Effects of Temperature, Plant Age, Inoculum Concentration, and Cultivar on the Incubation Period and Severity of Bacterial Canker of Tomato

R. J. CHANG, S. M. RIES, and J. K. PATAKY, Department of Plant Pathology, University of Illinois, Urbana 61801

ABSTRACT

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The incubation period and severity of bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* were influenced by temperature, plant age, inoculum concentration, and cultivar. The incubation period was longer and symptom development was less severe with cooler temperatures, older plants, lower concentrations of inocula, and moderately resistant cultivars. The time required before leaves wilted or cankers developed at the edge of inoculated petioles varied from 12 to 34 days, depending on conditions. Symptoms developed fastest on 2-wk-old susceptible seedlings grown at 25 C and inoculated with 8×10^8 cfu/ml. Similar factors affected the length of incubation period and severity of bacterial canker. The optimum conditions for appearance of symptoms also favored development of severe symptoms.

Bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis et al, is an important disease to the tomato (*Lycopersicon esculentum* Mill.) transplant industry. Control of bacterial canker has been based primarily on the use of certified seed and transplants (12,25). Nevertheless, infected, symptomless transplants can be shipped despite plant certification procedures, because incubation periods can be long and sampling methods to detect low populations of *C. m. michiganensis* in bedded seedlings are inadequate (16). For example, an epidemic of bacterial canker that devastated tomato crops throughout the midwestern United States and several Canadian provinces in 1984 was traced to certified tomato transplants from Georgia (16).

Phytopathogenic corynebacteria may exist on apparently healthy propagative materials as epiphytes or as latent infections (7,16,17,34). Generally, wilt symptoms appear on systemically infected plants 30–40 days after transplanting. Because of the long incubation period of *C. m. michiganensis*, it is conceivable that systemically infected plants could produce symptomless but infected fruits (25). Infested seed from such fruits could provide primary inocula for outbreaks in beds of seedlings. Corynebacteria have been found within healthy-appearing tomato fruits (24). Although the species of these bacteria were not identified, there is reasonable evidence that seed certified as disease-free may be contaminated with *C. m. michiganensis* (16).

The complex symptoms of bacterial canker of tomato can be differentiated into systemic and localized infections (25,27). A minimum incubation period of 3–5 days is required for the appearance of localized symptoms, such as white, blisterlike spotting of leaflets or bird's-eye spotting of fruit (1,23). The incubation period for systemic symptoms varies from 7 to 84 days, depending on the interactions among host, pathogen, and environment (4,26,31,33). The effects of plant age, temperature, host nutrition, and inoculum concentration on the development of bacterial canker in resistant and susceptible tomato accessions have been published (2,9,13,14,35). Also, the effects of predisposition on the progress of bacterial canker (35) and the effects of inoculation methods on severity have been evaluated (26). However, little or no attention has been given to the effects of these factors on the incubation period of bacterial canker.

Factors that affect the incubation period of bacterial canker in tomato might be useful in the development of improved plant certification programs. The objectives of this research were to evaluate the effects of temperature, plant age, inoculum concentration, and host cultivar on the incubation period and severity of bacterial canker of tomato seedlings.

MATERIALS AND METHODS

Bacterial strains and preparation of inoculum. Ten strains of *C. m. michiganensis* were obtained from diverse geographic locations (7). All strains produced a typical one-sided wilt in inoculated tomato plants (cv. Heinz 1810) and a hypersensitive reaction on leaves of four-o'clock (*Mirabilis jalapa* L.) (15). These strains were stored in King's B broth at −80 C and revived prior to each experiment to avoid repeated subculturing. Inoculum was prepared by transferring frozen cultures to King's B broth and incubating the cultures at room temperature on shakers for 48 hr. Broth cultures then were streaked on nutrient-broth-yeast extract agar medium. A single colony was selected and streaked on nutrient-broth-yeast extract agar plates, and plate cultures were incubated at 25 C for 48 hr and used as inoculum. Bacteria were suspended in sterile phosphate buffer (0.01 M, pH 7.2) containing 0.85% NaCl and adjusted to approximately 2×10^8 cfu/ml ($A_{590nm} = 0.16$) unless stated otherwise. The inoculum was a mixture of equal proportion of the 10 strains to account for variation in virulence that may have existed among strains (3,28,32). A modified CNS medium (18) (minus lithium chloride and polymyxin B sulfate) was used to reisolate the pathogen from inoculated plants.

