Survival and Dissemination of *Clavibacter michiganensis* subsp. *michiganensis* in Tomatoes

M. L. Gleason, E. J. Braun, W. M. Carlton, and R. H. Peterson

Department of Plant Pathology, Iowa State University, Ames 50011.
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ABSTRACT

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Rifampicin-resistant strains of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomato, were used to investigate the dynamics of epiphytic populations and overwintering survival of the pathogen. Epiphytic populations stabilized at 10^6–10^8 cfu per leaflet and 10^5–10^6 cfu per green fruit. After $\log_{10}$ transformation, epiphytic populations of the pathogen on leaflets and fruit approached a normal distribution. *C. m. michiganensis* survived for at least 24 mo in infested debris at the soil surface, but for only 7 mo in buried debris. Infested debris that was allowed to overwinter served as primary inoculum for establishment of epiphytic populations of *C. m. michiganensis* on a subsequent processing-tomato crop. These plants became diseased and their yields were significantly reduced. The establishment of epiphytic populations of the pathogen and local survival can be important in the disease cycle of bacterial canker.

Additional keywords: bacterial diseases.

Infested seed and infected transplants are well recognized as major sources of primary inoculum of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomato, in the north central United States (1,5,6,11,35). The canker pathogen also overwinters in tomato debris in many parts of the world (5,9,15,24,31), including the north central United States (7,10), and infects plants in the next year's crop (31). However, experimental evidence relating overwintered inoculum to potential disease losses is lacking.

The role of epiphytic populations of *C. m. michiganensis* in disease development in production fields is poorly understood. Dissemination in production fields has been reported to occur by rainsplash, overhead irrigation, machinery, pruning, and pesticide spraying (1,11,32,33). Spraying inoculum on foliage of very young tomato seedlings led to infection through substomatal chambers (30) and vascular tissue (19), but in other experiments, foliar-applied inoculum did not result in disease development (1,15). Basu (2) found that at least 10^6 cfu/ml of sprayed inoculum was needed to cause disease in 2- to 3-wk-old seedlings and that foliar symptoms were more distinct at high (87–97%) than at lower relative humidity. In Ohio, dissemination from inoculated, systemically infected transplants to adjacent transplants in a production field did not significantly reduce yield of processing tomatoes (25). Despite evidence that *C. m. michiganensis* can survive epiphytically (6,7,26) and that epiphytic populations can give rise to disease (25), the ecology of the pathogen in production fields has received little study.

The present study was undertaken to monitor the development and survival of epiphytic populations of *C. m. michiganensis* in tomato plantings and to relate overwinter survival of the pathogen in debris to both disease development and yield suppression in a subsequent tomato crop. Preliminary reports of the results have been published (13,14).

MATERIALS AND METHODS

Strains and media. Several media were tested to determine their suitability for isolating the pathogen from plants in field plots. Acceptable selectivity for *C. m. michiganensis*, as determined by leaf prints and by extracts from field-grown plants inoculated with the pathogen (M. L. Gleason, *unpublished data*), was not provided by KBT agar (8), KBT agar amended with 100 μg/ml of cycloheximide, or SCM agar (12). Three naturally occurring and rifampicin-resistant strains, derived from wild-type strains of *C. m. michiganensis* from Iowa (Cmm-1-R2) and Michigan (BR4-R1 and DR60-R1), were used in all experiments. The rifampicin-resistant strains were selected by plating dilutions of wild-type strains in 0.02 M, pH 7.0, phosphate buffer onto nutrient broth-yeast extract agar amended with rifampicin (50 μg/ml) and cycloheximide (100 μg/ml) (Sigma Chemical Co., St. Louis, MO) (NBYCR). Isolations of the rifampicin-resistant strains on NBYCR from leaflets of spray-inoculated, field-grown tomato plants were almost always free of bacterial interference and usually free of fungal interference.

