Research

Impact of Inoculum Concentration on Goss's Wilt Development in Corn and Alternative Hosts

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Abstract

Goss's bacterial wilt and blight of corn (*Zea mays* L.) is caused by *Clavibacter nebraskensis* (Cn). Research on Cn has confirmed alternative hosts for the disease, yet little is known about disease development in these alternative hosts. A greenhouse study was conducted to determine the inoculum concentration at which disease occurred for annual ryegrass (*Lolium multiflorum* Lam.), giant foxtail (*Setaria faberi* Herm.), and johnsongrass (*Sorghum halepense* L.), compared with Goss's wilt susceptible and moderately resistant corn hybrids. Inoculum concentrations from 1×10^0 to 1×10^7 CFU/ml were compared for each individual species. Time for initial lesion development to occur was measured and compared among the hosts and inoculum concentrations. At

21 days after inoculation, both corn hybrids had higher disease severity than the alternative hosts, and disease severity increased for both corn hybrids when inoculum concentrations increased. Alternative hosts had no differences in disease severity as inoculum concentrations increased. Days required for initial lesion development to occur were similar for all hosts, and lesion development occurred sooner when inoculum concentration increased. Results demonstrate corn contributes more to the inoculum potential of Cn, but alternative hosts can still be a refuge for Cn.

Keywords: *Clavibacter nebraskensis*, grasses, weeds, disease development

Goss's bacterial wilt and blight in corn (*Zea mays* L.), caused by *Clavibacter nebraskensis* (Cn), was first observed on hybrid corn in 1969 in Dawson County, Nebraska (Wysong et al. 1973), and was soon found in surrounding counties and regularly observed in Nebraska over the next 10 years. From 1980 until 2006, only sporadic cases of the disease were confirmed in fields where susceptible hybrids were planted (Jackson et al. 2007), but since 2006, Goss's wilt has reemerged and is confirmed in 17 states in the United States (Ikley et al. 2015).

Corn is the primary host, and infected plants and residue are thought to be the primary inoculum source for Cn (Biddle et al. 1990). Several studies have determined the role of Cn inoculum in disease development. Eggenberger et al. (2016) determined that corn residue infected with Cn contributed more to the incidence and spread of Goss's wilt compared with living infected plants. Mallowa et al. (2016) determined that Cn does not require wounds to enter host plants, but instead can enter through natural openings such as stomata or trichomes. This challenges the idea that Cn requires open wounds created from windblown sand, severe storms, or equipment to infect hosts plants (Claflin 1999). These events, however, can still aid in spreading Cn throughout the field, and no-till and continuous corn have a greater potential to increase the inoculum potential of Cn in the field, because they cause a buildup of corn residue (Eggenberger et al. 2016).

Research on the amount of inoculum required to cause disease is still limited. Schuster et al. (1972) and Calub et al. (1974a) evaluated different corn hybrids for susceptibility to Cn, and Calub et al. (1974b) determined what inoculum concentrations were required to distinguish between corn hybrids with different levels of susceptibility. Calub et al. (1974b) determined that disease severity ratings of plants inoculated with concentrations of Cn below 1×10^6 CFU/ml could not be used to differentiate resistance among genotypes. In this study, concentrations of 1×10^2 CFU/ml did not result in symptoms of Goss's wilt, indicating that inoculum concentrations must be higher than this level to cause disease. Other experiments conducted on corn have used inoculum concentrations of 1×10^7 or 1×10^8 CFU/ml (Calub et al. 1974c; Carlson et al. 1979; Pataky 1985). These inoculum concentrations are similar to inoculum concentrations that have been reported in fields with a history of Goss's wilt. Smidt and Vidaver (1986) reported 1×10^{10} CFU per gram of fresh weight at midwinter in infected corn residue and 1×10^5 CFU per gram of fresh weight at the end of the following season.

In addition to corn, some alternative hosts for Cn are known. Schuster (1975), Langemeier et al. (2014), and Ikley et al. (2015) discovered teosinte (*Euchlaena mexicana* Schrad.), eastern gamagrass (*Tripsacum dactyloides* [L.] L.), green foxtail (*Setaria viridis* L.), shattercane (*Sorghum bicolor* [L.] Moench ssp. *arundinaceum* [Desv.] de Wet & Harlan), grain sorghum (*Sorghum bicolor* [L.] Moench ssp. *drummondii* [Nees ex Steud.] de Wet & Harlan), sugarcane (*Saccharum officinarum* L.), yellow foxtail (*Setaria pumila* [Poir.] Roemer & J.A. Schultes), giant foxtail (*Setaria faberi* Herm.), bristly foxtail (*Setaria verticillata* [L.] Beauv.), large crabgrass (*Digitaria sanguinalis* [L.] Scop.), annual ryegrass (*Lolium multiflorum* Lam.), and johnsongrass (*Sorghum halepense*

