foxtail	20,19	5,6	12,10
Setaria verticillata bristly foxtail	18,17	5,5	8,5
Setaria pumila yellow foxtail	14,14	5,6	5, <1
Zea mays Golden Cross Bantam sweet corn	37,35	5,6	25,20

[z]Average height measured at the top of the whorl.

[y]Trial 1 and 2, respectively.

Figure 1. Modified multiple needle inoculation tool, designed to inoculate small, narrow leaved plants horizontally.

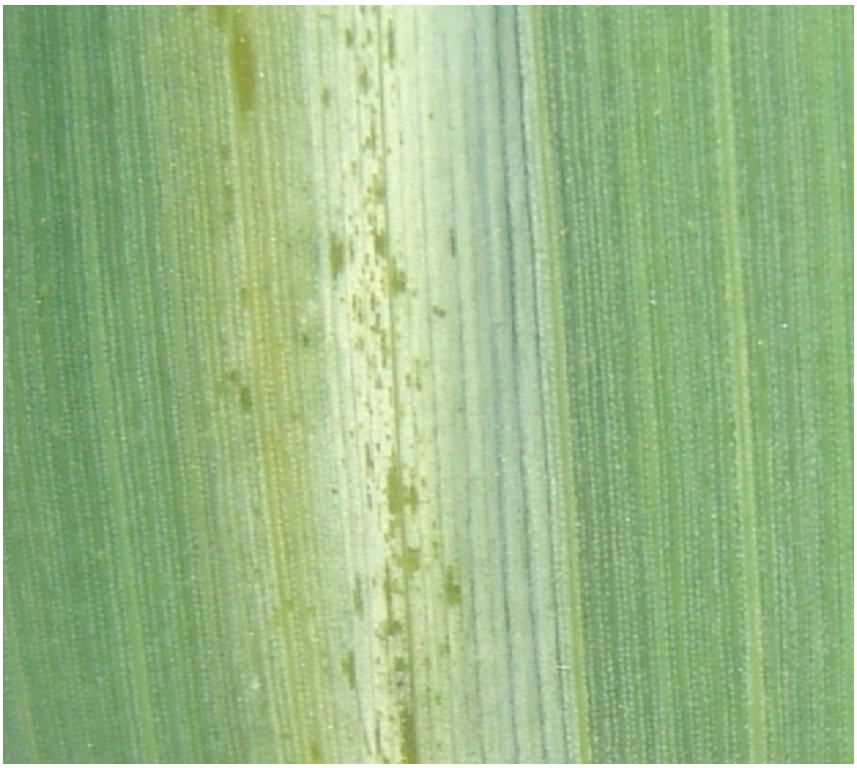

Figure 2. Typical symptoms (freckling and water soaking) observed on GCB.

Figure 3. Symptoms observed on *S. faberi* Herrm. Symptoms observed on the other three species were similar to the symptoms observed above.

