Population Dynamics of *Clavibacter michiganense* subsp. *nebraskense* in Field-Grown Dent Corn and Popcorn

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ABSTRACT

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Clavibacter michiganense subsp. *nebraskense* (=*Corynebacterium michiganense* subsp. *nebraskense*), causal agent of Goss's bacterial wilt and blight of corn, was recovered from field-grown popcorn and dent corn plants and residue throughout 1982 and 1983. All collection sites were in fields near Imperial, NE. The population of the pathogen in residue was highest just after harvest in October and declined a total of four to five $\log_{10}$ units over the winter and summer. Conversely, bacterial populations from live corn plants increased three to four $\log_{10}$ units throughout the growing season. The pathogen was isolated from the surfaces of plants in early June before disease symptoms were observed, suggesting an epiphytic phase for the bacterium in the field. Symptoms were observed after mid-July, when the population of the pathogen exceeded about 10^7 cfu/g fresh weight of leaf tissue. Severity of Goss's bacterial wilt and blight symptoms was evaluated near the end of the growing season and compared with similar evaluations made in previous years. Disease severity appeared to be associated with daily mean temperatures during late May and June, which were 3–5 C cooler in 1982 and 1983 than in 1980 and 1981. Corn plants grown under controlled conditions were inoculated with *Clavibacter michiganense* subsp. *nebraskense* strain CN72-2 and grown at 12, 16, 21, 26, 32, and 38 C while the bacterial populations were monitored. The optimum temperature for bacterial growth in corn plants was 27 C. At lower and higher temperatures, the growth rate dropped until at 12 C, the doubling time was >9 hr compared with 3.5 hr at the optimum temperature. At 38 C, the bacteria died. When inoculated plants were shifted from a temperature regime that favored bacterial growth (day/night regime of 32/25 C) to one that retarded bacterial growth (day/night regime of 40/20 C), the growth rate rapidly decreased. Conversely, when inoculated plants were shifted from the restrictive temperature to the permissive one, bacterial growth increased and rapidly reached its maximal rate.

Since its discovery in Nebraska in 1969, Goss's bacterial wilt and blight of corn has been found throughout most of the Midwest (18,19). Losses as great as 50% attributable to this disease have been mitigated in field corn in recent years through the use of resistant germ plasm (14). However, little resistance to this disease is available in inbreds used to make commercial popcorn hybrids (21). Initiation and development of Goss's bacterial wilt and blight symptoms are affected by many factors including the type and cultivar of corn planted, the presence of inoculum, and favorable weather, especially wind or hail, which damage the plants and provides avenues for invasion (18).

In 1979, a severe outbreak of Goss's bacterial wilt and blight occurred on popcorn in Chase County in western Nebraska. Some of these fields were replanted to popcorn for the next 3 or 4 yr. In this area, there are constant and sometimes severe winds and blowing sand that damage the plants, providing conditions essential for entry of bacteria and subsequent disease development (11,13). Adequate moisture for disease increase was present either from rainfall or from center-pivot irrigation. Because relatively susceptible popcorn hybrids were grown in successive years, bacterial inoculum was present in the previous year's residue. Consequently, the population dynamics of the pathogen were studied in several fields in this area.

The Goss's bacterial wilt and blight pathogen, *Clavibacter michiganense* subsp. *nebraskense* (4,17) (=*Corynebacterium michiganense* subsp. *nebraskense*), has been recovered from diseased crop residue by other workers (1,13). We examined the population dynamics of *C. m.* subsp. *nebraskense* from growing plants and residue over a 2-yr period. Epiphytic survival and growth of the pathogen on corn plants grown in the greenhouse have been reported (15). We were interested in determining if there was an epiphytic phase for the pathogen in the field.

The relationships between temperature and the development of other bacterial plant diseases have generally been derived empirically (2,3,8,9,12) and are usually presumed to reflect the effects of temperature directly on the growth of the pathogen. In some cases, temperature is an important factor in forecasting disease severity (8,12). Goss's bacterial wilt and blight of corn has been called a "warm weather disease" because the syptoms do not appear until midseason to late-season, when mean daily temperatures are relatively high (D. Wysong and J. Watkins, *personal communication*). However, no data were available regarding the relationship between bacterial populations and disease in plants grown at different temperatures.

In this paper, we report the temperature limits for growth of *C. m.* subsp. *nebraskense* in corn plants under controlled conditions. Temperature shift experiments were done to assess the responses of bacterial growth rates in plants to changes in external temperature. The relationship between disease severity and temperatures for field-grown plants was also examined.