Epiphytic populations following spray inoculation. Experiments were at the Curtiss Farm of Iowa State University, located near Ames in fields where tomatoes or other solanaceous crops had not been grown previously. In 1987, 20 tomato (*Lycopersicon esculentum* Mill.) seedlings (cv. Jet Star) were transplanted on 18 May and placed in wire cages in mid-June. Plants were sprayed to runoff on 10 July with 5×10^6 cfu/ml of a mixture (1:1:1) of strains Cmm-1-R2, BR4-R1, and DR60-R1 in 0.02 M phosphate buffer (pH 7.0), using a trigger-type hand-spray bottle. To allow spray droplets to settle evenly on plant surfaces, each plant was surrounded with a portable wind shelter during inoculation. Plots were fertilized in late June with 13-13-13 fertilizer and watered with overhead sprinklers as needed. Plots were sprayed weekly after mid-June with chlorothalonil and carbaryl at labeled rates, to control early blight (caused by *Alternaria solani*), Septoria blight (caused by *Septoria lycopersici*), anthracnose (caused by *Colletotrichum coccodes*) and tomato hornworm (*Manduca quinquemaculata*). At weekly intervals from 13 July to 22 August, six samples, including four asymptomatic, fully expanded leaflets and two asymptomatic green fruits (3–5 cm in diameter), were sampled from each plant and stored in individual plastic bags on ice. Approximately 2–5 h after sampling, each sample was shaken individually on a rotary shaker at 140 rpm for 1 h in 25 (leaflets) or 50 ml (fruits) of phosphate buffer (0.02 M, pH 7.0) containing 0.1% peptone. Serial 10-fold dilutions were prepared, and 0.1-ml aliquots of appropriate dilutions were spread

on plates of NBYCR agar. Plates were incubated at room temperature, and colonies were counted 5–7 days after plating. In 1988, 120 seedlings (cv. Heinz 6004) were planted on 18 May and inoculated on 16 June with 10^5 cfu/ml of an inoculum mixture, as described for 1987. A fully expanded terminal leaflet was sampled from midcanopy height on each of 30 randomly selected plants on 13, 16, 23, and 30 June, 14 and 28 July, and 11 August, and was processed as in 1987. No fruits were sampled in 1988. Means of populations per leaflet or fruit were calculated. Samples with no detectable *C. m. michiganensis* were arbitrarily assumed to have a population of zero.

Distribution of epiphytic populations. The distribution of epiphytic pathogen populations on individual leaflets was determined by two statistical tests for normality, the Shapiro-Wilk test (29) and the rankit test (22,36). The rankit test was conducted by arranging the population estimates in a data set in order of increasing size and assigning an appropriate rankit value to each of the rank-ordered observations. The rankits were then plotted against the corresponding population estimates. Both tests were carried out on both raw and log-transformed (base 10) data.

Production of infected debris for overwintering experiments. Four-hundred tomato seedlings (cv. Heinz 6004) were planted in a block of six rows, with 0.3 m between plants in a row and 1.2 m between rows, at Curtiss Farm on 18 May 1988. An equal mixture of strains Cmm-1-R2, BR4-R1, and DR60-R1, at a total concentration of 10^9 cfu/ml, was prepared in sterile phosphate buffer. Scissors dipped in the bacterial suspension were used to excise three leaflets on each plant on 8 June and three leaves per plant on 24 June and 7 July. All plants were inoculated on each date. The planting was irrigated as needed using overhead sprinklers and was sprayed weekly with chlorothalonil and carbaryl at labeled rates.

Marginal necrosis of leaflets appeared by early July, followed by vascular wilt 2 wk later. Recovery of *C. m. michiganensis* on NBYCR agar from symptomatic tissue confirmed the presence of the strains used in the study. By late August, wilting and necrosis caused by bacterial canker were severe throughout the planting.

Survival in debris. In early November 1988, air-dried leaflets and petioles were separated from stems, and both types of litter were cut into 4- to 7-cm segments. Nylon screen (1.0 mm mesh) was used to make litter bags (15 × 22 cm). Each bag was filled with 6 g dry weight of either leaf plus petiole segments or stem segments; the bag was stapled shut. On 8 November, bags were placed in a plot at Curtiss Farm more than 200 m from sites where tomatoes had been grown. Half the bags were held on the soil surface with metal clips, and the other bags were buried 10 cm deep in the soil.

At 2-mo intervals, three randomly selected bags of each type of litter (leaf plus petiole or stems) were sampled from surface and buried treatments. The contents of each bag were homogenized separately in a blender at high speed for 4 min with sterile phosphate buffer plus 0.1% peptone. Serial 10-fold dilutions were made from this extract, and 0.1-ml aliquots of each dilution were spread on replicate NBYCR plates. Colonies on these plates were counted after 4–6 days, and mean population of the pathogen per litter bag was determined. Populations below the limit of detection were assumed to equal zero.