Test plants and inoculation. Susceptible (Heinz 1810) and moderately resistant (Heinz 7417) tomato cultivars were used in these studies (6). Seeds were obtained from D. A. Emmatty (Heinz USA, OH). Tomatoes were sown at a depth of 1.5 cm in a sterilized mixture of equal portions (by volume) of soil, peat, and vermiculite in $35 \times 50 \times 9.5$ cm flats. Seedlings were thinned 1 wk after emergence to 6 cm apart within rows and about 7–10 cm between rows. Each flat contained 20–40 seedlings. Flats were kept in a greenhouse at about 25 C day and 20 C night prior to each experiment. Seedlings were watered daily to maintain moisture at a level favorable for tomato plant development. Granular potash 5-23-27 (FS Special, Growmark Inc. Bloomington, IL) was applied every 2 wk. Seedlings were inoculated by cutting the petiole of the first true leaf at its point of attachment using scissors dipped in inoculum as described by Thyr (30).

Disease assessment. The severity of bacterial canker symptoms was rated with a modified disease assessment key (13), where 0 = no symptoms, 1 = a few cankers present and/or one lower leaf wilted, 2 = two to fewer than one-half

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of the leaves wilted, 3 = one-half to three-quarters of the leaves wilted, 4 = more than three-quarters of the leaves wilted but terminal leaves on the main shoot not wilted, and 5 = terminal leaves of the main shoot and most other leaves wilted or dead.

Temperature. Tomato seedlings (cv. Heinz 1810) grown in the greenhouse at 25 C day and 20 C night were inoculated 4 wk after emergence by methods described previously. Inoculated seedlings were placed in growth chambers at 15, 20, 25, or 30 C with 14 hr of light and 10 hr of darkness. Each experimental unit consisted of four rows of five inoculated seedlings per row incubated in the same growth chamber. Seedlings were observed daily. Incubation period was recorded for each seedling as the time from inoculation to the appearance of symptoms with a disease rating of 1. The disease assessment key (13) described above was used to rate severity at 3- to 7-day intervals after inoculation. The experiment was done twice with separate randomization of temperature for the four growth chambers. The two trials were analyzed as replicates.

Plant age. Five rows of 10 seeds each of cv. Heinz 1810 were sown in flats (35 × 50 × 9.5 cm) at weekly intervals to obtain seedlings that were 2, 3, 4, 5, and 6 wk old. The five treatments were arranged in a randomized complete block design with four replicates. Each flat was a block. Each experimental unit consisted of one row of five seedlings. Seedlings were inoculated by methods described previously. Inoculated seedlings were grown in the greenhouse at about 25 C day and 20 C night. Seedlings were observed daily and rated as described above. The experiment was done twice. Data from the two trials were combined.

Inoculum concentration. In the greenhouse, 4-wk-old seedlings were inoculated with *C. m. michiganensis* at a concentration of 8×10^1, 8×10^2, 8×10^4, 8×10^6, or 8×10^8 cfu/ml. There also was a noninoculated control. Concentrations were determined by dilution plate analysis of the freshly prepared inocula as described previously. The highest concentration yielded an OD of 0.3 at 590 nm. The inocula were used within 30 min of preparation. Treatments were arranged in a randomized complete block design with three replicates. Each flat was a block. Each experimental unit consisted of one row of five seedlings. Inoculated seedlings were kept in a greenhouse at 25 C day and 20 C night throughout the experiment. Seedlings were observed daily and rated as described above. The experiment was done twice. Data from the two trials were combined.