MATERIALS AND METHODS

Field locations. All study fields were located near the city of Imperial in Chase County, Nebraska. In 1982, observations were made in a popcorn field (A) that had also been planted to popcorn from 1979 through 1981. In 1983, observations were made in two other popcorn fields (B and C) as well as in field A, which was planted to dent corn. All fields were center-pivot-irrigated and ranged from about 4 to 20 ha (10–50 acres). Fields were planted between 15 and 25 May.

Sampling of field-grown plants. Samples were taken from either a series of plots 18 × 18 m (60 × 60 ft) arranged along the radii of the pivots in a north-south line in fields A and B and in an east-west line in field C or from the approximate centers of each of the four quadrants of the fields and from near the centers of the fields. The first sampling scheme resulted in four replicates; the second scheme resulted in five replicates per field per sample date. Samples of live plants were transported in plastic bags on ice from the field to the laboratory and processed immediately. Crop residue was transported in the same way from June through October. In November through May, samples were shipped via commercial bus.

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Crop residue was collected from the soil surface. From November through March, residue consisted of leaf, husk, and stalk pieces; from April through October, residue samples consisted primarily of pieces of stalks. Each 50-g sample of residue was ground to a coarse powder in a Waring Blendor. The ground residue was suspended in 500 ml of half-strength nutrient broth-yeast extract (NBY) (16) without $MgSO_4$ or glucose and agitated on a gyrotory shaker at 100 rpm at 25 C for 3–4 hr. Aliquots were withdrawn from each suspension, serially diluted in 12.5 mM potassium phosphate buffer, pH 7.1 (KPi buffer), and plated on solidified CNS medium (5), which is semiselective for *C. m.* subsp. *nebraskense*.

Live plant material collected in June consisted of all aboveground parts of four plants per replicate. In July and ensuing months, the samples from each replicate consisted of four whole leaves picked at random from each of four plants. Leaves were fully expanded but without evidence of senescence. In 1982, the plants were first washed in sterile water to recover some of the bacteria adhering to the exteriors of the plants. The amount of sterile water used varied with each sample to ensure that sufficient volume was used to completely immerse the sample. Flasks containing the immersed plants were placed on a gyrotory shaker for about 1 hr at 25 C. Aliquots of the wash water were diluted serially and plated on CNS agar. Residual bacterial populations were measured by homogenizing the washed plants in sterile KPi buffer, diluting, and plating on CNS agar. In 1983, the plants were not washed before homogenizing so that the homogenates contained the total bacterial populations. Aliquots of the homogenates were serially diluted and plated on CNS agar. Some were also plated on NBY agar to compare the numbers of total bacteria that grew on NBY and CNS agar.

Disease severity in the field. The severity of Goss's bacterial wilt and blight was rated in September on a scale of 0–6, where 0 = no symptoms noted, 1 = trace to <1% of total leaf area affected, 2 = 1–10%, 3 = 11–25%, 4 = 26–50%, 5 = >50% of total leaf area affected, and 6 = all plants dead. In 1982 and 1983, disease was rated by visually inspecting plants in the area immediately surrounding the sampling sites as described before. Disease ratings in 1980 and 1981 were performed by Wysong and Linscott (20) and Wysong et al (21), respectively, using the same rating scale.

Weather data. Daily high, low, and mean temperatures at Imperial were obtained from the Department of Climatology and Meteorology, UNL, Lincoln, NE.

Inoculation and sampling of growth-chamber-grown plants. *C. m.* subsp. *nebraskense* CN72-2, a highly virulent strain, was used in these studies. Cultures were grown on NBY medium (16) or CNS medium (5) modified by omitting LiCl. Both media were solidified with 1.3% agar (Difco or Sigma).

Dent corn of the hybrid A619 × A632 was grown in growth chambers until the five- to six-leaf stage. Plants were then inoculated by injecting 10 µl of a bacterial suspension containing 10^5–10^6 cfu/ml of log-phase cells through the leaves into the whorl approximately at the growing

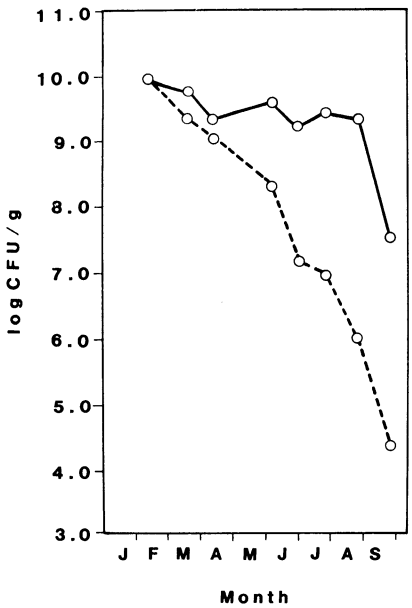

Fig. 1. Populations of bacteria in corn residue from 1981 growing season. Corn residue was collected in field A from February to October 1982. Solid line shows total number of bacteria that grew on CNS agar and dashed line shows number of *Clavibacter michiganense* subsp. *nebraskense*. Bacterial counts are expressed in $\log_{10}$ units per gram fresh weight. Letters represent first initial of each month.

