Dissemination of Goss's Wilt of Corn and Epiphytic *Clavibacter michiganensis* subsp. *nebraskensis* from Inoculum Point Sources

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Abstract

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Goss's wilt of corn, caused by *Clavibacter michiganensis* subsp. *nebraskensis*, has reemerged since 2006 as an economically important disease of corn in the Midwestern United States. In 2012 and 2013, field plot studies were conducted with a pathogenic, rifampicin-resistant *C. michiganensis* subsp. *nebraskensis* isolate and a Goss's wilt-susceptible corn hybrid to monitor epiphytic *C. michiganensis* subsp. *nebraskensis* population densities and the temporal and spatial spread of Goss's wilt incidence originating from inoculum point sources. The randomized complete block trial included three treatments: noninoculated control, inoculum point sources established by wound inoculation, and inoculum point sources consisting of *C. michiganensis* subsp. *nebraskensis*-infested corn residue. Epiphytic *C. michiganensis* subsp. *nebraskensis* was detected on asymptomatic corn leaves collected up to 2.5 m away from inoculum sources at 15 days after inoculation in both years. The

percentage of asymptomatic leaf samples on which epiphytic *C. michiganensis* subsp. *nebraskensis* was detected increased until mid-August in both years, and reached 90, 55, and 35% in wound-, residue-, and non-inoculated plots, respectively, in 2012; and 50, 11, and 2%, respectively, in 2013. Although both growing seasons were drier than normal, Goss's wilt incidence increased over time and space from all *C. michiganensis* subsp. *nebraskensis* point sources. Plots infested with *C. michiganensis* subsp. *nebraskensis* residue had final Goss's wilt incidence of 7.5 and 1.8% in 2012 and 2013, respectively; plots with a wound-inoculated source had final Goss's wilt incidence of 16.6 and 14.0% in 2012 and 2013, respectively. Our findings suggest that relatively recent outbreaks of Goss's wilt in new regions of the United States may be the result of a gradual, nondetected buildup of *C. michiganensis* subsp. *nebraskensis* inoculum in fields.

Since 2006, Goss's bacterial wilt and blight (Goss's wilt) of corn has presented a growing threat to corn production in the western Corn Belt. In recent years, the geographical extent of the disease in the United States has expanded to include Indiana (Ruhl et al. 2009), Minnesota (Malvick et al. 2010, 2012), Texas (Korus et al. 2011), Louisiana (Singh et al. 2015), North Dakota (Friskop et al. 2014), and Missouri (Sweets and Hosack 2014). The known range of Goss's wilt now extends from the southern states of Texas and Louisiana to the Canadian provinces of Alberta, Manitoba, and Ontario; as far west as Wyoming and Colorado; and as far east as Michigan and Indiana (Howard et al. 2015; Jackson-Ziems et al. 2012). Causes for the current outbreaks and pathogen spread have not been identified, but likely factors include planting of susceptible hybrids, weather conditions conducive for infection and disease spread during the growing season, increased continuous corn production, and increased adoption of reduced tillage practices (Givens et al. 2009; Jackson et al. 2007; Langemeier 2012).

The causal agent of Goss's wilt is a gram-positive bacterium, *Clavibacter michiganensis* subsp. *nebraskensis* (Vidaver and Mandel 1974), which was first found in Nebraska in 1969. Lesions develop in 7 to 14 days following inoculation (Calub et al. 1974) (A. E. Robertson, unpublished data) or plant injury from wind, hail, or windborne sand (Clafflin 1999; Rocheford et al. 1985). Warm temperatures (26 to 32°C) favor pathogen growth and lesion development (Smidt and Vidaver 1986). Early-season infection is associated

with higher levels of disease severity and greater yield loss (Pataky 1985).

Recent reports of Goss's wilt outbreaks in new states are of concern, particularly because it is not known how the pathogen was introduced to new states. Although seed-to-seedling transmission can occur, the rate of transmission is low (0.1 to 0.4%) (Biddle 1990; Biddle et al. 1990; Shepherd 1999), and *C. michiganensis* subsp. *nebraskensis*-infested residue is generally regarded as the primary source of inoculum in epidemic outbreaks (Biddle et al. 1985; Jackson et al. 2007; Schuster 1975; Smidt and Vidaver 1986). *C. michiganensis* subsp. *nebraskensis* survives for at least 10 months in corn crop residue left on the soil surface but pathogen survival in buried corn residue is greatly reduced (Biddle et al. 1985; Schuster 1975; Smidt and Vidaver 1986).

In 2008, an outbreak of Goss's wilt in Indiana occurred in multiple fields spread over two counties and 6,800 ha (Wise et al. 2009). Small, localized outbreaks of Goss's wilt were reported for the first time in northeast Louisiana in 2013 (Hollier et al. 2014). Seed transmission may be one plausible explanation for these outbreaks but it is also possible that the bacterium was introduced into these states in aerosols or *C. michiganensis* subsp. *nebraskensis*-infested residue. Storms have been shown to move plant pathogens in aerosols or on infested crop residue (Hirano and Upper 2000; Irely et al. 2006; Lindemann and Upper 1985; Pérombelon 1992).