Cultivar. Two processing tomato cultivars, Heinz 1810 and Heinz 7417, were grown in a greenhouse at 25 C day and 20 C night, and 4-wk-old seedlings were inoculated as described previously. Cultivars were arranged in a randomized complete block design with three replicates. Each flat was a block. Each experimental unit consisted of 10 seedlings of the same cultivar in a flat. Inoculated seedlings were observed daily and rated as described above. The experiment was done twice. Data from the two trials were combined.

Statistical analyses. Data from each experiment were analyzed by ANOVA with temperature, plant age, and inoculum concentration as quantitative independent variables and with cultivar as a qualitative independent variable. Mean incubation periods from each trial were regressed on temperature, plant age, and inoculum concentration. Severity of bacterial canker (0–5 scale) was regressed against time (days after inoculation) by treatment. Slope coefficients (B_1) were compared with t statistics ($P < 0.05$). F statistics ($P < 0.05$) were used to test the significance of the regression models and independent variables. Coefficients of determination (r^2) were calculated to determine the variation explained by the model. Residuals from the regression models were evaluated for lack of fit and outliers to test the appropriate models. Treatment means for cultivars were compared by Fisher's least significant difference (LSD) test.

RESULTS

Temperature. Growth of the inoculated seedlings was influenced by temperature. Seedlings grew better at higher temperatures than at lower temperatures. Maximum growth occurred at 25 C. Growth was poor at 15 C throughout the experiment. Most of the inoculated seedlings were stunted, and growth slowed as wilting progressed. Some seedlings grown at 25 and 30 C died 35 days after inoculation.

The incubation period was longer at 15 C than at 20, 25, or 30 C (Fig. 1). Wilted leaves or cankers were first observed on seedlings grown at 20, 25, or 30 C about 10 days after inoculation and on seedlings grown at 15 C about 20 days after inoculation. Generally, wilting was observed more frequently at higher temperatures and cankers were more prevalent at lower temperatures. The optimum temperature for appearance of symptoms based on incubation period appeared to be about 25 C (Fig. 2). The best estimates of incubation periods at the different temperatures were 26, 16, 14, and 19 days for 15, 20, 25, and 30 C, respectively. The relationship was curvilinear.

Regressions of severity on time were described best by linear models with slopes of 0.11, 0.12, 0.16, and 0.15 at 15, 20, 25, and 30 C, respectively (Fig. 3). Based on t tests, slope coefficients were significantly different between the lower temperatures (<20 C) and the

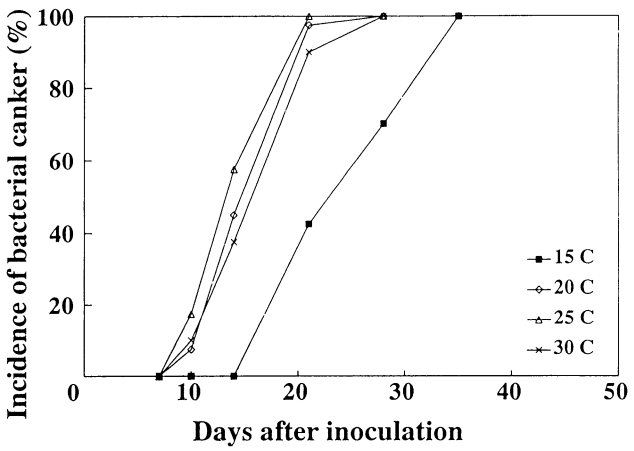

Fig. 1. Incidence of bacterial canker after the petiole of the first true leaf of 4-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 2×10^8 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held in growth chambers at 15, 20, 25, and 30 C. Each point represents the mean of 40 seedlings.

