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Goss's Bacterial Wilt Development and *Clavibacter michiganensis* subsp. *nebraskensis* Interactions with Spray Adjuvants

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GOSS'S BACTERIAL WILT DEVELOPMENT AND *CLAVIBACTER MICHIGANENSIS* SUBSP. *NEBRASKENSIS* INTERACTIONS WITH SPRAY ADJUVANTS

by

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Goss's bacterial wilt and leaf blight of corn (*Zea mays* L.), causal agent *Clavibacter michiganensis* subsp. *nebraskensis*, was first confirmed in Dawson County, NE in 1969. Disease incidence decreased in the 1980's due to various management strategies and disease developed sporadically until the early 2000's when it re-emerged and was economically important. A Midwest, multistate survey conducted in 2011 suggested farming practices that may have contributed to the pathogen's re-emergence. The use of agricultural pesticides was associated with Goss's wilt. Since spray adjuvants are often used with pesticides, and physical characteristics of these adjuvants may enable infection of the leaf by epiphytic *C. michiganensis* subsp. *nebraskensis*, greenhouse and field studies were conducted to assess the effect of adjuvants on Goss's wilt severity. A preliminary greenhouse study was conducted to determine if an epiphytic population of *C. michiganensis* subsp. *nebraskensis* could be established and initiate infection. The population became established and disease severity was higher when plants were less mature. To evaluate the effect of adjuvants on disease development with adjuvants, a greenhouse study was established. Adjuvants tested did not cause a consistent increase in

disease severity between trials. An inhibition test was designed to determine if spray adjuvants inhibited bacterial growth *in vitro* at different concentrations. Results showed minimal inhibition at label rate for NIS, and consistent inhibition at 10X label rate for NIS with some inhibition for MSO and COC. Field studies were conducted in southwest Nebraska in 2014 and 2015. Disease severity was lower in adjuvant treatments in 2014. In 2015, no differences for disease severity or systemically infected plants were detected among treatments. These data indicate that spray adjuvants commonly used in corn production are not causing an increase in Goss's wilt severity. Rather, adjuvants at higher rates may reduce the population of epiphytic *C. michiganensis* subsp. *nebraskensis* to below levels required for infection. Further research is needed before recommending non-label rate of these adjuvants under field conditions.

DEDICATION

I would like to dedicate this thesis to my parents, Donald and Barbara Schlund. They have always taught me to be persistant and diligent in my work and to never give up, even when the road gets rough.

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CHAPTER I

LITERATURE REVIEW

History of Corn

In 1492, *Zea mays* (L.) was first discovered by Columbus' men in Cuba and has been classified as dent corn, popcorn, waxy corn, flint corn, sweet corn, pod corn, and flour corn (Gibson and Benson 2002). While corn may be grown throughout the world, it possesses different names. Corn is also called maize or Indian corn in the United States, but may be referred to as wheat in England or oats in Scotland and Ireland (Gibson and Benson 2002). It is believed to have been domesticated in the Tehuacan Valley, Mexico where 80,000 year old pollen grains were discovered (Gibson and Benson 2002). Historically, corn has been grown as far north as North Dakota, south to Argentina and Chile, on both sides of the St. Lawrence Valley, and located in the high valleys of the Andes Mountains (Gibson and Benson 2002). Once settlers arrived and cleared wooded areas, corn was grown in Ohio, Iowa, Indiana, and Illinois and neighboring states (Gibson and Benson 2002).

Yield potential was low when corn was first grown in the United States. According to Gibson and Benson (2002), 62 million acres of corn were grown in the 1880's. This escalated to 95 million acres by 1900, 100 million acres by 1910, and a record total of 111 million acres in 1917 (Gibson and Benson 2002). Prior to 1940, U.S. yield averaged 31.7 bushels per acre (Gibson and Benson 2002). Mass production of corn was not as abundant until technological advances occurred. Yield increases began in the 1940's and 1950's, which rose to an average 109.5 bushels per acre in 1979 (Gibson and Benson 2002). Increased yield potential is due not only to breeding efforts, but the production and use of fertilizers after World War II. Excess nitrogen left over from bomb assemblies was applied as ammonium nitrate pellets in the 1940's (Ganzel and Reinhardt

n.d.). Due to its explosive nature, pellet use declined and anhydrous ammonia was applied in the 1960's (Ganzel and Reinhardt n.d.). After these developments, agricultural fertilizers became accessible and essential to reach high yield potential.

Corn prices have fluctuated over time depending on supply and demand, economy, and the environment. Corn prices averaged $2.00 per bushel from 1972 to 2005 (United States Department of Agriculture 2010) and increased to $5.00 from 2006 to 2009 (Wise and Mueller 2011), particularly with the use of corn for ethanol production. There are many industrial uses for corn including grain products, silage, food, and industrial products (Gibson and Benson 2002). Corn is an important commodity in our society and without it, many of our household items would not be available. Unfortunately, plant diseases can be detrimental to corn production if not managed. One of the most important diseases of corn during recent years is Goss's bacterial wilt and leaf blight.

Goss's Bacterial Wilt and Blight

Goss's bacterial wilt and leaf blight was first identified in 1969 in Dawson County, Nebraska near Lexington (Wysong et al. 1973). Goss's wilt has also been referred to as Nebraska leaf freckles and wilt, bacterial leaf freckles and wilt, bacterial leaf blight and wilt, Nebraska wilt and leaf freckles (Schuster 1975; Jackson et al. 2007a), and leaf freckles and wilt (Jackson et al. 2007a). Goss's wilt received its official name after former Department of Plant Pathology chair, and first dean of the Graduate College at the University of Nebraska-Lincoln, Dr. R.W. Goss, who was considered to pioneer modern plant pathology in Nebraska (Jackson et al. 2007a).

Since its discovery, the abundance of susceptible corn hybrids in corn growing areas made it easy for the pathogen to spread. By 1975, the disease was confirmed in neighboring states (Vidaver et al. 1981). Disease was present in 54 counties in Nebraska and five of the six bordering states including Iowa, Kansas, Colorado, South Dakota, Wyoming, Illinois, and Wisconsin (Jackson et al. 2007a). Goss's wilt was reported in western Iowa in 1971, Kansas in 1973, Colorado and South Dakota in 1974 (Schuster, 1975), Illinois in 1980, and Wisconsin in 1981 (Wysong et al. 1981). Management practices allowed for a reduction in disease incidence and severity, but it continued to develop sporadically in susceptible varieties (Jackson et al. 2007b). However, after nearly two decades this pathogen re-emerged in the U.S. Corn Belt. The reasons for Goss's wilt re-emergence is not fully understood, but a combination of favorable conditions are probable causes (Jackson et al. 2007b). Historically, reduced tillage and continuous corn were heavily utilized prior to 1969 (Schuster 1975). Goss's wilt had not been confirmed state-wide across Nebraska for nearly two decades prior to 2008 (Jackson 2009). It is possible that its re-emergence was a result of a lack of hybrid screening for disease resistance after the seeming disappearance of the disease for decades (Jackson et al. 2007a; b).

Since its re-emergence, the disease has developed in the Midwest U.S. corn growing states, as well as the Canadian provinces of Alberta (Howard et al. 2015), Manitoba, and Ontario (Desjardins 2010). Goss's wilt has been the predominant disease since 2005 in western Nebraska, southeast Wyoming, and northeast Colorado (Jackson 2008). Reports of disease development in new areas have become increasingly common during recent years. In August 2008, leaf samples from hybrid corn and popcorn from

Pulaski and Jasper counties, respectively, in Indiana were confirmed to be infected with the Goss's wilt pathogen (Ruhl et al. 2009). This was the first report of Goss's wilt in Indiana (Ruhl et al. 2009). In Nebraska, the pathogen was confirmed in 24 counties in August 2009 (Jackson 2009). Goss's wilt was also reported in two Minnesota fields in Stephens and Chippewa counties in 2009 (Malvick et al. 2010). Samples submitted to the Plant and Pest Diagnostic Clinic at the University of Nebraska-Lincoln from Dallam County, Texas presented symptomatic lesions in 2009. This was also the first report of Goss's wilt in Texas (Korus et al. 2011). North Dakota State University Plant Diagnostic Lab confirmed the Goss's wilt pathogen in 2011 (Friskop et al. 2014). It has also been confirmed in areas of Michigan (Jackson-Ziems et al. 2012; Howard et al. 2015). Goss's wilt was confirmed in Montana (2013) and Louisiana in 2013 (Singh et al. 2015). This pathogen was also found in Holt County, Missouri when samples were submitted to the University of Missouri Plant Diagnostic Clinic for testing (Sweets and Hosack 2014). While symptoms may be quite characteristic, diagnosis can be difficult due to other diseases with similar appearances. Goss's wilt is commonly misidentified in the field as Stewart's wilt or northern corn leaf blight (Robertson and Jesse 2008). Thus, it is quite possible that farmers were unable to differentiate the disease and made misdiagnoses.

The common and scientific name has changed since this pathogen was first reported. *Corynebacterium michiganense* subsp. *nebraskense* (Vidaver and Mandel 1974) Carlson and Vidaver 1982, comb. nov. had its name changed to *Clavibacter michiganensis* subsp. *nebraskensis* corrig (Vidaver and Mandel 1974) Davis et al. 1984, comb. nov. The pathogen was reclassified based on the presence of these phytopathogenic coryneform bacteria having peptidoglycans with 2,4-diaminobutyric

acids (Davis et al. 1984). The genus *Corynebacterium* was changed to *Clavibacter* by regrouping the bacteria (Eichenlaub and Gartemann 2011). *Clavibacter* only contains one species within *Microbacteriaceae*, being *Clavibacter michiganensis* (Eichenlaub et al. 2006; Evtushenko and Takeuchi 2006). *C. michiganensis* is divided into five subspecies based on host specificity: *C. michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *nebraskensis*, and *C. michiganensis* subsp. *tessellarius* (Waleron et al. 2011). Questions still remain on the origin of *C. michiganensis* subsp. *nebraskensis*. Schuster (1975) speculated that *Corynebacterium insidiosum*, causal agent of bacterial wilt of alfalfa, could have mutated or transformed from pesticide use or other farming practices.

Pathogen Biology and Ecology

Morphological and physical characteristics of *C. michiganensis* subsp. *nebraskensis* have been described. The bacteria are gram-positive, catalase-positive, oxidase-negative (Vidaver and Mandel 1974), coryneform-shaped, non-motile rods which produce fluidal orange-yellow colonies on nutrient broth yeast extract (NBY) and *Corynebacterium nebraskense* selective (CNS) agars (Gross and Vidaver 1979). They have also been described as non-acid fast, pleomorphic rods, lacking endospore formation, non-motile, obligate aerobes, not producing tyrosinase, lipase, or urease, not utilizing nitrite, and not reducing nitrate (Davis et al. 1984). According to Eichenlaub and Gartemann (2011), there is also no type three secretion system used by other bacterial pathogens.

Bacterial morphology includes circular, convex, butyrous, glistening colonies with entire margins, which may differ in diameter or presence of an outer margin

(Vidaver and Mandel 1974). Carotenoid pigments give the bacteria an orange color, and exopolysaccharides make them appear mucoid (Evtushenko and Takeuchi 2006). It has also been reported that thiamine is an essential component for orange pigmentation and growth of the bacteria (Schuster 1975). Morphology is not the same for all bacterial strains. Smidt and Vidaver (1987) reported four types of bacterial strains that differed in morphology, fluidity, and pigmentation. Differences in bacteria morphology may be contributed to the time span between collections (Smidt and Vidaver 1987).

Symptoms and Epidemiology

Strain variability may be related to host range. Although bacteria in *C. michiganensis* are typically host specific, they may infect and present similar symptoms on other hosts (Waleron et al. 2011). *C. michiganensis* subsp. *nebraskensis* infects popcorn, sweet corn, dent corn, and food-grade corn (Jackson et al. 2007a). Alternative hosts include teosinte (*Euchlaena mexicana* Schrad.), green foxtail (*Setaria viridis* (L.) Beauv.), grain sorghum (*Sorghum bicolor* (L.) Moench), sudangrass (*Sorghum bicolor* (L.) Moench ssp. *drummondii* (Nees ex Steud.) de Wet and Harlan), sugarcane (*Saccharum officinarum* L.), shattercane (*Sorghum bicolor* (L.) Moench ssp. *arundinaceum* (Desv.) de Wet and Harlan) (Schuster 1975), and barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) (Wysong et al. 1981; Jackson et al. 2007a; Robertson and Jesse 2008).

There is no known vector for transmitting *C. michiganensis* subsp. *nebraskensis* (Jackson et al. 2007a; b), but wounds are often utilized to initiate infection (Schuster 1975). Wounding may occur from heavy rains, hailstorms, sandblasting, or wind (Rocheford et al. 1985; Claflin 1999). However, infection is most prevalent following a

hail storm (Jackson et al. 2007a; b). While injury allows for infection, other ways are possible. Stomata are a natural entry for the bacteria (Schuster 1975; Mallowa et al. 2012; Mallowa et al. 2014; Mallowa et al. 2015). Typically, water is desired for disease development, but Goss's wilt can be present in both irrigated and dryland fields (Jackson et al. 2007a). The bacteria are not able to travel long distances on their own, so it is assumed they are present when wounding occurs (Jackson 2009). This conclusion is supported by Smidt and Vidaver (1986) who reported leaf washings from asymptomatic plants harbored the bacteria, suggesting an epiphytic living strategy. Schuster et al. (1983) reported that greenhouse grown plants had epiphytic bacteria. In more recent studies, Marcell and Beattie (2002) demonstrated that *C. michiganensis* subsp. *nebraskensis* adhered to leaf surfaces. Therefore, the epiphytic living strategy of the pathogen has been well supported.

Not all bacteria cause a similar effect during infection. Bacteria infecting the vascular system can cause systemic infection and premature death of the host plant (Jackson et al. 2007a; b; Robertson and Jesse 2008). While this pathogen typically resides in the vascular tissue, it is suggested that it may invade surrounding parenchyma cells which leads to the formation of "freckles" (Schuster 1975), or discontinuous water-soaked spots (Schuster 1975; Wysong and Doupnik 1984; Wysong et al. 1981). Wilting may be attributed to vascular tissue impediment, but other plant defense responses also occur. Wounding triggers a xylem-blocking response by secreting phenolic compounds and mucilage into the xylem (Crews et al. 2003) in an effort to slow the spread of bacteria in the plant.

Systemic wilt and leaf blight are two major phases of disease (Jackson et al. 2007a; b; Agarkova et al. 2011). The leaf blight phase is more common, but systemic wilt is severe and can cause plant death (Jackson 2008). According to Calub et al. (1974) and Schuster (1975), infected plants may appear stunted. These characteristics are not the same for all infected plants. Goss's wilt development is affected by hybrid, inoculum present, and favorable weather conditions (Wysong and Doupnik 1984). Systemic infection can kill seedlings and mature plants any time during infection, and may result in 50% yield loss (Claflin 1999).

The bacteria have been isolated from all parts of the plant including roots, stems, leaves, sheaths, tassels, husks, silks, cobs, and kernels (Schuster 1975). Bacteria have been reported in the vascular bundles of the cob, ear shank, and the inner and outer vascular bundle ring which can be transported to the developing kernel (Schuster 1975). Bacterial ooze and water-soaked lesions may be found in the husks of infected ears (Schuster 1975). While bacteria may be carried on seeds, it can also be found inside the kernel between the scutellum and endosperm, and near the embryo where it can remain viable for up to 12 months (Schuster 1975). Typically, bacteria are spread via infected residue, but other ways are possible. Seed transmission of the bacteria is possible, but the rate of transmission is typically 0.1-0.4% (Biddle 1990; Biddle et al. 1990; Shepherd 1999). This might explain how *C. michiganensis* subsp. *nebraskensis* was reported in new areas.

Leaf infections are the most common and exhibit distinct symptoms. Gray to yellow lesions, wavy margins, water-soaking, dark green to black discontinuous water-soaked spots (Schuster 1975; Wysong and Doupnik 1984; Wysong et al. 1981) that may

be parallel to the leaf venations (Calub et al. 1974), shiny bacterial exudate (Calub et al. 1974; Schuster 1975; Treat and Tracy 1990; Jackson et al. 2007a; b), in addition to brown root and stalk rot, wilting, and stunting (Treat and Tracy 1990) have all been reported as potential symptoms. Bacteria have been confirmed in the vascular bundles, intercellular spaces, adjacent to vessels, and on the leaf surface (Schuster 1975). Leaf lesions are large and may be elliptical or V-shaped (Robertson 2009). As these lesions continue to grow, they coalesce to blight leaves (Jackson et al. 2007a; Jackson 2009). It is important to recognize all of these symptoms for correct diagnosis.

Inoculation Techniques

C. michiganensis subsp. *nebraskensis* infection can occur in different ways. Corn stubble is a primary source of inoculum (Schuster 1975). However, bacteria are not capable of entering the host without either a natural opening or wound. Schuster (1975) found that inoculated seedlings did not show any symptoms until they were puncture-wounded. There are many ways of inoculating plants to initiate infection. According to Schuster (1975), spraying cut leaves with a bacterial suspension or injecting seedlings one inch above the soil line with a hypodermic syringe resulted in infection. Disease incidence may differ between seedlings and more mature plants. Schuster (1975) found that older leaves may be less susceptible than younger leaves with the cut-spray method. Epiphytic populations are natural inoculum sources since the bacteria can wait until wounding occurs or infect through stomata (Mallowa et al. 2015).

Other inoculation methods are more direct. Smidt and Vidaver (1987) performed pathogenicity tests by stabbing sterile toothpicks with *C. michiganensis* subsp. *nebraskensis* colonies into four- to five-leaf stage Golden Cross Bantam (GCB) sweet

corn plants. Vidaver (1977) used the needle-eye method by inserting the eye of a number eight sewing needle with inoculum into the stem. Other methods include multiple-needle inoculators. Laurence and Aluisio (1981) inoculated plants by wounding the second leaf with a small grouping of needles. Schuster (1975) infected 10 day old plants with a multi needle inoculator submerged in a bacterial suspension. All methods resulted in different amounts of disease severity.

Plant age at inoculation is another important characteristic to consider. Plant age is important when analyzing resistant and susceptible hybrids with a syringe inoculation (Calub et al. 1974). Infection rate was greater for seedlings compared to more mature plants (Suparyono and Pataky 1986). Suparyono and Pataky (1989) found that tolerant plants had less disease compared to susceptible plants when inoculated at the three- to five-leaf stage of development. They also reported that plants inoculated at the five- to seven- or seven- to nine-leaf stage did not have as severe disease as the three- to five-leaf stage plants. Inoculation site may also contribute to disease severity. Calub et al. (1974) showed that plants inoculated in the upper half of the stem exhibited symptoms earlier than those inoculated in the lower half. They also found that higher inoculum density had greater disease incidence. Thus the method, application timing, inoculum placement, and bacterial density may alter disease severity.

The epiphytic strategy of *C. michiganensis* subsp. *nebraskensis* is important for disease development. Different methods have been used to simulate the epiphytic population (Schuster et al. 1983; Mallowa et al. 2012; Mallowa et al. 2014; Mallowa et al. 2015). Infection rates may have been low in these studies, but evidence of stomatal entry may be possible for the bacteria to initiate infection (Mallowa et al. 2012; Mallowa

et al. 2014; Mallowa et al. 2015). Thus, more research may be needed to study the importance of epiphytic populations on disease development.

Management

Use of crop rotation to non-hosts, tillage to promote degradation of infected crop debris, and planting partially resistant hybrids are effective management strategies for this pathogen (Schuster 1975; Jackson et al. 2007a; b; Jackson 2009). The bacteria overwinter in infected crop residue and will continue to be problematic in following growing seasons if not managed properly (Schuster 1975; Jackson et al. 2007a; Jackson 2009). This infected residue provides an inoculum source in continuous corn (Jackson et al. 2007b). A reduction in tillage practices may have contributed to increased disease incidence during recent years. Reduced tillage minimizes residue breakdown which has contributed to inoculum availability (Jackson et al. 2007b). Historically, disking and cultivation controlled available inoculum and reduced disease severity in subsequent years (Wysong and Doupnik 1984). Schuster (1975) found that infected leaf residue buried eight inches deep for 10 months did not allow bacterial survival. At different soil depths, bacteria are more likely to survive in stems, ears, and cobs instead of leaves (Schuster 1975).

Fungicides have been beneficial in controlling many fungal plant pathogens. However, seed treatments and fungicides are not beneficial in controlling *C. michiganensis* subsp. *nebraskensis* (Schuster 1975; Agarkova et al. 2011). The use of partially resistant hybrids is the most effective management strategy for this pathogen (Jackson et al. 2007a; b; Agarkova et al. 2011; Langemeier et al. 2015) which can be difficult when companies do not advertise Goss's wilt ratings (Jackson et al. 2007a; b).