Relationships between population densities of epiphytic pathogens and the incidence or severity of bacterial disease on leaves have been modeled in some plant pathosystems (Daugrois et al. 2014; Rouse et al. 1985) but not for Goss's wilt in corn. Smidt and Vidaver (1986) suggested that *C. michiganensis* subsp. *nebraskensis* has an epiphytic phase. They recovered *C. michiganensis* subsp. *nebraskensis* from the phyllosphere of asymptomatic, field-grown popcorn plants in June, approximately 4 weeks after planting, whereas symptom development was not noted until mid-July (Smidt and Vidaver 1986). Relationships between disease incidence and seasonal trends

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in epiphytic *C. michiganensis* subsp. *nebraskensis* populations throughout the growing season have not been quantified or described.

To improve our understanding of the epidemiology of Goss's wilt, the objectives of this study were to monitor (i) the spatial spread of epiphytic *C. michiganensis* subsp. *nebraskensis* and (ii) the development of Goss's wilt symptoms from inoculum point sources established within plots.

Materials and Methods

Bacterial strains and inoculum production. A pathogenic, rifampicin-resistant strain of *C. michiganensis* subsp. *nebraskensis* (isolate 91-R) was obtained from Charles Block (United States Department of Agriculture Plant Introduction Station, Ames, IA). The strain was grown at 24°C on solidified nutrient broth yeast extract agar amended with 50 mg of rifampicin, 100 mg of cycloheximide (Gleason et al. 1991), and 20 mg of chlorothalonil (Bravo Ultrex WDG; Syngenta Crop Protection LLC, Greensboro, NC) per liter (NBYCR+B). Inoculum was prepared from 5-day-old colonies that were suspended in 10 mM phosphate-buffered saline + Tween 20 (1 drop liter⁻¹; pH 7.4) (PBST) (Saalau-Rojas and Gleason 2012). Inoculum concentration was measured with a spectrophotometer at 600 nm and adjusted to approximately 1×10^6 CFU ml⁻¹ according to an optical density curve obtained previously (A. Robertson, unpublished data). Pathogen-infested residue for 2012 field studies was obtained from greenhouse-grown corn plants that had been artificially inoculated with *C. michiganensis* subsp. *nebraskensis* 91-R. Leaves with well-developed Goss's wilt lesions were air dried and stored at room temperature until needed. In 2013, symptomatic leaves saved from the previous year's 91-R-inoculated field trial were used as *C. michiganensis* subsp. *nebraskensis*-infested residue. One week prior to inoculation, the presence of *C. michiganensis* subsp. *nebraskensis* 91-R in the stored leaves from 2012 was confirmed by suspending leaf tissue in PBST and plating aliquots on NBYCR+B.

Within-field dispersal of epiphytic *C. michiganensis* subsp. *nebraskensis* and Goss's wilt from inoculum point sources. A Goss's wilt-susceptible hybrid, DeKalb DKC55-09 (rated 7 on a 1-to-9 scale, where 1 is excellent and 9 is poor) was planted at the Iowa State University Horticulture Research Station in Gilbert on 8 June 2012 and 21 June 2013. In both years, trials were established in newly tilled areas that had been used for grass hay production in previous years. Plots were 6 m wide by 9 m in length and contained approximately 400 plants, equivalent to 50 plants in eight rows, with 76 cm between rows and approximately 15-cm plant spacing within rows (86,500 plants ha⁻¹). Plots were bordered on all sides by four rows (3 m) planted with a Goss's wilt-resistant hybrid (DeKalb DKC56-55; rated 2 on a 1-to-9 scale, where 1 is excellent and 9 is poor). The experimental design was a randomized complete block design with three treatments: a noninoculated control, an inoculum point source established by wound inoculation (to simulate seedling infection resulting from seed transmission of *C. michiganensis* subsp. *nebraskensis*), and an inoculum point source consisting of *C. michiganensis* subsp. *nebraskensis*-infested corn residue. Point sources of inoculum were established at crop development stage V3 (Abendroth et al. 2011) on 25 June 2012, 17 days after planting (DAP), and at crop development stage V4 on 10 July 2013, 19 DAP. The four plants in the center of each wound-inoculated plot were inoculated by pinpricking with modified tongs (Calub et al. 1974) dipped in a suspension of *C. michiganensis* subsp. *nebraskensis* strain 91-R at 10^6 CFU ml⁻¹. For the residue-inoculated treatment, approximately 50 g of dry 91-R-infested corn leaves were placed on the ground in a 0.4-m² area at the base of the center four plants, and spread to cover at least 80% of the soil surface. Leaf residue was held in place with 1.3-cm mesh hardware cloth and pinned with wire landscape-cloth staples.

Visual assessments of disease incidence. Assessments were made on five dates in 2012 (10, 20, and 24 July and 17 and 30 August; crop development stages V8 to R5) and seven dates in 2013 (11 and 26 July; 7, 15, and 26 August; and 4 and 19 September; crop

development stages V4 to R5) (Table 1). All plants within plots were assessed visually for the presence or absence (incidence) of Goss's wilt on each date, and the presence or absence of symptoms on each plant was recorded.

Sample collection to determine epiphytic *C. michiganensis* subsp. *nebraskensis* population densities. In 2012, epiphytic *C. michiganensis* subsp. *nebraskensis* population densities were quantified using leaves sampled from 16 individual corn plants in each plot. A cross-sampling pattern was used to monitor both across-row and within-row spread of epiphytic *C. michiganensis* subsp. *nebraskensis* from point sources. Leaves were collected on the same five dates when visual assessments for disease incidence were made. On the first two dates, two leaves (one from the upper canopy and one from the lower canopy) were collected from the center plant in all eight rows (i.e., the 25th plant in each row), and the remaining eight plants were randomly selected (using a random number generator) from within the center two rows (i.e., the fourth and fifth rows). On the three subsequent dates, across-row samples were collected from the 24th, 26th, and 27th plants, respectively, in all eight rows. Within-row samples were located close to plot centers at the beginning of the season but, as the season progressed, leaf samples were collected from random plants located along the entire length of the plot. Leaves were placed in plastic bags and transported in coolers with ice. In the lab, leaf samples were kept at 5°C until processing, which was completed within 2 days.