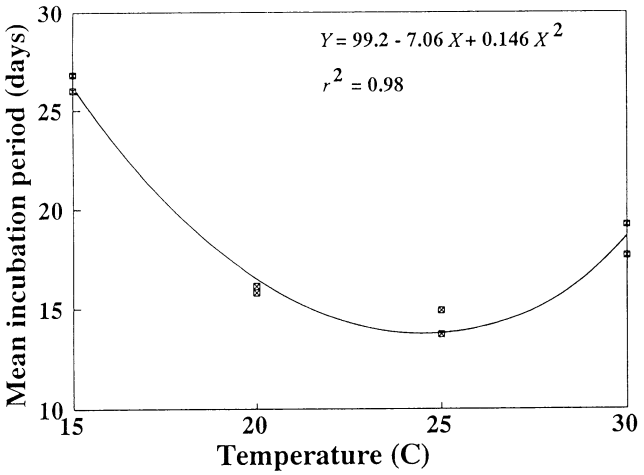

Fig. 2. Mean incubation period of bacterial canker after the petiole of the first true leaf of 4-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 2×10^8 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held in growth chambers at 15, 20, 25, and 30 C.

higher temperatures (>25 C). Generally, symptoms developed faster on seedlings grown at warmer temperatures.

Plant age. Height of tomato seedlings was influenced by plant age. Older seedlings were generally taller than younger seedlings. Usually, 2- and 3-wk-old seedlings died by 28 days after inoculation.

Generally, incubation period increased and severity decreased as plant age increased (Figs. 4–6). Wilted leaves were first observed about 10 days after inoculation on 2- and 3-wk-old seedlings and about 14 days after inoculation on 4-, 5-, and 6-wk-old seedlings (Fig. 4). The optimum plant age for appearance of symptoms based on incubation period appeared to be about 2 wk (Fig. 5). The best estimates of incubation periods for 2-, 3-, 4-, 5-, and 6-wk-old seedlings were 12, 14, 16, 17, and 19 days, respectively. The relationship was linear.

Regressions of severity on time were explained by quadratic equations for 2-, 3-, and 4-wk-old seedlings and by linear equations for 5- and 6-wk-old seedlings (Fig. 6). Generally, leaves wilted faster on younger seedlings. Slope coefficients were similar for 5- and 6-wk-old seedlings based on *t* tests.

Inoculum concentration. Growth of the inoculated seedlings was influenced by inoculum concentration. For seedlings receiving 8×10^4 cfu/ml or more inoculum, growth slowed by 14 days after inoculation and seedlings started to die 42 days after inoculation.

Incubation period decreased and severity increased as inoculum concentration increased (Figs. 7–9). Wilted leaves were first observed about 10–14 days after inoculation on seedlings inoculated with concentrations of 8×10^4 cfu/ml or higher. All seedlings inoculated with at least 8×10^4 cfu/ml showed wilt symptoms 28 days after inoculation (Fig. 7). Wilted leaves were first observed 28 days after inoculation on seedlings inoculated with 80 and 800 cfu/ml of *C. m. michiganensis*. Only 60 and 87% of those seedlings became systemically infected. The optimum inoculum concentration for appearance of symptoms based on incubation period appeared to

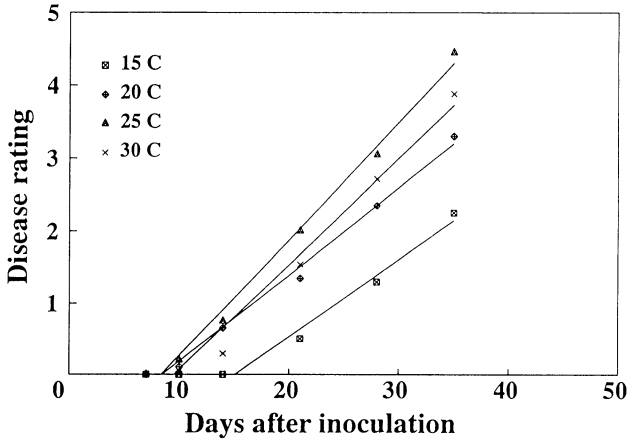

Fig. 3. Severity of bacterial canker after the petiole of the first true leaf of 4-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 2×10^8 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held in growth chambers at 15, 20, 25, and 30 C. Each point represents the mean of 40 seedlings. Relationships were described by linear equations: $Y = -1.63 + 0.11\ X$ ($r^2 = 0.98$), $Y = -1.02 + 0.12\ X$ ($r^2 = 0.99$), $Y = -1.35 + 0.16\ X$ ($r^2 = 0.81$), and $Y = -1.39 + 0.15\ X$ ($r^2 = 0.73$) for 15, 20, 25, and 30 C, respectively.