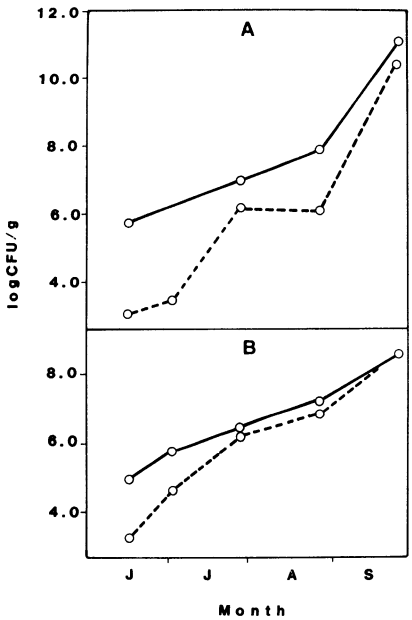

Fig. 2. Populations of bacteria on popcorn plants during 1982 growing season. Plants were sampled from field A. Bacterial populations in **(A)** plant homogenates and **(B)** wash water were determined as described in text. Solid lines show total number of bacteria that grew on CNS agar and dashed lines show number of *Clavibacter michiganense* subsp. *nebraskense* from the same plants. Bacterial counts are expressed in $\log_{10}$ units per gram fresh weight. Letters represent first initial of each month.

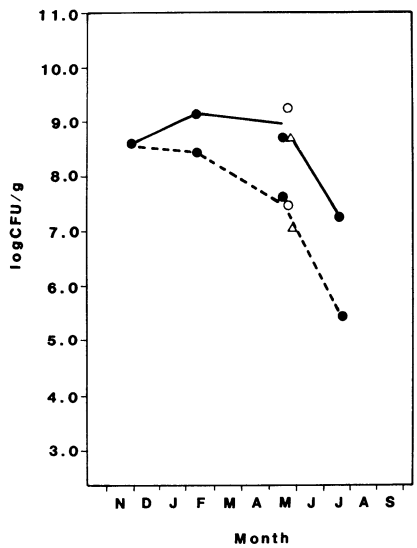

Fig. 3. Populations of bacteria in popcorn residue from 1982 growing season. Residue was collected in fields A (•), B (o), and C (Δ). Bacterial populations were determined as described in text. Solid line shows total number of bacteria and dashed line shows number of *Clavibacter michiganense* subsp. *nebraskense* that grew on CNS agar. Bacterial counts are expressed in $\log_{10}$ units per gram fresh weight. Letters represent first initial of each month: N and D in 1982, J through S in 1983.

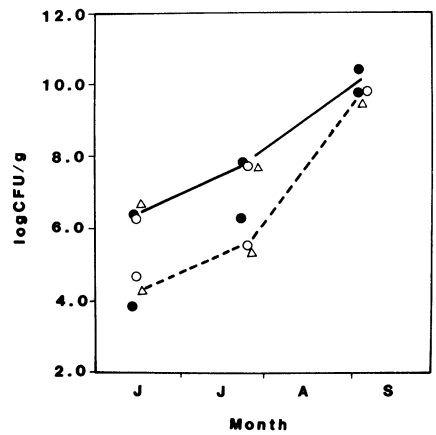

Fig. 4. Bacterial populations from popcorn and dent corn plants during 1983 growing season. Plants were sampled in fields A (•), B (o), and C (Δ). Field A was planted in dent corn; fields B and C, in popcorn. Solid line shows total number of bacteria that grew on CNS agar and dashed line shows number of *Clavibacter michiganense* subsp. *nebraskense* in and on those same plants. Bacterial counts are expressed in $\log_{10}$ units per gram fresh weight. Letters represent first initial of each month.

point. Injections were made with a Pipetman (Gilson) fitted with an 18-gauge hypodermic needle. After inoculation, plants were placed at the appropriate test temperatures. In all cases, the same growth chamber was used to obtain a 12-hr photoperiod with fluorescent illumination at 200–250 μE m^{-2} s^{-1}. Plant samples were collected on the day of inoculation and at various intervals for up to 8 days after inoculation. Each sample consisted of six to nine plants that were excised at soil level. Plants were pooled and homogenized with a Waring Blendor in KPi buffer. Serial dilutions of the homogenates were plated on modified CNS agar. Each plating was replicated two or three times per repetition.