Epiphyte colonization, disease progress, and yield. For an experiment investigating disease progress and yield, entire dried plants were harvested in October 1988 from the clip-inoculated planting described previously and placed on three plots (2 × 7 m). Approximately twice the amount of debris that would be generated by a planting density of 30 cm within rows and 1.2 m between rows was incorporated into the plots on 25 October to a depth of approximately 15 cm, using a rotary tiller. Three control plots (2 × 7 m) also were cultivated, but received no tomato debris. Control and debris-infested plots were separated from each other by 12 m. Treatments were arranged in a completely randomized design. The site was located more than 200 m from areas in which tomatoes had been grown.

To minimize movement of the pathogen among plots, 12 rows of field corn were planted between each plot in May 1989. All plots were cultivated with a rotary tiller again in late May. On 30 May, greenhouse-grown tomato seedlings (cv. Heinz 6004) were transplanted into each plot in two rows, with a spacing of 30 cm within rows and 1.2 m between rows. In late June, plants were fertilized with approximately 33–44 kg/ha of nitrogen in the form of 13-13-13 fertilizer. Chemical sprays for pest control were as described previously. Overhead irrigation was provided as needed.

An additional 2 × 7 m plot, located 12 m from the above experiment, was designated for the study of epiphytic populations of *C. m. michiganensis*. Debris infested with the pathogen was incorporated on the same date and at the same density as described previously. Plant cultivar, spacing, and care in 1989 were also as described previously. The two experiments were separated from each other by 12 rows of field corn. At 2-wk intervals from 9 June until harvest, a single, asymptomatic, terminal leaflet was collected from midcanopy of each of 30 plants. Leaflets were shaken individually for 1 h in 50 ml of sterile phosphate buffer containing 0.1% peptone. Serial 10-fold dilutions of extracts were made, and plating was accomplished as described previously.

In the disease development and yield experiment, symptom development was monitored at intervals of 10–14 days from transplanting until harvest. Observations of disease development were qualitative rather than quantitative. All ripe fruits were picked on 11 and 25 September, separated into cull and marketable categories, and weighed. Yields were summed and converted to estimates of kilograms per hectare. Symptom development and yield were not monitored in the epiphytic population experiment because of possible confounding effects of wounding during leaflet sampling.

RESULTS

Epiphyte populations after spray inoculation. Epiphytic populations of *C. m. michiganensis* on leaflets in 1987 and 1988 and on half-size green fruits in 1987 increased in the weeks after inoculation (Fig. 1). Mean populations stabilized at approximately 10^6–10^8 cfu per leaflet and 10^5–10^6 cfu per fruit. The increase in mean populations was due to a combination of epiphytic colonization of an increasing proportion of the leaflets and fruits and larger population sizes per colonized leaflet or fruit. There were no obvious relationships among epiphytic population changes and the timing and amount of rainfall or overhead irrigation (M. L. Gleason, *data not shown*). The time interval of 1–2 wk between plant samples may have been too long to detect such relationships, however. Marginal necrosis of leaflets appeared during the last week of August in 1987 and during the last week of July in 1988 and became widespread by mid-August in 1988. In both years *C. m. michiganensis* was isolated from necrotic leaflets using NBYCR medium. No wilt or stem-canker symptoms appeared during either year.

Overwintered debris: Pathogen survival and dissemination. Populations of *C. m. michiganensis* declined more rapidly in buried debris than in debris at the soil surface (Fig. 2). Because of excessive growth of soil microorganisms on NBYCR, the pathogen could not be quantified on buried debris after May 1989. By July 1989, buried leaf-petiole tissue had disappeared and buried stem debris had decayed to a spongy, fibrous texture. On the same date, both types of debris at the soil surface were structurally intact and recognizable. In November 1990, populations of the pathogen from stem and leaf-petiole debris at the soil surface could still be quantified; stems and petioles had retained structural integrity in these samples, but leaf blades were no longer evident.

C. m. michiganensis appeared on leaflets within 1 wk after transplanting into a debris-infested plot (Fig. 3). Five to 7 wk after transplanting, mean epiphytic populations of the pathogen had reached 10^6–10^8 cfu per leaflet, the approximate equilibrium level reached after spray inoculation in the field (Fig. 1). As in other experiments, increases in mean epiphytic populations were caused by increases in both the percentage of leaflets colonized by the pathogen and populations on individual leaflets.