Widespread use of management practices led to a decrease in disease incidence after 1974 (Vidaver et al. 1981). Disease developed sporadically with most reports coming from susceptible and injured popcorn, sweet corn, and field corn (Jackson et al. 2007b). However, complete resistance to this pathogen has not been identified in any hybrid or inbred (Schuster 1975). Disease severity depends on plant susceptibility. Suparyono and Pataky (1989) found that disease development was not as severe in plants moderately susceptible to infection opposed to ones highly susceptible. Many hybrids are grown for their exceptional qualities including germination, vigor, ear shape, kernel development, and kernel weight (Suparyono and Pataky 1989) which contribute to yield potential (Pataky et al. 1988). However, these varieties may be more susceptible to *C. michiganensis* subsp. *nebraskensis* infection and Goss's wilt development (Suparyono and Pataky 1989). Resistant hybrids often yield 10 to 15 bushels per acre less than susceptible hybrids (Vidaver et al. 1981). This may have increased disease incidence since farmers were more likely to plant susceptible hybrids with better yield potential (Vidaver et al. 1981). Disease severity has a direct correlation with yield potential. Infection of the Goss's wilt pathogen may reduce yield by 50% in severe cases (Wysong and Doupnik 1984). Plants may become infected at any time and die (Schuster 1975), but susceptibility based on maturity is not uniform for all varieties (Calub et al. 1974). Therefore, no single management strategy provides complete disease control.

Misdiagnosis of Goss's wilt is common, and allows the disease to spread and increase in severity and incidence, resulting in greater yield loss. Lesions can be mistaken for drought injury (Schuster 1975) since it has often been reported with early senescence (Jackson 2008). Goss's wilt is often confused with Stewart's wilt and other disease due to

similar symptoms (Jackson et al. 2007a; b). And, the bacteria was first mistakenly described as a more virulent form of the bacteria causing Stewarts's wilt (Schuster 1975). Northern corn leaf blight could easily be mistaken for Goss's wilt due to the presence of elliptical lesions, except the causal agent is a fungal pathogen (Robertson 2009). Therefore, proper disease identification is essential for effective disease management.

Langemeier et al. (2015) conducted a multistate survey on the re-emergence of the Goss's wilt pathogen in the Midwest. The objective of the survey project was to identify farming practices that had the greatest impact on disease development, including crop rotation, tillage, irrigation, nutrient management, field history, pesticide use, etc. In addition to some of the management practices mentioned throughout this literature review, pesticide use was moderately ranked among other farming practices in the survey, especially the use of common herbicides containing the active ingredient glyphosate and foliar fungicides (Langemeier et al. 2015). This correlation needs to be investigated in further depth to understand what may be causing an increase in Goss's wilt incidence.

Glyphosate Background and Mode of Action

Glyphosate [(N-phosphonomethyl)glycine] is a post-emergence, broad-spectrum, non-selective herbicide (Kremer and Means 2009) that targets the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Steinrücken and Amrhein 1980; Herrmann 1995; Dill 2005). The inhibition of EPSPS starves amino acid synthesis (Jaworski 1972; Grossbard and Atkinson 1985; Cerdeira and Duke 2006; Tuffi Santos et al. 2011). Different methods were utilized when producing glyphosate resistant products, but the introduction of an insensitive EPSPS was eventually used (Dill 2005). Changes in

the amino acid sequence make it impossible for glyphosate to bind properly (Dill 2005) which makes this mode of action quite effective.

It has been said that plants are more susceptible to disease with the inhibition of the shikimate pathway (Johal and Rahe 1988). The shikimic acid pathway is used to make precursors of plant defense amino acids, such as phenolic compounds (Moesta and Griesbach 1982; Sharon et al. 1992; Dill 2005) including coumarins, tannins, isoflavonoids, anthocyanins, flavonoids, phytoalexins, lignin, and salicylic acid (Srivastava 2001). Glyphosate chelates manganese, a cofactor for the EPSPS enzyme, and inhibits this pathway in plants and microorganisms (Jaworski 1972; Grossbard and Atkinson 1985; Cerdeira and Duke 2006). Fungi and bacteria may also possess this pathway and have adverse effects with glyphosate (Kishore and Shah 1988; Feng et al. 2005). Thus, it is possible that glyphosate may affect plants, fungi, and bacteria.

There are many different compounds used when applying glyphosate, but the active ingredient is essential for weed control. Glyphosate acid is the active ingredient that results in herbicide activity (Armstrong and Lancaster n.d.). According to Martin and Green (2004), there are more than 30 different glyphosate formulations which may have an additional surfactant or use different salts. Formulations may include ammonium, diammonium, dimethylammonium, isopropylamine, and potassium salts (Armstrong n.d.; Armstrong and Lancaster n.d.). It is not the type of salt which affects weed control, but rather the formulation (Armstrong n.d; Armstrong and Lancaster n.d.). There are many different glyphosate products on the market, and each have unique formulations. Products labeled as ‘fully loaded’ mean that an adjuvant has already been added (Armstrong and

Lancaster n.d.). This is the most common method for applying glyphosate, but some farmers still prefer tank mixing.

Tank mixing and alternating application times have an increased profit return on corn production (Blandino et al. 2012). Tank mixing allows for effective burndown of existing weeds in no-till or reduced-till corn (Liebl et al. 2008). One of the disadvantages to using glyphosate is herbicide resistance. Fortunately, resistance can be avoided if properly managed. Wilson et al. (2007) reported that herbicide rotation, crop rotation, herbicide tank-mixing, and soil applied herbicides are recommended strategies for managing weed shifts for glyphosate resistance.

Glyphosate tolerant crops are desirable for broad use applications. Since glyphosate is harmful to crops, it was necessary to develop plants that could withstand applications (Dill et al. 2008). This was accomplished by inserting a glyphosate-resistant clone CP4-EPSPS to allow proper function of the shikimate pathway (Dill et al. 2008). Most of the commercial glyphosate-resistant corn has CP4, a bacterial EPSPS which was isolated from a species of *Agrobacterium* (Dill 2005). These products have been continuously scrutinized over the years. Over-expression, expression of insensitive target enzymes, and detoxification of glyphosate were all tested before introducing these crops (Dill 2005).

Broad spectrum applications were popularized with herbicides in the 1990's (Wise and Mueller 2011). These applications were more economical to farmers and allowed them to spray herbicides when needed while reducing pre- and post-emergence weed control (Kremer and Means 2009). According to Monsanto (2007), glyphosate products are approved in more than 100 crops, and are registered in more than 130

countries. The adoption of glyphosate-resistant crops occurred for production efficiency, economic benefits, and conservation tillage efforts (Dill 2005). Since its release in 1996, glyphosate-resistant crops have become an important management practice for cotton (*Gossypium* spp. L.), soybeans (*Glycine max* L.), canola (*Brassica* spp. L.), corn (Dill 2005), and sugar beets (*Beta vulgaris* L.) (Gianessi 2005). Glyphosate-resistant corn became available to farmers in 1997, and estimates have shown that approximately 50% of all corn in the U.S. is sprayed with glyphosate (United States Department of Agriculture 2007). Glyphosate-resistant corn is the second highest commodity of these crops grown (Dill et al. 2008).

There are many components that affect glyphosate activity. Environment, weed susceptibility, rates of application (Zhou et al. 2007), herbicide delivery, deposition, retention, and penetration are all important characteristics for herbicide efficacy (Beckett et al. 1992). Absorption and active ingredient adhesion increase with a longer wetting period for systemic pesticides (Knoche and Bukovac 1994; Knoche et al. 2000). Glyphosate is a systemic herbicide, so a longer wetting period would increase herbicide absorption. Weeds should be sprayed when they are less than four inches tall for optimal herbicide efficacy (Armstrong and Lancaster n.d.). Early applications allow crops to be more competitive against late emerging weeds by killing early season weeds first (O'Sullivan and Bouw 1997). Glyphosate has no residual activity (Sprankle et al. 1975; Noruma and Hilton 1977), so late-emerging weeds may be problematic before harvest (Ferrell and Vencill 2003). It is important to use glyphosate properly for effective weed control.

Some studies have shown that glyphosate use can cause a reduction in certain plant disease. Tuffi Santos et al. (2011) reported that rust severity on eucalyptus (*Eucalyptus grandis*. Hill ex Maiden) plants decreased with increased glyphosate dose. A reduction in urediniospore formation was caused by blocking the shikimic acid pathway (Tuffi Santos et al. 2011). Kuklinshy-Sobral et al. (2005) reported that pre-plant applications of glyphosate on soybean fields can change endophytic bacterial communities. Other studies have shown that glyphosate applications have increased disease incidence.

Glyphosate use has been considered a causal agent for the re-emergence of pathogens (Campbell and Altman 1977; Mekwatanakarn and Sivasithamparam 1987). Metabolic inhibition and destruction of plant defense mechanisms make tomatoes more prone to Fusarium crown and root rot after glyphosate use (Bramhall and Higgins 1988). Glyphosate is available to rhizosphere and soil microbes which increases activity and biomass (Haney et al. 2000; Wardle and Parkinson 1990). Therefore, its activity may be expanding beyond weed control.

The systemic nature of glyphosate allows for the root system to be infected more easily. After glyphosate applications, root colonization of soil pathogens aid herbicidal activity (Johal and Rahe 1984), which may increase plant susceptibility (Baley et al. 2009). This is known as a ‘glyphosate synergistic response’ and has been reported in *Pythium* species (Lévesque and Rahe 1990; Lévesque and Rahe 1992; Lévesque et al. 2000) in addition to *Fusarium* species (Johal and Rahe 1984; Levésque et al. 1993). These compounds remain intact while being translocated throughout the plant, allowing it to target action sites and cause impact during translocation before degradation or

symptom development (Cerdeira and Duke 2006). Glyphosate can have a negative impact on nutrient uptake through the roots. According to Eker et al. (2006), glyphosate reduces uptake and translocation of soil nutrients including copper, iron, and manganese. This adds to its excellent weed control by reducing nutrient uptake.

Fungicide Background

Many factors have increased the use of foliar fungicides. Hybrids are often selected for yield potential instead of disease resistance (Wise and Mueller 2011), higher market prices in 2007 lowered yield response for application costs (Bradley and Ames 2010), higher demands for ethanol production and industrial uses (Wise and Mueller 2011), reports of enhanced yields, disease control, increased growth, and stress tolerance at VT applications have all caused an increase in fungicide applications (Elmore 2000). According to Wise and Mueller (2011), over four million corn hectares planted in 2011 were sprayed with fungicides in the U.S. Some farmers are using fungicides in the absence of disease. "Insurance applications" of fungicides disregard integrated pest management (IPM) strategies (Wise and Mueller 2011). Many factors are used to determine if fungicide applications are necessary. Corn markets, fungicide cost, yield potential, hybrid susceptibility, and disease severity are all important factors (Blandino et al. 2012). Applications may also increase production costs (Mulrooney 2009). Hybrid susceptibility is important, and if environmental conditions are suitable for disease development, a properly timed fungicide application may be profitable (Wegulo et al. 1997).

Application timing, fungicide type, and foliar coverage are all important factors for disease control (Edwards 1992). Growth stage at application, fungicide cost, disease

intensity, and number or frequency of applications are important to consider (Wegulo et al. 1997). Studies on soybean rust have shown that fungicides are most economical when applied during reproductive development (Miles et al. 2003; Yang and Robertson 2005). Shah and Dillard (2010) reported that New York sweet corn processors benefit from a single fungicide application for common rust and northern corn leaf blight control if disease severity thresholds exceed 20% leaf coverage. Fungicide trials in Nebraska between 2005 and 2009 indicate that applications made between VT and R4 result in a positive yield increase in about 90% of fungicides tested (Wise and Mueller 2011). Fungicide applications with low disease severity may encourage resistance and selection pressure (Mulrooney 2009). In an effort to reduce fungicide resistance, it is recommended to limit applications, use recommended dosages, and mix modes of action (Brent 1995).

One of the most difficult decisions is deciding when fungicides should be applied. Timing is crucial since grain fill lasts approximately 55-65 days (Robertson 2008). It is recommended to apply fungicides as soon as disease is detected (Munkvold 2006). Later applications of fungicides have also caused a larger decline in disease pressure compared to earlier applications (Blandino et al. 2012). These decisions may depend on application cost and diseases present.

Strobilurin Background and Mode of Action

Strobilurin fungicides are Quinone outside Inhibitors (QoI) and are produced from fungicidal derivatives including strobilurin A, oudemansin A, and myxothiazol A (Bartlett et al. 2002). Azoxystrobin and kresoxim-methyl were first introduced in 1992 (Godwin et al. 1992), but it wasn't until 1996 when these products were available for public use (Mavroeidi and Shaw 2006). QoI fungicides were available to corn growers in

the mid-2000's (Wise and Mueller 2011). Initially discovered as fungicidal derivatives, companies like BASF began to synthesize analogues of these products (Leroux 1996). Early applications are efficacious as preventive fungicides and can be beneficial if applied early for curative affects (Bartlett et al. 2002). Application timing is critical for effective control and avoiding resistance. QoI fungicides are a concern for developing disease resistance from selection pressure and fungal population shifts (Walker et al. 2009). Studies have also shown potential plant benefits in addition to disease control. According to Mulrooney (2009), strobilurins increase disease tolerance and improve plant health. Therefore, applicators may be applying them for more reasons than disease control.

Strobilurins move translaminar (Godwin et al. 1992; Yepma and Gold 1999) to provide disease control within the plant (Wong and Wilcox 2001). Strobilurins are mitochondrial respiration inhibitors (Mavroeidi and Shaw 2006; Mizutani et al. 1995). Mitochondrial inhibition occurs in the inner membrane of the Qo site of cytochrome b in cytochrome bc1 complex by inhibiting electron transport at the quinol oxidation site preventing 5'-triphosphate formation to disrupt energy production (Ammermann et al. 2000; Bartlett et al. 2002).

Strobilurins have been beneficial in disease management. They have been used on wheat (*Triticu aestivum* L.) for extended flag leaf greenness to increase yield potential (Dimmock and Gooding 2002a) and protein content in grain (Dimmock and Gooding 2002b). However, extensive use may lead to resistance. The Fungicide Resistance Action Committee (FRAC) rated QoI fungicides as being high risk when analyzing possible resistance development (Wise and Mueller 2011). Preventive fungicides are best applied

early before infection occurs to inhibit spore germination (Bartlett et al. 2002; Miles et al. 2007).

It has been suggested that strobilurins have plant health benefits. Modifications to ethylene and auxin production and delayed leaf and canopy senescence are associated with early fungicide applications (Grossmann and Retzlaff 1997; Grossmann et al. 1999) due to the inhibition of respiration and by-product formation (Grossmann and Retzlaff 1997). Pyraclostrobin has increased leaf greenness, photosynthesis, chlorophyll content, water usage, and delayed senescence (Grossmann and Retzlaff 1997; Grossmann et al. 1999). In the absence of disease, applications have increased wheat and barley (*Hordeum vulgare* L.) yield potential (Grossman and Retzlaff 1997; Grossman et al. 1999). Therefore, it is important to understand the properties and uses of these products for disease control.

Triazole Background and Mode of Action

Triazoles are also used to control plant pathogens. Triazoles are sterol demethylation inhibitors (DMI) that were introduced in the late 1960's (Mavroeidi and Shaw 2006). They act on gibberellin synthesis, ethylene production, and increase cytokinin production by inhibiting the isoprenoid pathway in plants (Graebe 1987; Zhou and Leul 1998). Like strobilurins, there are many benefits to applying triazoles. Fungicides that inhibit ergosterol synthesis, such as triazole, imidazole, or triazolinthione, were beneficial when applied for Fusarium head blight and are active against DON contamination (Haidukowski et al. 2005; Ioos et al. 2005). Applications of chlorothalonil, mancozeb, or propiconazole for common rust of corn increased yield potential (Wegulo et al. 1998).

It is common for farmers to tank mix fungicides with different modes of action. One recommended practice in particular is to mix triazoles with strobilurins. However, researchers who study the effects of fungicides either mixed or separate are finding interesting results. Triazoles performed better than strobilurins when applied alone instead of being combined (Scherm et al. 2009). There are still benefits to mixing fungicide modes of action though. To combat fungicide resistance, alternating fungicides may be beneficial (Kable and Jeffery 1980). Skylakakis (1981) preferred mixing fungicides to slow selection pressures, as was confirmed by Mavroeidi and Shaw (2006). Physiological effects with triazoles and strobilurins include: increase production of chlorophyll, thicker tissues, delayed senescence, increased alkaloid production, enlarged chloroplasts, increased ratio of roots to shoots, and increased antioxidant activity (Ruske et al. 2003a; Ruske et al. 2004; Zhang et al. 2010). Strobilurins and triazoles delay leaf senescence and reduce oxidative stress (Wu and von Tiedemann 2001). Therefore, there are many benefits to applying a combination product.

Less defoliation and rust infection on soybean occurs when applying a combination product (Miles et al. 2007). Soybean rust was controlled better with applications of triazole or strobilurin + triazole which increased yield (Miles et al. 2007). Soybean rust severity was higher in fields that used strobilurins compared to triazoles, but yields were also higher (Miles et al. 2007). Application timing is also important for fungicides with mixed modes of action and may have an impact on yield potential. Applications of DMI and QoI fungicides between mid-stem elongation and flowering increased yield potential (Blandino et al. 2012).

Fungicide use to delay leaf senescence of wheat can increase nitrogen uptake and use for grain fill (Ruske et al. 2003b). The majority of products available for corn are only effective for 2-3 weeks (Robertson 2008). In addition to timing, the number of applications are important for disease management. Disease control is best if fungicides are applied early, and consecutively at least three times in corn (Wegulo et al. 1998). Before disease is visible, the first application should be made for maximum yield potential (Scherm et al. 2009). Yield increases after fungicide application have been reported (Mulrooney 2009) which may be attributed to crop standability (Wise and Mueller 2011). Yield results from fungicide applications vary from study to study. Bradley and Ames (2010) showed that corn yield was not affected significantly from fungicide applications. Swoboda and Pedersen (2009) reported that addition of fungicides with the absence of disease did not help soybean physiology or yield potential. This may depend on crop or disease pressure.

While there are many benefits to applying fungicides, specific formulations or tank mixes may cause crop damage. Phytotoxicity can be related to temperature during applications (Robertson 2008). If plants are severely damaged, it can decrease yield potential. Phytotoxicity, absence of disease, or low disease incidence can result in profit loss (Wegulo et al. 1997). Also, since the plant's main objective is to reproduce, it will utilize its own resources to complete grain fill. Cannibalism is possible if fungal infection occurs during grain fill, which can reduce stalk strength (Mulrooney 2009). A loss in stalk strength decreases standability, which could reduce yield potential. Therefore, it is important to consider all possible outcomes before making a fungicide application.

Pesticide Spray Additives Background and Efficacy

Spray additives are often added to pesticides to increase their efficacy. These additives often include different kinds of adjuvants. Adjuvants are used with pesticides to increase efficacy or operation (Khan et al. 2007). They allow chemicals to remain in solution, and consequently aid in penetration and absorption (Xu et al. 2010b). Increased pesticide uptake is correlated with increased droplet spread (Xu et al. 2010b), which enhances rainfastness and translocation (Shaner et al. 2006). Activator adjuvants include surfactants, nitrogen fertilizers, and oils (Xu et al. 2010b). Efficacy can be influenced by the surfactant concentration (Xu et al. 2010b) and type (Wang and Liu 2007). Adjuvants may increase efficacy while reducing pesticide rates, which is desirable from an environmental and economic standpoint (Harker 1995; Xu et al. 2010a).

Adjuvants are used to modify physical properties or increase spray performance (Hazen 2000; Thacker 2001). They can either be special-purpose or activator adjuvants (Xu et al. 2010b) and are classified by their class, function, or affect (Spanoghe et al. 2007). Examples include buffers, deposition aids, stabilizers, drift retardants, and defoaming agents (Xu et al. 2010b). These are special-purpose adjuvants that are used to modify the spray's physical characteristics (Hazen 2000). Functional adjuvants include stabilizers, buffers, spreaders, antifoaming agents, defoaming agents, stickers, or wetting agents (Hazen 2000; Woznica 2001; Heinrichs 2002). These additives are also classified by application and are described as utility modifiers, spray modifiers, or activators (Spanoghe et al. 2007). There are many benefits to using spray additives. They can lower surface tension, increase spray retention time, and adjust solution pH (Stein and Storey 1986; Stock and Holloway 1993; Liu 2004; Singh and Singh 2008). Droplet size and

characteristics may be altered by spray adjuvants. Droplet size is important for spray mixtures, and its spread may increase with petroleum oils, water-insoluble emulfisiers, crop oils, and surfactants (Spanoghe et al. 2007). Organosilicones are used to lower droplet contact angle and surface tension to promote water adhesion (Sharma and Singh 2000), and increase droplet spread (Monaco et al. 2002).

Surfactants increase wetting, spreading, dispersal, and absorption while decreasing phototransformation (Penner 2000). Gimenes et al. (2013) reported that wettability was highest with methylated seed oil (MSO), followed by crop oil concentrate (COC), nonionic surfactant (NIS), and oil surfactant blend (OSB).

Surfactants are amphiphilic molecules with a hydrophilic and hydrophobic end (Rodriguez-Cruz et al. 2006). A lipophilic surfactant has a low hydrophilic-lipophilic balance (HLB) number, and a hydrophilic surfactant has a high HLB number (Griffin 1949; 1954). Surfactants with low HLB numbers are emulsifiers, while ones with higher values are detergents (Griffin 1949). Each adjuvant has different characteristics that make them desirable to use.