Bacterial growth rates were determined in plants grown at constant temperatures ranging from 12 to 38 C. At the two lowest temperatures, experiments were repeated once; at the highest four temperatures, experiments were repeated up to five times. To determine the responses of bacterial growth rates to temperature shifts, plants were grown in two day/night temperature regimes: one with 40 C during the day and 20 C at night and the other with 32 C during the day and 25 C at night. Two growth chambers were used, both with photoperiods and illumination as described previously. Inoculated plants were placed in both regimes on the day of inoculation. Two or 3 days after inoculation, some of the plants were shifted to the other temperature regime. The shift occurred during the period of illumination and higher temperatures. Samples were taken as described. Plants were sampled both from groups that were kept in one temperature regime and those that were subjected to the temperature shifts. Both types of shift experiments were repeated once.

RESULTS

Populations of *C. m.* subsp. *nebraskense* and total bacteria in corn residue and plants. In 1982, the population of *C. m.* subsp. *nebraskense* in residue was about 10^{10} cfu/g fresh weight in midwinter and slowly declined throughout the winter and spring. The decline was more rapid during the summer (Fig. 1). At the season's end, it was about 10^5 cfu/g fresh weight, which was barely detectable within the background of other bacteria that grew on the plates. Over the same time period, the population of total bacteria declined slowly from about 10^{10} to 10^8 cfu/g fresh weight. In contrast, populations of *C. m.* subsp. *nebraskense* from living popcorn plants increased about seven $\log_{10}$ units throughout the growing season (Fig. 2). The number of total bacteria increased about five $\log_{10}$ units. Bacterial populations in the plant homogenates paralleled those in the wash water and exceeded them by one to two $\log_{10}$ units. In 1983, the population of *C. m.* subsp. *nebraskense* and total bacteria in popcorn residue again declined throughout the sampling period (Fig. 3), and populations from live plants increased throughout the growing season (Fig. 4). Population levels and trends were very similar in both years.

The term "total bacteria" refers to all bacteria that were able to grow on CNS

Table 1. Number of total bacteria from corn recoverable on CNS and NBY agar media

	Total no. bacteria/g tissue	
Collection site[a]	CNS[b]	NBY
Field A	5.80×10^7	1.12×10^9
Field B	3.86×10^7	6.95×10^8

[a] Field A was planted in dent corn; field B was planted in popcorn.

[b] Of the bacteria that grew on CNS, about 23% were subsequently determined to be *Clavibacter michiganense* subsp. *nebraskense*.