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[L.] Pers.) to all be susceptible to *Cn*. These alternative hosts were evaluated with inoculum concentrations of 1×10^6 to 1×10^8 CFU/ml in greenhouse experiments. Langemeier et al. (2014) and Ikley et al. (2015) were able to demonstrate alternative hosts respond differently to *Cn* in terms of the level of disease severity observed on the infected leaves at a single inoculum concentration. Corn appears to be more susceptible compared with the alternative hosts, except for giant foxtail, which had similar disease severity to the tested corn hybrids. These alternative hosts are important in Goss's wilt management because they potentially serve as a refuge for *Cn* when farmers rotate to a nonhost crop. Although corn did not have Goss's wilt symptoms when inoculated at 1×10^2 CFU in a previous study, there is no information regarding inoculum levels less than 1×10^6 that can result in disease symptoms in alternative hosts (Calub et al. 1974b).

Because research involving Goss's wilt has focused mainly on corn, there is a lack of information on how alternative hosts influence the epidemiology of *Cn*, specifically how much inoculum is needed to cause disease in these hosts, and how the disease develops over time at different inoculum concentrations. Information on alternative host interactions with *Cn* could give a better understanding of how to limit inoculum potential in areas of the Midwest affected by Goss's wilt, because the presence of alternative hosts may reduce the effectiveness of management measures, such as tillage and crop rotation. Even if farmers are proactive and try to reduce the inoculum potential of *Cn* by removing corn residue, there still may be enough inoculum in the field for infection to occur. With limited research examining the impact of lower inoculum concentrations on disease severity, there is a need to determine how sensitive alternative hosts are to *Cn* infection. The purpose of this research was to determine the inoculum concentration at which *Cn* infection can occur in corn and alternative hosts and to determine the time required for symptoms to appear on alternative hosts compared with corn.

Effect of *Cn* Inoculum Concentration on Alternative Hosts

The hosts selected for comparison were annual ryegrass, giant foxtail, and johnsongrass, along with a susceptible corn hybrid and a moderately resistant corn hybrid. The *Cn*-susceptible corn hybrid (DKC55-09RIB Brand Blend, Dekalb Corn, Monsanto Company, St. Louis, MO) is rated as an eight on a 1 to 9 scale, with 9 being susceptible, and the *Cn* moderately resistant corn hybrid (33D42, Pioneer Corn, Dupont, Johnston, IA) is rated as a seven on a 1 to 9 scale, with 9 being resistant.

Plants were arranged in a randomized complete block design in a greenhouse in West Lafayette, IN. Each plant species had 10 replicate plants per inoculation treatment and a noninoculated treatment for a total of 96 plants per species. The experiment was conducted twice. Greenhouse temperatures were maintained at 26 to 31 and 21 to 25°C for experiments 1 and 2, respectively. Greenhouse temperatures were different between the experiments owing to temperature requirements of other projects being conducted in the greenhouse. *Cn* has been reported by Smidt and Vidaver (1986) to grow rapidly at 16 to 32°C, with the optimum temperature for maximum *Cn* occurring at 27°C. Supplemental light was provided with 400-W high-pressure sodium lamps used to produce 16 h of light per 24-h period. The two corn hybrids and johnsongrass were directly seeded into 655-cm³ Cone-tainers (Deepest, Stuewe and Sons, Tangent, OR) filled with potting soil (Redi-Mix, Sun-Gro Horticulture, Bellevue, WA). Annual ryegrass was directly seeded into 164-cm³ Cone-tainers (Ray Leach SC-10 Super Cell Cone-tainers; Stuewe and Sons) filled with potting soil. Giant foxtail was sown in 26 × 26 × 6-cm flats containing potting soil and transplanted into 164-cm³ Cone-tainers once two true leaves were present.

Inoculum was prepared from a confirmed strain of *Cn* found in Indiana and used by Ikley et al. (2015). The isolate was grown on nutrient broth yeast medium for 3 days at 24°C. The bacterial colonies were then suspended in sterile double-distilled water. A stock inoculum concentration of 1×10^7 CFU/ml was prepared by adjusting the initial inoculum with a spectrophotometer (DU 530, Beckman Coulter, Brea, CA) set at 600 nm. The inoculum concentrations of 1×10^0 to 1×10^6 CFU/ml were then prepared by performing stepwise dilutions from the 1×10^7 CFU/ml inoculum.