Distribution of epiphytic pathogen populations. Distributions

were analyzed once the pathogen populations had stabilized at stationary phase levels and all the leaflets sampled supported detectable populations of the pathogen. These criteria were met about 4 wk after spray inoculation or 5–7 wk after transplanting into plots containing infested debris. Data sets from leaflets collected 7, 9, and 11 wk after transplanting into plots containing infested debris were selected for analysis.

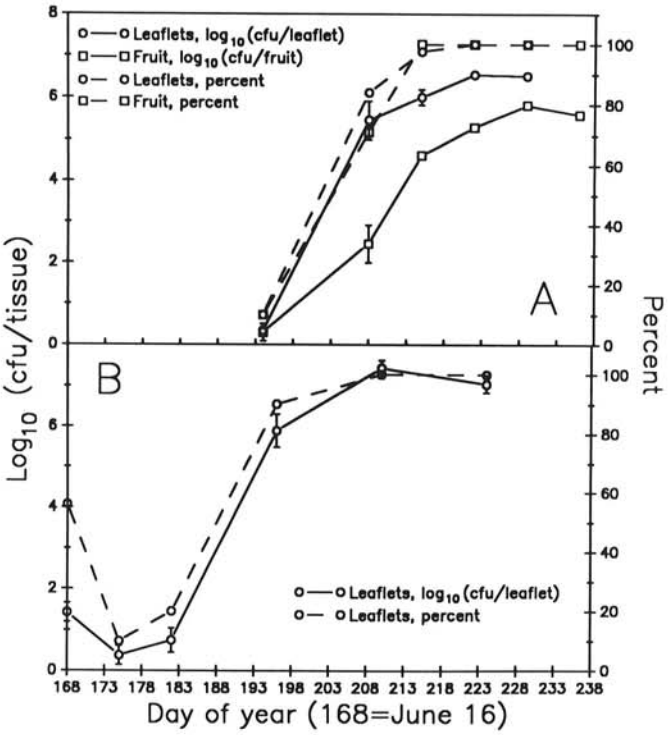

Fig. 1. Mean epiphytic populations of *Clavibacter michiganensis* subsp. *michiganensis* on leaflets or fruits of field-grown, fresh market tomatoes (cv. Jet Star) in 1987 (**A**) and leaflets of processing tomatoes (cv. Heinz 6004) in 1988 (**B**), and percentage of samples with detectable populations of the pathogen. Plants were spray-inoculated with 5×10^6 cfu/ml of a mixture of three rifampicin-resistant strains on 10 July 1987 (day 191) and 10^5 cfu/ml on 16 June 1988 (day 168). The limit of detection was 1.25×10^2 for individual leaflets and 2.5×10^2 for individual fruits. In 1987, $n = 80$ (four samples per plant $\times$ 20 plants) for leaflets and $n = 40$ (two samples per plant $\times$ 20 plants) for fruits; in 1988, $n = 30$ (one sample per plant $\times$ 30 plants). Error bars = $\pm$1 SEM; bars are smaller than the symbols where bars are not shown.

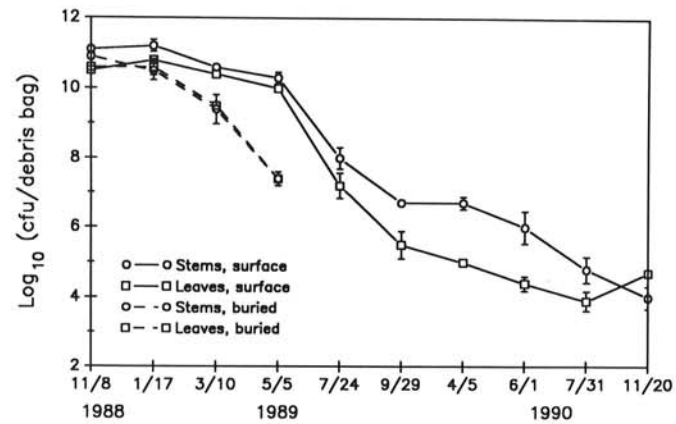

Fig. 2. Survival of a mixture of three rifampicin-resistant strains of *Clavibacter michiganensis* subsp. *michiganensis* in infested tomato debris. In November 1988, 6 g dry weight of leaf-petiole debris ("leaves") or stem debris were sealed in nylon screen bags and placed at the soil surface or buried at 10-cm depth. The limit of detection was 5×10^2 cfu per debris bag. $n = 3$, except on 20 November 1990, when $n = 5$ for stems and $n = 1$ for leaves. Error bars = $\pm$1 SEM; bars are smaller than the symbols where bars are not shown.