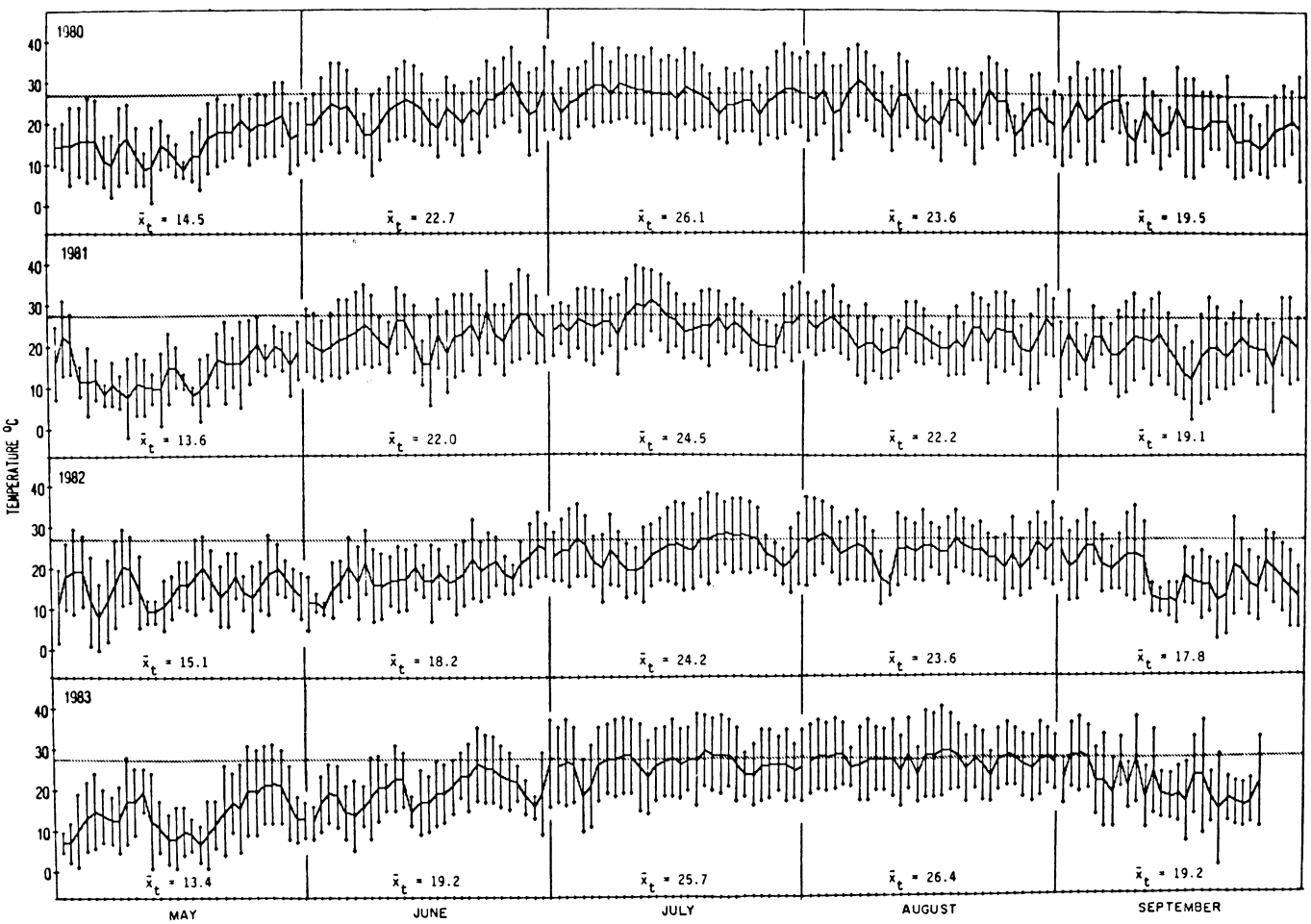

Fig. 5. Temperature data from Imperial, NE, for the growing seasons from 1980 through 1983. For each year, solid line shows daily mean temperature and bars show daily high and low temperatures. Dashed line at 27 C is the optimum temperature for growth of *Clavibacter michiganense* subsp. *nebraskense* in corn tissue. Overall mean temperature is shown for each month.

agar medium; these are confined to a limited number of gram-positive bacteria including *C. m.* subsp. *nebraskense* (5). Plating homogenates on NBY gave erratic results because frequently one type of bacterium would be present in large numbers in one homogenate but not in another. These opportunistic bacteria were often fast-growing and would completely obscure or inhibit other bacteria on the plate. Growth of some fungi on NBY also often obscured bacterial colonies. NBY agar supported growth of about 20 times more bacteria than CNS agar in one experiment where counts could be done (Table 1), indicating about that level of recoverable gram-negative bacteria. This was during early to midseason, when *C. m.* subsp. *nebraskense* was 23% of the total bacteria growing on CNS. By the end of the season, when disease symptoms were apparent, more than 90% of the total bacteria that grew on CNS were *C. m.* subsp. *nebraskense* (Figs. 2 and 4) and the proportion of gram-negative bacteria would likewise be altered.

Table 2. Relationship between severity of Goss's bacterial wilt and blight of corn and temperature in June

Year	Av. daily mean temperature (C) in June	Av. disease rating[a]
1980	22.7	2.5 (2.1, 2.9)
1981	22.0	2.7 (2.3, 3.1)
1982	18.2	1.8 (1.5, 2.0)
1983	19.2	1.5 (1.3, 1.7)

[a] Disease severity was rated in early September on a scale of 0–6, where 0 = no symptoms noted, 1 = trace to <1% of total leaf area affected, 2 = 1–10%, 3 = 11–25%, 4 = 26–50%, 5 = >50% of total leaf area affected, and 6 = all plants dead. Each rating was the mean of 15 or 20 separate evaluations. Numbers in parentheses are the lower and upper limits, respectively, of the 95% confidence interval.

Climatological measurements. A 4-yr record of daily high, low, and mean temperatures at Imperial for May through September is illustrated in Figure 5. The dashed line represents the temperature at which *C. m.* subsp. *nebraskense* grows at its maximum rate. The mean temperatures for June were 3–5 C cooler in 1982 and 1983 than in the preceding 2 yr. There was less than a 2 C difference in the mean temperatures for May, July, and September. The higher mean temperature in August 1983 did not correlate with differences in disease severity. However, there was an association between mean daily temperature in June and ultimate disease severity (Table 2). The 95% confidence intervals calculated for each mean disease rating indicate that severity was similar in 1980 and 1981 and in 1982 and 1983 but that the severity in the first 2 yr was greater than in the second 2 yr.