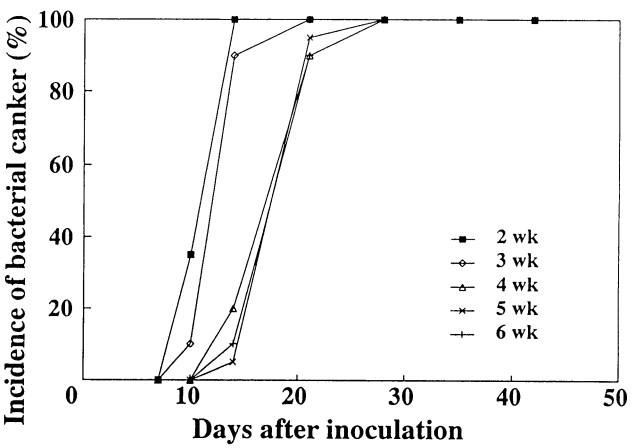

Fig. 4. Incidence of bacterial canker after the petiole of the first true leaf of 2-, 3-, 4-, 5-, or 6-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 2×10^8 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held at 25 C day and 20 C night. Each point represents the mean of 40 seedlings.

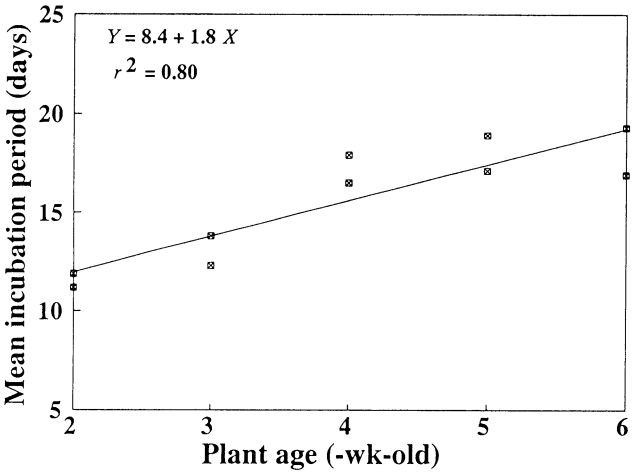

Fig. 5. Mean incubation period of bacterial canker after the petiole of the first true leaf of 2-, 3-, 4-, 5-, or 6-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 2×10^8 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held at 25 C day and 20 C night.

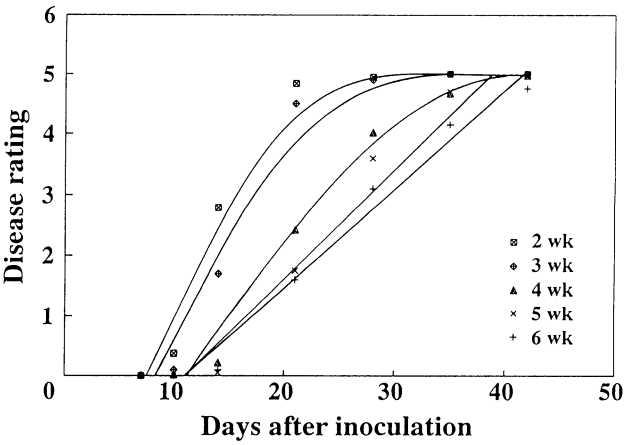

Fig. 6. Severity of bacterial canker after the petiole of the first true leaf of 2-, 3-, 4-, 5-, or 6-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 2×10^8 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held at 25 C day and 20 C night. Each point represents the mean of 40 seedlings. Relationships were described by quadratic equations: $Y = -3.70 + 0.56\ X - 0.009\ X^2$ ($r^2 = 0.96$), $Y = -3.77 + 0.52\ X - 0.007\ X^2$ ($r^2 = 0.96$), and $Y = -3.88 + 0.39\ X - 0.004\ X^2$ ($r^2 = 0.98$) for 2-, 3-, and 4-wk-old seedlings, respectively. For 5- and 6-wk-old seedlings, relationships were described by linear equations: $Y = -1.93 + 0.18\ X$ ($r^2 = 0.96$) and $Y = -1.80 + 0.16\ X$ ($r^2 = 0.98$), respectively.