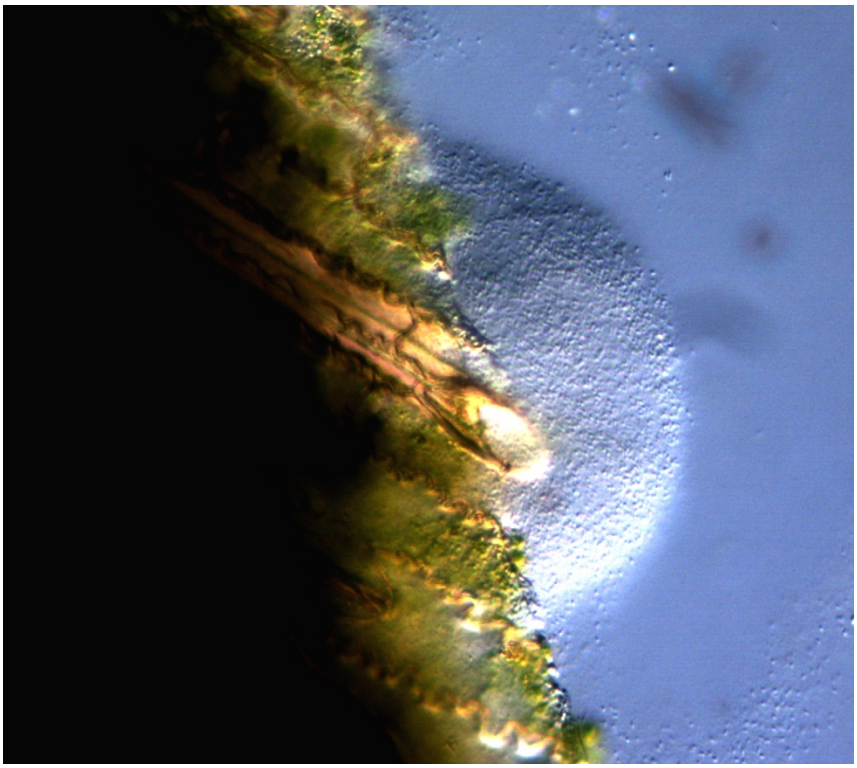

Figure 4. Bacterial streaming observed on GCB.

APPENDIX

Company and Representative Name:_____
Address: _____
City:_____State:_____Phone number:_____

UNL, ISU, and Industry Collaborative Goss's Wilt Survey 2011

Please complete all portions of the survey by filling in the blanks and/or marking the appropriate box.

Name of producer: _____ Date:_____
County, state:_____ Phone number:_____
Field location or I.D.:_____ GPS or T/R/S* coordinates:_____
Corn hybrid and Brand:_____ Hybrid Goss's wilt rating: _____
Planting population:_____ Planting date:_____
*Township/Range/Section

Field History:

What is the cropping rotation in this field?
Continuous corn: ☐ Corn-soybean: ☐ Corn-corn-soybean: ☐ Wheat-corn-fallow: ☐ Sugarbeet-corn-dry bean: ☐
If corn-corn-soybean, which year of corn production? First year corn: ☐ Second year corn: ☐
List any alternative crops that appear in your rotation (Select all that apply.):
Sunflower: ☐ Proso millet: ☐ Potato: ☐ Alfalfa: ☐ Sugarbeet: ☐ Dry bean: ☐
Other or most common rotation (Please describe.): _____

What is the current tillage practice in this field?
Conventional till: ☐ Strip-till: ☐ No-till: ☐ Ridge-till: ☐
Other (Please describe.): _____

How long has this been the tillage practice in this field?
1-2 Years: ☐ 3-5 Years: ☐ 6-10 Years: ☐ >10 Years: ☐

What was the estimated residue cover at the time of the Goss's wilt development this year?
0%: ☐ 1-15%: ☐ 15-30%: ☐ 30-45%: ☐ 45-60%: ☐ 60-75%: ☐ >75%: ☐

What is the estimated yield history for corn in this field in bu./acre?
<100: ☐ 100-150: ☐ 150-200: ☐ 200-250: ☐ >250: ☐

Was livestock manure applied in the fall, spring, or were livestock allowed to graze?
Fall: ☐ Spring: ☐ Grazing: ☐ Grazing and fall application: ☐ Grazing and spring application: ☐
Type of manure applied? Poultry: ☐ *Swine:* ☐ *Cattle:* ☐ Other: _____

Irrigation:

What type of irrigation was used on this field this season, and how many inches were applied?
Center pivot: ☐ Sub-surface: ☐ Gravity/flood: ☐ None: ☐
Inches of water applied per acre:_____

Disease History and Severity:

Have you ever confirmed Goss's wilt in this field?
No: ☐ 1-2 Years: ☐ 3-5 Years: ☐ 6-10 Years: ☐ >10 Years: ☐

What is the percent estimated yield loss in the previous corn crop due to Goss's wilt?
0%: ☐ 1-10%: ☐ 10-20%: ☐ 20-30%: ☐ 30-40%: ☐ 40-50%: ☐ >50%: ☐

If you have had Goss's wilt in the past, what would best describe the severe weather leading up to the disease? (Select all that apply.)
None: ☐ Hard rainfall: ☐ Sandblasting: ☐ High winds: ☐ Hail: ☐

