Seed Transmission of *Clavibacter michiganense* subsp. *nebraskense* in Corn

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ABSTRACT

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Clavibacter michiganense subsp. *nebraskense*, the Goss's wilt pathogen, was detected in seeds, ear shanks, and stalks of a susceptible corn inbred, A632Ht, after leaves were inoculated in the field one, two, or three times with a rifampicin-tolerant strain of *C. m.* subsp. *nebraskense*. Pathogen populations tended to increase in all plant parts with increasing numbers of inoculations. In a second year of the study, in which the same inbred was inoculated three times, internal populations of the bacterium in seedlots were similar to those in the previous year, and the percentage of seeds infected ranged from 17.1 to 30.7. Internal populations of the pathogen and the percentage of seeds with these populations were not affected by either moisture content at harvest (38% or 25%) or by drying at 35 C. Transmission of the pathogen into seedlings did not occur when nine 1,000-seed samples from infested seed lots were planted in sterile soil in the greenhouse or when three 1,000-seed samples were planted in the field. The pathogen was transmitted to seedlings at rates of 0.1–0.4% from seeds that had been inoculated with *C. m.* subsp. *nebraskense* by vacuum infiltration.

Goss's bacterial wilt and leaf blight was first discovered in south central Nebraska in 1969 (9). Since then, the disease has spread into several midwestern states but has not been reported elsewhere in the United States or in other countries. Crop losses associated with the disease usually have been minor, with occasional reports of severe losses in individual fields (15,16). Schuster (9) demonstrated that the pathogen, *Clavibacter michiganense* subsp. *nebraskense* (Vidaver and Mandel) Davis et al was seedborne on field corn. He reported that in heavily infected kernels, the pathogen could be seen in the chalazal region, the area between the scutellum and the endosperm, and in the vicinity of the embryo. He also demonstrated transmission of the pathogen from infected seeds to seedlings grown in autoclaved soil in the greenhouse. Although seeds could be an important means of disseminating the pathogen, the limited spread of the disease over the past 20 yr is not consistent with efficient transmission of the pathogen by seeds. The present study was carried out to clarify the role of infected seed in the epidemiology of Goss's wilt by examining the process of transmission of *C. m.* subsp. *nebraskense* from diseased plants in the seed-production field to the plants grown from infected seeds.

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MATERIALS AND METHODS

Bacterial strains and inoculum production. A rifampicin-tolerant strain of *C. m.* subsp. *nebraskense* (CNR) was obtained by gradient plate methods (13) from strain CN 9-1, which originated from corn with typical Goss's wilt and blight (14). In pathogenicity tests, the two strains were similarly aggressive on corn. A stock culture of CNR was stored in liquid nitrogen and used in all inoculation experiments.

For inoculum production, the bacteria were rapidly thawed and grown for 24–48 hr at 27 C on a nutrient broth-yeast extract medium (NBY; 8). Bacteria were then suspended in a phosphate-magnesium-sulphate buffer containing 7 g of K_2HPO_4, 2 g of KH_2PO_4 and 0.25 g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water, adjusted to pH 7.2. Inoculum concentrations were standardized based on spectrophotometric measurements and verified by standard dilution plating techniques.