Surfactants can be anionic, organosilicones, nonionic, silicones, or cationic (Xu et al. 2010b). COC products are paraffinic, non-phytotoxic oils with 15 to 20% surfactant (Bunting et al. 2004). MSO products are esterified fatty acids extracted from seed oils (Miller and Westra 1996). NIS products are commonly used to reduce surface tension of a spray solution (Bunting et al. 2004). Adjuvant use is driven from their desired effect.

Adjuvants are added to increase diffusion through the plant cuticle, and may have sticking, wetting, or spreading capabilities (Noack et al. 2011). The desired effect

depends on pesticide type and plant species. Wetted areas on waxy leaves were improved with adjuvants compared to droplets without additives (Xu et al. 2010b). Oil-based adjuvants aid in absorption and penetration through thick, waxy cuticles (Xu et al. 2010b). MSO and NIS adjuvants are more effective in droplet spread, which decreased evaporation and increased absorption (Xu et al. 2010b). Oil-based adjuvants, including crop oils, seed oil concentrates, and crop oil concentrates are used for penetration through waxy cuticles (Xu et al. 2010b).

Surfactants may be added for modifying the plant cuticle, or preparing an infection court (Weaver et al. 2009). Surfactants, oils, and adjuvants work as solvents to dissolve waxy plant cuticles that act as hydrophobic barriers (Gimenes et al. 2013). Marcell and Beattie (2002) reported that surface wax directly affects bacterial colonization in a species-dependent manner. The cuticle is thinnest over the stomatal pores (Currier and Dybing 1959) which may allow better absorption of foliar pesticides. Penetration is enhanced with the cuticle removed (Jenks and Ashworth 1999; Kirkwood 1999). No single adjuvant, spray volume, or rate will increase spread, delay drying, or increase cuticle permeability (Noack et al. 2011). This explains why pesticide formulations typically contain multiple adjuvants.

Different methods can be used to apply pesticides, which may include tank mixing or formulated solutions. Formulated pesticides ensure proper adjuvant volumes compared to manual additions (Mulqueen 1990). Even though formulated pesticides are commonly used, some need to be diluted for proper application. Adjuvants must be added in a particular order to a tank mix. Careless tank mixing leads to crop injury, reduced

efficacy, and possible off-target effects (Campbell 1978; Hancock et al. 1984; Grichar and Boswell 1987).

Herbicide Activity with Adjuvants

Water is the most common carrier for pesticide applications. However, carrier ions may interfere with pesticide efficacy. Hard water ions such as calcium, magnesium, and other cations interact with glyphosate molecules forming complexes the plant cannot absorb (Ellis et al. 2002). Additives can be used with tank mixing to reduce the effect of hard water ions. AMS is an important additive for its ability to overcome antagonistic cations such as calcium, sodium, potassium, and magnesium (Nalewaja and Matysiak 1993a; b; Nalewaja et al. 1995).

Positively charged salts interact with negatively charged glyphosate molecules forming antagonistic glyphosate-salt complexes (Nalewaja and Matysiak 1991; Thelen et al. 1995). Addition of AMS reduces antagonistic ion activity interacting with glyphosate molecules (Thelen et al. 1995). For example, AMS binds with calcium ions to form calcium—sulfate while the ammonium combines with glyphosate to form a glyphosate—ammonium salt that can be absorbed by the plant (Thelen et al. 1995). Glyphosate—ammonium complexes are absorbed more readily compared to glyphosate—calcium or glyphosate—sodium complexes (Nalewaja et al. 1992). This adjuvant is beneficial since a lot of the U.S. water probably has hard water ions.

Adjuvants are typically used when making glyphosate applications. Surfactants allow pesticides to permeate the cuticle and move into the apoplast (Riechers et al. 1994). Modified epidermal waxes could aid in herbicide absorption (McWhorter and Barrentine

1988; Shaner et al. 2006). Some studies have shown that adjuvants have not increased pesticide efficacy. Deen et al. (2006) found that neither crop tolerance nor weed control were affected from tank mixing glyphosate with adjuvants. This could depend on many variables though.

Adjuvants either aid in spreading and wetting of herbicides, or penetration through cuticles, waxes, or membranes (Harker 1995). Adjuvants used to reduce surface tension of droplets and increase retention and spread of herbicides include surfactants, crop oils, and ammonium fertilizers (Sanyal et al. 2006). Surfactants are necessary for optimal absorption and activity (Sanyal et al. 2008) and they are ideal for avoiding herbicide desorption (Rodriguez-Cruz et al. 2006). Surfactant type may affect herbicide activity.

Pesticide enhancement with a surfactant depends on formulation and active ingredient (Sanyal et al. 2008). Some glyphosate products may not have additional surfactants, but one may be needed to increase product efficacy (Armstrong and Lancaster n.d.). The three most common adjuvants for agricultural purposes are NIS, MSO, and COC (Bunting et al. 2004). Other adjuvants like organosilicons may also be used (Vanhaecke 2000). NIS products are typically polyethoxylated aliphatic oils, but they may have fatty acids which aid in herbicide penetration (Bunting et al. 2004). Nonionic surfactants have linear or non-phenyl alcohols with or without fatty acids that are both lipophilic and hydrophilic, and can interact with similar herbicides (Monaco et al. 2002). These surfactants are recommended when applying glyphosate, whereas COCs and MSOs are not, due to a lack in weed control (Armstrong and Lancaster n.d.). Crop oil concentrates are used with clethodim, atrazine, sethoxydim, quizalofop, bentazon,

fluazifop, and fenoxaprop (Monaco et al. 2002). COCs delay herbicide crystallization, enhance absorption, and reduce volatile and photodegredative losses (Monaco et al. 2002).

Adjuvants are added to increase herbicide efficacy (Khan et al. 2007), including glyphosate (Leaper and Holloway 2000). Efficacy is related to adjuvant use, concentration, comparison, composition, and weed species controlled (Chen and Penner 1985), which may aid in absorption (Weed Science Society of America 1982), penetration, and translocation (Weed Science Society of America 1982). Specific adjuvants may have an effect on herbicide action. Knezevic et al. (2010) reported that weed control was highly influenced by adjuvant type and application timing. It is possible that there may be an interaction occurring between specific herbicides and surfactants which directly affect weed species (Molin and Hirase 2005). Surfactants not only decrease surface tension of the solution, but they allow permeability into the cuticle, plasma membrane, or both, which increases absorption and potential phytotoxicity (Riechers et al. 1994).

Certain types of surfactants may perform differently than others when applying herbicides. The result from increased contact between the solution and plant surface may disrupt the cuticle. Surfactants increase penetration and movement of glyphosate by disrupting the cuticle and epidermal cells (Kirkwood et al. 2000). Once contact has been made, not all surfactants have the same spreading capabilities. Xu et al. (2010b) reported that surfactant adjuvants did not have as much uniformity in spread compared to oil-based adjuvants.

Adjuvant combinations may have a different effect on herbicide activity. Zhou et al. (2007) found that the addition of MSO and AMS to glyphosate did not aid in velvetleaf control compared to AMS alone, but that AMS with NIS did control stressed velvetleaf. Addition of MSO and petroleum oil concentrate to glyphosate did not improve efficacy when used for velvetleaf control (Zhou et al. 2007). Liu (2004) demonstrated that glyphosate mixed with AMS and NIS improved uptake. There are multiple components to consider in an herbicide solution for effective control.

In some cases, adjuvants are not beneficial for herbicide activity. Nurse et al. (2008) found that AMS added to glyphosate did not affect the control of redroot pigweed (*Amaranthus retroflexus* L.) or common lambsquarters (*Chenopodium album* L.). The environment could also directly affect herbicide activity. A study on quackgrass (*Elytrigia repens* L.) by de Ruiter et al. (1996) demonstrated that field plots sprayed with glyphosate and AMS had less control compared to plants sprayed in the greenhouse. Herbicide rate may cause an increase or decrease in herbicide activity. The effect of AMS on plants may decrease as glyphosate rate increases (de Ruiter et al. 1996).

While there are many benefits with adjuvants, damage of non-target species can occur. Toxic effects cannot be placed solely on the active ingredient, carrier, or the adjuvants for many herbicides (Weaver et al. 2009). Phytotoxicity may be species-specific and depend on adjuvant use (Khan et al. 2007). Feng et al. (1998) reported that tissue necrosis was visible at the application site 24 hours after treatment due to surfactant use. Pesticide damage can lead to decreased yield potential.

Not all adjuvants cause the same amount of damage. Surfactants and AMS increase phytotoxicity with glyphosate application while oil adjuvants are antagonistic

(Nalewaja and Matysiak 1993a; Leaper and Holloway 2000). According to Harker (1995), AMS can increase herbicide phytotoxicity. In environmental chambers, phytotoxicity caused by sethoxydim and AMS marginally increased (Smith and Born 1992) whereas sethoxydim caused more phytotoxicity under greenhouse conditions (Hazen and Krebs 1992). Wyrill and Burnside (1977), in addition to Riechers (1992), reported that cationic surfactants are more effective in increasing phytotoxicity compared to nonionic surfactants.

Specific weed species are affected more than others when applying pesticides. Leaf damage and glyphosate efficacy increased with ethoxylated tallow amine on velvetleaf (*Abutilon theophrasti* Medic.) (Feng et al. 1998; Ryerse et al. 2004) suggesting that the cuticle and epidermal layer may have been damaged (Shaner et al. 2006). Damage has also been reported on corn plants when herbicides are used with adjuvants. Soltani et al. (2009) reported that saflufenacil applied post-emergence to two- to three-leaf corn with adjuvants caused injury and yield loss. However, Nurse et al. (2008) found that alone or with AMS, glyphosate did not cause visible damage or injury to corn plants (Nurse et al. 2008). When applications of formasulfuron with MSO were made, plant injury was more apparent, which indicates that adjuvant selection had more impact on corn production than herbicide rate (Bunting et al. 2004). It is crucial to understand adverse effects that can happen before making these applications.

Fungicide Activity with Adjuvants

Fungicides enhanced with adjuvants have been beneficial in controlling plant pathogens. Adjuvants added to pyraclostrobin may provide better control when disease is present (Khan et al. 2000). Adjuvants added to pyraclostrobin had better disease control

on sugar beets than applications of pyraclostrobin without adjuvants (Khan et al. 2007). Some adjuvants have adverse effects on fungal growth (Steiner and Watson 1965; Lacy 1974) including cationic surfactants that inhibit fungal and bacterial growth (Grant et al. 1990). Below et al. (2009) described that adjuvants added to strobilurins may affect hollow husk symptoms in corn from its impact on ethylene production. Adjuvants may cause a negative impact on plant growth and yield potential too. Khan et al. (2007) found that increased adjuvant rates caused higher phytotoxicity on sugar beets than pyraclostrobin applied alone. This caused a reduction in yield potential in some instances.

Blunt ear syndrome in corn had been reported from post-emergence herbicide, insecticide, fungicide, or adjuvant use (Nielsen 2007). Blunt ear syndrome may be caused by chemical or environmental stresses present on corn plants between V7 through V15 (Nielsen 1996), and is different from kernel tip back (Thomison and Geyer 2007). Another name for blunt ear syndrome is arrested ear development. More recently, Schmitz et al. (2011) determined that arrested ear development is caused by alkylphenol ethoxylate, a chemical component found in many NIS formulations. This affects yield potential when applied between vegetative stages V10 and V14, when ear length is being determined. These negative impacts have an undesirable effect on yield potential.

Summary of Literature Review

Historically, corn has been grown throughout the world and is produced heavily in the United States. Since its initial discovery in Nebraska, *C. michiganensis* subsp. *nebraskensis* spread to other corn growing areas. The pathogen often requires a wound to initiate infection, but this might not always be necessary as the bacteria can overwinter in infected residue, live epiphytically, and be introduced through natural openings. Lesions

develop after infection occurs, characterized with a shiny bacterial exudate and discontinuous water-soaked spots. Symptoms can either be localized lesions, leaf blight, or systemic wilt. Research has been conducted to understand the bacteria's biology, ecology, and re-emergence. A multistate survey on the pathogen's re-emergence showed a correlation between pesticide use, specifically fungicides and herbicides, and disease incidence.

Glyphosate was created as a broad spectrum herbicide to ease chemical applications. This product has been effective in controlling undesirable weed species while having minimal effects on crops. Other effects have been reported including disease impacts from plant pathogens, increasing glyphosate resistance in weeds, and phytotoxicity from glyphosate misuse or adjuvant use.

Farmers did not rely on fungicides until the 2000's because they were neither profitable nor affordable. An increase in demand for corn products caused an increase in fungicide use. Strobilurins and triazoles are two commonly used fungicide classes. These fungicides have been successful in controlling fungal pathogens and, in some cases, providing plant benefits. However, some studies have reported fungal resistance, phytotoxicity, and yield decreases caused by application timing, rate, fungicide, and adjuvant use.

Questions remain about this pathogen and more have been raised from results obtained by a Langemeier et al. (2015) survey, conducted in 2011. The purpose of this research is to observe Goss's wilt development in the field and greenhouse while studying *C. michiganensis* subsp. *nebraskensis* in the lab to fully understand if disease is correlated to spray adjuvant use.

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CHAPTER II

FIELD STUDY ON EFFECTS OF SPRAY ADJUVANTS USED IN CORN PRODUCTION ON THE DEVELOPMENT OF GOSS'S BACTERIAL WILT AND LEAF BLIGHT

Abstract

A field study was conducted in 2015 to investigate the effect of spray adjuvants used in corn (*Zea mays* L.) production on disease severity of Goss's bacterial wilt and leaf blight, caused by *Clavibacter michiganensis* subsp. *nebraskensis*. Three field locations in southwest Nebraska were used that had a history of this disease; two in Madrid and one in Gothenburg. Infected residue served as a source of inoculum. One susceptible hybrid with a 111 day relative maturity (RM) was used to test the effect of spray adjuvants on Goss's bacterial wilt and leaf blight development. These plots were surrounded by a resistant hybrid (115 RM) to reduce disease spread to neighboring fields at all three locations. Plots were sprayed with one of five spray adjuvants, one fungicide (azoxystrobin), one herbicide (glyphosate), one bactericide (copper hydroxide), or a negative control (water) at V6 growth stage. Separate plots were sprayed at R1 with one of two spray adjuvants, three different fungicides (azoxystrobin, propiconazole, or azoxystrobin + propiconazole), copper hydroxide, or a negative control. Visual estimates of disease severity, systemically infected plants, and yield data were all collected. Results for the V6 and R1 applications showed that there were no differences in disease severity among adjuvant treatments at all three locations for disease severity over time. Systemic infection results at Gothenburg V6 applications neared significance for nonionic surfactant (NIS) treatment ($P = 0.1151$), but this was the only treatment at any of the spray times or locations to near significance. Yield data showed no treatment effect from adjuvant use. These results are important because they disagree with the initial hypothesis that spray adjuvants increase disease severity. Since spray adjuvants are widely used in corn production, further research is needed to determine if environmental conditions were the only contributing factor to disease severity in this study.

Introduction

Goss's bacterial wilt and leaf blight, which is caused by *Clavibacter michiganensis* subsp. *nebraskensis*, is an economically important disease of corn (*Zea mays* L.) (Vidaver and Mandel 1974). As the name suggests, there are two phases commonly observed with this diease. Typical symptoms of Goss's leaf blight include chlorosis, necrosis, water-soaked margins, shiny bacterial exudate (Jackson et al. 2007a; b), and discontinuous water-soaked spots (Wysong and Doupnik 1984; Wysong et al. 1981). Less common symptoms of the systemic phase of infection include stunting (Calub et al. 1974), lack of ear production, plugged vascular tissue, and even death (Claflin 1999).

C. michiganensis subsp. *nebraskensis* can be spread by seed (Biddle 1990; Biddle et al. 1990; Shepherd 1999), rain splash from infected residue, or leaf rubbing (Wysong et al. 1981). The pathogen also has the capability of living epiphytically without initiating infection (Schuster et al. 1983; Smidt and Vidaver 1986; Marcell and Beattie 2002). Therefore, the pathogen may not be evident until symptoms develop.

It was first reported in Dawson County, Nebraska in 1969 (Wysong et al. 1973). By 1975, the disease was confirmed in neighboring states, infecting corn growing areas (Vidaver et al. 1981). The pathogen was present in 54 counties in Nebraska and five of the six bordering states including Iowa, Kansas, Colorado, South Dakota, and Wyoming in addition to Illinois and Wisconsin (Jackson et al. 2007a).

During the early 1980's, farmers began implementing tillage to promote degradation of crop debris, crop rotation to non-hosts, and selecting partially resistant

hybrids to manage *C. michiganensis* subsp. *nebraskensis*. These methods were effective for the next 20 years as disease developed sporadically in susceptible popcorn or sweetcorn fields (Jackson et al. 2007b). During the early 2000's, farming practices changed to support the increased need of corn production for ethanol and the growing population (Wise and Mueller 2011). Less crop rotation was implemented, and the use of no-tillage for soil and water conservation was more popular (Jackson et al. 2007b). Farmers started using high yielding varieties that did not contain the resistance package against the bacterial pathogen that causes Goss's wilt (Jackson et al. 2007b). These factors probably allowed for the re-emergence of the disease in the Midwest.

While we understand some of the farming practices that were being used before the pathogen re-emerged, we are uncertain what other practices were being implemented that allowed for its rapid spread to other corn producing states and provinces. A Midwest, multistate survey by Langemeier et al. (2015) was conducted in 2011 to assess production agriculture practices that might contribute to the re-emergence of the pathogen. Results from the survey mentioned hybrid selection, tillage, rotation, planting density, pesticide use, etc. The use of agricultural pesticides was interesting because its use has increased since the late 1990's to early 2000's; about the time when *C. michiganensis* subsp. *nebraskensis* re-emerged. It may be possible that the use of these pesticides or additives, including adjuvants, may be contributing to disease development.

Many pesticides are used in agricultural production. The introduction of genetically modified crops has allowed for easier pesticide use without detrimental crop damage. For example, Roundup Ready® corn was introduced in 1997 and resulted in increased production efficiency, improved weed control, conservation tillage, and

economic benefits (Dill 2005; Gianessi 2005). The adoption of glyphosate resistant crops has saved U.S. growers billions of dollars while reducing herbicide use by millions of pounds every year (Gianessi 2005).

Fungicides have also impacted corn production since the early 2000's. The active ingredients of most foliar fungicides currently in use in corn come from two fungicide classes, the strobilurins and triazoles. The increased need for corn production during the early 2000's and profitable corn prices made it appealing for farmers to spray fungicides (Wise and Mueller 2011).

When pesticides are applied, an adjuvant is typically added to increase pesticide efficacy (Khan et al. 2007). Some of the more common adjuvants used in corn production include nonionic surfactant (NIS), ammonium sulfate (AMS), crop oil concentrate (COC), high surfactant oil concentrate (HSOC), and methylated seed oil (MSO). Each adjuvant has different properties that make them desirable for use with specific pesticides or applications. For example, oils are typically used to allow pesticide penetration through cuticle layers (Xu et al. 2010). Thus, it is possible that bacteria living epiphytically may gain easier access when oil-based adjuvants damage the cuticle layer. Surfactants like NIS are added to reduce surface tension of the spray mix for better coverage (Bunting et al. 2004). This reduction in surface tension could facilitate entry of *C. michiganensis* subsp. *nebraskensis* into the plant if epiphytic populations are washed towards stomata through which they could enter (Mallowa et al. 2012; Mallowa et al. 2014; Mallowa et al. 2015). Another additive that is more commonly used with herbicides is AMS. It reduces antagonistic ion activity interacting with glyphosate

molecules (Thelen et al. 1995) and consequently allows for better uptake and weed control.

However, there have also been negative effects associated with adjuvants. For example, Schmitz et al. (2011) determined that arrested ear development is caused by alkylphenol ethoxylate, a chemical component found in many NIS formulations. Crop damage, such as phytotoxicity, may also occur with the use of spray adjuvants (Knezevic et al. 2010).

The objectives of this experiment was to evaluate the affect of spray adjuvants in corn production on Goss's wilt severity under field conditions.

Methods and Materials

Field description, management, and preparation. A field experiment was conducted at three location: two were located in southwest Nebraska near Madrid, and one was located at the Monsanto Water Utilization Learning Center in Gothenburg. The two location in Madrid, NE will be referred to as Madrid North and Madrid South, and the location in Gothenburg, NE will be referred to as Gothenburg for the remainder of this paper. *C. michiganensis* subsp. *nebraskensis* infected residue from the previous growing season was used as a source of inoculum at all three locations. Each field was irrigated.