If observations come from a normal distribution, then a rankit plot should approximate a straight line. Systematic departure of rankit plots from such a linear trend suggests non-normality. Rankit diagrams constructed using log-transformed data approximated a straight line, whereas those constructed using raw data were curved (Fig. 4). Such a result suggests that the pathogen populations were approximately lognormally distributed. Results of the Shapiro-Wilk test also suggested that the populations approximated a lognormal distribution. In the Shapiro-Wilk test (29), if data are from a normally distributed population, the value of the Shapiro-Wilk statistic, W, should approach one. As a data set departs from normality, the value of W approaches zero. Probabilities (P), with which given values of W could be calculated from normally distributed data sets of various sizes, are given in Shapiro and Wilk (29). The values of the Shapiro-Wilk statistic for the raw data obtained from leaflets collected 7, 9, and 11 wk after transplanting were 0.5689 ($P < 0.01$), 0.6794 ($P < 0.01$), and 0.7368 ($P < 0.01$), respectively. The values for the log-transformed data collected 7, 9, and 11 wk after transplanting were 0.9667 ($P = 0.50$), 0.9098 ($0.01 < P < 0.02$), and 0.9659 ($P = 0.50$), respectively. Similar results were obtained for four data sets of 30–80 leaflets each, and four data sets of 33–40 fruits each, from field-grown tomato plants spray-inoculated with suspensions of *C. m. michiganensis* (data not shown).

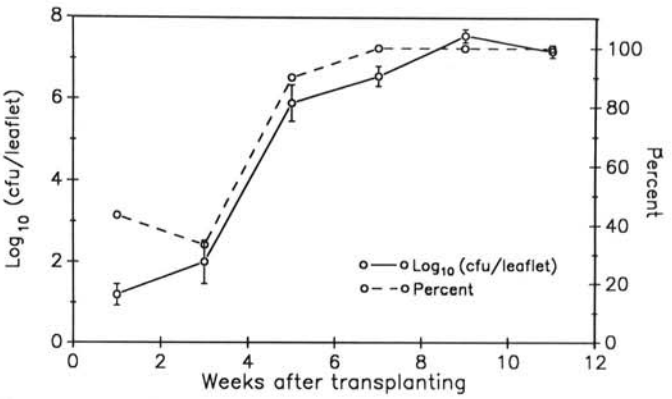

Fig. 3. Mean epiphytic populations of *Clavibacter michiganensis* subsp. *michignensis* on tomato leaflets (cv. Heinz 6004) and percentage of sampled leaflets with detectable populations of the pathogen in 1989. Tomato debris infested with the pathogen had been incorporated in a field plot in October 1988. The limit of detection was 1.25×10^2 cfu per leaflet. Transplanting was done on 30 May 1989. $n = 30$. Error bars = $\pm$1 SEM; bars are smaller than the symbols where bars are not shown.

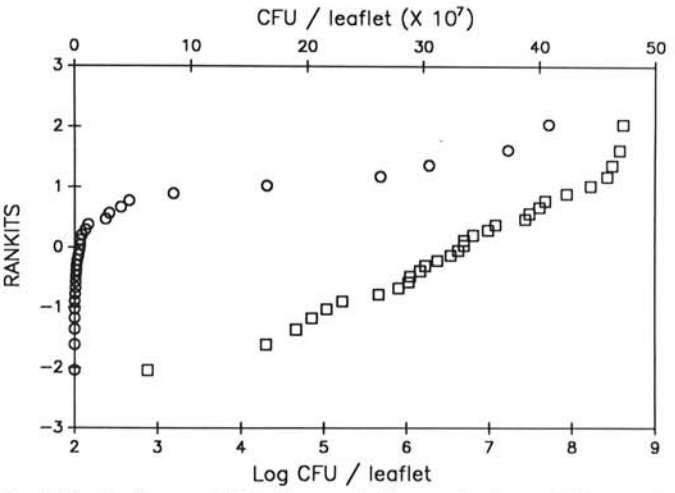

Fig. 4. Rankit diagram of *Clavibacter michiganensis* subsp. *michiganensis* populations on 30 tomato leaflets on 7 July 1989. Plants were grown in a plot containing infested debris as an inoculum source. Symbols: O = cfu per leaflet ($\times 10^7$); □ = $\log_{10}$ cfu per leaflet.