be about 8×10^8 cfu/ml (Fig. 8). The best estimates of incubation period for the seedlings inoculated with 8×10^8, 8×10^6, 8×10^4, 8×10^2, and 8×10^1 cfu/ml of the pathogen were 12, 13, 18, 27, and 34 days, respectively. The relationship was curvilinear.

Regressions of severity on time were explained best by linear models for inoculum concentrations of 800 and 80 cfu/ml and by quadratic models for concentrations of 8×10^4, 8×10^6, and 8×10^8 cfu/ml (Fig. 9). Generally, leaves wilted faster on seedlings inoculated with higher concentrations. However, for seedlings inoculated with concentrations of 8×10^4 cfu/ml or higher, disease ratings were not significantly different 35 days after inoculation and thereafter. Therefore, 8×10^4 cfu/ml appeared to be the critical concentration for disease development.

Cultivar. Wilted leaves were first observed about 10 days after inoculation on the susceptible cultivar, Heinz 1810, and about 17 days after inoculation on the moderately resistant cultivar, Heinz 7417. The mean incubation period was 14 days for the susceptible cultivar and 20 days for the moderately resistant cultivar.

Regressions of severity on time were described by a linear model, $Y = -1.57 + 0.12\ X$ ($r^2 = 0.99$), for the moderately resistant cultivar and by a quadratic model, $Y = -1.98 + 0.28\ X - 0.003\ X^2$ ($r^2 = 0.99$), for the susceptible cultivar. Symptoms always were more severe for the susceptible cultivar than for the moderately resistant cultivar.

DISCUSSION

Incubation period and development of bacterial canker on tomato seedlings were influenced by temperature, host age, inoculum concentration, and host resistance. Cooler temperatures, older plants, lower concentrations of inoculum, and moderately resistant cultivars increased the incubation period and decreased severity. The mean incubation period ranged from 12 to 34 days. Optimum conditions for appearance of symptoms also were favorable for development of severe symptoms. Obviously, incubation period and severity are affected by interactions among tomato cultivars, *C. m. michiganensis*, and enviromental conditions, as others have shown (2,13,26).

Traditionally, certification of tomato seedlings has been done by detection of symptomatic transplants (21). Although stem printing on semiselective medium has been successful in detecting the pathogen from symptomless transplants (16), problems exist with methods and timing of sampling fields and the time required for confirmation of bacteria in the laboratory assays. Normally, transplants produced in the southeastern United States are harvested for shipment within 8 wk of emergence (21). Clipping tomato seedlings is the major method of spreading the pathogen from infected to healthy seedlings (7). The initial clipping usually is done when seedlings are 4–5 wk old. Therefore, on the basis of our results, bacterial canker symptoms should be observed 16–17 days after the first clipping, if susceptible seedlings are clipped in the presence of 2×10^8 cfu/ml of the pathogen and seedlings are growing at 25 C. However, systemically infected plants often appear about 30 days after transplanting, which suggests that seedlings might be exposed to lower concentrations of inoculum or seedlings are older than 4–5 wk when inoculated. Because 7 days of incubation are required for high recovery of *C. m. michiganensis* using the stem printing method and because 7–10 days are required for confirmation (16), intensive sampling should be done 7 days after the first clipping.