In 2013, the sampling procedure was modified in order to increase the number of plants sampled on each date and to quantify the increase of epiphytic populations over time at distances relative to inoculum point sources. The center two rows of the plots were divided into 10 0.9-m-long quadrats, which were sampled to quantify the spatial spread of *C. michiganensis* subsp. *nebraskensis* within rows, as described by Nutter et al. (1998). Two additional 0.9-m quadrats were designated at the centers of rows 2 and 3 and rows 6 and 7, to monitor the spread and increase of epiphytic *C. michiganensis* subsp. *nebraskensis* populations across rows. Four asymptomatic leaves were collected from each quadrat (one upper-canopy leaf and one lower-canopy leaf from two plants) and processed as a bulk sample for dilution plating. Plants in quadrats with inoculum sources were left intact early in the season to avoid removing leaves in the area that served as the inoculum source. Leaves were collected from all quadrats on the last sample date. Repeat sampling of previously sampled plants was avoided until later in the season, when all plants in the center two rows had been sampled once. Workers donned fresh disposable gloves before moving from one plot to the next. Samples were collected on seven dates in 2013, from 24 July to 16 September (crop development stages V8 to R5; Table 1). Quadrat samples were bagged separately and placed in a cooler with ice for transport to the lab. Samples were stored at 5°C and processed within 2 days.

Dilution plating to determine *C. michiganensis* subsp. *nebraskensis* population densities. In 2012, pieces of healthy, green leaf tissue, approximately 10 to 12 cm long, were taken from both the tip and the middle of each sampled leaf. Fresh weight of the leaf pieces (combined weight = 2.5 to 6.0 g) was recorded. All four pieces from a plant were combined and placed in test tubes with 20 ml of sterile PBST (Beattie and Marcell 2002). Tubes were sonicated for 7 min in a B200 ultrasonic cleaner (Branson Ultrasonics Corp., Danbury, CT) filled with ice water, then packed in crushed ice. Tubes were vortexed for 15 s; and 0.1-ml aliquots were transferred immediately to sterile 1.5-ml microcentrifuge tubes filled with 0.9 ml of sterile 1× PBST buffer. Single 0.1-ml aliquots of the original suspension and a 10⁻¹ dilution were plated on NBYCR+B agar plates and spread with sterile glass beads. Cultures were inspected 5 or 6 DAP, and the presence or absence of typical *C. michiganensis* subsp. *nebraskensis* colonies was recorded. The lower detection limit for this procedure, based on buffer volumes used and plated, was 2.0×10^2 CFU sample⁻¹.

In 2013, leaf pieces approximately 10 to 15 cm long were taken from the tip and middle of the four leaves from each quadrat. Pieces were combined, placed in a 118-ml Whirl-Pak bag (Nasco, Fort Atkinson, WI), and weighed. The average fresh sample weight was

6 to 7 g. PBST (20 ml) was added to each bag and samples were shaken briefly, then sonicated and packed in ice as in 2012. Prior to plating, bagged samples were squeezed rapidly 10 times and then shaken vigorously for 10 s to ensure adequate mixing of the suspension. Ten-fold dilutions were prepared by transferring 0.9 ml of the original suspension to an empty well in a sterile, deep-well polypropylene plate (similar to PlateOne, 96 2.0-ml wells/plate; USA Scientific, Ocala, FL), and then transferring 0.1-ml aliquots to wells filled with 0.9 ml of sterile PBST. At each step, suspensions were mixed by pipetting 10 times with fresh pipette tips. Six 10-ml drops of the 10^0 , 10^{-1} , and 10^{-2} dilutions were placed on two plates of NBYCR+B media using an electronic multichannel pipette. A total volume of 0.12 ml per dilution was drop plated. The lower detection limit for the procedure was 1.7×10^2 CFU sample $^{-1}$. The protocol was adapted from the procedure described by Chen et al. (2003). Agar plates were incubated in the dark at room temperature.

Colonies on NBYCR+B were counted 5 DAP and recounted on day 6. Colony counts were expressed as CFU g $^{-1}$ fresh leaf tissue (log $_{10}$ scale). The proportion of plant samples (in 2012) or quadrat samples (in 2013) with epiphytic *C. michiganensis* subsp. *nebraskensis* populations was calculated for each plot and sample date and recorded as the incidence of epiphytic *C. michiganensis* subsp. *nebraskensis*.

Data analysis. Disease incidence was defined as the number of plants with Goss's wilt symptoms/the total number of plants per plot ($n = 400$) $\times 100$ (Campbell and Madden 1990). Treatment differences in disease incidence on individual sampling dates were analyzed using PROC MIXED (SAS Institute, Cary, NC). Significant treatment-sample date interactions ($P \leq 0.05$) were observed in both years; therefore, incidence data from each sample date were analyzed separately. In both years, one or more treatments had fewer than five assessment dates with symptoms present; therefore, change in disease incidence over time was not modeled (Campbell and Madden 1990; Madden et al. 2007; Nutter 1997). Instead, average rates of disease increase over time were calculated and treatment differences were compared using *t* tests. Unless stated otherwise, all treatment means were compared using Tukey's test for mean separations ($P = 0.05$).