Demonstration of transmission of *C. m.* subsp. *nebraskense* from plant to seed. Plant-to-seed transmission was tested in a field experiment with three inoculation treatments and appropriate uninoculated controls in a randomized complete block design with three replications. The corn inbred line A632Ht, known to be susceptible to Goss's wilt (2,3), was planted in the field near Ames, Iowa, on 15 May 1984. Individual plots consisted of four rows, 4.5 m long and 76 cm wide. Plots were separated by four border rows of plants composed of three genotypes, Mo17Ht × B73Ht, W153R, and N28, resistant to Goss's wilt (15). Plants in one set of plots were inoculated with strain CNR at the three-leaf, six-leaf, and tasselling growth stages on 28 June, 12 July, and 26 July, respectively. Plants in a second set of plots were inoculated at the six-leaf and tasselling stages, and in a third set at the tasselling stage only. Leaves on 100 plants per plot were inoculated with 2×10^7 cfu/ml of CNR in a buffer solution with a pinprick-injection device (4). For the three- and six-leaf growth stages, all leaves on a plant were inoculated, whereas only the ear leaf and those above were inoculated for the tasselling growth stage. For each inoculation treatment, plants in corresponding control plots were injected with buffer solution alone. Lesion areas on ear leaves of 10 randomly selected plants in each plot were estimated at the late milk/early dough growth stage by measuring the length and width of the lesions. At harvest maturity (30 September), the ear and attached stalk extending 10 cm above and 10 cm below the ear were harvested from another set of 10 randomly selected plants in each plot. Twenty seeds were shelled from each ear and pooled to provide 200 kernels from each plot. To estimate populations of *C. m.* subsp. *nebraskense* within seeds, 100 seeds from each plot were surface-sterilized for 1 min in 0.5% NaOCl, rinsed in sterile, distilled water, and ground for 60 sec in sterile buffer in a Stein laboratory mill (Fred Stein Laboratories, Atchison, KS). Duplicate platings of serial 10-fold dilutions were made onto two media: NBY containing 0.05 g of rifampicin and 0.04 g of cycloheximide/L (Rif-A), and *Corynebacterium nebraskense*-selective (CNS) modified by reducing the LiCl concentration to 0.1 g/L (CNS-A; 5,11). Plates were incubated at 25 C and colonies were counted after 5–7 days. This procedure was also used to measure populations of the pathogen in 10 sections of shank (0.5 cm in length) and stem (1 cm in length) from each plot. Pathogen populations on seed surfaces were determined for the remaining 100 seeds per plot by shaking seeds in 30 ml of sterile buffer for 30 min, diluting the washings, and plating as described.

The experiment was repeated in 1985 at the same location, with the following modifications. All plants were inoculated with CNR three times, at the three-leaf, six-leaf, and tasselling growth stages on 5 June, 1 July, and 25 July, respectively, and plants in control plots received buffer alone. At seed moistures of 38% and 25%, 30 ears were harvested from each plot. One hundred and twenty seeds were removed from each ear and pooled

to provide a composite sample of 3,600 seeds for each replicate of each treatment. The remaining ears were dried at 35 C to 10% or 12% moisture content, and a second sample of 3,600 seeds was then taken from each replicate. Internal populations of *C. m.* subsp. *nebraskense* were estimated for 200 seeds from each replicate by plating diluted suspensions on Rif-B and CNS-B media (identical to CNS-A and Rif-A, except for the addition of 0.02 g of the fungicide benomyl [Benlate 50W] per liter). Infection of individual seeds was determined by surface-sterilizing seeds for 1 min in 0.5% NaOCl, rinsing in sterile, distilled water, and plating 1,000 seeds from each replicate on both CNS-B and Rif-B media. Plates were incubated at 22 C for 7–14 days. Approximately 10% of the colonies with cultural characteristics resembling those of C. m. subsp. *nebraskense* were streaked onto CNS-B and Rif-B media and the subcultures were inoculated to A632Ht × A619Ht seedlings in the greenhouse. Plants were evaluated for typical Goss's wilt symptoms over a period of 14 days.

Demonstration of transmission from seed to plant. Samples of 1,000 seeds per replicate from each treatment in the 1985 field experiment were planted in pasteurized soil and grown at 24 ± 3 C in a greenhouse. The seedlings were examined for the presence of Goss's wilt symptoms each week for 6 wk. For detection of symptomless infections, 100 seedlings per replicate were cut into 2-cm lengths, surface-disinfested in 0.5% NaOCl for 60 sec, rinsed in sterile, distilled water, and ground for 60 sec in buffer solution in a Stein laboratory mill. Duplicate serial 10-fold dilutions in buffer were plated onto CNS-B and Rif-B media. Plates were incubated at 25 C and colonies were counted after 5–7 days. Colonies with cultural characteristics similar to *C. m.* subsp. *nebraskense* were streaked onto CNS-B, Rif-B, and NBY media, and colony identity was confirmed by Gram reactions and pathogenicity tests on susceptible seedlings. Seeds harvested at 38% moisture and not dried were not included in the test because of their poor germination potential.