Others have observed that bacterial canker developed slower and the incubation period was longer under unfavorable conditions, including cold temperature or low nutrients (22,35). Blood (4) reported that bacterial canker progressed most rapidly when air and soil temperatures were 28 C. Similar studies (13,22) confirmed these results. In our study, wilted leaves appeared first and the symptoms developed fastest at 25 C. The optimum temperature for growth of *C. m. michiganensis* ranges from 24 to 27 C (20). When inoculated seedlings were placed at 15 C, we also observed that symptoms occurred most frequently as open cankers rather than as various degrees of wilting. Seedlings grew poorly at 15 C throughout the experiment. Growth of the inoculated seedlings was improved and wilting was more frequent at temperatures higher than 15 C. Thus, cooler temperatures, which reduced vigor of the inoculated seedlings, may have slowed disease development and resulted in stem cankers rather than wilting (35).

The incubation period increased and the rate of symptom development declined with increasing plant age. Kendrick and Walker (22) reported that the symptoms developed at about the same rate but wilted leaves appeared earlier in younger plants. In our study, symptoms developed faster on 2- to 4-wk-old seedlings than on 5- to 6-wk-old seedlings. Plant age may not affect canker development after plants are 5 wk old, but age is an important factor affecting the length of incubation period.

Thyr (29) reported that tomato seedlings can be successfully inoculated with as few as five cells and probably with a single cell. We observed systemic infections with as few as 80 cfu/ml, although symptoms did not appear for 28 days;

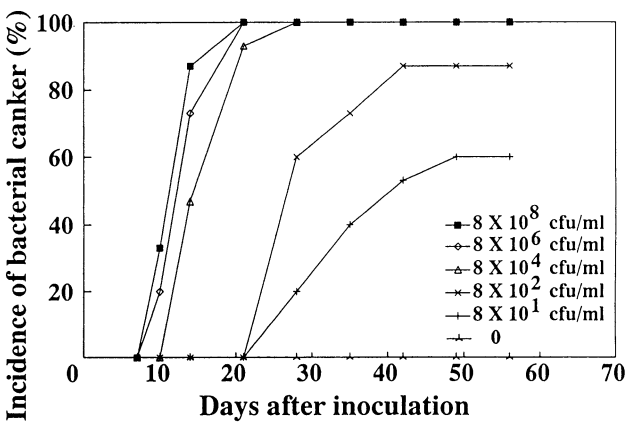

Fig. 7. Incidence of bacterial canker after the petiole of the first true leaf of 4-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 8×10^8, 8×10^6, 8×10^4, 8×10^2, 8×10^1, or 0 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held at 25 C day and 20 C night. Each point represents the mean of 30 seedlings.

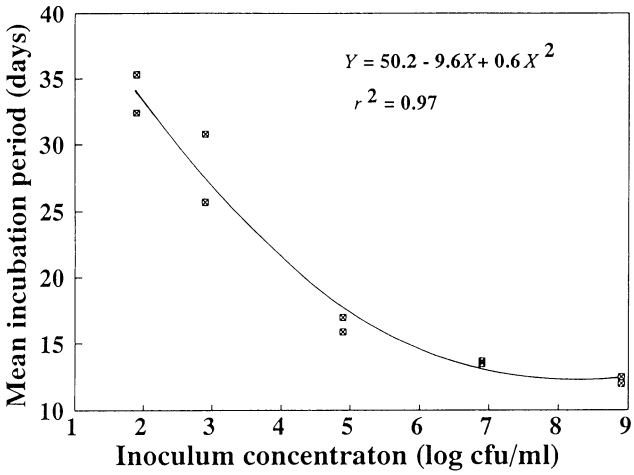

Fig. 8. Mean incubation period of bacterial canker after the petiole of the first true leaf of 4-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 8×10^8, 8×10^6, 8×10^4, 8×10^2, or 8×10^1 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held at 25 C day and 20 C night.