Growth of *C. m.* subsp. *nebraskense* at different temperatures in inoculated plants. *C. m.* subsp. *nebraskense* grew rapidly in 10- to 14-day-old corn plants between 16 and 32 C. At 38 C, the bacteria died, and at 12 C, their growth rate was retarded (Fig. 6). The shortest doubling time measured occurred at 26 C and was about 3.9 hr. Similarly, cultures in NBY broth grew at 10 C but not at 37 C, and the optimum temperature for growth was 26 C with a doubling time of about 3.5 hr (17). Doubling times in plants were calculated from the logarithmic phase of growth for each temperature and are shown in Table 3. The optimal temperature for growth, interpolated from a graph of these growth rates (plot not shown), was 27 C. At 21, 26, and 32 C, the bacterial growth rate began to level off after about 4 days. At that time, the plants showed very severe wilting symptoms and would have been rated 5 on a scale of 0–6 (20). Plants grown at 12, 16, and 38 C showed no symptoms before the end of the experiment.

Bacterial responses to temperature shifts. The bacteria grew slowly in plants in a day/night regime of 40/20 C, whereas the day/night regime of 32/35 C favored rapid bacterial growth. When plants were shifted from the restrictive temperature (40 C) to the permissive one (32 C), bacterial growth increased and reached its maximal rate rapidly (Fig. 7). Conversely, shifting from the growth-permissive temperature (32 C) to the restrictive one (40 C) resulted in a rapid cessation of growth (Fig. 8).

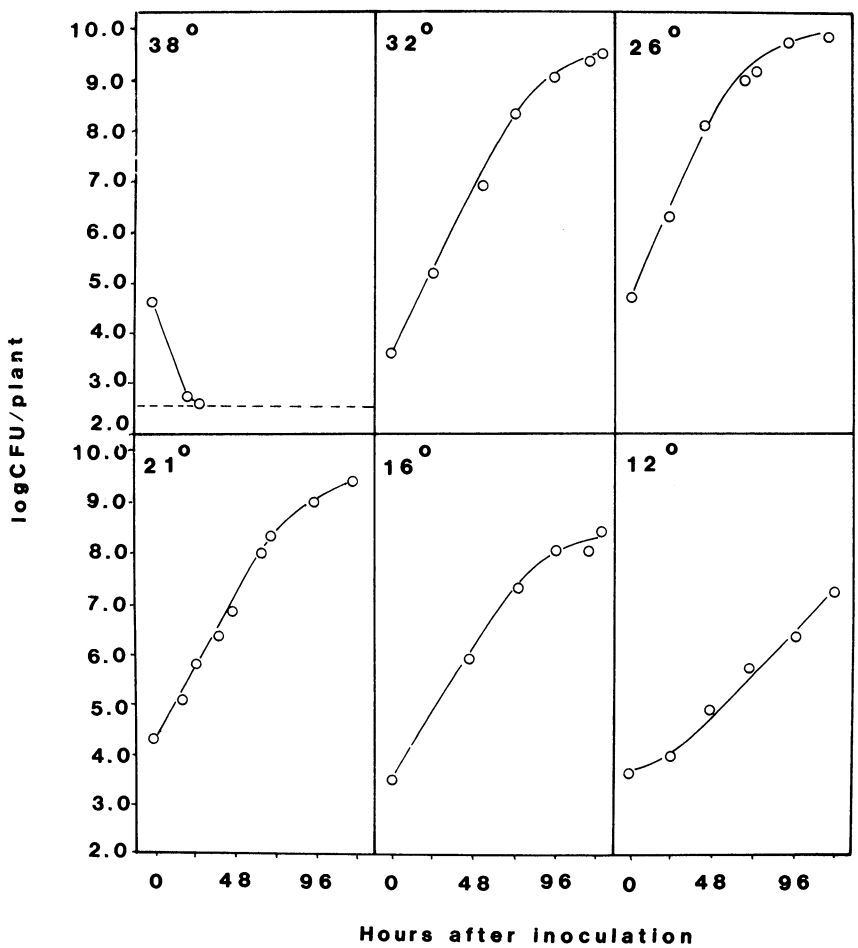

Fig. 6. Growth of *Clavibacter michiganense* subsp. *nebraskense* in corn plants at different temperatures. Plants of susceptible dent corn hybrid A619 × A632 were inoculated as described in text. After inoculation, plants were maintained at constant temperature in a growth chamber. Plants were sampled at various times after inoculation and bacterial concentration was determined as described in text. Each panel shows growth of *C. m.* subsp. *nebraskense* at the temperature (C) shown in upper left corner. Differences in bacterial concentrations at 0 hr reflect the differences in the inoculum prepared for each experiment at different times. Dashed line in upper left panel (38 C) shows lower limit of bacterial detection by our methods. Data are from a representative experiment.