Epiphytic *C. michiganensis* subsp. *nebraskensis* incidence was graphed using percentages but incidence of *C. michiganensis* subsp. *nebraskensis* in samples was recorded as count data. Initial analyses of binomial count data detected significant treatment-days after inoculation interactions; therefore, data for specific sample dates were analyzed separately using SAS PROC GLIMMIX, with treatment and

replication as fixed effects. Total counts of *C. michiganensis* subsp. *nebraskensis*-positive samples for the growing season were also compared using PROC GLIMMIX. Mean epiphytic *C. michiganensis* subsp. *nebraskensis* population densities on individual sampling dates in 2013 and season-long averages were compared using SAS PROC MIXED.

Spatial pattern analyses. Three methods were used to analyze the spatial dynamics of Goss's wilt within corn plots. Ordinary runs analysis and black-white joins analysis (Byamukama et al. 2011) were used to determine aggregation in the spatial pattern of Goss's wilt-infected plants within plots. A distance-based analysis was used to describe the spread of Goss's wilt incidence within the plots (Gougherty and Nutter 2015).

Ordinary runs analyses were conducted using data for individual plots and visual assessment dates to assess the spatial aggregation of symptomatic plants in both years (Byamukama et al. 2011; Madden et al. 1982). A run is defined as a series of like events (i.e., a series of healthy plants or a series of Goss's wilt-infected plants). Statistical significance was determined by calculating a *z*-statistic, which compares the actual number of runs in a plot to the number of runs expected if the infected plants were randomly distributed. This analysis tests the null hypothesis that infected plants are randomly distributed within the plot. Significant spatial aggregation of Goss's wilt-infected plants within a plot was identified when the *z*-statistic was less than -1.64 ($P \leq 0.05$) (Byamukama et al. 2011).

Black-white joins analysis (Byamukama et al. 2011; Sawada 1999) was also conducted for each sampling period in both years. A Microsoft Excel macro, Rookcase, was used to perform the black-white joins analysis (Sawada 1999). This analysis also assesses the aggregation of similar classes but black-white joins analysis can be used to measure relationships in two dimensions simultaneously (e.g., within and across rows). A black-white join is an adjacency between two different classes (i.e., an infected plant and a healthy plant). The analysis simulates 1,000 randomizations of the observed distribution within a plot in order to determine whether the observed pattern is significantly different from a randomized pattern. As with ordinary runs analysis, a *z*-statistic is calculated which compares the observed number of black-white joins to the expected number of joins under the null hypothesis of randomization.

Distance-based analyses. In order to get a measure of the spread of Goss's wilt incidence within plots, we used a nearest-neighbor analysis to determine the distance from newly infected plants to the nearest infected plant detected during a previous sampling period (Gougherty and Nutter 2015). All nearest-neighbor distances for individual plots and visual assessment dates were used for analysis, and distances to

Table 1. Timeline of activities performed in inoculum-point-source field studies conducted at the Iowa State University Horticulture Research Farm, Gilbert, in 2012 and 2013^a

Activities	2012			2013		
	Date	GS	DAP	Date	GS	DAP
Planting	8 June		0	21 June (0)	...	0
Inoculation	25 June	V3	17	10 July (19)	V4	19
Sampling for epiphytic bacteria	10 July–30 August (5 dates)	V8 to R5	32 to 83	24 July–16 September (7 dates)	V4 to R5	33 to 87
Date bacteria first detected in						
Inoculated treatment	10 July	V8	32	24 July	V8	33
Residue treatment	10 July	V8	32	24 July	V8	33
Noninoculated treatment	24 July	V13	46	7 August	V11	47
All plots	17 August	R3	70	n/a	–	n/a
Visual assessments	10 July–30 August (5 dates)	V8 to R5	32 to 82	11 July–19 September (7 dates)	V4 to R5	20 to 90
Date lesions first recorded in						
Inoculated treatment	10 July	V8	32	22 July ^b	V7	31
Residue treatment	24 July	V13	46	7 August	V11	47
Noninoculated treatment	24 July	V13	46	n/a	...	...
All plots	30 August	...	83	n/a	...	...

^a *Clavibacter michiganensis* subsp. *nebraskensis*, the causal agent of Goss's wilt and leaf blight of corn, was introduced at plot centers either by leaf-prick inoculation or by placing dried, *C. michiganensis* subsp. *nebraskensis*-infested corn leaves on the soil surface. GS = corn growth stage (Abendroth et al. 2011), DAP = days after planting, and n/a = not applicable.

^b Targeted inspection of inoculated plants was made on 22 July but not all plants were inspected on this date. The second census assessment was made on 26 July.

50 and 95% of newly infected plants (D_{50} and D_{95} , respectively) within plots were calculated. Analysis of nearest-neighbor distances was performed using sequential visual assessment dates, as well as on the final cumulative dataset. Treatment differences in D_{50} and D_{95} distances were assessed by analysis of variance.

Weather data. Precipitation and daily high and low temperature data for all locations were downloaded from the Iowa Environmental Mesonet (Fig. 1; <http://mesonet.agron.iastate.edu/agclimate/>). In both years, monthly precipitation in June, July, and August was 40 to 80% below 30-year historical averages. Daily mean temperatures were warmer than average in June and July 2012 and June 2013. A wind and rain storm on 25 July 2012 (47 DAP) caused considerable leaf damage and plant lodging in research plots at the Horticulture Station.