A Goss's wilt susceptible hybrid with a 111 day relative maturity (RM) was bulk planted at Madrid North and Madrid South on 30 April 2015 at 79,100 plants ha^{-1}. A resistant hybrid with a 115 RM was planted as a boarder around the susceptible plots to reduce the spread of the pathogen to surrounding fields. Previous crop rotation at Madrid

North (40.889046°N, 101.513116°W) was corn-on-corn for many years and irrigation was provided via a Reinke center pivot irrigation system (Reinke Manufacturing Company, Inc., Deshler, NE) in addition to natural rainfall. The field was irrigated 12 times over the course of the growing season by applying 2.54 cm each date. Strip tillage was also implemented for residue management. Additional field preparation for fertilizer included 319 kg of 3200 nitrogen with no starter fertilizer. Previous crop rotation at Madrid South (40.799722°N, 101.622884°W) was corn-on-corn and the field was irrigated via a Reinke center pivot irrigation system (Reinke Manufacturing Company, Inc., Deshler, NE) in addition to natural rainfall. The field was watered 10 times over the course of the growing season by applying 2.54 cm each date. Field preparation included chisel plowing and additional fertilizer included 79 kg of actual nitrogen, 49 kg of 10-34-0 pre-plant ammonia, and 48 kg of 32-0-0 nitrogen fertilizer. No starter fertilizer was used at planting. Pre-emergence herbicides were applied to both locations on 19 May 2015 with 1.88 kg ha^{-1} S-metolachlor, 0.19 kg ha^{-1} mesotrione, and 0.71 kg ha^{-1} atrazine (Lumax® EZ).

The same field layout was used at the Monsanto Water Utilization Learning Center in Gothenburg (40.882556°N, 100.149728°W) and was planted on 13 May 2015 with a population density of 84,000 plants ha^{-1}. Previous crop rotation was corn-on-corn. This location received 12.7 cm of water in addition to natural rainfall during the growing season. Water was applied via a T-L lateral irrigation system (T-L Irrigation Co., Hastings, NE) on five dates, applying 2.54 cm of water each time. Field preparation included fertilizer applications of 90.72 kg nitrogen prior to strip tillage, then added 18.14 kg phosphorus and 9.07 kg sulfur by strip tillage on 2 May 2015. Pre-plant herbicides

were applied with 0.11 kg ha^{-1} mesotrione (Callisto®), 1.12 kg ha^{-1} atrazine (Atrazine® 4L), 2.45 kg ha^{-1} acetochlor (Harness®), and 1.12 kg ha^{-1} glyphosate (Roundup®).

Experimental design and spray applications. Experimental design was a repeated measure with treatments arranged in randomized complete blocks with eight replications per treatment. Three separate locations were used to replicate the field trial in space. Plot dimensions were cut to 3 m wide and 9 m long at Madrid North and Madrid South. Plot dimensions at Gothenburg were 3 m wide and 6 m long. Differences in plot size were due to planting equipment at the Monsanto Learning Center. Plots were four row plots with 76.2 cm row spacing. All four rows were sprayed, but data was only collected from the middle two rows. The V6 treatment list can be found in Table 1, and the R1 treatment list can be found in Table 2. All V6 treatments were on one half of the field and all R1 treatments were located at the other end of the field. Treatments were applied at maximum label rate. Copper hydroxide was added as a treatment due to differing results from previous studies on Goss's wilt control and product efficacy (Korus et al. 2010; Oser et al. 2013; Wise et al. 2014; Mehl et al. 2015). Commonly used pesticdes will also be used for observational purposes.

All V6 applications were made with a CO_2-pressurized backpack sprayer with a six nozzle TeeJet® AIXR110025 (Spraying Systems Co., Wheaton, IL) boom spaced 50.8 cm apart at 94 L ha^{-1}, 0.016 L sec^{-1}, and 159 kPa. All R1 applications were made with a CO_2-pressurized backpack sprayer with a six nozzle TeeJet® AIXR110015 (Spraying Systems Co., Wheaton, IL) boom spaced 50.8 cm apart at 94 L ha^{-1}, 0.016 L sec^{-1}, and 159 kPa.

Spray applications for V6 and R1 were made at Madrid North on 17 June 2015, and 21 July 2015 respectively. Applications for V6 and R1 were made at Madrid South on 17 June 2015 and 22 July 2015. Both applications made at Gothenburg were done on 18 June 2015 and 23 July 2015 for V6 and R1, respectively.

Disease assessment, data collection, and statistical analysis. Disease severity was measured on a continuous scale (0 to 100%) as a percentage of leaf damage per plot (Pataky et al. 2011). Disease data were collected 14, 28, and 42 days after spraying at each location. The number of systemically infected plants was also recorded at each assessment time. This was done with visual assessment by identifying key symptoms that indicate systemic infection described earlier. After visually calibrating for systemically infected plants, data was collected for statistical analysis. Yield data was collected at each location by harvesting the middle two rows of each plot. Harvest occurred on 9 November 2015 at Madrid North, 9 November 2015 at Madrid South, and 16 November 2015 at Gothenburg.

Disease severity and the number of systemically infected plants were analyzed using area under the disease progress curve (AUDPC) to determine differences between treatments over the growing period. Mean values were separated using a one-way analysis of variance (ANOVA) with PROC GLIMMIX. Yield was subjected to a one-way analysis of variance (ANOVA) using PROC GLIMMIX to determine if yield was affected by spray adjuvant use. Blocks were treated as random and disease severity collected at different rating times was used for the repeated measure. Statistical analyses were tested at $\alpha = 0.10$ and were completed using SAS 9.4 (SAS Institute Inc., Cary, NC).

Results

Goss's wilt developed at all locations and disease severity increased throughout the growing season after infection occurred. AUDPC results from Madrid North showed that there were no differences between treatments for disease severity over the growing season for V6 application times ($P = 0.2560$; Table 3; Figure 1). There were also no treatment differences for the number of systemically infected plants ($P = 0.2404$). Results from the R1 applications showed that there was a signficiant treatment effect over time for disease severity ($P = 0.0831$). The COMB treatment had the greatest disease severity (133.00), but none of the adjuvants tested had significant amount of disease present (Table 4; Figure 2). There were no differences between treatments over the growing season for plants that became systemically infected ($P = 0.7122$).

AUDPC results from Madrid South showed that there were no differences between treatment for disease severity over the growing season for V6 application times ($P = 0.9218$; Table 5; Figure 3). There were also no treatment differences for the number of systemically infected plants ($P = 0.9781$). For the R1 application times at Madrid South, there were no differences between treatment for disease severity over the growing season ($P = 0.2506$; Table 6; Figure 4). There were also no treatment differences for the number of systemically infected plants ($P = 0.2395$).

Field results using AUDPC at the Gothenburg location showed that there were no differences between treatment for disease severity over the growing season for V6 application times ($P = 0.2389$; Table 7; Figure 5). Results from the systemic infection data neared significance ($P = 0.1551$) with NIS treated plots having the highest number of systemically infected plants throughout the growing season (26.25; Table 8; Figure 6).

For the R1 application times at Gothenburg, there were no differences between treatment for disease severity over the growing season (P = 0.5435; Table 9; Figure 7). There were also no treatment differences for the number of systemically infected plants (P = 0.5971).

Yield data collected from Madrid North showed that there were no differences between treatments for yield potential for V6 (P = 0.9242) and R1 (P = 0.2195), respectively . Madrid South also showed no significant differences between treatments applied at either V6 (P = 0.5567) or R1 (P = 0.1228) application time. Finally, yield data at Gothenburg showed that there were no significant differences between treatments for either V6 (P = 0.6844) or R1 (P = 0.4770) application time.

Discussion

No treatment effects were reported with the use of spray adjuvants for increasing disease severity at either application time. Once the bacteria entered the plant, they were able to initiate infection. The plants may have been more susceptible to bacterial invasion at V6 compared to R1 application times. Vegetative characteristics of younger, less mature plants include short, narrow leaves, no trichomes or bulliform cells, and a thin cuticle layer with an epicuticular wax (Abedon et al. 1996). Characteristics of an older, more mature corn plant include long, wide leaves, bulliform cells, trichomes, and a thick cuticle with no epicuticular wax (Abedon et al. 1996). Therefore, even though the V6 plants were grown in a natural environment and exposed to environmental conditions, they still may not have been developed enough to avoid bacterial infection when they were less mature.

Similarities between treatment results indicate that spray adjuvants did not cause differences in the bacteria’s capability to infect the plants based on which adjuvant was

applied. Adjvuants also did not decrease bacterial virulence once inside the plant. There were no differences between adjuvant treatments tested for systemically infected plants at V6 applications, but the number of systemically infected plants increased during the growing season at all three locations. This is understandable because Madrid North had sandy soil texture that would allow for microabrasions and potential entry of bacteria (Rocheford et al. 1985). Madrid North and South both experienced wind and hail damage during the growing season. Early hail damage, in addition to high winds, may have contributed to disease development (Rocheford et al. 1985; Claflin 1999). Wind damage was apparent at the Madrid South location, but the main concern at this field was hail damage shortly before V6 applications. When an epiphytic population has become established, wounding enables bacterial entry (Wysong et al. 1981; Rocheford et al. 1985; Claflin 1999). Hail damage especially allows for bacterial entry and the start of infection (Jackson et al. 2007a; b). While there was no severe hail damage reported in 2015, Gothenburg also had an increase of infection over time for both disease severity and systemically infected plants per plot. These results are understandable since the adjuvants did not impact bacterial virulence once inside the plant.

There were some differences in disease severity present between field locations that may have allowed for more systemic infection at one location compared to another. The Gothenburg field site was used during the previous growing season and was spray inoculated with *C. michiganensis* subsp. *nebraskensis* inoculum. The plants were not wounded during inoculation to establish an epiphytic population. However, this field sustained significant hail damage at least three times in 2014. Heavy disease severity caused early senescence. The 2015 field preparation only included strip tillage, which did

not allow for sufficient residue degradation. However, this residue was desirable to establish an epiphytic population by planting corn into this infected residue. This agrees with Eggenberger et al. (2015), which stated that point sources of infected residue on the soil surface may establish an epiphytic population and spread of the pathogen.

The use of a susceptible hybrid and available inoculum from the previous growing season allowed for optimal infection to develop. Studies have shown that susceptible varieties harbor higher epiphytic populations than resistant ones (Schuster et al. 1983). Hybrids that are more susceptible to *C. michiganensis* subsp. *nebraskensis* infection (Suparyono and Pataky 1989) may often yield 10 to 15 bushels per acre more than resistant hybrids (Vidaver et al. 1981), which is why farmers often plant them. High inoculum density and potential environmental factors would allow for more systemic infection to occur at Gothenburg compared to the other two locations. Even though significant hail damage was not reported, this field may have experienced wind damage, which would allow for bacterial entry (Rocheford et al. 1985; Claflin 1999).

Madrid North and Madrid South had *C. michiganensis* subsp. *nebraskensis* the previous growing season, however, it is undetermined how severe infection was in 2014. Madrid North has a sandy soil texture with a history of planting susceptible hybrids for many years. Inoculum build up is also substantial at this location because only strip tillage is used for soil conservation purposes. Eggenberger et al. (2015) stated that gradual buildup of inoculum with minimal tillage, continuous corn, and using susceptible hybrids allow for an increase in disease incidence. It is difficult to make assumptions from these field results at Madrid North because hail damage was sustained during the 2015 growing season. Hail damage may have contributed to the lack of differences

between treatments at Madrid North. Madrid South had more tillage for residue management which may have contributed to less disease severity. Disking and cultivation often control inoculum levels (Wysong and Doupnik 1984) because the bacteria are probably not able to live in shredded, buried residue. Mehl et al. (2013) found that deeper tillage methods allowed for better control of infected residue and decreased the amount of Goss's wilt present. Personal communication with the cooperator indicated uncertain amounts of disease pressure from the previous growing season in the current study location. The cooperator was able to confirm that the field had experienced Goss's wilt in 2014 though. Percent residue at planting was not collected at any of the locations used in 2015.

Results from yield data collected show that at both Madrid locations, there were no significant differences in yield between treatments applied. It is difficult to determine from these locations how effective these adjuvants were due to hail damage. Madrid North had wind and minimal hail damage when the plants were smaller, and the field sustained hail damage about one week prior to R1 spray applications. Madrid South had substantial hail damage immediately before V6 applications. The data from the R1 application at this field showed that copper hydroxide had the highest yield followed by WATER. This is to be expected because copper hydroxide is a contact product that might control the epiphytic population (Kennelly et al. 2007). WATER would allow for more disease than copper hydroxide, and if the hypothesis of adjuvants increasing disease severity is true, yield would be higher for WATER and lower for the adjuvants tested. However, WATER was not statistically different than the other adjuvants tested. Muddy conditions and deep pivot tracks made this field difficult to harvest with a two row plot

combine. However, with all of the harvesting errors, the data still showed no significant differences in disease severity between treatments applied. Yield data from Gothenburg also showed no significant differences in disease severity in yield between treatments applied. This field sustained a lot of hail damage during 2014 and disease incidence was relatively uniform across the field. Minor hail and wind damage was reported for 2015 and natural inoculum from infected field residue was used, allowing for even inoculum distribution. The uniformity between all three locations for yield purposes indicate that adjuvants are not causing an increase in disease severity.

While disease was present at each location used in 2015, the amount of available inoculum varied at each location due to farming practices. Differences between tillage methods may have reduced the amount of inoculum available compared to a field that had minimum tillage. Also, hail damage sustained at different locations also contributed to differences between disease severity or the amount of systemic infection present at certain locations. These factors contributed to the differences that may be occurring between field locations.

This study showed that the use of agriculture spray adjuvants commonly applied in corn production are not having a major impact on Goss's wilt development. There have been some negative effects reported with the use of adjuvants (Knezevic et al. 2010; Schmitz et al. 2011), but in this particular study, nothing was reported. It is unfortunate that hail damage was sustained on two field locations which may have impacted disease severity. Differences in residue management may have contributed to disease uniformity within each respective field. Soil texture may have also contributed to disease spread as in the Madrid North field location where sandblasting might allow bacterial entry. A

greenhouse study with a more controlled environment may be needed to successfully test this hypothesis of increased infection from *C. michiganensis* subsp. *nebraskensis* with spray adjuvants.

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Table 1. Chemical list for V6 spray applications at Madrid, NE and Gothenburg, NE.

Trade Name	Active Ingredient	Reference Name	Rate	Manufacturer Information
	V6-V7 Application			
DuPont™Kocide® 3000	copper hydroxide	KOCIDE® 3000	4.26 kg ha^{-1}	DuPont, Wilmington, DE
Water	H_2O	WATER	93.53 L ha^{-1}	N/A
Touchdown HiTech®	glyphosate	GLY	1.20 kg ha^{-1}	Syngenta Crop Protection, LLC Greensboro, NC
Quadris®	azoxystrobin	AZO	0.26 kg ha^{-1}	Syngenta Crop Protection, LLC Greensboro, NC
Bronc®	ammonium sulfate	AMS	5.0% v/v	Wilbur-Ellis Company, Fresno, CA
ROC®	crop oil concentrate	COC	1.0% v/v	Wilbur-Ellis Company, Fresno, CA
High Load®	high surfactant oil concentrate	HSOC	0.5% v/v	Wilbur-Ellis Company, Fresno, CA
Super Spread MSO®	methylated seed oil	MSO	1.0% v/v	Wilbur-Ellis Company, Fresno, CA
R11®	nonionic surfactant	NIS	0.25% v/v	Wilbur-Ellis Company, Fresno, CA

Table 2. Chemical list for R1 spray applications at Madrid, NE and Gothenburg, NE.

Trade Name	Active Ingredient	Reference Name	Rate	Manufacturer Information
	R1 Application			
DuPont™Kocide® 3000	copper hydroxide	KOCIDE® 3000	4.26 kg ha^{-1}	DuPont, Wilmington, DE
Water	H_2O	WATER	93.53 L ha^{-1}	N/A
Quilt Xcel®	azoxystrobin + propiconozole	COMB	0.14 kg ha^{-1} + 0.13 kg ha^{-1}	Syngenta Crop Protection, LLC Greensboro, NC
Quadris®	azoxystrobin	AZO	0.26 kg ha^{-1}	Syngenta Crop Protection, LLC Greensboro, NC
Tilt®	propiconozole	PRO	0.13 kg ha^{-1}	Syngenta Crop Protection, LLC Greensboro, NC
ROC®	crop oil concentrate	COC	1.0% v/v	Wilbur-Ellis Company Fresno, CA
R11®	nonionic surfactant	NIS	0.25% v/v	Wilbur-Ellis Company Fresno, CA

Table 3. AUDPC values for all treatments tested for Goss's wilt severity over the growing season for V6 spray applications at the Madrid North field location.

Treatment	AUDPC
KOCIDE®3000	55.13[b*]
WATER	56.00[b]
GLY	65.63[a]
AZO	54.25[b]
AMS	65.63[a]
COC	58.00[ab]
HSOC	58.63[ab]
MSO	63.00[ab]
NIS	60.00[ab]

*Means separated by letters are significant at $\alpha = 0.10$.

Table 4. AUDPC values for all treatments tested for Goss's wilt severity over the growing season for R1 spray applications at the Madrid North field location.

Treatment	AUDPC
KOCIDE®3000	105.88[b*]
WATER	111.00[b]
COMB	133.00[a]
AZO	114.63[b]
PRO	113.00[b]
COC	113.75[b]
NIS	109.38[b]

[*]Means separated by letters are significant at $\alpha = 0.10$.

Table 5. AUDPC values for all treatments tested for Goss's wilt severity over the growing season for V6 spray applications at the Madrid South field location.

Treatment	AUDPC
KOCIDE®3000	55.13[b*]
WATER	56.00[b]
GLY	65.63[a]
AZO	54.25[b]
AMS	65.63[a]
COC	58.00[ab]
HSOC	58.63[ab]
MSO	63.00[ab]
NIS	60.00[ab]

*Means separated by letters are significant at $\alpha = 0.10$.

Table 6. AUDPC values for all treatments tested for Goss's wilt severity over the growing season for R1 spray applications at the Madrid South field location.

Treatment	AUDPC
KOCIDE®3000	105.88[b*]
WATER	111.00[b]
COMB	133.00[a]
AZO	114.63[b]
PRO	113.00[b]
COC	113.75[b]
NIS	109.38[b]

[*]Means separated by letters are significant at $\alpha = 0.10$.

Table 7. AUDPC values for all treatments tested for Goss's wilt severity over the growing season for V6 spray applications at the Gothenburg field location.

Treatment	AUDPC
KOCIDE®3000	55.13[b*]
WATER	56.00[b]
GLY	65.63[a]
AZO	54.25[b]
AMS	65.63[a]
COC	58.00[ab]
HSOC	58.63[ab]
MSO	63.00[ab]
NIS	60.00[ab]

*Means separated by letters are significant at $\alpha = 0.10$.

Table 8. AUDPC values for all treatments tested for systemic Goss's wilt development over the growing season for V6 spray applications at the Gothenburg field location.

Treatment	AUDPC
KOCIDE®3000	55.13[b*]
WATER	56.00[b]
GLY	65.63[a]
AZO	54.25[b]
AMS	65.63[a]
COC	58.00[ab]
HSOC	58.63[ab]
MSO	63.00[ab]
NIS	60.00[ab]

*Means separated by letters are significant at $\alpha = 0.10$.

Table 9. AUDPC values for all treatments tested for Goss's wilt severity over the growing season for R1 spray applications at the Gothenburg field location.

Treatment	AUDPC
KOCIDE®3000	105.88[b*]
WATER	111.00[b]
COMB	133.00[a]
AZO	114.63[b]
PRO	113.00[b]
COC	113.75[b]
NIS	109.38[b]

[*]Means separated by letters are significant at $\alpha = 0.10$.

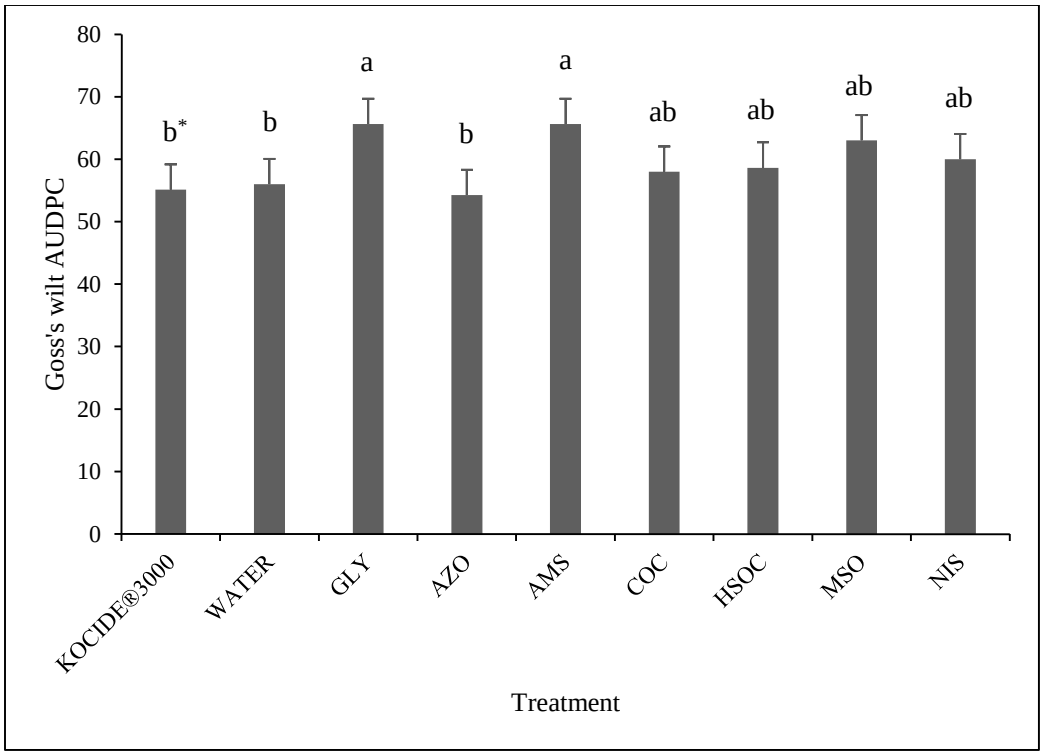

Figure 1. AUDPC with means separation for Goss's wilt disease severity over the growing period for V6 spray applications at the Madrid North field location.
*Means separated by letters are significant at $\alpha = 0.10$.