Ears harvested in 1985 at 25% moisture and dried to 10% were stored over winter at 10 C and 50% relative humidity (RH). Samples of 200 and 1,000 seeds per replicate were removed from ears in the following spring and tested for internal and external populations of the pathogen, respectively, as described. A second 1,000-seed sample per replicate was planted in the field in four-row plots. Plants were examined periodically for symptoms of Goss's wilt until the tasselling growth stage.

Seed-to-plant transmission of *C. m.* subsp. *nebraskense* was also tested on inoculated seeds of the hybrid A632Ht × A619Ht. Seeds were drawn from a lot that had been previously determined by culture plate tests to be free of the pathogen. Seeds were vacuum-infiltrated at 380-mm Hg vacuum for 5 min with a suspension of CNR (10^7 cfu/ml) in a buffer solution or with buffer alone. A second control treatment consisted of seeds that had not been infiltrated. All treatments were applied to both dry seeds and to seeds that had imbibed sterile water for 16 hr on damp blotters. Lots of 1,000 seeds for each inoculation/imbibition treatment were planted in pasteurized soil in the greenhouse and grown at 24 ± 3 C, either uncovered or at 85% RH under plastic covers. The percentage of seeds internally colonized with the pathogen was determined for 500 seeds per treatment immediately before planting. Plant emergence was recorded and the seedlings were examined for characteristic Goss's wilt symptoms until 5 wk after planting. When symptoms were found, the pathogen was isolated and its identity was confirmed by pathogenicity tests as described. The experimental design was a randomized complete block, replicated twice over time.

An analysis of variance was carried out on data in each of the above experiments.

RESULTS

Infected seeds on inoculated plants. Symptoms typical of Goss's wilt developed on plants inoculated with strain CNR in the field in 1984. Lesions on ear leaves, measured at the late milk/early dough growth stage, averaged 7.4, 50.2, and 119.0 cm^2 in plots inoculated with the bacterium one, two, and three times, respectively. No symptoms were detected on plants that received buffer alone. The pathogen was recovered consistently from seeds (externally and internally), and from ear shanks and stalks in inoculated plots (Table 1). In general, populations of the pathogen increased with numbers of inoculations. It was not clear, however, whether the increase was in response to numbers of inoculations or to growth stage at which inoculation took place. The pathogen was not recovered from plants treated with buffer alone, except for the detection of CNR in seeds from plants injected three times. Similar results were obtained on Rif and CNS media in this and subsequent experiments. Therefore, only data for the Rif media are presented (Tables 1–3).

Populations of *C. m.* subsp. *nebraskense* within seeds in the 1985 field experiment were similar to those detected in plants inoculated three times in 1984 (Table 2). The pathogen was detected in

Table 1. Populations[a] of *Clavibacter michiganense* subsp. *nebraskense*, measured at harvest maturity, in plant parts of corn inoculated different numbers of times in 1984

	Number of inoculations[b]		
Plant part	**1**	**2**	**3**
Seed			
External	0.0	3.4	6.1
Internal	0.6	0.0	6.7
Shank	2.5	4.6	7.2
Stem	0.0	4.5	6.7

[a] Log ([cfu/g fresh weight] + 1). Serial 10-fold dilutions of plant extracts were plated onto Rif-A (NBY + 0.05 g of rifampicin + 0.04 g of cycloheximide/L). Plates were incubated at 25 C and colonies were counted after 5–7 days. Values are the means of three replicates.