Plants were inoculated when they had three exposed leaf collars. The newest emerged leaf was inoculated using a modified version described in Korus (2011). This method of inoculation involves first cutting off the end of the leaf and then submerging it into a beaker containing the bacterial suspension for approximately 5 s (Ikley et al. 2015).

Data Collection and Statistical Analysis

After inoculation, plants were rated for symptoms of Goss's wilt every 3 days for 3 weeks. Lesions were measured (millimeters) from the cut tip to the end of the lesion. Days required for initial lesion development to occur was recorded for each plant when symptoms first appeared on the inoculated leaves. One plant from each species and treatment was tested after the 21 days rating to ensure the observed symptoms were caused by *Cn*. The infected leaf was examined under a microscope to look for bacterial streaming and then was tested with Agdia ImmunoStrip test kits (Agdia, Elkhart, IN) designed for the detection of *Clavibacter michiganensis*, which are also successful in detecting *Cn* (Korus 2011).

Lesion length and days for initial lesion development were checked for normality using PROC UNIVARIATE in SAS 9.3 (SAS Institute, Cary, NC). Lesion length ratings were nonnormal, and data were log transformed ($\log_{10}$ transformed). Data were back-transformed for presentation in figures. Two-way ANOVAs were conducted to compare the susceptibility of the five hosts and the days required for initial symptoms to occur using the PROC GLIMMIX procedure in SAS 9.3. There was a significant ($P < 0.0001$) effect of experiment and inoculation, and therefore data from experiments were analyzed separately. A significant interaction ($\alpha = 0.05$) was detected between species and inoculum concentrations at $P < 0.0001$, and thus they were pooled for analysis. Mean separations were obtained using Fisher's protected least significant difference at $P \leq 0.05$.

Effect of Inoculum Concentration on Lesion Development

In experiment 1, no lesions developed at the lowest inoculum concentration of 1×10^0 CFU/ml on any host, and less than 6 mm of lesion development was observed on all hosts at 1×10^1 CFU/ml (Fig. 1A). The susceptible corn hybrid had the longest lesions, followed by the moderately resistant corn hybrid. All alternative hosts responded similarly for all inoculum concentrations. When comparing the different inoculum concentrations within an individual species, both susceptible and moderately resistant hybrids had significant increases in lesion length compared with alternative hosts beginning at concentrations of 1×10^2 CFU/ml inoculum in the susceptible hybrid and 1×10^3 CFU/ml in the moderately resistant hybrid. No differences in lesion length were observed for alternative hosts at increased inoculum concentrations.

In experiment 2, the susceptible corn hybrid and johnsongrass expressed symptoms of Goss's wilt at the 1×10^0 CFU/ml inoculum concentration (Fig. 1B). Moderately resistant corn and annual ryegrass expressed symptoms at the 1×10^1 CFU/ml inoculum concentration, and giant foxtail expressed symptoms at the 1×10^4

CFU/ml inoculum concentration. The susceptible corn had the longest lesions, followed by the moderately resistant corn hybrid. All alternative hosts responded similarly for all inoculum concentrations. When comparing the different inoculum concentrations within a single species, both susceptible and moderately resistant hybrids had significant increases in lesion length compared with alternative hosts beginning at inoculum concentrations of 1×10^2 CFU/ml. Inoculum concentration did not affect lesion length in any alternative hosts.

Days Required for Initial Lesion Development

In experiment 1, days required for initial lesion development varied by 1 to 3 days across all hosts when inoculated with inoculum concentrations of 1×10^4 to 1×10^7 CFU/ml (Table 1). At inoculum concentrations of 1×10^5 to 1×10^7 CFU/ml, symptom development occurred 1 to 2 days sooner on all hosts compared with annual ryegrass. At inoculum concentrations below 1×10^4 CFU/ml, lesion development occurred 2 to 7 days sooner on annual ryegrass compared with the four other hosts. When comparing the

FIGURE 1 Mean lesion length in millimeters 21 days after inoculation of *Clavibacter nebraskensis* on host plants: **A**, experiment 1; and **B**, experiment 2. Mean separation was conducted using Fisher’s protected least significant difference with species ($P < 0.0001$), inoculum ($P < 0.0001$), and a significant interaction ($P < 0.0001$). Error bars indicate standard error of the mean.