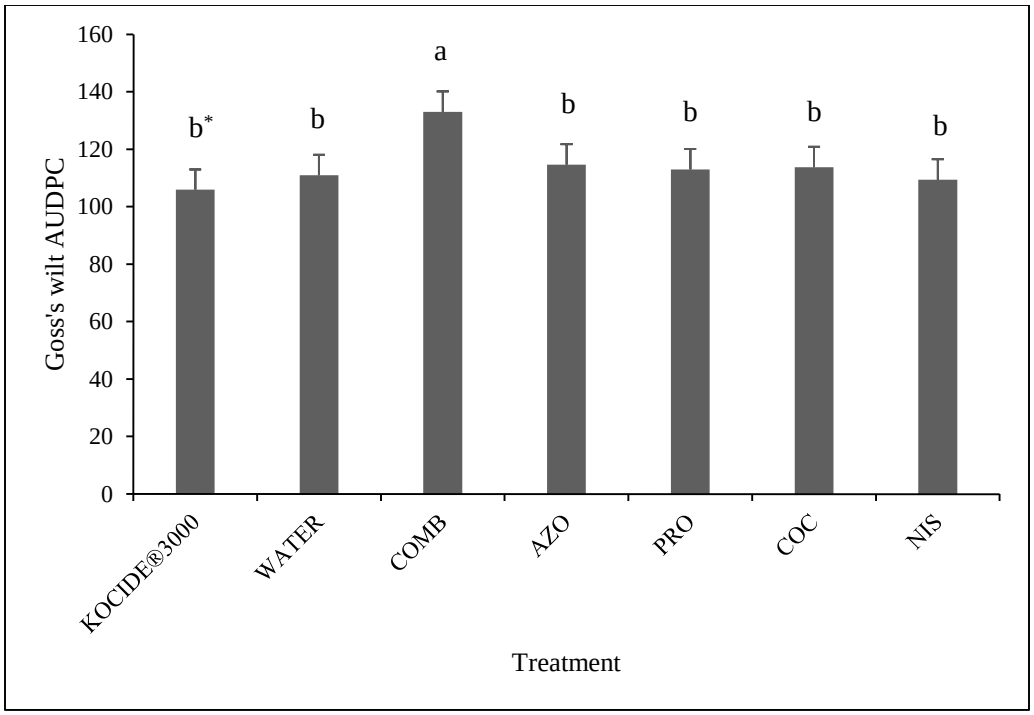

Figure 2. AUDPC with means separation for Goss's wilt disease severity over the growing period for R1 spray applications at the Madrid North field location.
*Means separated by letters are significant at $\alpha = 0.10$.

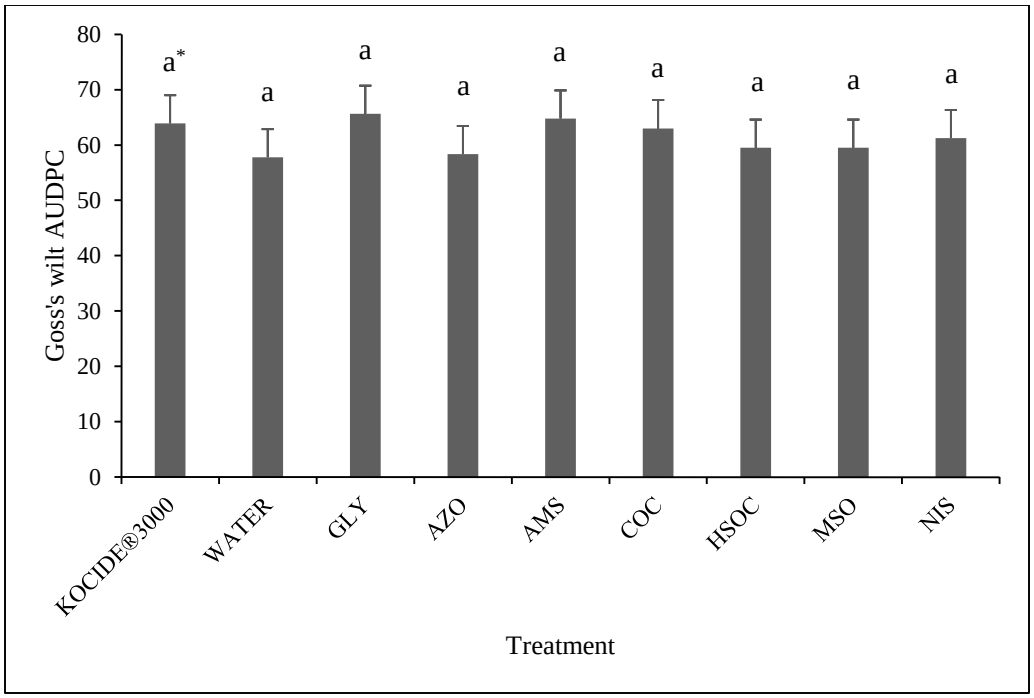

Figure 3. AUDPC with means separation for Goss's wilt disease severity over the growing period for V6 spray applications at the Madrid South field location.
*Means separated by letters are significant at $\alpha = 0.10$.

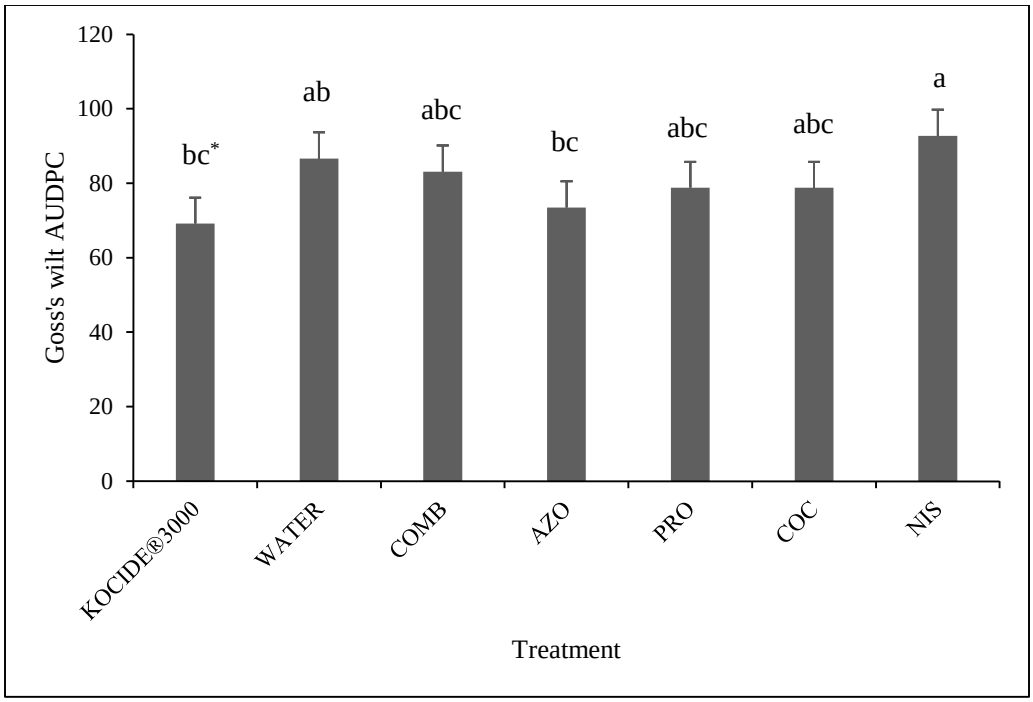

Figure 4. AUDPC with means separation for Goss's wilt disease severity over the growing period for R1 spray applications at the Madrid South field location.
*Means separated by letters are significant at $\alpha = 0.10$.

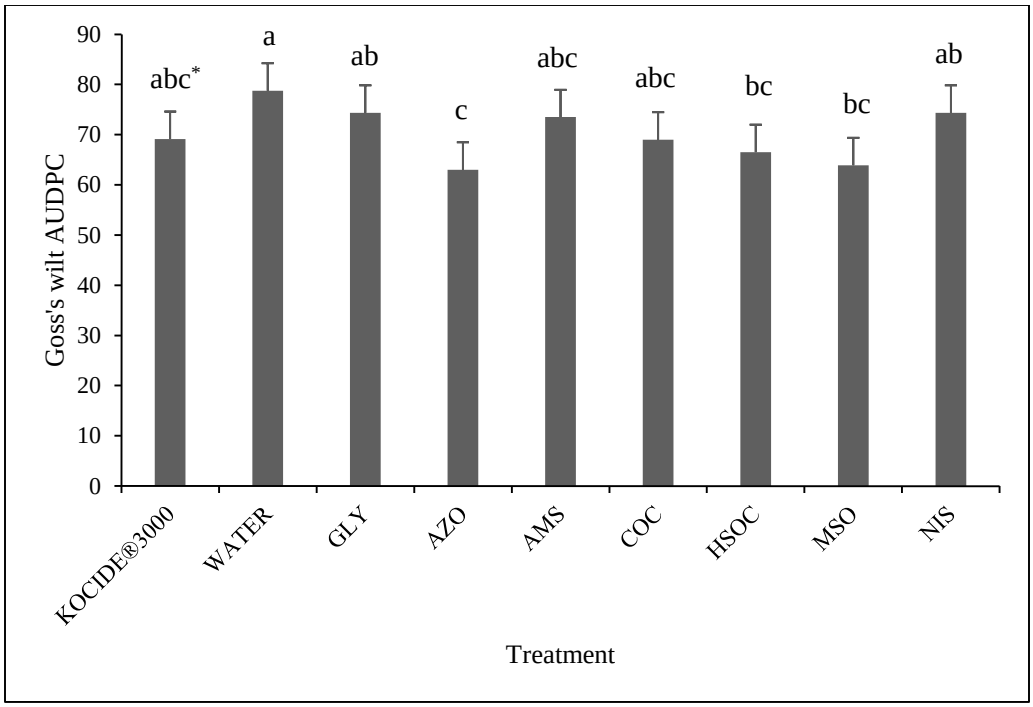

Figure 5. AUDPC with means separation for Goss's wilt disease severity over the growing period for V6 spray applications at the Gothenburg field location.
*Means separated by letters are significant at $\alpha = 0.10$.

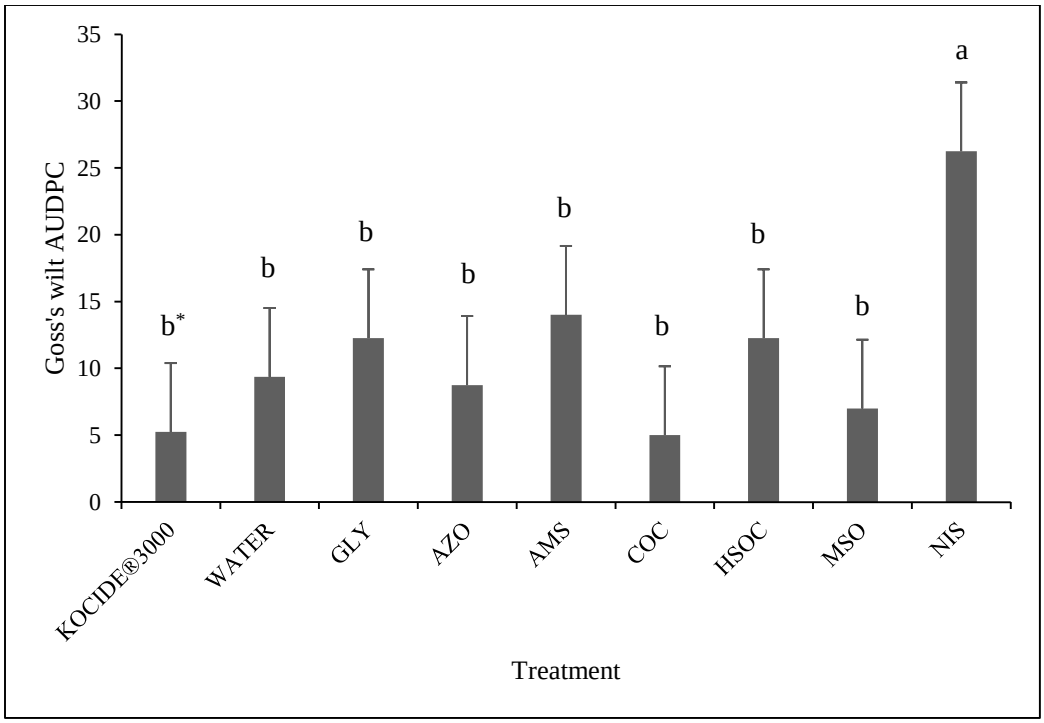

Figure 6. AUDPC with means separation for systemic Goss's wilt development over the growing period for V6 spray applications at the Gothenburg field location.
*Means separated by letters are significant at $\alpha = 0.10$.

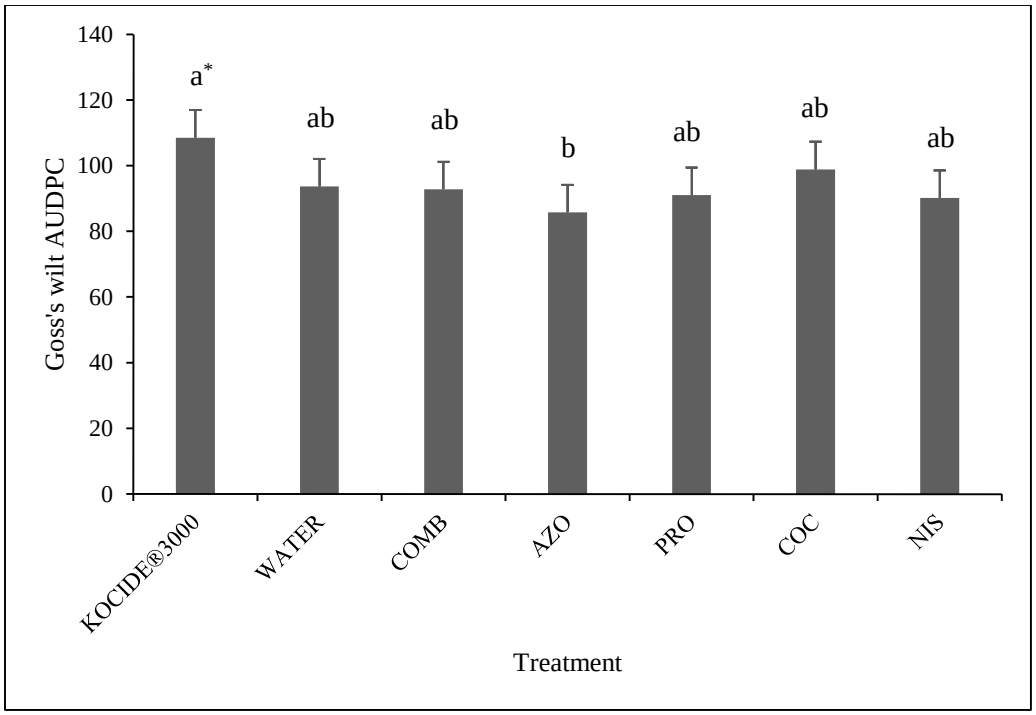

Figure 7. AUDPC with means separation for Goss's wilt disease severity over the growing period for R1 spray applications at the Gothenburg field location.
*Means separated by letters are significant at $\alpha = 0.10$.

CHAPTER III

THE EFFECT OF PESTICIDE ADJUVANTS ON GOSS'S BACTERIAL WILT AND LEAF BLIGHT DEVELOPMENT UNDER GREENHOUSE CONDITIONS

Abstract

A greenhouse study was conducted to investigate the potential impacts of pesticide adjuvants commonly used in corn (*Zea mays* L.) production on disease severity of Goss's bacterial wilt and leaf blight, caused by *Clavibacter michiganensis* subsp. *nebraskensis.* A bacterial suspension of *C. michiganensis* subsp. *nebraskensis* at 1 x 10^9 CFU ml^{-1} in 10 mM potassium phosphate buffer was spray-inoculated on Golden Cross Bantam (GCB) sweetcorn plants using an air-pressurized canister at V1. An inoculated wound (WOUND) control was to ensure bacterial virulence. A non-wounded, spray inoculated control (SPRAY) was used, but these plants were not treated with adjuvants. Inoculated plants were sprayed with one of five spray adjuvants 48 hours after inoculation. A negative control (WATER), a positive control (copper hydroxide), and a potassium phosphate control (BUFFER) were also used. Percent disease severity was estimated as a repeated measure at 7, 10, 13, 17, and 21 days after spray applications. The experiment was repeated once. Results from area under the disease progress curve (AUDPC) indicated that there were differences between treatments applied in disease severity over time. The WOUND treatment consistently had the greatest disease severity over time while the BUFFER control had the lowest disease severity for both experimental trials. However, all of the adjuvant treatments had similar disease severity throughout the experiment compared to the SPRAY control. These results are important because of the widespread use of spray adjuvants with pesticides and the potential for impacts on disease severity in production agriculture. Overall, it does not appear that adjuvants are contributing to Goss's wilt development in the greenhouse. Further research is needed to determine if differences in chemical composition within adjuvant groups or varying adjuvant concentrations have similar impacts.

Introduction

Goss's wilt and leaf blight, caused by *Clavibacter michiganensis* subsp. *nebraskensis*, is an economically important disease of corn (*Zea mays* L.) (Vidaver and Mandel 1974). Physical wounding such as hail, wind, sandblasting, or mechanical damage will often facilitate infection if bacteria are present (Wysong et al. 1981; Rocheford et al. 1985; Claflin 1999). There are currently no known vectors that transmit the bacteria to initiate infection (Jackson et al. 2007a; b).

Symptom development can vary depending on the variety, hybrid, or type of corn. Typical symptoms include chlorosis, necrosis, lesions with water-soaked margins (Jackson et al. 2007a; b), and discontinuous water-soaked spots (Wysong and Doupnik 1984; Wysong et al. 1981). The bacteria often exude out the lesions, dry on the leaf surface, and appear shiny (Jackson et al. 2007a; b). This strategy allows for the bacteria to reside on the leaf surface until they spread to another host through water splashing or leaf rubbing (Wysong et al. 1981). However, the presence of bacteria does not always ensure infection. Smidt and Vidaver (1986) found that *C. michiganensis* subsp. *nebraskensis* can live epiphytically without initiating infection and suggested the population density needs to reach an optimal threshold for infection and disease development to occur. Symptom development may depend on hybrid genetics where infection might not be as severe in moderately susceptible hybrids compared to ones that are highly susceptible (Suparyono and Pataky 1989). Disease severity usually refers to the leaf blight phase depending on partial hybrid resistance. If bacteria enter the vascular system, they can move systemically and mimic drought stress or injury (Wise et al. 2010). Systemically infected plants are typically stunted (Calub et al. 1974) and may not

produce an ear (Claflin 1999). If an ear is produced, harvested yield is typically less than for plants not infected with the pathogen.

Wounding is not the only way for bacterial introduction. Studies have shown that the epiphytic nature of this bacteria allow them to get into the plant through natural openings, like stomata, and initiate infection (Schuster et al. 1983; Mallowa et al. 2012; Mallowa et al. 2014; Mallowa et al. 2015). Disease may develop slower when infection occurs via stomata (Mallowa et al. 2012; Mallowa et al. 2014), but it is important to understand that wounding may not be required for infection and that *C. michiganensis* subsp. *nebraskensis* may go undetected until a disease outbreak occurs (Eggenberger et al. 2015).

Goss's wilt was first confirmed in Dawson County, Nebraska in 1969 (Wysong et al. 1973), and the pathogen eventually spread to neighboring counties and states (Wysong et al. 1973; Jackson et al. 2007a). In the 1980's, new farming practices were implemented to manage inoculum levels. Bacterial spread and disease pressure slowed until it developed sporadically within susceptible varieties for the next 20 years (Jackson et al. 2007b). During the early 2000's, management practices changed to increase corn production. As a result, the pathogen re-emerged in western Nebraska, southeastern Wyoming, and eastern Colorado (Jackson 2008). The bacteria then spread to other corn growing areas of the Great Plains and Midwest (Ruhl et al. 2009; Malvick et al. 2010; Korus et al. 2011). It is not fully understood what farming practices were being used that allowed for pathogen re-emergence.