DISCUSSION

C. m. subsp. *nebraskense* was recovered from corn residue and plants throughout

Table 3. Doubling time of *Clavibacter michiganense* subsp. *nebraskense* in corn plants at different temperatures

Temperature (C)	Doubling time (hr)
12	9.03
16	5.86
21	5.25
26	3.90
32	4.60

the year. The quantitative results reported here confirm earlier qualitative findings (13). As the growing season progressed, the population in residue dropped while that from live plants increased. Disking and cultivation redistributed residue throughout the soil and probably contributed to this decline in the population (18) because *C. m.* subsp. *nebraskense* is short-lived in pulverized, buried residue (10). The pathogen has not been detected in the soil in the absence of residue (10,13).

C. m. subsp. *nebraskense* was found in washings from symptomless plants on the earliest sampling date (about 4 wk after planting), suggesting an epiphytic phase for the bacterium in the field. Such epiphytic survival and increases of population have been reported for several other bacteria and were discussed in a recent review (7). Symptoms of Goss's bacterial wilt and blight were not observed in the field before mid-July when the population exceeded about 10^7 cfu/g fresh weight. The highest populations were reached just before physiological maturity.

The population levels reported here may be skewed from the actual population levels because of at least two sources of experimental error in our sampling process. Because our samples were composites of several leaves, the concentrations of bacteria were higher than if measurements had been taken assuming a log-normal distribution (6). Correction of this error requires sampling of individual leaves. Conversely, CNS medium suppresses the growth of some *C. m.* subsp. *nebraskense* colonies isolated from plant parts (M. L. Smidt and A. K. Vidaver, *unpublished*) and thus gives lower estimates of actual bacterial concentrations.

We attempted to understand the reason(s) for the mild symptom development during 1982 and 1983. Our data showed that 1) inoculum was present in the residue throughout the growing seasons, 2) susceptible popcorn plants were present, and 3) the wind damage was sufficient to enable infection to occur. Adequate water from rainfall or center-pivot irrigation was maintained in each growing season to optimize plant growth. Daily temperature records for the growing seasons at Imperial revealed a correlation between disease severity and the average temperatures during late May and June. In June of 1980 and 1981, average daily mean temperatures were 3–5 C higher than in 1982 and 1983.

By examining the effects of temperature on growth of the pathogen in corn plants under controlled conditions, we showed that the bacteria grew most rapidly in plants at 27 C. (It is interesting to note that the maximal bacterial growth rate exhibited in plant tissue was nearly the same as that in NBY broth at 25 C, suggesting that the nutritional composition of the corn tissue is as favorable for growth as that of culture medium.) At lower temperatures, the growth rate dropped gradually, until at 12 C, the doubling time was 2.3 times greater than at 27 C. At temperatures higher than 27 C, growth rates also dropped, and at 38 C, the bacteria died after about 1 day. Thus the relationship between growth rate and temperature was roughly a bell-shaped curve. The amount of bacterial growth that occurred in any one day could be calculated as a function of each temperature throughout the day. In May and June, when the temperatures were mostly below the optimum, an average temperature increase of 3–5 C would have had a greater effect on bacterial growth rate than a similar average increase in July or August, when the daily average temperatures were closer to the optimum for growth.

The effects of temperature on growth of *C. m.* subsp. *nebraskense* in plant tissue shown by these studies can explain bacterial growth in corn plants in the field. We showed that bacterial populations in plants growing at day/night regimes of 40/20 C increased at a rate that was considerably lower than one would expect at the mean temperature of 30 C. Thus, the bacteria did not respond to the mean daily temperature, but probably, some died during the day at 40 C and others grew during the night at 20 C for a net increase in population. By shifting infected plants between temperatures, we demonstrated that the bacterial growth rate could change