For lab use only. Sample: Yes ☐ No ☐ Goss's wilt: Positive ☐ Negative ☐
Date received _____ Date completed_____

Were other diseases present in this field this season, and if so, what?
Diseases:_____

Was there any severe weather this season? (Select all that apply.)
None: ☐ **Hard rainfall:** ☐ **Sandblasting:** ☐ **High winds:** ☐ **Hail:** ☐

Approximately what percent of plants in this field appear to have Goss's wilt now?
0%: ☐ 1-10%: ☐ 10-20%: ☐ 20-40%: ☐ 40-60%: ☐ 60-80%: ☐ >80%: ☐

Approximately what percent of leaf area in this field is damaged by Goss's wilt lesions now?
0%: ☐ 1-10%: ☐ 10-20%: ☐ 20-40%: ☐ 40-60%: ☐ 60-80%: ☐ >80%: ☐

What is the estimated yield loss in the current crop due to Goss's wilt?
0%: ☐ 1-10%: ☐ 10-20%: ☐ 20-30%: ☐ 30-40%: ☐ 40-50%: ☐ >50%: ☐

What is the approximate growth stage of the crop?
V1-V3: ☐ V4-V6: ☐ V7-V9: ☐ V10-Tasseling: ☐ **Silking**-milk: ☐ **Dough-maturity**: ☐

Insects, Weeds, Nutrients and Pesticides:

List the 2 most important insect/arthropod pests in this field this season, estimate the damage each caused, and to what plant part(s):

Insect 1:_____ Root: ☐ Ear: ☐ Stalk: ☐ Leaf: ☐

0%: ☐ 1-10%: ☐ 10-20%: ☐ 20-30%: ☐ 30-40%: ☐ 40-50%: ☐ >50%: ☐

Insect 2:_____ Root: ☐ Ear: ☐ Stalk: ☐ Leaf: ☐

0%: ☐ 1-10%: ☐ 10-20%: ☐ 20-30%: ☐ 30-40%: ☐ 40-50%: ☐ >50%: ☐

*What are your two most problematic grassy weeds **this season**, and at what density do they occur in plants/square yard?*
Weed 1:_____ **Density:**_____/sq. yd. **Weed 2:**_____ **Density:**_____/sq. yd.

*What were your two most problematic grassy weeds **last season** and at what density did they occur in plants/square yard?*
Weed 1:_____ **Density:**_____/sq. yd. **Weed 2:**_____ **Density:**_____/sq. yd.

Have any additional foliar or soil insecticides been applied this season, and if so at what rate?
Insecticide:_____ **Rate:**_____

Have any herbicides been applied this season, if so when and at what rate?
Pre-plant: ☐ At planting: ☐ **Post plant:** ☐ **Pre and post plant applications:** ☐
Herbicide:_____ **Rate:**_____
Herbicide:_____ **Rate:**_____
Herbicide:_____ **Rate:**_____

Have any additional foliar fungicides been applied this season, and if so at what rate(s) and when?
Fungicide:_____ **Rate and timing:**_____
Fungicide:_____ **Rate and timing:**_____

Has nitrogen, phosphorus, potassium or other nutrients been applied this season and if so, how?

Nitrogen	**Pre**-plant: ☐	At planting: ☐	In season side-dress: ☐	In season fertigate: ☐
Phosphorus	**Pre**-plant: ☐	At planting: ☐	In season side-dress: ☐	In season fertigate: ☐
Potassium	**Pre**-plant: ☐	At planting: ☐	**In season side**-dress: ☐	In season fertigate: ☐
Other nutrients	**Pre**-plant: ☐	At planting: ☐	In season side-dress: ☐	In season fertigate: ☐

N-P-K ratio (example 10-34-0):_____
Other nutrients in lbs./acre (example 10 lbs. Zn.):_____

IOWA STATE UNIVERSITY

University of Nebraska Lincoln

Dear Client, May 1, 2011

Thank you for participating in this very important survey to help us to better understand the disease, Goss's bacterial wilt and blight of corn. As part of this multi-state research project between the University of Nebraska-Lincoln and Iowa State University, we are conducting a survey to collect information about your experience with the disease. The results of the survey will be statistically analyzed to determine the most important agronomic factors for disease development. In exchange for completing our survey, we will provide you with diagnostic testing of a leaf sample from that field at NO COST.