A Midwest multistate survey was conducted by Langemeier et al. (2015) in 2011 on farming practices for Goss's wilt and bacterial re-emergence in the Midwest. There

were many management and farming practices used including disease ratings, planting population, crop rotation, etc. One interesting practice reported was the use of agricultural pesticides. Pesticides have been used for many years, especially with the introduction of Roundup Ready® corn in 1997 (Dill 2005). The increased use of pesticides and spray adjuvants since the late 1990's and early 2000's is interesting because this occurred approximately when the Goss's wilt pathogen re-emerged. It is possible that these products may have contributed to pathogen re-emergence in the Midwest.

While herbicides have advanced in the industrial market, fungicides have also become more prevalent for disease control since the early 2000's (Munkvold et al. 2008). There are two main fungicide classes which are heavily relied upon for disease management: strobilurins and triazoles. Fungicides have been beneficial controlling diseases during the growing season.

Spray adjuvants are commonly used to improve pesticide efficiency (Khan et al. 2007). Numerous adjuvants can aid in pest control. These include, but are not limited to, nonionic surfactant (NIS), ammonium sulfate (AMS), crop oil concentrate (COC), high surfactant oil concentrate (HSOC), and methylated seed oil (MSO). Depending on pesticide application or crop restrictions, these adjuvants have different properties that contribute to pesticide efficacy. According to Wang and Liu (2007), adjuvants allow for better coverage, uptake, and movement throughout the plant with minimal crop damage.

However, some negative effects, like crop injury resulting in yield loss (Knezevic et al. 2010) and arrested ear development from NIS derivatives (Schmitz et al. 2011) have been reported. Epiphytic bacteria may be introduced more easily into the plant due to

adjuvant physical properties if the bacteria are being moved towards stomata or gain access through damaged cuticles.

A field study was conducted in 2015 to determine if adjuvants could affect disease severity. If *C. michiganensis* subsp. *nebraskensis* is living epiphytically, and adjuvants enable infection via stomata or damage to the cuticle, it may explain why the pathogen re-emerged and spread after evident inactivity. Micro-abrasions may allow bacterial entry if they are living epiphytically (Rocheford et al. 1985). Although a preliminary field study in 2014 indicated reduced disease severity with the use of adjuvants, a field trial in 2015 that was conducted at three locations in Nebraska indicated that surfactants had no affect on Goss's wilt disease development. However, since wind, hail, and heavy rain damage occurred at these field sites, this may have confounded the data. Therefore, the hypothesis of increased disease severity from spray adjuvants must be tested in a controlled greenhouse environment.

Results from the Langemeier et al. (2015) survey and our current knowledge on Goss's wilt development, management, re-emergence, and changes in farming practices have raised questions on the effect of pesticide, specifically adjuvant, use on disease development. Therefore, a greenhouse experiment was designed to evaluate the affect of spray adjuvants on Goss's wilt development. Greenhouse studied would avoid environmental factors that occur in the field and confound the data.

Methods and Materials

Bacterial isolate selection and inoculum preparation. An isolate of *C. michiganensis* subsp. *nebraskensis* collected in 2011 from Hall County, NE (225C) was used for this experiment. This isolate was identified as *C. michiganensis* subsp.

nebraskensis based on an ImmunoStrip® (ELISA) test kit (Agdia Inc. Elkhart, IN) (Korus et al. 2010b) for *C. michiganensis* subsp. *michiganensis*, AFLP and BOX-PCR analysis (Langemeier et al. 2014) for *C. michiganensis* subsp. *nebraskensis*, and greenhouse bioassays to acertain virulence of the isolate.

Isolate 225C was stored on beads in a Microbank™ vial (Pro-Lab Diagnostics Inc. Toronto, Canada) in cold storage (Langemeier et al. 2015). It was removed from cold storage at -80°C and streaked onto *Corynebacterium nebraskense* selective medium (CNS). This medium was developed to selectively isolate *C. michiganensis* subsp. *nebraskensis* (Gross and Vidaver 1979). The bacteria were subcultured to ensure culture purity and then transferred onto nutrient broth yeast extract (NBY) agar (Vidaver 1967) approximately every 4-6 days until a full plate of bacteria was grown. Full plates of bacteria were gently scraped from the agar surface with a sterile plastic scraper, suspended in 10 mM potassium phosphate buffer (pH 7.1) (Carlson et al. 1979), read on a Spectrophotometer (Helios α Spectophotometer, Thermo Electron Corporation, Beverly, MA, USA) at OD 600 nm (Beattie and Marcell 2002), and adjusted to reach an average concentration of 1 x 10^9 CFU ml^{-1} across trials.

Sweet corn seed. Golden Cross Bantam (GCB) sweet corn with a relative maturity of 76 days was planted, two seeds per 15.2 cm pot in a steam pasteurized soilless mix. This particular sweet corn hybrid was selected because it is very susceptible to *C. michiganensis* subsp. *nebraskensis*, and because it has been used in previous Goss's wilt studies. The seed was planted at a depth of 3.75 cm. Pots were planted 16 February 2015 and 23 March 2015 for each respective trial, and thinned to one plant per pot once they grew to V1.

Greenhouse conditions and experimental design. The experimental design for this study was a repeated measure with treatments arranged in randomized complete blocks. There were 10 treatments tested, 5 adjuvants and 5 controls, as can be found in Table 1. This experiment was conducted at the University of Nebraska-Lincoln Plant Pathology greenhouse in Lincoln, NE in Spring 2015 and was repeated during that same season. Greenhouse day / night temperature was 28 / 22°C with 16 hours of artificial light and eight hours of darkness within a 24 hour period. Pots were fertilized every day when watered. Plants were initially watered once daily and then twice as needed. Several controls were included in the trial. A buffer (BUFFER) control was to confirm bacterial virulence; a spray (SPRAY) control was to confirm that infection of corn by *C. michiganensis* subsp. *nebraskensis* can occur in the absence of wounding; copper hydroxide (positive control) was used to manage disease; a negative (WATER) control was used to test the effect of the adjuvants on Goss's wilt development, and a wound-inoculated control (WOUND) was used to also confirm bacterial virulence. Copper hydroxide was added as a treatment due to conflicting results from previous studies on Goss's wilt control and product efficacy (Korus et al. 2010a; Oser et al. 2013; Wise et al. 2014; Mehl et al. 2015).

Inoculations and spray applications. Once plants reached V1, they were inoculated with a suspension of *C. michiganensis* subsp. *nebraskensis* (1×10^9 CFU ml^{-1}). The growth stage at which inoculation was performed was determined from preliminary greenhouse studies conducted on GCB at the University of Nebraska-Lincoln Plant Pathology greenhouse and the University of Nebraska West Central Research and Extension Center in North Platte, NE (*data not shown*). The *C. michiganensis* subsp.

nebraskensis suspension was applied to the plants with an air pressurized canister (Sure Shot® Sprayer, Milwaukee Sprayer Manufacturing Co., Inc., Menomonee Falls, WI), pressurized with a Zéfal Air Max floor pump (Todson, Inc, Attleboro, MA), and calibrated between 517 kPa and 690 kPa to give an output of approximately 10 ml for 3 sec. The spray canister was held 30.5 cm to 45.7 cm away from leaf canopy. Trial 1 inoculations took place on 4 March 2015 and Trial 2 inoculations occurred on 31 March 2015.

Plants were sprayed with different adjuvants 48 hours after inoculation using a CO_2 pressurized backpack sprayer at 172 kPa, 94 L ha^{-1}, 5.6 kph to 6.4 kph, and a two nozzle boom on 76.2 cm centers with TeeJet® AIXR110025 nozzles (Spraying Systems Co., Wheaton, IL). This time frame was selected using results from a study by Beattie and Marcell (2002) who reported that the epiphytic population levels increase 48 hours after inoculation. Spray applications were made on 6 March 2015 for Trial 1 and 2 April 2015 for Trial 2.

Data collection and statistical analysis. Disease severity was observed by collecting visual estimates of the percentage of the leaf area showing Goss's wilt symptoms 7, 10, 13, 17, and 21 days after adjuvant applications. This was done by visually estimating disease severity as the percent lesion area of total leaf area on a continuous percentage scale (0 to 100%) (Mueller et al. 2009) with the help of a training program (Corn Pro version 3.4, F. W. Nutter Jr., Iowa State University, Ames, IA) (Bhatia and Munkvold 2002). Measurements were averaged based on the vegetative growth stage. After 21 days, leaves were randomly selected from different treatments to confirm infection by observing bacterial streaming and re-isolation onto NBY.

Measurements were analyzed using area under the disease progress curve (AUDPC) to determine differences between treatments over the growing period. Mean values were separated using a one-way analysis of variance (ANOVA) with PROC GLIMMIX. Adjuvant treatments were compared against one another and the SPRAY control. Blocks were treated as random and disease severity collected at different rating times was used for the repeated measure. Statistical analyses were tested at $\alpha = 0.05$ using SAS 9.4 (SAS Institute Inc., Cary, NC).

Results

Goss's wilt developed in both experimental trials. Disease was first observed anywhere from 5-8 days after inoculation with minimal symptom development initially. Results from Trial 1 showed that there were differences between treatments in disease severity during the growing period (P <0.0006). WOUND had the greatest disease severity (127.07) and BUFFER had the lowest disease severity (0). The SPRAY control (99.06) had a similar amount of disease compared to all of the other treatments tested (Figure 1). All AUDPC values for Trial 1 are summarized in Table 2.

Results from Trial 2 also showed that there were differences between treatments in disease severity during the growing period ($P < 0.0075$). WOUND had the greatest disease severity (332.87) and BUFFER had the lowest disease severity (0). NIS had interesting an interesting amount of disease severity over the growing season (232.31), but it was similar to the SPRAY control (129.96). All other treatments tested were similar to the SPRAY control (Figure 2). All AUDPC values for Trial 2 are summarized in Table 3.

Slight phytotoxicity also developed on the lower plant leaves after adjuvants were applied. Only two of the treatments applied showed phytotoxicity: HSOC and NIS. However, this damage did not appear to have an effect on disease development.

Discussion

Field observations by agronomists in Nebraska and Iowa, and data from a survey of Goss's wilt in 2011 (Langemeier et al. 2015) suggested that adjuvants used in pesticides commonly applied to corn during production may increase the risk of Goss's wilt developing. In this current study, spray adjuvants had no effect on Goss's wilt development in the greenhouse. It is possible that the method of inoculation used in this study allowed for bacterial entry prior to adjuvant use. Spray droplet size of the Sure Shot® Sprayer may have been small enough to allow for bacterial entry. The average size of this cornyform bacteria is 0.5 μm in width and 2.5 μm in length (Claflin 1999). This would average to around 1.25 μm^2. According to Kiesselbach (1949), the average stomatal aperture size when fully turgid is 51.3 μm^2 for most Nebraska hybrids. There are an average of 50,000 stomata per square inch on the upper leaf surface and 60,000 stomata per square inch on the lower leaf surface of corn plants (Kiesselbach 1949). Therefore, if the stomata were open, and the droplet size was small enough, the bacteria could have been directly introduced during inoculation.

Wounding techniques including sterile toothpicks (Smidt and Vidaver 1987), needle-eye method (Vidaver 1977), and multi-needle inoculators (Laurence and Aluisio 1981) all provide great ways of initiating infection in the greenhouse. SPRAY inoculations to observe the epiphytic population and infection development have been

performed and disease incidence is not typically as high compared to the wound-type inoculations (Mallowa et al. 2012; Mallowa et al. 2014).

It is possible that the bacteria could have accumulated around stomata, trichomes, or leaf hairs (Mallowa et al. 2012; Mallowa et al. 2014; Mallowa et al. 2015) that would have allowed for entry during early morning guttation (Eichenlaub et al. 2006), watering, or adjuvant use. Adjuvants like HSOC and NIS may allow for the bacteria to flood into the stomata, since the surface tension of the carrier is being compromised (Curran and Lingenfelter 2009). Further testing is desirable to confirm or deny this hypothesis.

Cuticle thickness of greenhouse grown plants may not be hardy enough to resist bacterial introduction. Vegetative characteristics of juvenile plants include short, narrow leaves, no trichomes or bulliform cells, and a thin cuticle layer with an epicuticular wax (Abedon et al. 1996). Characteristics of more mature corn plant include long, wide leaves, bulliform cells, trichomes, and a thick cuticle with no epicuticular wax (Abedon et al. 1996). Plants might be hardier in the field compared to the greenhouse due to environmental conditions. This might explain why adjuvant phytotoxicity was seen for some of the treatments tested in the greenhouse, but not in the field (Figure 3). Plant age at inoculation and spray adjuvants may have contributed to visible phytoxicity. This might also contribute to higher disease incidence and severity than expected. Some of the products tested have oil properties, including MSO, COC, and HSOC, that would allow for cuticle penetration (Curran and Lingenfelter 2009). If the bacteria were living epiphytically 48 hours after infestation, the adjuvants may have damaged the cuticle and allowed the bacteria to gain access into the plant. Further testing with leaf microscopy would be desirable to test this hypothesis.

Treatments may not have had any effect on bacterial entry. There was sufficient infection in the SPRAY control, and there were not consistent differences between other treatments in both trials. This is important because it shows that once the bacteria get into the plant, these adjuvants do not have an effect on pathogenicity or virulence. Korus et al. (2010a) found that copper hydroxide reduced disease development, but it was not statistically significant compared to the non-treated plots that were wound inoculated. Copper hydroxide is a contact product (Kennelly et al. 2007) which may have an effect on the epiphytic population. However, once the bacteria get into the plant, copper hydroxide may not be effective in controlling its growth (Kennelly et al. 2007).

Higher disease severity was observed in this greenhouse trial compared to a field trial conducted in 2015 that also evaluated the effects of adjuvants on Goss's leaf blight severity. The most plausible reason for this is the sweet corn hybrid used in the greenhouse study was more susceptible to infection by *C. michiganensis* subsp. *nebraskensis* than the hybrid used in the field trials.

Adjuvants are commonly added to pesticides that are applied to field crops. Data from a survey of Goss's wilt in 2011 (Langemeier et al. 2015) as well as observations by agronomists suggested applications of pesticides may increase the risk of Goss's wilt. There are more than 36.4 million corn hectares grown in the United States (United States Department of Agriculture 2015). In 2011 alone, four million of those hectares had foliar fungicide applications (Wise and Mueller 2011). In 2007, approximately 877 million pounds of active ingredients used in pesticides were applied to U.S. crops, costing $7.9 billion (United States Department of Agriculture 2012). Although adjuvants have many beneficial qualities, it is important to understand any damaging effects that may occur

(Knezevic et al. 2010; Schmitz et al. 2011). These greenhouse studies, as well as field studies conducted in 2015, suggest that Goss's wilt severity does not increase by the use of spray adjuvants.

Overall, spray adjuvants tested in this study do not show an increase in disease severity. Therefore, it is difficult to conclude if spray adjuvants contributed to Goss's wilt re-emergence. It is desirable, however, to understand why there is a slight decline in disease severity from preliminary field data with these same adjuvants (*data not shown*). Further research is needed to determine if spray adjuvants are decreasing disease severity instead of increasing it, as originally hypothesized.

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Table 1. Chemical list for adjuvant treatments used to determine increased disease severity for Goss's wilt.

Trade Name	Active Ingredient	Reference Name	Manufacturer Information	Rate	*Per 1 L Mix
Bronc®	ammonium sulfate	AMS	Wilbur-Ellis Company, Fresno, CA	5.0% v/v	50 ml mix^{-1}
ROC®	crop oil concentrate	COC	Wilbur-Ellis Company, Fresno, CA	1.0% v/v	10 ml mix^{-1}
High Load®	high surfactant oil concentrate	HSOC	Wilbur-Ellis Company, Fresno, CA	0.5% v/v	5 ml mix^{-1}
R11®	nonionic surfactant	NIS	Wilbur-Ellis Company, Fresno, CA	0.25% v/v	2.5 ml mix^{-1}
Super Spread MSO®	methylated seed oil	MSO	Wilbur-Ellis Company, Fresno, CA	1.0% v/v	10 ml mix^{-1}
[a]Water	H_2O	WATER	N/A	94 L ha^{-1}	1000 ml mix^{-1}
[b]DuPont™ Kocide®3000	copper hydroxide	KOCIDE® 3000	DuPont, Wilmington, DE	4.26 kg ha^{-1}	0.046 kg mix^{-1}
[c]Spray	N/A	SPRAY	N/A	N/A	N/A
[d]Wound	N/A	WOUND	N/A	N/A	N/A
[e]Buffer	10mM potassium phosphate buffer	BUFFER	N/A	N/A	N/A

[a]Water as a negative (-) control.
[b]DuPont™ Kocide®3000 as a positive (+) control.
[c]Inoculation control, no wounding, no adjuvants applied.
[d]Inoculation spray control, wounding, no adjuvants applied.
[e]10mM potassium phosphate buffer control, no bacterial spray, no wounding, no adjuvants applied (also a negative control).
*All applied with a CO_2 backpack sprayer at 94 L ha^{-1}, 172 kPa with an AIXR110025 nozzle.

Table 2. AUDPC values for all treatments tested for Goss's wilt development over the growing period in Trial 1 greenhouse study.

	Trial 1
Treatment	AUDPC
SPRAY	99.06[ab*]
BUFFER	0.00[c]
WOUND	127.07[a]
KOCIDE®3000	71.34[b]
WATER	76.64[b]
AMS	87.50[ab]
COC	84.48[ab]
HSOC	58.22[b]
MSO	94.33[ab]
NIS	92.41[ab]

*Means separated by letters are significant at $\alpha = 0.05$.

Table 3. AUDPC values for all treatments tested for Goss's wilt development over the growing period in Trial 2 greenhouse study.

	Trial 1
Treatment	AUDPC
SPRAY	129.96[bc*]
BUFFER	0.00[c]
WOUND	332.87[a]
KOCIDE®3000	130.26[bc]
WATER	119.26[bc]
AMS	112.09[bc]
COC	154.77[b]
HSOC	128.61[bc]
MSO	155.59[b]
NIS	232.31[ab]

*Means separated by letters are significant at $\alpha = 0.05$.

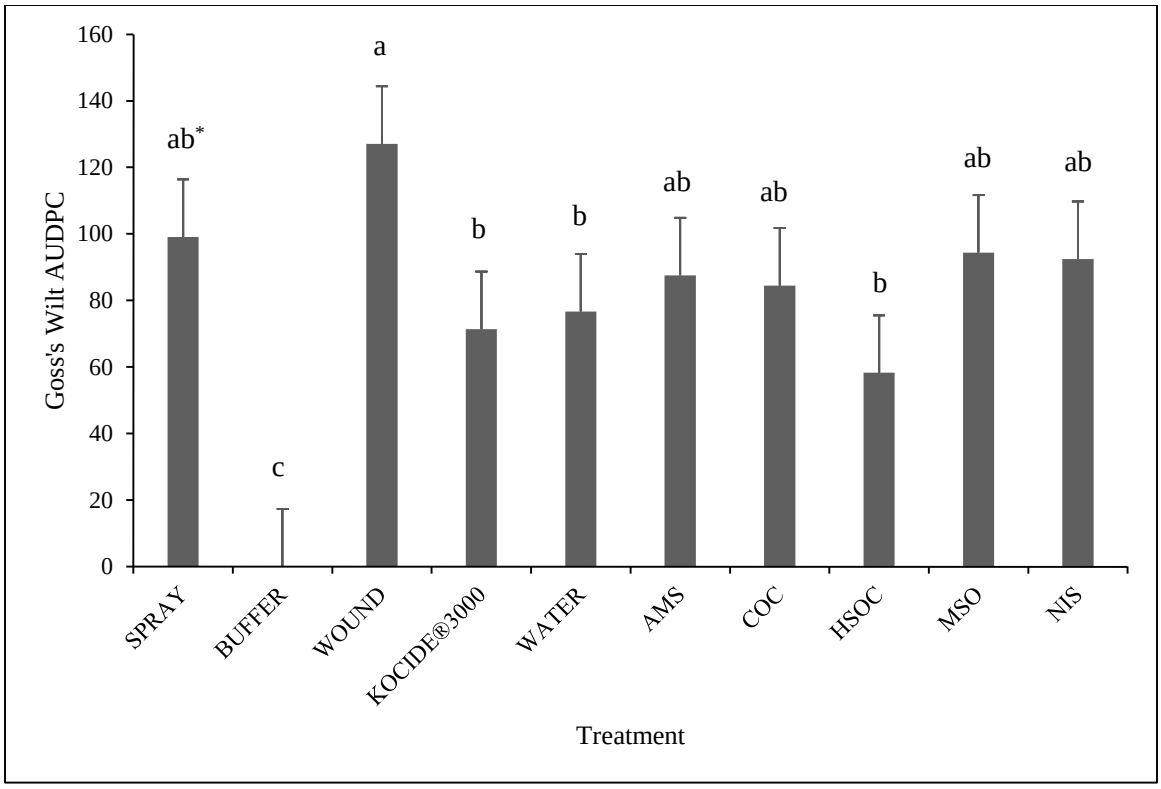

Figure 1. AUDPC with means separation for Goss's wilt disease severity over the growing period in Trial 1 greenhouse study.
*Means separated by letters are significant at $\alpha = 0.05$.