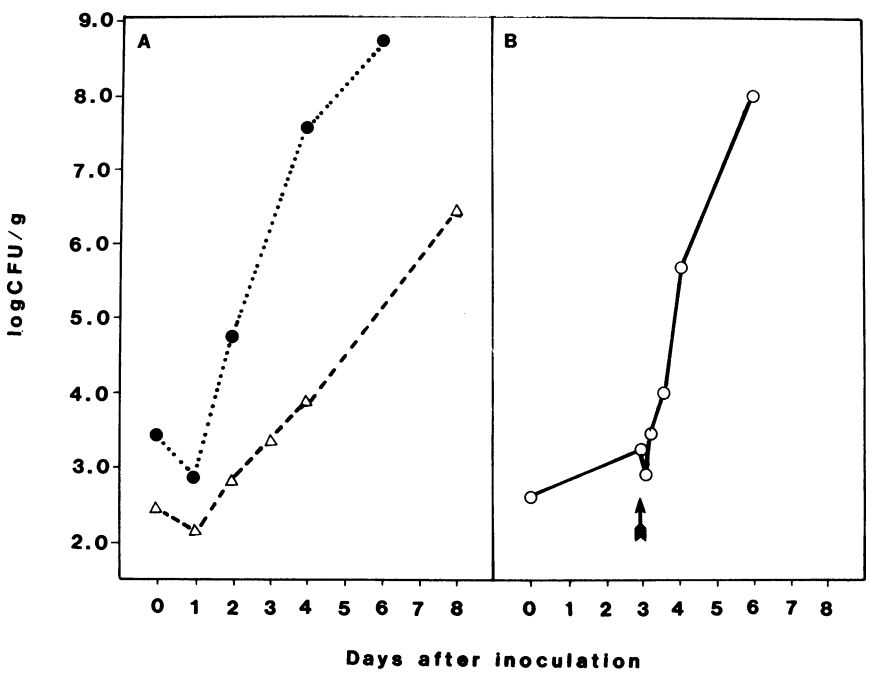

Fig. 7. Sensitivity of growth of *Clavibacter michiganense* subsp. *nebraskense* in corn plants to temperature shifts. Corn plants of hybrid A619 × A632 were inoculated with *C. m.* subsp. *nebraskense* strain CN72-2 as described in text. After inoculation, plants were grown in two temperature regimes up to 8 days after inoculation. **(A)** Bacteria recovered from plants grown in day/night regimes of 32/25 C (●···●) and 40/20 C (Δ—Δ). **(B)** Bacteria in plants that were shifted from the restrictive regime (40/20 C) to the permissive regime (32/25 C) rapidly began growing at a faster rate (o——o). Arrow indicates when shift occurred.

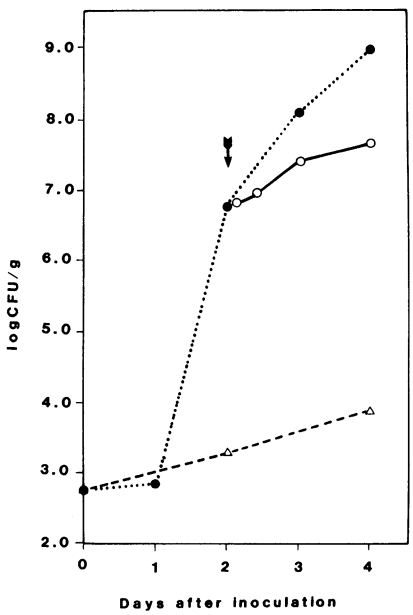

Fig. 8. Growth of *Clavibacter michiganense* subsp. *nebraskense* in corn plants grown in different temperature regimes and shifted between regimes. *C. m.* subsp. *nebraskense* strain CN72-2 was inoculated into corn plant hybrid A619× A632 as described in text. After inoculation, plants were grown in two temperature regimes. Plants were sampled up to 4 days after inoculation. On the second day, some plants were shifted from 32 to 40 C. Bacterial growth in day/night regimes of 32/25 C (●···●) and 40/20 C (Δ—Δ). Bacteria in plants that were shifted from 32/25 to 40/20 C rapidly adapted the slower growth rate (o——o). Arrow indicates when temperature shift occurred.

rapidly with a change in temperature. In the environment of field-grown corn plants, temperatures vary not only throughout the day and night but may vary throughout the plant depending on the location and thickness of the infected tissues.

The lower average temperatures in June of 1982 and 1983 may have retarded bacterial growth enough to affect the severity of symptoms that ultimately developed in late July and August. Unfortunately, bacterial population data for 1980 and 1981 were not obtained, so we do not know whether bacterial populations were higher or built up more quickly during those years. By the end of the season in 1982, population levels were similar to those in early samples of residues from the 1981 growing season. This may have occurred because temperatures during July, August, and September were similar and nearer the optimum in all 4 yr, allowing bacterial populations to reach a maximum level by the season's end. These results suggest that events early in the season contribute more to the development of disease severity than those occurring later in the season and also suggest the possibility of a predictive system for development of Goss's bacterial wilt and blight. Such data should be useful for plant breeders and others interested in the epidemiology of this disease.

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