TABLE 1 Days after inoculation required for initial symptoms to develop on host plants infected with <i>Clavibacter nebraskensis</i> in experiment 1 inoculated with the leaf tip removal technique					
Inoculum concentration ^v	S. corn ^w	M.R. corn ^x	Annual ryegrass	Giant foxtail	Johnsongrass
1×10^0	— ^y	—	—	—	—
1×10^1	16 a ^z	13 b–d	9 ef	15 a–c	12 d
1×10^2	12 d	13 cd	9 ef	16 ab	13 cd
1×10^3	8 fg	8 fg	6 g–j	17 a	10 e
1×10^4	6 g–j	6 g–j	7 g–i	7 gh	9 ef
1×10^5	4 j–m	4 j–m	6 g–j	5 i–m	4 j–m
1×10^6	4 j–m	3 m	5 h–k	3 m	4 j–m
1×10^7	3 m	3 m	5 h–l	3 m	3 m

^v Colony forming units per milliliter.
^w Susceptible corn hybrid.
^x Moderately resistant corn hybrid.
^y Dash (—) indicates no symptoms observed.
^z Mean separation was conducted using Fisher’s protected least significant difference with species ($P < 0.0001$), inoculum ($P < 0.0001$), and a significant interaction ($P < 0.0001$). Values followed by different letters within both columns and rows are significantly different ($P \leq 0.05$).

different inoculum concentrations within a single species, all hosts required fewer days for initial lesion development as inoculum concentrations increased.

In experiment 2, initial lesion development occurred in all hosts at a similar time for all inoculum concentrations except annual ryegrass, which required at least 2 days or more for initial lesions to develop (Table 2). When comparing the different inoculum concentrations within a single species, all hosts required fewer days for initial lesion development to occur as inoculum concentrations increased.

Influence of Inoculum Levels on Alternative Hosts and Management of Goss's Wilt

Our study provides more detailed evaluation of the role of inoculum level on Goss's wilt severity and demonstrates that disease can occur at lower inoculum levels than previously reported in corn. In our study, low levels of Goss's wilt symptoms were observed consistently in corn at 1×10^1 in both experiments, which is lower than the previously reported limit of symptom development at 1×10^2 (Calub et al. 1974c). As expected, we observed higher levels of Goss's wilt in corn with inoculum concentrations over 1×10^2 CFU/ml, and days from inoculation to initial symptom detection decreased with increasing inoculum concentrations.

Our results also found that alternative hosts consistently expressed measurable disease symptoms at the 1×10^4 CFU/ml and higher inoculum concentrations. Alternative hosts still had lesion development occur at lower inoculum concentrations, but disease severity was not consistent for each experiment. These low inoculum concentrations may occur more commonly in fields with a history of Goss's wilt, based on findings of Smidt and Vidaver (1986), which indicated that residue from previously infected fields had 1×10^5 CFU of *Cn* per gram of fresh weight after one year out of corn. This implies that inoculum concentrations much lower than the 1×10^6 to 1×10^8 CFU/ml inoculum concentrations commonly used in *Cn* experiments could provide inoculum for both alternative hosts and corn under field conditions.

All alternative hosts tested had similar final disease severity across all inoculum concentrations, proving that low levels of inoculum can result in similar disease severity as higher concentrations over time in these species. In fact, *Cn* was confirmed in hosts after inoculation using the Agdia ImmunoStrip test kits, which

have a lower detection limit of 1×10^7 CFU/ml, meaning that at least that level of *Cn* was present at the final disease severity rating. This result could be partly influenced by plant age at inoculation. Calub et al. (1974c) demonstrated that corn hybrids inoculated 2 weeks after planting were more susceptible to *Cn* than plants infected at 4, 6, and 8 weeks after planting. Inoculating alternative hosts at the younger three-collar stage in our experiment could have allowed lower inoculum concentrations to cause similar amounts of disease severity as higher inoculum concentrations in alternative hosts. Additionally, Meletzus et al. (1993) showed that hindering *C. michiganensis*'s ability to infect tomatoes by removing two plasmids from the bacteria did not reduce the amount of bacteria found in infected tomato plants, and all treatments produced bacterial concentration of 1×10^9 CFU per gram of plant tissue after 9 days of infection. This demonstrates that *C. michiganensis* thrives once infection occurs in the plant and could explain how low inoculum concentrations of *Cn* result in similar disease concentrations in alternative hosts compared with higher inoculum concentrations. More research on how *Cn* develops in alternative hosts is needed to help understand the role of inoculum concentration on initial and final disease levels.