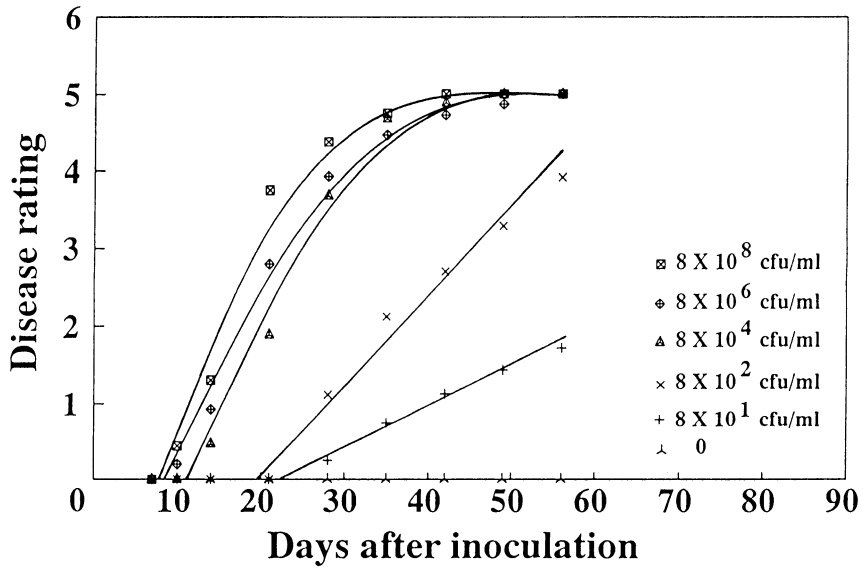

Fig. 9. Severity of bacterial canker after the petiole of the first true leaf of 4-wk-old tomato seedlings (cv. Heinz 1810) was removed with scissors that had been dipped into 8×10^8, 8×10^6, 8×10^4, 8×10^2, 8×10^1, or 0 cfu/ml of *Clavibacter michiganensis* subsp. *michiganensis*. Seedlings were then held at 25 C day and 20 C night. Each point represents the mean of 30 seedlings. Relationships were described by quadratic equations: $Y = -2.65 + 0.37\ X - 0.005\ X^2$ ($r^2 = 0.98$), $Y = -2.37 + 0.31\ X - 0.003\ X^2$ ($r^2 = 0.99$), and $Y = -3.45 + 0.35\ X - 0.004\ X^2$ ($r^2 = 0.98$) for inoculum concentrations of 8×10^8, 8×10^6, and 8×10^4 cfu/ml, respectively. For inoculum concentrations of 8×10^2 and 8×10^1 cfu/ml, relationships were described by linear equations: $Y = -2.24 + 0.12\ X$ ($r^2 = 0.98$) and $Y = -1.17 + 0.05\ X$ ($r^2 = 0.99$), respectively.

60% of seedlings inoculated with 80 cfu/ml became systemically infected. Generally, susceptible tomato seedlings inoculated with lower concentrations of bacteria have longer incubation periods and slower development of severe symptoms (11,13,33), but this association may not be true with resistant tomato cultivars (13).

Several tomato cultivars with partial resistance to *C. m. michiganensis* have been introduced (3,8). Thyr (31) and Van Steekelenburg (33) found that multiplication of the pathogen was slowed by partial resistance. We previously reported (6) that the incubation period was normally distributed among 84 cultivars and may be a criterion by which to classify levels of resistance to canker. Although the difference in the mean incubation period between the susceptible (Heinz 1810) and the moderately resistant (Heinz 7417) tomato cultivars was only 6 days, this might sufficiently delay the results of tests used in certification. Further research is needed.

Latent infections may result from a prolonged period of incubation because of host resistance or unfavorable temperatures for growth of the pathogen (19, 34). Latency is common under conditions that are unfavorable for the pathogen. For example, under cool conditions, *C. m. sepedonicus*, the causal organism of potato ring rot, may be present in a susceptible host without visible symptoms in tubers or foliage for at least 1 yr (5). Appearance of bacterial canker symptoms is closely related to populations of the pathogen in the host (31,33). Multiplication of the pathogen is favored only in appropriate hosts, under favorable environmental conditions, and with adequate nutrition (10). Thus, under favorable environmental conditions, symptoms of systemic infection in 4-wk-old susceptible seedlings can be seen 2 wk after inoculation with 2×10^8 cfu/ml of *C. m. michiganensis*. This incubation period can be increased to 34 days or longer under cool conditions, in older plants, or in plants inoculated with concentrations of bacteria less than 80 cfu/ml.

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