Results

Incidence of Goss's wilt symptoms. In 2012, Goss's wilt lesions (large, yellow to gray-green lesions, with necrotic centers and characteristic "freckles" on lesion edges) were present on all wound-inoculated plants on 10 July, 15 days after inoculation (DAI; Table 1). Symptomatic plants were observed in two residue-inoculated plots and one noninoculated plot on 24 July (29 DAI, crop development stage V13). On the last sample date (30 August, approximate crop development stage R5), all 12 plots contained plants with Goss's wilt lesions. Mean incidence of Goss's wilt at the end of the season was 16.6, 7.5, and 0.9% in the wound-inoculated, residue-inoculated, and noninoculated treatments, respectively (Fig. 2A). Orthogonal contrasts of least-squares means confirmed that disease incidence in the residue-inoculated plots was lower than incidence

in the wound-inoculated plots on all dates in 2012 ($P \leq 0.0001$ on the first four dates and $P = 0.0705$ on 30 August). Mean disease incidence for the entire growing season was lowest in the noninoculated plots ($P = 0.0677$); however, on any individual sampling date, disease incidence in noninoculated control plots was not significantly different from incidence in residue-inoculated treatments ($P > 0.10$).

In 2013, the timing of early symptom development in wound-inoculated and residue-inoculated treatments was very similar to that seen in 2012 (Table 1); however, Goss's wilt symptoms were never observed in the noninoculated control plots. At the end of the 2013 growing season, 14.0, 1.8, and 0.0% of the plants in the wound-inoculated, residue-inoculated, and noninoculated plots, respectively, had Goss's wilt lesions (Fig. 3A). In 2013, disease incidence in the wound-inoculated treatment was higher ($P < 0.01$) than incidence in the residue-inoculated treatment on all visual assessment dates. Mean incidence in the residue-inoculated plot was not significantly different from zero and hence, not different from the noninoculated treatment on any date. Mean incidence for the entire growing season was lowest in the noninoculated treatment ($P = 0.0853$).

In 2012, disease incidence in wound-inoculated plots increased at the average rate of $0.30\% \text{ day}^{-1}$. This was twice as fast as the rate of increase in residue-inoculated plots ($0.15\% \text{ day}^{-1}$), and 15 times faster than the rate of increase in noninoculated plots ($0.02\% \text{ day}^{-1}$). Rates of increase fluctuated through during the season, and the fastest rates for disease increase were observed at the end of the season (Fig. 2A). In general, slower rates of disease increase were observed in 2013. In the second year of the study, the incidence of plants with symptoms increased at the average rate of $0.24\% \text{ day}^{-1}$ in wound-inoculated

Fig. 1. Daily maximum temperature and precipitation at the Iowa State University Horticulture Research Farm near Gilbert, IA during the A, 2012 and B, 2013 corn growing seasons.

plots, which was seven times faster than the rate of increase in residue-inoculated plots ($0.03\% \text{ day}^{-1}$; Fig. 3A). The fastest rates of disease increase in 2013 occurred during the period from 26 August to 4 September.

Incidence of epiphytic *C. michiganensis* subsp. *nebraskensis* on asymptomatic samples. In both years, *C. michiganensis* subsp. *nebraskensis* was detected on asymptomatic green leaves collected from wound-inoculated and residue-inoculated plots on the first sampling dates, which occurred 15 and 14 DAI in 2012 and 2013, respectively (Table 1). On these dates, *C. michiganensis* subsp. *nebraskensis*-positive samples were collected as far as 2.5 m from inoculum locations.

In 2012, the incidence of plants with detectable populations of epiphytic *C. michiganensis* subsp. *nebraskensis* increased until 17 August (Fig. 2B), when rifampicin-resistant *C. michiganensis* subsp. *nebraskensis* was recovered from 90, 55, and 35% of samples collected

from wound-inoculated, residue-inoculated, and noninoculated plots, respectively ($P \leq 0.05$). The incidence of *C. michiganensis* subsp. *nebraskensis*-positive samples in wound-inoculated and residue-inoculated inoculated plots was similar on the first three collection dates ($P > 0.10$), but greater in wound-inoculated plots on the last two dates ($P < 0.05$) (Fig. 2B). Epiphytic *C. michiganensis* subsp. *nebraskensis* was detected on 41.5%, 25.7%, and 10.4% of all samples collected during the growing season from wound-, residue-, and noninoculated treatments, respectively ($P < 0.0004$).

In the noninoculated control treatment, rifampicin-resistant *C. michiganensis* subsp. *nebraskensis* was first detected on one plant in a single noninoculated control plot on 20 July 2012. On 17 and 30 August, the pathogen was detected in all four control plots, and sample incidence within plots ranged from 6 to 73% on these two dates. Throughout the 2012 season, asymptomatic-tissue samples from noninoculated plots had the lowest incidence of *C.*

Fig. 2. A, Incidence (%; $n = 400$) of corn plants (DeKalb DKC55-09) with Goss's wilt symptoms and **B,** incidence of asymptomatic plants on which epiphytic *Clavibacter michiganensis* subsp. *nebraskensis* was detected in 2012. On 25 June (17 days after planting), inoculum point sources were established in plots near Gilbert, IA by pinprick inoculation of four plants or by placing *C. michiganensis* subsp. *nebraskensis*-infested residue at the base of four plants.