In addition, for every field you submit a completed survey (all questions answered) and a leaf sample for Goss's wilt testing, you will be entered into a random drawing for a $100 gift card from Cabelas. This is a special thank you for helping us complete this project that will help us to make better management recommendations for Goss's wilt! Please note that it is NOT necessary to have a corn sample with the disease to submit a survey and to be entered for the gift card drawing.

Your participation in this survey is voluntary. You are not under any obligation to complete this survey. However, participants who complete the survey will be entering into a drawing for one of two $100 Cabelas gift cards for each the producer and ag-business representative. The overall odds of receiving a gift card depends upon the number of people who participate and the number of fields for which you choose to submit survey information for, but you have at least a 1 in 500 chance of receiving a gift card. It should take about 10-15 minutes to complete this survey. There are no known risks involved in participating. Please see the back side of this letter for additional information and brief instructions for completing the survey and submitting a sample.

The confidentiality of the participants is kept in paper folders and through Microsoft Excel documents in a section of our laboratory. The paper documents are kept where only people directly involved in the project will know their location and have access to them. Use of your name on the survey will be removed from the statistical analyses, final data and results summary.

We greatly appreciate your time in helping us better understand the disease Goss's bacterial wilt and blight, which will help us to improve future management recommendations. Please respond to this survey between July 1, 2011 and September 1, 2011. Results of the Goss's wilt test on your sample(s) will be reported back to you from the UNL Plant and Pest Diagnostic Clinic. You may contact us with any questions you have.

Thank you for your time and assistance.

Sincerely,

Tamra A. Jackson
Extension Plant Pathologist
(402) 472-2559

Greg Kruger
Cropping Systems Specialist
(308) 696-6740

Alison Robertson
Assistant Professor
(515) 294-1741

If you have any questions about the research, or wish to report any concerns, please contact the UNL IRB office at (402) 472-6965.

IOWA STATE UNIVERSITY

Nebraska Lincoln

Instructions for completing the Goss's wilt survey:

- Please check the box that best describes your situation for each question.
- Only check one box per question, unless otherwise directed.
- Complete only one survey per field. Please complete an additional survey for each field for which you want a sample tested. You may use the same self-addressed postage paid envelope for submitting several surveys and samples simultaneously. Please only submit up to 5 surveys/samples per producer.
- If you wish to submit more than one survey and sample representing multiple fields, please attach the survey to the appropriate Ziploc bag.
- Fill in blanks to the best or your knowledge (for example - 125 lbs/ acre N)
- Feel free to attach any questions you have regarding Goss's wilt.
- It is NOT necessary to have confirmed Goss's wilt in the field to complete a survey.
- When submitting a sample, please place at least 1 leaf inside the Ziploc bag, seal it, and insert it in the self-addressed postage paid envelope with your completed survey.
- Please place up to 4-5 leaves in a ziploc bag.
- Only add a DRY paper towel if samples are wet. Do NOT add water.
- Please mail early in the week so plant material doesn't degrade over the weekend.
- We will contact you with the testing results for all fully completed surveys.
- Please do not return samples before July 1, 2011

Samples will be shipped to the UNL Plant & Pest Diagnostic Clinic at the address below:

UNL Plant and Pest Diagnostic Clinic
c/o Goss's Wilt Survey
448 Plant Science Hall
Lincoln, NE 68583-0722

If you have any questions about the research, or sample submission, please contact The UNL Plant and Pest Diagnostic Clinic at (402) 472-2559.

Funding for this project is provided by a competitive grant from the North Central Regional Integrated Pest Management Center which is sponsored by the United States Department of Agriculture, Cooperative State Research, Education and Extension Service.

We sincerely thank our industry partners for helping us conduct this survey.