[b] A suspension of a rifampicin-tolerant strain of *C. m.* subsp. *nebraskense* (CNR) in buffer solution was inoculated into leaves of the corn inbred line A632Ht with a pinprick device, once (at the tasselling growth stage), twice (at the six-leaf and tasselling stages), and three times (at the three-, six-leaf, and tasselling stages). Plants in corresponding control plots received buffer solution alone, and strain CNR was not detected in them except for a 3.27 population value in seeds of plants injected three times.

Table 2. Infection by *Clavibacter michiganense* subsp. *nebraskense* of corn seeds harvested from inoculated[a] plants as influenced by harvest moisture content and drying in 1985[b]

		Internal seed population[c]		Seeds infected (%)[d]	
Seed harvest	**Drying treatments**	**Inoculated**	**Control**	**Inoculated**	**Control**
Harvested at 38% moisture content	Not dried	7.0	0.0	17.5	0.0
	Dried to 10%	6.5	0.0	17.1	0.0
Harvested at 25% moisture content	Not dried	7.0	3.5	30.7	0.0
	Dried to 12%	6.1	1.8	23.0	0.0

[a] A suspension of a rifampicin-tolerant strain of *C. m.* subsp. *nebraskense* (CNR) in buffer solution was inoculated into leaves of the corn inbread line A632Ht with a pinprick device, at the three-leaf, six-leaf, and tasselling growth stages. Corresponding control plants received buffer solution alone.

[b] Seeds were dried at 35 C.

[c] Log ([cfu/g fresh weight] + 1). Serial 10-fold dilutions of ground seeds were plated onto Rif-B. Plates were incubated at 25 C and colonies were counted after 5–7 days. Values are the means of three replicates of 200 seeds.

[d] Seeds were surface-sterilized for 1 min in 0.5% NaOCl, rinsed in sterile, distilled water, and plated on Rif-B. Plates were incubated at 22 C and seed infection was determined after 7–14 days. Values are the means of three replicates of 1,000 seeds.

control plants but at significantly lower levels than in inoculated plants. Symptoms of Goss's wilt were found on some plants in buffer-treated plots. The pathogen was readily recovered from these but not from asymptomatic plants in the same plots. Infection of control plants most likely resulted from spread of bacteria from inoculated plots. Results based on the percentage of seeds infected followed a similar pattern. Neither harvest moisture nor drying treatment had any significant effect on internal populations of the pathogen or the percentage of seeds infected.

Transmission of *C. m.* subsp. *nebraskense* from seeds to seedlings. Goss's wilt symptoms did not develop on seedlings grown in pasteurized soil from any of the seed lots produced in the 1985 field experiment, nor was *C. m.* subsp. *nebraskense* recovered from any of the symptomless seedlings. The disease also was not found in field plantings of infected and uninfected seedlots in 1986. Seed infection levels in the latter seedlots, determined immediately before planting, were similar to those that had been detected after harvest. All seedlots had germination rates greater than 90% in pasteurized soil and over 80% in the field. Transmission of the bacterium from seed to plant was achieved from inoculated seeds planted in the greenhouse. However, despite induction of seed infestation levels as high as 73.2%, transmission rates of *C. m.* subsp. *nebraskense* to seedlings averaged only 0.1 to 0.4% (Table 3). Symptoms on seedlings were highly characteristic of Goss's wilt, with deep green freckles on the leaves, leaf wilting, and stunting. The pathogen was recovered from diseased tissues and its identity was confirmed. High relative humidity during seedling growth had no detectable effect on transmission rate of the pathogen or on symptom expression.

DISCUSSION

Transmission of *C. m.* subsp. *nebraskense* from plant to seeds was demonstrated by the recovery of the pathogen from stems, shanks, and seeds of inoculated plants. The rifampicin-tolerant strain used in the study was similar in pathogenicity to the strain from which it was derived. Its stability was demonstrated by ease of recovery from inoculated plants in the field and from infected seeds stored over winter. Although the mechanism for transmission of the pathogen from mother plant to seeds was not elucidated, the transmission process was relatively efficient. Seeds harboring *C. m.* subsp. *nebraskense*, therefore, are likely to occur frequently on plants with Goss's wilt.