Fig. 3. A, Incidence (%; $n = 400$) of corn plants (DeKalb DKC55-09) with Goss's wilt symptoms and **B,** incidence of asymptomatic leaf samples on which epiphytic *Clavibacter michiganensis* subsp. *nebraskensis* was detected in 2013. Inoculum point sources were established in plots near Gilbert, IA on 10 July (19 days after planting) by pinprick inoculation of four plants or by placing *C. michiganensis* subsp. *nebraskensis*-infested residue at the base of four plants. Asymptomatic leaves from two plants were sampled from 12 quadrat locations per plot.

michiganensis subsp. *nebraskensis*-positive samples (Fig. 2B), although epiphytic incidence was significantly lower than that in residue-inoculated plots only on 20 July ($P = 0.0062$) and 17 August ($P = 0.0517$).

In 2013, the incidence of *C. michiganensis* subsp. *nebraskensis*-positive samples peaked in mid-August (19 August, crop development stage R1), as in 2012. However, peak incidence (50, 11, and 2% in wound-, residue-, and noninoculated control plots, respectively; Fig. 3B) was much lower than in 2012. In wound-inoculated plots, incidence of *C. michiganensis* subsp. *nebraskensis*-positive samples increased over time during the first five sample dates. In residue-inoculated and noninoculated treatments, the incidence of *C. michiganensis* subsp. *nebraskensis*-positive samples fluctuated throughout the 2013 growing season. On all sample dates in 2013, epiphytic *C. michiganensis* subsp. *nebraskensis* was not detected in one or more residue-inoculated inoculated plots; therefore, incidence of *C. michiganensis* subsp. *nebraskensis*-positive samples in residue-inoculated and noninoculated plots was not significantly different on individual sampling dates ($P > 0.10$). However, total incidence of *C. michiganensis* subsp. *nebraskensis*-positive samples over the season was greater in residue-inoculated plots than in noninoculated control plots ($P = 0.0170$). On the last sample date in 2013, epiphytic *C. michiganensis* subsp. *nebraskensis* was recovered from the wound-inoculated treatment but not from the residue-inoculated or noninoculated treatments. Epiphytic *C. michiganensis* subsp. *nebraskensis* was recovered from 29.0% ($\pm 2.6\%$), 6.5% ($\pm 1.3\%$), and 0.9% ($\pm 0.5\%$) of all samples collected from wound-inoculated, residue-inoculated, and noninoculated plots, respectively, during the 2013 season. In 2013, *C. michiganensis* subsp. *nebraskensis* was detected on only three individual samples from the noninoculated treatment: from two noninoculated plots on 7 August and one noninoculated plot on 19 August.

Early-season incidence of *C. michiganensis* subsp. *nebraskensis*-positive samples on 10 July 2012 was correlated with disease incidence on three subsequent sampling dates in 2012 (24 July, 17 August, and 30 August; $r = 0.66$ to 0.96 , $P > r < 0.05$) but most of the association was explained by early symptoms in the four wound-inoculated plots, and linear relationships were not identified

(data not shown). In 2013, epiphytic incidence on 7, 14, 19, and 26 August (growth stages V11, V13, R1, and R2, respectively) was correlated with Goss's wilt incidence on all assessment dates from 31 July to the end of the season ($r = 0.74$ to 0.97 ; $P < 0.05$). In both years, final disease incidence in individual plots was correlated with the number of days between planting and dates when lesions were first noted in plots ($r = -0.65$ and -0.75 in 2012 and 2013, respectively; $P < 0.05$).

Epiphytic population density. In 2013, the mean population density of epiphytic *C. michiganensis* subsp. *nebraskensis* detected on asymptomatic leaf tissue was 1.6×10^3 CFU g^{-1} . Epiphytic population densities detected on individual samples ranged from 1.6×10^1 CFU g^{-1} fresh leaf tissue to 4.4×10^7 CFU g^{-1} . Considerable variation in epiphytic population densities was seen in all three treatments (Fig. 4). Treatment differences were not evident on most sample dates ($P > 0.10$) but, on 31 July, mean *C. michiganensis* subsp. *nebraskensis* population densities in wound-inoculated plots exceeded mean densities in residue-inoculated plots by a factor of 10 (1 unit on the $\log_{10}$ scale; $P > t = 0.0727$). Averaged over the entire season, epiphytic population densities were higher (3.39 ± 0.15 on the $\log_{10}$ scale) in the wound-inoculated treatment than in the residue-inoculated treatment (2.58 ± 0.32 ; $P = 0.0333$). However, mean epiphytic population densities in residue-inoculated plots increased from 2.1 ± 0.4 (before lesions were present) to 3.6 ± 0.5 (after lesions were present) (data not shown). Mean population density in noninoculated controls (2.10 ± 0.84) was not different from early-season or season-long mean densities in the residue-inoculated treatment ($P > 0.10$; Fig. 5).

Spatial pattern. In both years and both inoculated treatments, the spatial pattern of plants with Goss's wilt symptoms was generally aggregated (Figs. 6 and 7). In 2012, plants with Goss's wilt symptoms were aggregated in 90.0% of the instances when ordinary runs could be calculated. In 2013, 79.4% of the plot-date instances had significantly aggregated spatial patterns of symptomatic plants. Black-white joins analysis results largely agreed with the ordinary runs results. Across all sampling periods, symptomatic plants were present in aggregated patterns in 93% of the instances in 2012 and in 72.2% of the instances in 2013. In 2012, several control plots also had plants with Goss's wilt lesions. The spatial pattern in these plots, however, was random.