Disease development and yield. In the overwintered debris experiment, marginal necrosis of leaflets was first noted in debris plots on 10 July. By 22 July, marginal necrosis was widespread in these plots, and localized areas of yellowing and wilting were observed. Isolations from symptomatic tissue consistently indicated that rifampicin-resistant *C. m. michiganensis* was present. Wilting was scattered throughout the debris plots by 1 August, and the canopy had become open in many areas. Most plants, however, continued to produce new shoots until harvest. Marginal necrosis appeared on a few leaflets in one control plot on 22 July and in all three control plots by 1 August. Wilt appeared in the control plots by mid-August. On all observation dates, however, disease development in control plots appeared to be much less severe than in debris plots.

Mean total yield was significantly higher in the control treatment (14,508 kg/ha) than in the debris treatment (10,467 kg/ha) (Student's t test, $P < 0.01$). Mean harvestable yield was also significantly higher in the control (13,700 kg/ha) than in the debris treatment (9,917 kg/ha) (Student's t test, $P < 0.01$). There was no significant difference between the treatments in terms of cull weight.

DISCUSSION

Three years of field experiments verified that tomato leaflets and fruit can support large epiphytic populations of *C. m. michiganensis*. Mean epiphytic populations stabilized between 10^6 and 10^8 cfu per leaflet in all field trials, whether the May–August period had above average rainfall (61 cm in 1987) or below average rainfall (34 cm in 1988 and 30 cm in 1989), whether inoculum was sprayed on or derived from overwintered debris, and whether a fresh market cultivar or a processing cultivar was used. Chang et al (6) reported epiphytic populations of similar magnitude (10^7–10^9 cfu/g fresh weight of leaflets) in field studies of susceptible and resistant processing-tomato cultivars in Illinois. This seeming "carrying capacity" may result from limitations imposed by the phylloplane environment, such as availability of trichomes (19,20, 21,28) or other microsites favorable for epiphytic survival of *C. m. michiganensis*, microbial antagonism, nutrient depletion (34), a combination of these factors, or some other factor (4,27). The long latent period of 5–7 wk between the establishment of epiphytic *C. m. michiganensis* and development of marginal necrosis on leaflets is consistent with an earlier observation (23) that disease development on plants adjacent to systemically infected plants is often delayed by 4–6 wk.

It was not our intent to rigorously determine the nature of the distribution of epiphytic populations of *C. m. michiganensis*. Analysis of complete data sets indicated that the lognormal distribution described the populations better than the normal distribution, as observed for certain other epiphytic phytopathogenic bacteria (16). More work would need to be done to determine if some other distribution described the populations more precisely (17).

The debris study provides the first experimental confirmation that the canker pathogen can survive in debris for 2 yr in the north central region of the United States. The canker pathogen survived in tomato debris under field conditions for 7 mo (November–June) in Ohio (10) and for 6 mo (November–May) in Illinois (7). As noted by Basu (3), pathogen populations in debris declined relatively slowly in frozen soil (December–February in our study) compared to warm soil. The meager available evidence suggests that soil moisture influences survival of the pathogen less than temperature (3,24). Soil type also has been reported to affect survival of the pathogen in buried debris (24).

The finding that the pathogen survived longer in soil-surface debris than in buried debris is in agreement with results of Chang et al (7). In our study, particles of buried debris underwent much more rapid physical degradation than they did at the soil surface. Physical breakdown may have exposed the pathogen to intensified attack by soil microorganisms. Results of both the present study and that of Chang et al (7) emphasize the potential value of fall cultivation after the occurrence of a bacterial canker epidemic to reduce the overwintering population of the pathogen.

The present study provides the first experimental evidence that a canker epidemic originating from overwintered, infested debris can significantly reduce yield in the subsequent tomato crop. Therefore, rotating away from tomatoes for at least one year after a canker outbreak can lead to substantial economic benefits in terms of yield. The relative contributions of foliar epiphytic populations and other possible avenues of debris-derived infection, such as root wounds (18), to disease development are unclear at this time.

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