Transmission of the pathogen from seeds to seedlings was not demonstrated with naturally infected seeds. This is counter to the findings of Schuster (10) in Nebraska. One major difference in the two studies was that the Nebraska seeds were described as having an orange discoloration or bacterial exudate (9). Thus, they may have carried a much higher inoculum load than the seeds used in the present study. Because we did demonstrate transmission in artificially inoculated seeds in which the incidence of infestation was high, the data from the two studies would support the conclusion that *C. m.* subsp. *nebraskense* can be transmitted from seeds to seedlings but that high levels of inoculum are necessary.

Infested corn residues are the major inoculum source for Goss's wilt (1,12,15). Seedborne inoculum, therefore, should be of minor concern as an inoculum source in an area where the disease is already established. Infected or contaminated seeds could, however, introduce the pathogen into new areas, and it is to this possibility that any efforts to reduce or eradicate seedborne inoculum of *C. m.* subsp. *nebraskense* should be directed. Because seed treatments are not available (6), and drying had no influence on seed infection, eradication of the pathogen does not seem to be an option. Seeds could be tested for presence of the pathogen, however. With further research, particularly on the specificity of selective media and the sensitivity of tests made with ground and whole seeds, the laboratory procedures described here could be the basis for a reliable test method.

The efficiency of transmission of the pathogen from plant to seeds indicates that seeds carrying *C. m.* subsp. *nebraskense* could have been planted extensively in the corn belt and introduced into other countries during the last 20 yr. Despite these possibilities, the disease has remained limited in distribution and has had little economic impact. These limitations could be attributed to the inefficiency of seed transmission, as in the present study, and/or the high degree of resistance to Goss's wilt that exists in field corn hybrids and inbreds (6,9,15). The fact that Goss's wilt persists as an important disease in Nebraska may be attributable to the presence of epidemiological factors favoring the establishment of the disease that are absent in other regions of the corn belt. Most of the corn in Nebraska is grown on sandy soils, and it has been shown that plant injury caused by blowing sand allows avenues of entry for the pathogen into the plants (7,10). Extensive overhead irrigation in that state also might exacerbate the disease problem.

We conclude from this study that the potential exists for the introduction of *C. michiganense* subsp. *nebraskense* into new geographical regions by seeds, but efforts to reduce seed infestation or eradicate the pathogen from seeds do not seem to be warranted because of the minimal risk of such introductions having significant economic impact.

Table 3. Transmission of *Clavibacter michiganense* subsp. *nebraskense* from artificially inoculated seeds to seedlings under low and high relative humidity (RH) in a greenhouse

Inoculation treatment	Infested seeds planted (%)[b]	Transmission rate to seedlings (%)[a]	
		Low RH	High RH
Dry seeds	0.0	0.0	0.0
Dry/buffer-infiltrated[c]	0.0	0.0	0.0
Dry/bacteria-infiltrated[d]	69.7	0.1	0.1
Imbibed[e]	0.0	0.0	0.0
Imbibed/buffer-infiltrated	0.0	0.0	0.0
Imbibed/bacteria-infiltrated	73.2	0.1	0.4

[a] Values for each humidity regime are based on the number of seedlings, of 1,000, showing Goss' wilt symptoms.
[b] Seeds were surface-sterilized for 1 min in 0.5% NaOCl, rinsed in sterile, distilled water, and plated on Rif-B medium. Plates were incubated at 22 C and seed infestation was determined after 7–14 days. Values are based on a sample of 500 seeds per treatment.
[c] Vacuum infiltration of buffer solution at 380-mm Hg vacuum for 5 min.
[d] Vacuum infiltration of a suspension of CNR (10^7 cfu/ml) in buffer solution at 380-mm Hg vacuum for 5 min.
[e] Seeds imbibed sterile water for 16 hr on damp blotters.

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