Fig. 4. Mean population densities of epiphytic *Clavibacter michiganensis* subsp. *nebraskensis* detected in washings of asymptomatic corn leaves sampled from a Goss's wilt-susceptible hybrid (DeKalb DKC55-09) in plots near Gilbert, IA, on seven dates during the 2013 growing season. Inoculum point sources were created on 10 July (19 days after planting) as wound-inoculated plants (circles) or *C. michiganensis* subsp. *nebraskensis*-infested residue (triangles).

Fig. 5. Correlations between final Goss's wilt incidence and number of days after planting when symptoms were first seen in plots in field studies conducted in 2012 and 2013 near Ames, IA. Pearson correlation coefficients are significant at $P < 0.05$.

Fig. 6. Plot maps of 2012 field trial showing plants with Goss's wilt symptoms. Each plot was planted to a Goss's wilt-susceptible hybrid (DeKalb DKC55-09) and measured eight rows wide by 50 plants long (6 by 9 m). A 3-m border of a Goss's wilt-resistant hybrid (DKC 56-55) surrounded each plot (not shown). W, R, and N indicate wound-inoculated, residue-inoculated, and noninoculated treatments, respectively. Colors indicate assessment date when symptoms were first recorded on each plant (red, 10 July; orange, 24 July; green, 17 August; light blue, 30 August).

Fig. 7. Plot maps of 2013 field trial showing plants with Goss's wilt symptoms. W, R, and N indicate wound-inoculated, residue-inoculated, and noninoculated treatments, respectively. Plot dimensions (6 m wide by 9 m long) and positions are drawn to scale; four-row borders are not shown. Colors indicate date when symptoms were first recorded on each plant (red, 26 July; orange, 7 August; yellow, 15 August; green, 26 August; light blue, 4 September; dark blue, 19 September).

Distance-based analysis. D_{50} and D_{95} varied by year and by treatment. In 2012, the average D_{50} distance for the wound-inoculated treatment was 0.56 m, whereas D_{50} for the residue-inoculated treatment was 1.24 m (Table 2). These values indicate that newly symptomatic plants in wound-inoculated plots tended to be nearer to previously symptomatic plants, whereas newly symptomatic and previously symptomatic plants were farther apart in residue-inoculated treatment plots ($P < 0.05$). Maximum distances between newly symptomatic and previously symptomatic corn plants and D_{95} showed similar patterns for wound-inoculated and residue-inoculated treatments, and differences in treatment means were not significant ($P \geq 0.05$). In 2013, D_{50} distances for residue-inoculated and wound-inoculated plots were not significantly different (Table 2).

Discussion

Our work demonstrates that *C. michiganensis* subsp. *nebraskensis*-infested corn residue on the soil surface is a source of inoculum for epiphytic populations of the pathogen, which can increase on asymptomatic plants throughout the growing season, and that the presence of infested residue can result in plants with Goss's wilt symptoms, even during dry growing seasons. Our findings suggest that the relatively recent expansion of Goss's wilt into new regions of the United States may be the result of a gradual, unnoticed buildup of inoculum in new areas, via movement of fauna, wind-blown residue, or aerosols from historically infested fields, which is further supported by susceptible hybrids, continuous corn production, and reduced-tillage practices. Residue management and rotation to nonhost crops may help growers manage the disease from one season to the next but may not completely eliminate inoculum sources and disease risk.

Our findings suggest that *C. michiganensis* subsp. *nebraskensis* from residue can quickly become established as an epiphyte on corn. Smidt and Vidaver (1986) detected epiphytic *C. michiganensis* subsp. *nebraskensis* in mid-June, approximately 4 weeks after planting, and Goss's wilt in mid-July. In both 2012 and 2013, we detected epiphytic *C. michiganensis* subsp. *nebraskensis* 2 weeks after establishing inoculum point sources within each plot. The use of inoculum point sources and targeted sampling (near point sources) most likely improved our chances of detecting *C. michiganensis* subsp. *nebraskensis* sooner than Smidt and Vidaver (1986) (Kinkel 1997). In both years of our study, rainfall events occurred 10 to 14 DAI, shortly before the first sample collection dates. These rain events likely facilitated dispersal of *C. michiganensis* subsp. *nebraskensis* from well-developed Goss's wilt lesions, which were present on wound-inoculated plants at 15 and 14 DAI. Epiphytic populations of *C. michiganensis* subsp. *nebraskensis* were also detected in residue-inoculated plots on these dates, and nonrandom patterns of Goss's wilt incidence within residue-inoculated plots suggest that disease in these plots developed from residue point sources. Although weather conditions in different locations and years influence the development and detection of epiphytic *C. michiganensis* subsp. *nebraskensis* populations, it is likely that commercial fields with *C. michiganensis* subsp. *nebraskensis*-infested residue contain plants with epiphytic *C. michiganensis* subsp. *nebraskensis* soon after corn seedling emergence.

The rates of disease increase observed in our study clearly document the type of disease increase that can occur in very dry years, which are usually unfavorable for disease development. Substantially faster rates occurred in plots with wound-inoculated plants than in plots where *C. michiganensis* subsp. *nebraskensis*-infested residue

was used to establish inoculum point sources. These rate differences can only be explained by timing of initial infection, because pathogen strain, host hybrid, and environmental conditions were the same in both treatments. After lesions are present, the bacteria ooze out of diseased tissue (Jackson et al. 2007), more inoculum is present, and disease increase proceeds at a faster rate. We observed that plots with earlier initial symptoms had higher levels of final disease incidence. Smidt and Vidaver (1986) noted that disease development in June was correlated with end-of-season disease severity levels. These data support the importance of delaying the onset of disease symptoms.