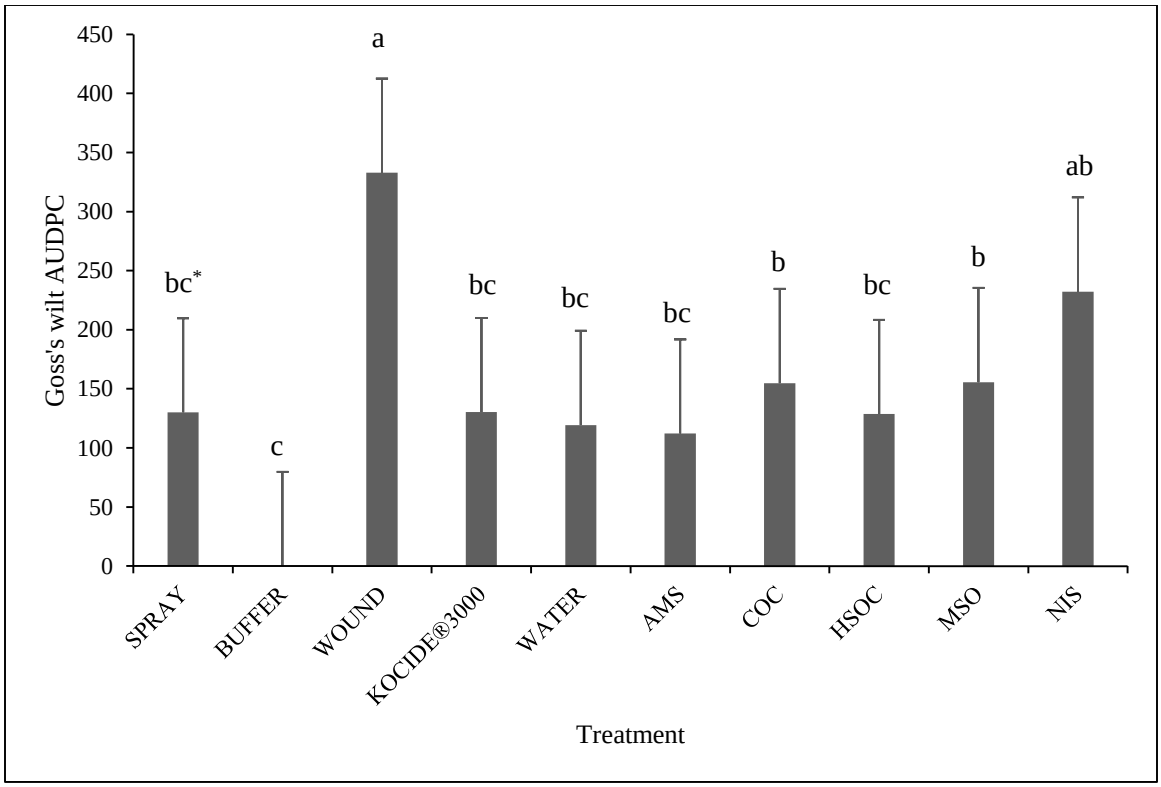

Figure 2. AUDPC with means separation for Goss's wilt disease severity over the growing period in Trial 2 greenhouse study.
*Means separated by letters are significant at $\alpha = 0.05$.

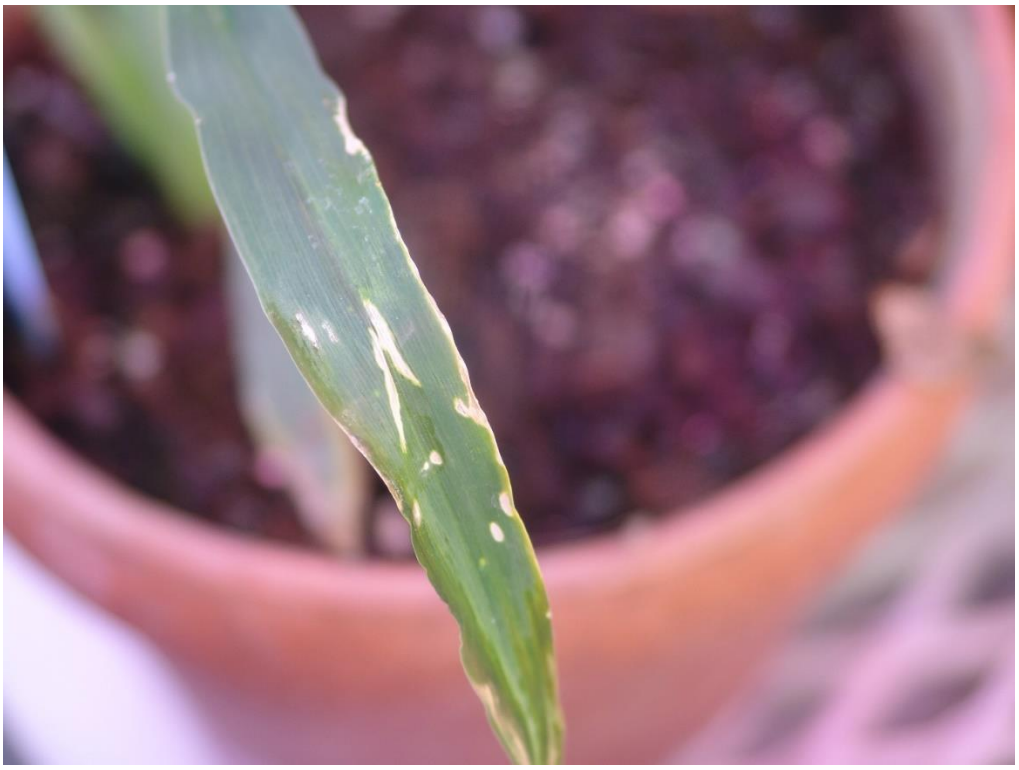

Figure 3. Phytotoxicity from spray adjuvants applied to Golden Cross Bantam sweet corn.

CHAPTER IV

INHIBITORY EFFECTS OF SPRAY ADJUVANTS TESTED ON *CLAVIBACTER MICHIGANENSIS* SUBSP. *NEBRASKENSIS IN VITRO*

Abstract

Field studies on disease severity of Goss's bacterial wilt and leaf blight of corn (*Zea mays* L.) with spray adjuvants yielded different results between experiments. A decrease in disease severity raised questions of spray adjuvants inhibiting *Clavibacter michiganensis* subsp. *nebraskensis* growth *in vitro*. The bacteria were grown in nutrient broth yeast extract (NBY) broth and aliquots of the bacterial suspension were plated onto NBY agar. Filter disks were soaked in one of five adjuvants at one of three concentrations (0.1, 1.0, and 10 x recommended rate) or two controls. Zones of inhibition around the filter disks were measured. Chi-square analyses indicated that the magnitude of inhibition varied for all treatments at each concentration for bacterial growth. A positive control (copper hydroxide) had the greatest frequency of bacterial inhibition among treatments at the three concentrations. Corn producers are encouraged to include adjuvants at 1.0 x recommended rate and results from this study indicate that the nonionic surfactant (NIS) decreased *C. michiganensis* subsp. *nebraskensis* growth *in vitro* for Trial 1 and Trial 2 compared to the rest of the adjuvants. Adjuvant concentrations at 10 x recommended rate inhibited bacterial growth the most for all three trials. NIS inhibited growth more than the positive control in Trial 2. There was some inhibition for other treatments including methylated seed oil (MSO) and crop oil concentrate (COC) as seen in Trial 2 and Trial 3, but they were not as consistent across trials as the inhibition caused by NIS. These suggest that epiphytic populations of the pathogen may be reduced by spray adjuvants commonly used in corn production. Further research is needed to determine if these effects can be demonstrated in the field and whether differences in chemical composition within an adjuvant group have an effect on *C. michiganensis* subsp. *nebraskensis* growth.

Introduction

Clavibacter michiganensis subsp. *nebraskensis* is a gram-positive bacterium and causal agent of Goss's bacterial wilt and blight of corn (*Zea mays* L.) (Vidaver and Mandel 1974). The bacteria can enter the plant in multiple ways, but a wounding event is usually associated with infection (Claflin 1999). Physical wounding can occur through hail, wind, sandblasting, or even mechanical damage (Wysong et al. 1981; Rocheford et al. 1985; Claflin 1999).

Bacteria are believed to be water splashed from infected residue, or moved by leaf rubbing and live ephiphytically (Wysong et al. 1981). This ephiphytic strategy allows for the bacteria to be present and may lead to infection if wounding occurs (Schuster et al. 1983). It has been reported that more susceptible varieties can support higher populations of epiphytic *C. michiganensis* subsp. *nebraskensis* (Schuster et al. 1983; Smidt and Vidaver 1986). Studies have also shown that the bacteria may be able to infect without physical injury by entering the stomata (Schuster et al. 1983; Mallowa et al. 2012; Mallowa et al. 2014; Mallowa et al. 2015).

Goss's wilt has not always been a severe problem since its first confirmation. This disease was first observed in Dawson County, Nebraska in 1969 (Wysong et al. 1973), and has gone through periods of evident inactivity where it developed sporadically in susceptible hybrids, popcorn, and sweet corn varieties where wounding occurred (Jackson et al. 2007). The pathogen's re-emergence in the early 2000's raised questions as to what farming practices were being used to increase disease incidence and severity. A 2011 survey, conducted by Langemeier et al. (2015), assessed the contribution of

farming practices to the re-emergence of Goss's wilt in the Midwest. Results of the study suggested that its re-emergence may be correlated with pesticide use.

Spray adjuvants are often mixed with pesticides to improve pest control (Khan et al. 2007). Common spray adjuvants in corn production include methylated seed oil (MSO), nonionic surfactant (NIS), crop oil concentrate (COC), ammonium sulfate (AMS), and high surfactant oil concentrate (HSOC). Adjuvants allow for better coverage, absorption, and translocation with minimal negative crop effects (Wang and Liu 2007). MSO, NIS, and COC adjuvants increased control of velvetleaf (*Abutilion theophrasti* Medic.) with foramsulfuron through better coverage and penetration (Bunting et al. 2004).

Adjuvants have also been beneficial in fungicide applications. Khan et al. (2000) reported applications of pyraclostrobin amended with adjuvants resulted in better control of Cercospora leaf spot (CLS), caused by *Cercospora beticola*, on sugar beets (*Beta vulgaris* L.). Similarly, Gent et al. (2003) reported the addition of adjuvants increased absorption of contact fungicides, such as azoxystrobin, when spraying onions (*Allium* spp. L.) and potatoes (*Solanum tuberosum* L.). Negative effects have also been associated with adjuvant use. Knezevic et al. (2010) found that POST applications of saflufenacil to winter wheat (*Triticum aestivum* L.) alone, or combined with COC or NIS, caused crop injury and yield loss. Arrested ear development of corn was shown to be caused by alkylphenol ethoxylate, a chemical component found in many NIS formulations (Schmitz et al. 2011).

In preliminary field trials conducted in 2014 in Nebraska, Goss's wilt severity was reduced when certain adjuvants were tested (*data not shown*). These data suggest

that adjuvants may directly affect *C. michiganensis* subsp. *nebraskensis*. Since the pathogen is capable of living epiphytically (Smidt and Vidaver 1986; Mallowa et al. 2012; Mallowa et al. 2014; Mallowa et al. 2015), populations would be exposed to pesticides and adjuvants. Since fungicides and herbicides have no affect on a bacterial pathogen like *C. michiganensis* subsp. *nebraskensis*, it is conceivable that the adjuvant may be having an impact on bacterial growth. However, there is little published research describing the effect of spray adjuvants on bacterial growth and development. Consequently, a lab-based experiment was designed to determine if spray adjuvants inhibit growth of *C. michiganensis* subsp. *nebraskensis in vitro*.

Methods and Materials

Bacterial isolate selection. An isolate of *C. michiganensis* subsp. *nebraskensis* collected in 2011 from Hall County, NE (225C) was used for this experiment. This isolate was identified as *C. michiganensis* subsp. *nebraskensis* based on an ImmunoStrip® (ELISA) test kit (Agdia Inc. Elkhart, IN) (Korus et al. 2010b) for *C. michiganensis* subsp. *michiganensis*, AFLP and BOX-PCR analysis (Langemeier et al. 2014) for *C. michiganensis* subsp. *nebraskensis*, and greenhouse bioassays to acertain virulence of the isolate.

Bacterial suspension preparation. Isolate 225C was stored on beads in a Microbank™ vial (Pro-Lab Diagnostics Inc. Toronto, Canada) in cold storage (Langemeier et al. 2015). It was removed from cold storage at -80°C and transferred onto nutrient broth yeast extract (NBY) agar (Vidaver 1967). Plates were incubated at 27°C for six days, one colony was selected, transferred to a new NBY plate, and incubated to ensure a pure culture was used. A single colony was selected and suspended into a 250

ml Erlenmeyer flask containing 100 ml of sterile NBY broth. The stock flask was placed on a rotary shaker at 160 rpm in a dark incubator and grown at 27°C for 29 hours. This experiment was repeated two more times.

A Spectrophotometer (Helios α Spectophotometer, Thermo Electron Corporation, Beverly, MA, USA) was used to measure the initial OD reading at 600 nm (Beattie and Marcell 2002) for each flask with an average reading of 1.818±0.097. Flasks of bacteria were not adjusted to reach a specific final concentration for this experiment. Full plates of bacteria were desired in order to observe any inhibitory effects from spray adjuvants, if present.

Experimental design, adjuvant treatments, and plate preparation. A split plot design was used for this experiment with concentration as the main plot and plate as the subplot. An experimental unit was each filter plate imbibed with different treatments, and 25 plates containing the individual treatments were used for each concentration tested. Adjuvants used in the study are listed in Table 1. All adjuvants were selected based on their physical properties and desired effects when applying pesticides within one chemical company (Wilbur-Elis Company, Fresno, CA). Adjuvants were diluted to reach a desired concentration in sterile distilled water. Concentrations of 0.1 x, 1.0 x, and 10 x recommended rates were used for this experiment. Dilutions were prepared this way to mimic tank clean-out or rain dilution after spraying (0.1 x recommended rate), recommended application (1.0 x recommended rate), and carrier evaporation after application (10 x recommended rate).

Modifying the methods of Prabuseenivasan et al. (2006), 500 µl aliquots of bacterial suspension were plated onto dry NBY plates using spread plate techniques. The

plates were allowed to dry with the bacterial suspension for four to five hours, and an additional 30 min with the lids cracked in a sterile fume hood to ensure sufficient drying. Using the disk diffusion method modified from Prabuseenivasan et al. (2006), filter paper disks were cut, 7 mm in size, autoclaved, and soaked in three separate adjuvant concentrations. Filter papers were aseptically transferred to labeled plates, and incubated for four days at 27°C. Treatments were color coded for easy identification (Table 2). Plates were not Parafilmed or turned upside down during incubation. Filter papers remained on the plates after drying for data collection.

Data collection and statistical analysis. After four days, zones of inhibition were measured with a ruler from the edge of the filter disk, to the edge of the inhibition zone in millimeters (mm). Chi-square analysis was used to report frequency of inhibition for each concentration. Measurements were subjected to a two-way analysis of variance (ANOVA) to report differences between adjuvant treatments and concentration. Statistical analysis was performed with $\alpha = 0.05$ using SAS 9.4 (SAS Institute Inc., Cary, NC).

Results

Inhibition of the growth of *C. michiganensis* subsp. *nebraskensis* varied across all concentrations tested for all three trials. Chi-square analysis detected significant inhibitory effects ($P < 0.0001$) for all tests (Table 3). This was due to the positive control (copper hydroxide) which consistently had the greatest frequency of inhibition. Copper hydroxide was maintained at maximum recommended rate throughout this experiment. None of the adjuvants showed inhibitory effects when tested at 0.1 x recommended rate for all trials. At 1.0 x recommended rate, a few treatments caused inhibition, but this was

not consistent across trials. The highest frequency of adjuvant inhibition was at 10 x recommended rate for most of the trials (Table 3).

For Trial 1, there is a significant interaction effect for concentration and treatment ($P = 0.0007$) with copper hydroxide having the most inhibition overall (Figure 1). The most significant interaction effect for adjuvants tested was NIS at 10 x label rate. This was the only adjuvant that showed significant inhibition when tested at any concentration (Figure 1). In Trial 2, there was a significant interaction effect for concentration and treatment ($P < 0.0001$) with NIS having the highest overall effect at 10 x recommended rate followed by copper hydroxide and MSO, which were statistically similar, and COC all at 10 x recommended rate (Figure 2). Trial 3 also had a significant interaction effect ($P < 0.0001$) with copper hydroxide having the largest overall effect followed by NIS and MSO maintained at 10 x recommended rate (Figure 3). Tabular description of these results can be found (Table 4). Plates with zones of inhibition can be seen in Figure 4.

Discussion

Copper hydroxide has consistent inhibitory effects on *C. michiganensis* subsp. *nebraskensis* growth *in vitro* when used at the maximum recommended rate. Since copper hydroxide was maintained at one rate across all trials, it shows that bacterial inhibition is consistent with the use of this product at maximum recommended rate. These findings are significant because copper hydroxide is used as a positive control in this lab study, but its efficacy in managing disease is not consistent in field trials. Both Oser et al. (2013) and Wise et al. (2014) did not find copper hydroxide to be efficacious in controlling Goss's wilt in field studies. However, Korus et al. (2010a) found that copper hydroxide reduced Goss's wilt severity. Copper hydroxide is a contact product

(Kennelly et al. 2007) and if the bacteria live epiphytically, bacterial growth may be affected. However, when the plant is wounded, the bacteria have a way to infect the plant, reducing copper hydroxide's efficacy (Kennelly et al. 2007). While these results are important for field studies, minimal work has been done *in vitro*. Further research needs to be conducted on copper hydroxide efficacy to find specific rates that would inhibit *C. michiganensis* subsp. *nebraskensis* growth *in vitro*.

Adjuvants tested in this study did not have consistent inhibitory effects at lower concentrations compared to the ones tested at recommended rate or 10 x recommended rate. The Prabuseenivasan et al. (2006) study on essential oils with antibacterial properties, determined that some oils had greater bacterial inhibition at higher concentrations compared to more diluted concentrations. Therefore, it may be necessary to use higher rates of spray adjuvants to have an effect on *C. michiganensis* subsp. *nebraskensis*.

These results are important because they show that some adjuvants have more inhibitory effects on this pathogen than others which are frequently used in corn production. It is also important because the bacteria can live epiphytically, which might lead to increased pesticide exposure. However, it is crucial to understand the chemical makeup of these adjuvants. NIS used in this experiment contains butyl alcohol and alkylphenol ethoxylate (Young 2012). Depending on the chemical company, these compounds may or may not exist in all NIS formulations. These compounds are often found in cleaning agents, detergents, surfactants, and industrial products (Ying et al. 2002), which may contribute to killing *C. michiganensis* subsp. *nebraskensis*. However, alkylphenol ethoxylate also contributes to arrested ear development, which can be

detrimental to yield (Schmitz et al. 2011). Also, as mentioned earlier, some spray adjuvants can cause phytotoxicity (Knezevic et al. 2010). These effects are not desirable for corn producers.

While inhibitory effects were observed *in vitro*, greenhouse studies did not show a decrease in disease severity (*data not shown*). Preliminary field results showed a decrease in disease present per plot, especially for the NIS treatment (*data not shown*). However, these results were not repeatable under greenhouse conditions. A field study did not show any differences between adjuvant treatments for disease severity either (*data not shown*). Contributing factors may include differences in hybrids, method of inoculation, lack of plant hardiness, or other environmental factors.

Minimal inhibition at recommended rate occurred for some treatments, but inhibition was not consistent across trials. Results obtained from the 10 x recommended rate treatment resulted in some adjuvants inhibiting bacterial growth. While these findings are important, they are not currently realistic due to potential crop damage (Knezevic et al. 2010; Schmitz et al. 2011). We can hypothesize that epiphytic populations exposed to spray adjuvants at recommended rate, may inhibit their growth and potential for disease development. It has been found that the bacteria may reside on the leaf surface, surrounding the stomata (Mallowa et al. 2014; Mallowa et al. 2015), on epidermal junctions, cuticle depressions, and around the base of trichomes (Mallowa et al. 2015). More research is needed to determine what treatments can be effectively applied to these areas to control the bacteria.

In conclusion, it is difficult to recommend the use of these adjuvants to decrease disease incidence in the field from a lack of consistency between studies even though

significant inhibition occurs *in vitro*. While the results are fascinating and encouraging that research is uncovering potential means of controlling this pathogen, it is uncertain what affect non-recommended adjuvant rates may have one corn yield. Further research is needed to determine inhibitory effects on products within similar chemical families of these treatments that showed bacterial inhibition. It would be necessary to test these adjuvants with different formulations across multiple chemical companies. It is also desirable to continue testing products that inhibit *C. michiganensis* subsp. *nebraskensis* growth *in vitro* to determine methods to decrease disease incidence and severity in the greenhouse and field.

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Table 1. Chemical list for spray adjuvant treatments used *in vitro*.