The time for disease development to occur is most likely influenced by the amount of bacteria found in the different inoculum concentrations. Mallowa et al. (2016) observed that when inoculating corn plants with 1×10^8 CFU/ml inoculum concentration, *Cn* colony numbers declined on the leaf surface in the first 24 h after infection and then steadily increased after 24 h. This rebound could take longer with less inoculum, although this is currently unknown. Our results also suggest that alternative hosts and corn have similar initial responses in time for disease development, because initial lesion development at low inoculum concentrations was observed at similar rates in all species.

In our experiment, time to initial symptom development occurred at different rates between the two experiments. The difference in time to lesion development could be owing to several factors, including temperature changes in the greenhouse settings. Temperatures for experiment 2 were adequate for *Cn* growth but were 5 to 6°C cooler than greenhouse temperatures in experiment 1. This temperature difference could explain the delay in symptom development in all plant species in experiment 2, particularly giant foxtail. Although experiment 2 was conducted at a temperature

TABLE 2
Days after inoculation required for initial symptoms to develop on host plants infected with *Clavibacter nebraskensis* in experiment 2 inoculated with the leaf tip removal technique

Inoculum concentration ^v	S. corn ^w	M.R. corn ^x	Annual ryegrass	Giant foxtail	Johnsongrass
1×10^0	18 ab ^y	—	—	—	18 ab
1×10^1	15 c	18 a–c	21 a	—	15 c
1×10^2	10 ed	16 bc	17 bc	—	12 cd
1×10^3	9 ef	10 e	14 c	12 c–e	11 de
1×10^4	8 e–h	8 e–h	12 cd	9 e–g	11 de
1×10^5	6 h–j	6 h–j	12 cd	9 e–g	7 f–i
1×10^6	6 h–j	6 h–j	11 ed	6 h–j	6 h–j
1×10^7	4 j	5 ij	6 g–j	7 g–j	5 ij

^v Colony forming units per milliliter.

^w Susceptible corn hybrid.

^x Moderately resistant corn hybrid.

^y Mean separation was conducted using Fisher's protected least significant difference with species ($P < 0.0001$), inoculum ($P < 0.0001$), and a significant interaction ($P < 0.0132$). Values followed by different letters within both columns and rows are significantly different ($P \leq 0.05$).

^z Dash (—) indicates no symptoms observed.

slightly lower than the optimal temperature for *Cn* growth, it still fell in the previously described range for adequate *Cn* growth (Smidt and Vidaver 1986). Additionally, final disease severity across all species was comparable to or greater than final disease severity observed in experiment 1, indicating that disease progressed rapidly in experiment 2, despite having a delayed start. These results indicate that further research is needed to better understand the role of temperature on symptom expression and disease development in *Cn* hosts.

The interaction observed between species and inoculum concentration was likely influenced by the low levels of disease severity observed at the lower inoculum concentrations. The two corn hybrids tested had lower disease severity than annual ryegrass at 1×10^1 and 1×10^2 CFU/ml; however, no alternative hosts had entire leaves affected, and none became systemically infected. Additionally, as inoculum concentrations increased, corn had higher disease severity than alternative hosts, likely resulting in the interaction observed. Regardless of interaction, both susceptible and moderately resistant corn hybrids tested were more susceptible to *Cn* compared with the alternative hosts, which is similar to previous findings of Langemeier et al. (2014) and Ikley et al. (2015) and, thus, indicates that corn has the greatest effect on the inoculum potential of *Cn*.

In conclusion, this greenhouse study demonstrates that *Cn* has the ability to cause disease consistently at the 1×10^4 to 1×10^7 CFU/ml inoculum concentration in both corn and alternative hosts. Our results also confirm that corn is a preferred host for *Cn* compared with the three alternative hosts tested. Alternative hosts, however, still expressed disease at lower inoculum concentrations and had similar amounts of disease severity compared with the two corn hybrids at low inoculum concentrations. Therefore, these hosts should not be ruled out as potential in-field sources of *Cn* inoculum. The inoculum potential from alternative hosts may be low, but over time we observed that low inoculum concentrations in alternative hosts can result in similar amounts of disease severity as higher inoculum concentrations. We recommend farmers select corn hybrids resistant to Goss's wilt and consider methods to reduce the amount of corn residue found in the field, such as tillage and crop rotation, because infected corn residue has the greatest effect on the inoculum potential of *Cn*.

Managing alternative hosts through pre- and postemergence herbicide programs is also important, not only in the corn crop but also when rotating away from corn, to reduce the potential of any carryover inoculum into the next corn crop.

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