We saw considerable variation in epiphytic population densities on asymptomatic leaves, and found no relationship between population density of epiphytic *C. michiganensis* subsp. *nebraskensis* with the incidence of *C. michiganensis* subsp. *nebraskensis*-positive samples. Mean population densities calculated using all samples (including zeroes; that is, samples without detected *C. michiganensis* subsp. *nebraskensis*) mimicked trends seen in plots of epiphytic incidence over time (data not shown). This suggests that sampling to determine the incidence of epiphytic *C. michiganensis* subsp. *nebraskensis* on leaves is an adequate measure of increase in epiphytic populations; quantification of detected populations by dilution plating may not yield much additional information.

Previous studies with other plant pathosystems have identified relationships between epiphytic population densities and disease severity. Lindemann et al. (1984a,b) identified relationships between epiphytic population densities and disease severity in the bean halo blight–common bean pathosystem (*Pseudomonas syringae* on *Phaseolus vulgaris*). Smidt and Vidaver (1986) recovered high epiphytic *C. michiganensis* subsp. *nebraskensis* populations (10^3 to 10^{11} CFU g⁻¹) from commercial popcorn fields and associated symptom development with a threshold population of 10^7 CFU g⁻¹ fresh weight. The leaf sampling criteria used in their study was not described. Because we restricted sampling to asymptomatic leaves in order to assess epiphytic population trends at potential infection sites, we were not able to identify relationships among epiphytic population density, epiphytic population incidence, and disease incidence. Population density estimates or incidence estimates based on entire individual leaves may potentially be more useful for estimating disease severity (Hirano et al. 1982). The corn leaves we sampled were large, and only a portion of each leaf was sonicated and used for determining population densities. Furthermore, we bulked leaf pieces from four leaves in each sample. We likely recovered much lower populations ($<10^7$ CFU g⁻¹) than Smidt and Vidaver (1986) because of our sampling method. Leaves with active infections would most likely have much higher *C. michiganensis* subsp. *nebraskensis* population densities, because the bacteria can often be observed oozing out of blighted tissue (Jackson et al. 2007).

Anecdotal reports describe “sudden” outbreaks of Goss's wilt following severe storms. Wise et al. (2009) suggested that storms that occurred early in the growing season contributed to the introduction and dispersal of the pathogen in northwest Indiana. In our 2012 study, the most severe weather event of the summer occurred on 25 July, when heavy rain and 40-plus mph (66 km h⁻¹) winds lodged many of the plants in the trial, just 1 day after 24 July, when low incidence of Goss's wilt was detected in inoculated plots. In 2013, *C. michiganensis* subsp. *nebraskensis* was detected in plots before storms occurred on 22 July 2013, although initial infections were limited to fewer plants than in 2012. Incidence of epiphytic *C. michiganensis* subsp. *nebraskensis* increased two- to fourfold in plots after these storms; higher incidence of Goss's wilt symptoms was

Table 2. Mean distances from plants with Goss's wilt symptoms, identified on previous assessment dates, to 50% (D_{50}), 95% (D_{95}), or 100% (D_{max}) of new plants with Goss's wilt symptoms in 2012 and 2013^a

Treatment	2012			2013		
	D_{50} (m)	D_{95} (m)	D_{max} (m)	D_{50} (m)	D_{95} (m)	D_{max} (m)
Wound-inoculated	0.56*	1.92	2.88	0.45	2.19	2.97
Residue-inoculated	1.24*	2.47	3.59	0.70	1.82	1.97

^a Asterisks (*) indicate that distances for wound- and residue-inoculated treatments are significantly different at $P = 0.05$.

observed on the next assessment dates, 17 August 2012 (23 days later) and 5 August 2013 (12 days later). These observations suggest that severe storms assisted with dispersal of *C. michiganensis* subsp. *nebraskensis* from corn leaves in aerosols and, consequently, increased disease incidence.

Despite the fact that we observed increased incidence of Goss's wilt after severe storms, our studies do not support the hypothesis that single storm events will result in sudden outbreaks with high disease incidence. Our data suggest that local *C. michiganensis* subsp. *nebraskensis* dispersal gradients can be steeper than expected. After the storm in 2012, symptoms of Goss's wilt increased from 4 to 7% (from 16 to 28 plants) in the wound-inoculated plots and 1 to 3% (from 4 to 12 plants) in the residue-inoculated plots. Symptomatic plants were clustered close to the source of inoculum or, in 2012, occurred as single plants in the control plots (Fig. 6). These levels of disease could easily go unnoticed, particularly if Goss's wilt occurred in a small area within a large field, yet the area could constitute an effective source of initial inoculum for subsequent corn crops, especially in fields with continuous-corn production and reduced tillage. Based on these data, we suggest that "sudden outbreaks" of the disease may involve multiple infection cycles over more than one growing season.

The fact that spatial patterns of Goss's wilt-infected plants in the field are mostly aggregated has important implications with regards to sampling and the quantification of yield losses (Madden and Nutter 1995; Nutter 2002). Because infected plants tend to be aggregated within cornfields, a systematic sampling design should be employed when disease intensity (e.g., incidence or severity) is assessed in corn fields (Byamukama et al. 2011; Madden and Nutter 1995). Because the spatial pattern of infected plants is aggregated, there is less opportunity for healthy plants to compensate for yield loss via increased seed number and increased seed weight (Hughes et al. 1989; Madden et al. 2007; Nutter 2001), because infected plants are more likely to be neighbored by other Goss's wilt-infected plants.

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