Trade Name	Active Ingredient	Reference Name	Rate	Manufacturer Information
Bronc®	ammonium sulfate	AMS	5.0% v/v	Wilbur-Ellis Company, Fresno, CA
ROC®	crop oil concentrate	COC	1.0% v/v	Wilbur-Ellis Company, Fresno, CA
High Load®	high surfactant oil concentrate	HSOC	0.5% v/v	Wilbur-Ellis Company, Fresno, CA
R11®	nonionic surfactant	NIS	0.25% v/v	Wilbur-Ellis Company, Fresno, CA
Super Spread MSO®	methylated seed oil	MSO	1.0% v/v	Wilbur-Ellis Company, Fresno, CA
N/A	H_2O	WATER	10 ml	N/A
DuPont™ Kocide®3000	copper hydroxide	KOCIDE®3000	0.21 g 10 ml^{-1}	DuPont, Wilmington, DE

Table 2. Chemical list for spray adjuvant treatments applied at 0.1 x, 1.0 x, and 10 x label rate *in vitro*.

Reference Name	Color	Trade Name	Manufacturer	0.1 x Rate*	1.0 x Rate**	10 x Rate***
AMS		Bronc®	Wilbur-Ellis	0.05 ml	0.5 ml	5 ml
COC		ROC®	Wilbur-Ellis	0.01 ml	0.1 ml	1 ml
HSOC		High Load®	Wilbur-Ellis	0.005 ml	0.05 ml	0.5 ml
NIS		R11®	Wilbur-Ellis	0.0025 ml	0.025 ml	0.25 ml
MSO		Super Spread MSO®	Wilbur-Ellis	0.01 ml	0.1 ml	1 ml
[a]WATER		N/A	N/A	10 ml	10 ml	10 ml
[b]KOCIDE®3000		DuPont™ Kocide®3000	DuPont	0.21 g $10ml^{-1}$	0.21 g $10ml^{-1}$	0.21 g $10ml^{-1}$

[a]WATER is a negative control.
[b]KOCIDE®3000 is a positive control. Rates remained constant across all concentrations.
*0.1 x rate denoted as C1.
**1.0 x rate denoted as C2.
***10 x rate denoted as C3.

Table 3. Chi-square analysis at 0.1 x, 1.0 x, and 10 x label rate for spray adjuvant concentration reported as percent inhibition of *C. michiganensis* subsp. *nebraskensis* growth ($\alpha = 0.05$).

	0.1 x Label Rate					
Treatment	Trial 1		Trial 2		Trial 3	
	N	Y	N	Y	N	Y
AMS	100	0	100	0	100	0
COC	100	0	100	0	100	0
HSOC	100	0	100	0	100	0
KOCIDE®3000	0	100	4	96	32	68
MSO	100	0	100	0	100	0
NIS	100	0	100	0	100	0
WATER	100	0	100	0	100	0
p-value	$P < 0.0001$		$P < 0.0001$		$P < 0.0001$	

	1.0 x Label Rate					
Treatment	Trial 1		Trial 2		Trial 3	
	N	Y	N	Y	N	Y
AMS	100	0	100	0	96	4
COC	100	0	76	24	100	0
HSOC	100	0	100	0	96	4
KOCIDE®3000	4	96	0	100	4	96
MSO	100	0	96	4	96	4
NIS	100	0	8	92	76	24
WATER	100	0	100	0	100	0
p-value	$P < 0.0001$		$P < 0.0001$		$P < 0.0001$	

	10 x Label Rate					
Treatment	Trial 1		Trial 2		Trial 3	
	N	Y	N	Y	N	Y
AMS	96	4	100	0	92	8
COC	100	0	4	96	76	24
HSOC	100	0	96	4	84	16
KOCIDE®3000	8	92	0	100	0	100
MSO	96	4	8	92	40	60
NIS	52	48	0	100	0	100
WATER	100	0	100	0	100	0
p-value	$P < 0.0001$		$P < 0.0001$		$P < 0.0001$	

Table 4. Inhibitory effects of *C. michiganensis* subsp. *nebraskensis* for spray adjuvant treatments in all three trials at 0.1 x, 1.0 x, and 10 x label rate.

Adjuvant	Trial 1		
Concentration	0.1 x	1.0 x	10 x
AMS	***-[d]	-[d]	0.10±0.05[d]
COC	-[d]	-[d]	-[d]
HSOC	-[d]	-[d]	-[d]
**KOCIDE®3000	0.89±0.38[b]	1.13±0.55[c]	0.88±0.66[b]
MSO	-[d]	-[d]	0.01±0.05[d]
NIS	-[d]	-[d]	0.21±0.27[c]
*WATER	-[d]	-[d]	-[d]

Adjuvant	Trial 2		
Concentration	0.1 x	1.0 x	10 x
AMS	-[f]	-[f]	-[f]
COC	-[f]	0.12±0.26[f]	1.71±0.73[d]
HSOC	-[f]	-[f]	0.08±0.4[f]
**KOCIDE®3000	1.74±0.72[d]	2.68±0.86[b]	2.61±0.78[b]
MSO	-[f]	0.04±0.2[f]	2.28±1.35[c]
NIS	-[f]	1.24±0.54[e]	3.16±1.29[a]
*WATER	-[f]	-[f]	-[f]

Adjuvant	Trial 3		
Concentration	0.1 x	1.0 x	10 x
AMS	-[e]	0.01±0.05[e]	0.02±0.07[e]
COC	-[e]	-[e]	0.06±0.11[de]
HSOC	-[e]	0.03±0.15[e]	0.04±0.09[e]
**KOCIDE®3000	0.75±0.68[c]	1.00±0.59[b]	1.26±0.55[a]
MSO	-[e]	0.06±0.22[de]	0.19±0.18[d]
NIS	-[e]	0.06±0.11[de]	0.93±0.36[b]
*WATER	-[e]	-[e]	-[e]

* WATER is a negative control.
**KOCIDE®3000 is a positive control. Rates remained constant across all concentrations.
***All values indicated by (-) are equivalent to no bacterial inhibition.
Values are mean inhibition zone (mm) ± standard deviation of all 25 replications.
Means within each respective trial separated by letters are significant at $\alpha = 0.05$.

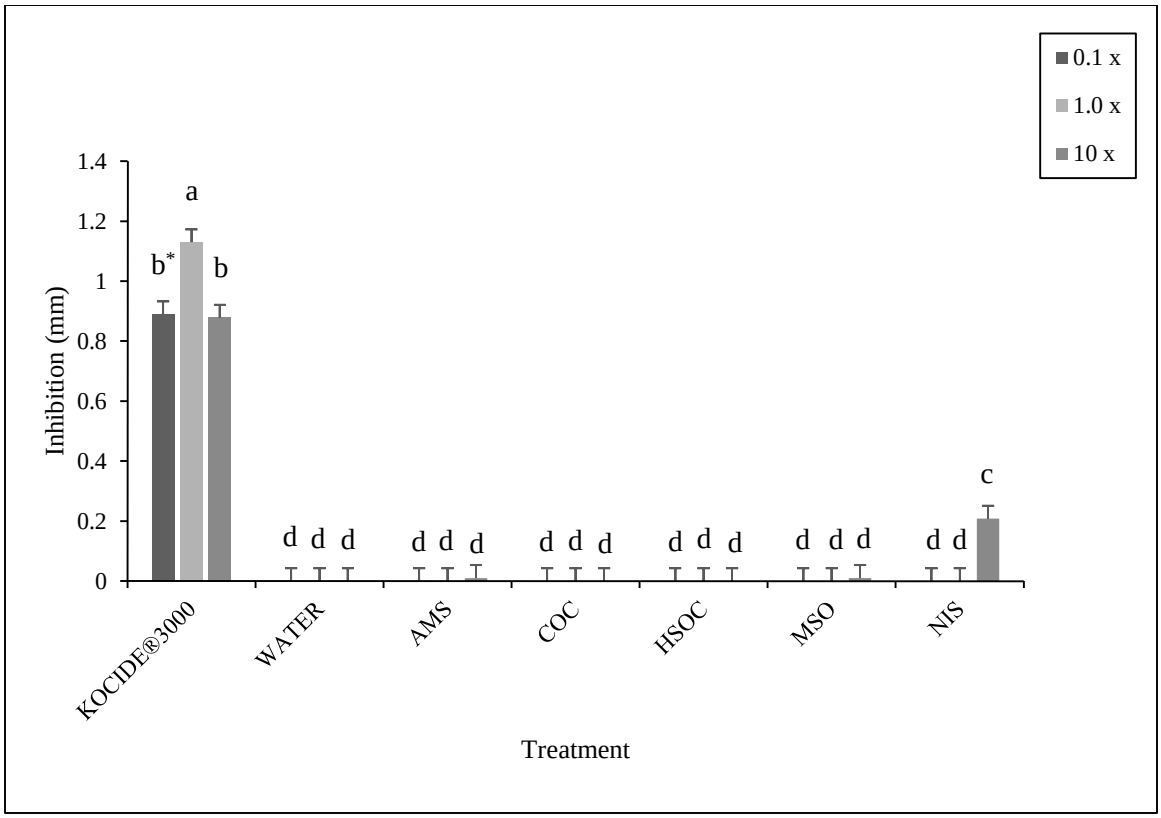

Figure 1. Inhibition results of *C. michiganensis* subsp. *nebraskensis* for all treatments in Trial 1 at 0.1 x, 1.0 x, and 10 x label rate.
*Means separated by letters are significant at $\alpha = 0.05$.

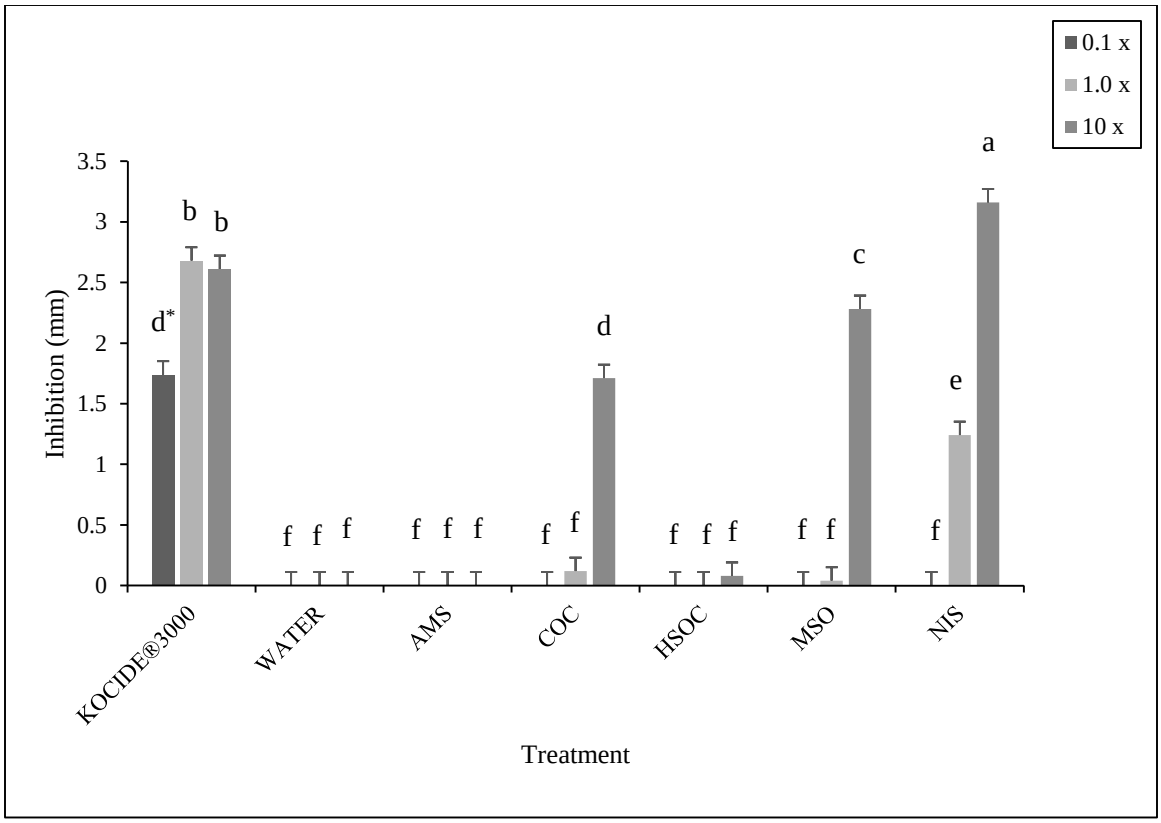

Figure 2. Inhibition results of *C. michiganensis* subsp. *nebraskensis* for all treatments in Trial 2 at 0.1 x, 1.0 x, and 10 x label rate.
*Means separated by letters are significant at $\alpha = 0.05$.

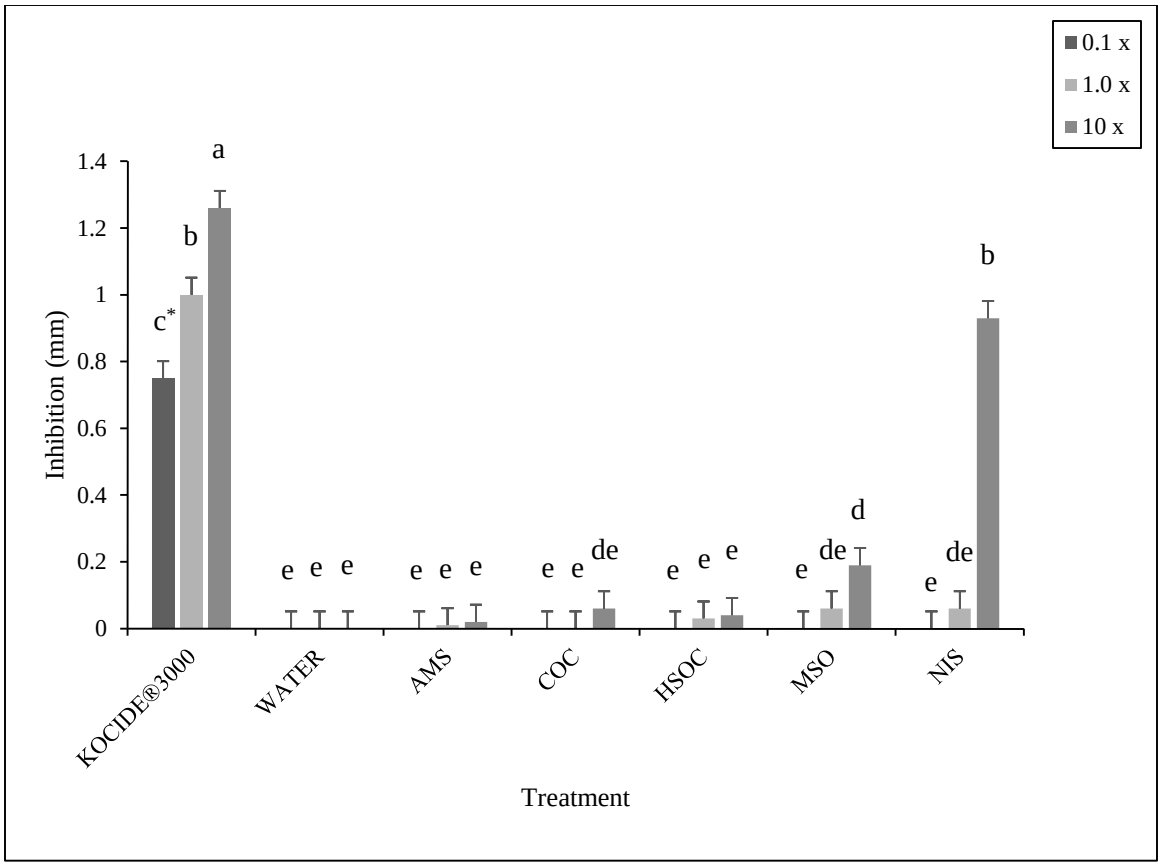

Figure 3. Inhibition results of *C. michiganensis* subsp. *nebraskensis* for all treatments in Trial 3 at 0.1 x, 1.0 x, and 10 x label rate.

*Means separated by letters are significant at $\alpha = 0.05$.

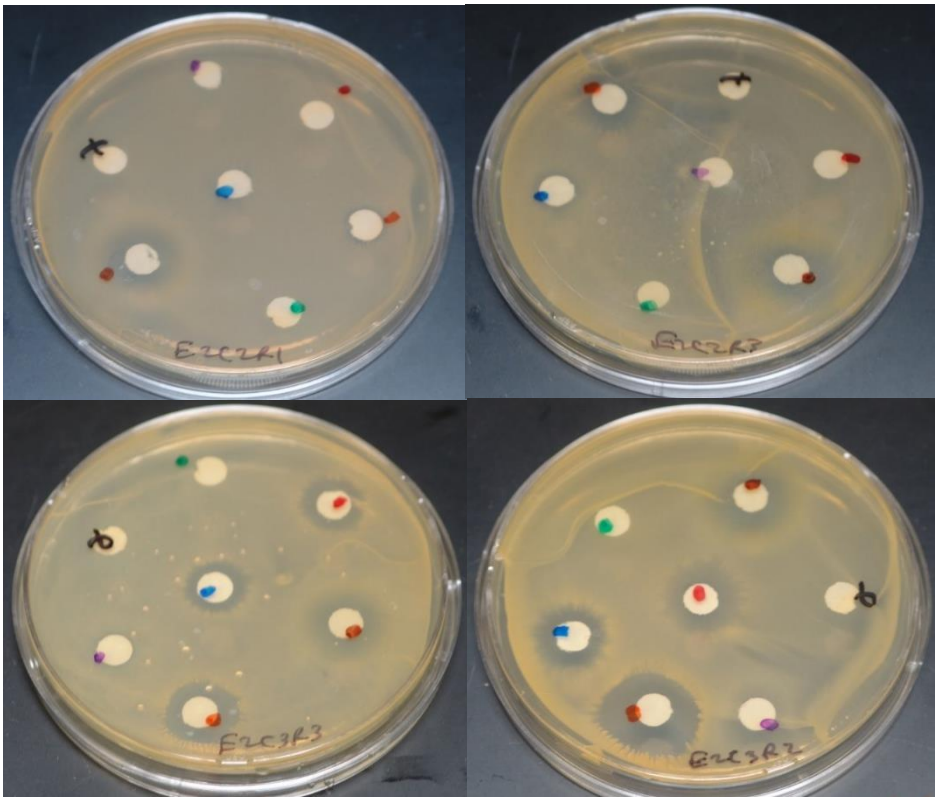

Figure 4. Inhibition plates for 1.0 x label rate (top) and 10 x label rate (bottom) for Trial 2 data.

APPENDIX I

PRELIMINARY FIELD STUDY INTERACTIONS OF GOSS'S WILT DEVELOPMENT AND SPRAY ADJUVANTS

Abstract

A preliminary field study was conducted to investigate any impacts on disease severity of Goss's wilt, causal agent *Clavibacter michiganensis* subsp. *nebraskensis*, when applying spray adjuvants and select active ingredients used in corn (*Zea mays* L.) production. Two field locations in southwest Nebraska were used. Two different susceptible hybrids were planted, an 87 day relative maturity (RM) hybrid at Madrid and a 107 RM hybrid at Gothenburg. Plots were sprayed with one of five adjuvants, one fungicide (azoxystrobin), one herbicide (glyphosate), or one negative control (water) at V6 growth stage. Separate plots were sprayed at R1 with one of two spray adjuvants, three separate fungicides (azoxystrobin, propiconazole, or azoxystrobin + propiconazole), or a negative control. Visual estimates of disease severity, systemically infected plants, and yield data were all collected for analysis. Results from Madrid indicated a decrease in disease severity with slight differences between treatments at V6 application timing. At R1 application timing, there were no differences between treatments in disease severity over the growing season. There were no difference between treatments for the number of systemically infected plants that developed for either application timing. There were also no differences between treatments for harvested yield. These results are important because they disagree with the initial hypothesis of spray adjuvants increasing disease severity. Results showed a decrease in disease severity compared to the control plots. There is a widespread use of spray adjuvants in corn production and further research is needed to determine if these results are repeatable, or if environmental factors were contributing to disease severity.

APPENDIX II

PRELIMINARY GREENHOUSE STUDY ON INOCULATION TIMING OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *NEBRASKENSIS*

Abstract

A preliminary greenhouse study was conducted to determine if inoculations of *Clavibacter michiganensis* subsp. *nebraskensis*, causal agent of Goss's wilt and leaf blight of corn (*Zea mays* L.), at specific growth stages allow for an increase in disease severity. Bacterial suspensions of *C. michiganensis* subsp. *nebraskensis* were made with either 10% tryptic soy broth (TSB) or 10 mM potassium phosphate buffer (Buffer) with bacterial density of 2.85 x 10^7 CFU ml^{-1} or 6.10 x 10^7 CFU ml^{-1}, respectively. Spray inoculations occurred on Golden Cross Bantam (GCB) sweetcorn plants with an air-pressurized canister at different growth stages. Disease severity was recorded 7 and 14 days after inoculation. Results indicated that disease severity was different between treatments and rating times, with TSB Trial 1 and Trial 2, having significant disease severity at the second rating time. For the Buffer experiment, both Trial 1 and Trial 2 also had significant disease pressure at the second rating time. These results are important because, it shows that plants can be infected with epiphytic populations of *C. michiganensis* subsp. *nebraskensis* when they are less mature, resulting in higher disease severity. This information can be used for future studies on the epiphytic population of *C. michiganensis* subsp. *nebraskensis*.