Dynamics and Spatial Distribution of *Xanthomonas campestris* pv. *citri* Group E Strains in Simulated Nursery and New Grove Situations

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ABSTRACT

Gottwald, T. R., Civerolo, E. L., Garnsey, S. M., Brlansky, R. H., Graham, J. H., and Gabriel, D. W. 1988. Dynamics and spatial distribution of *Xanthomonas campestris* pv. *citri* group E strains in simulated nursery and new grove situations. Plant Disease 72: 781-787.

The distribution and spread of strains of *Xanthomonas campestris* pv. *citri* group E were studied on Duncan grapefruit and Valencia orange in two nursery plots and two new grove plots, one each of the same cultivars at Frederick, MD, under strict security. Strains of *X. c.* pv. *citri* group E have been reported only in Florida and primarily on young citrus stock. Focal plants inoculated with Florida strains of *X. c.* pv. *citri* group E were placed in the center of each of the four plots. Epiphytic bacterial populations were detected 3 m from foci by days 49 and 79 in the two nursery plots by immunofluorescence microscopy and/or DNA-DNA hybridization probe techniques. In new grove plots, bacteria were detected at the 13.8-m limits of the plots by day 50 by both detection techniques. Bacterial spread and eventual disease development appeared to be nondirectional. Disease symptoms developed in the grapefruit nursery and grapefruit new grove plots by days 94 and 141, respectively. Epiphytic foliar populations of *X. c.* pv. *citri* group E decreased in all plots in the fall and dropped further after frosts. *X. campestris* was repeatedly recovered from soil at 10^3–10^4 cfu/g under the grapefruit nursery focal tree. Nonpathogenic *X. campestris* was recovered from under the orange and grapefruit nursery focal trees and the orange new grove focal tree. No *X. c.* pv. *citri* group E was detected in soil under asymptomatic plants. As temperatures decreased in the fall, *X. campestris* soil populations also decreased. Soil populations were not reduced further immediately following a freeze.

Citrus canker, caused by *Xanthomonas campestris* pv. *citri* (Hasse) Dye, occurs in many citrus-growing countries (15,16). The bacterium causes lesions on foliage, green wood, and fruit of many citrus cultivars and citrus relatives (17,22). There are presently four bacterial groups or strains recognized as causing different types of citrus canker and related diseases (12,25). Group A of the pathogen, often referred to as Asiatic citrus canker, is the most widely distributed worldwide and has the widest known host range (30). Strains of groups B and C are narrow in citrus host range and quite different in global distribution and symptom expression from strains of group A (30). Strains of group E have not been reported outside Florida, and their host range is also narrower than group A strains (25). Genetic tests of strains generally parallel geographic distribution and demonstrate that *X. c.* pv. *citri* is composed of three different clonal groups: A, B/C, and E (10).

In 1984, a disease similar to citrus canker was discovered in a nursery in central Florida. Subsequently, over 20 outbreaks of the disease have been discovered in nurseries in Florida. At least eight genetically unique *X. c.* pv. *citri* strains resulted from isolations from plants sampled at these infected nurseries (8,12,18,19). These nursery (*X. c.* pv. *citri* group E) strains are serologically, genetically, and physiologically distinct from the four previously known citrus canker pathogenic groups and are not susceptible to any of the phages commonly used to differentiate these groups (3–5,9; Civerolo, *unpublished data*). In addition, disease symptomatology on susceptible citrus cultivars is unique. Leaf lesions caused by *X. c.* pv. *citri* group E strains are usually flat, rather than the typical raised lesion normally associated with Asiatic citrus canker, and are water-soaked with chlorotic halos. Because of these differences, the disease has been referred to as the Florida form, nursery form, E form, citrus leaf spot, and recently citrus bacterial spot (CBS). It will be denoted as CBS in this paper. Citrus bacterial spot is presently known to occur only in Florida.

No field data exist for CBS. Although citrus canker was previously discovered in Florida in early 1911, the disease was eradicated by 1927 following the destruction of approximately 20 million nursery and grove trees; but little, if any, epidemiological data were collected. Therefore, information on field epidemiology of citrus canker has been extrapolated from epidemics caused by *X. c.* pv. *citri* group A strains elsewhere. Rates of disease increase (k) of Asiatic citrus canker in groves in Argentina were calculated from linearized Gompertz transformed data, to be 0.04–0.06, 0.1, 0.18, and 0.13–0.24 for mandarin, satsuma, and navel orange, respectively (7). Rates of disease increase were also affected by scion/rootstock combinations. Slopes of linearized disease gradients ranged from −0.21 to −4.13. However, discrete foci of inoculum were not determined (6). Rootstock/scion combination has a profound effect on the rate of disease increase (1). Recently, rates of disease increase from discrete foci of disease in orange and grapefruit new grove simulation were calculated from Gompertz-transferred data to be 0.005 and 0.009/day (11). Disease gradients of $-\ln(\ln[y]) = a - b \log_{10} m$ were 0.713 to 1.237 and 0.048 to −1.856 for orange and grapefruit plots, respectively, and directionality of spread was correlated to windblown rain events (11).

Temperatures ranging from 20 to 35 C (optimum of 30 C) combined with free moisture are conducive to infection and disease development of citrus canker (17,21,23). Bacterial dissemination and disease incidence have been directly correlated with windblown rain, especially when wind speeds exceed 8 m/sec (14,16,23,26,27,29,30). Bacterial concentrations in rainwater collected from citrus canker-infected foliage were 10^5–10^6 cfu/ml and have been detected in rainwater up to 32 m from infected foliage (6). Propagating activities and harvesting also have been implicated in pathogen dissemination as has movement of live plant parts and nursery stock (4,14,16,17,22,28).

Survival of *X. c.* pv. *citri* group A strains of the citrus canker bacterium in soil is variable and dependent on several factors including soil moisture, temper-

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Accepted for publication 22 April 1988.

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ature, type, and organic content. Soil survival of the bacteria has been estimated to be approximately 2–8 wk (27). Recent studies in Argentina, however, indicate that the bacteria did not survive in soil for more than 1–4 wk (Graham, *unpublished data*).

Spread of *X. c.* pv. *citri* group E strains between citrus nurseries and movement of the bacterium from nurseries to mature plantings on contaminated replant trees are of considerable concern to the Florida citrus industry and the Florida Division of Plant Industry. Therefore, information on disease spread in nursery, replant, and new grove situations was much needed to gain a greater understanding of the disease potential and its implications in the Florida environment.

The purpose of this study was to investigate the dynamics of phylloplane and soil bacteria of *X. c.* pv. *citri* group E in simulated nursery and new grove situations, to test the efficiency of detection assays under field conditions, and to follow soil populations over time under infected trees. Due to the eradication efforts against cirus canker at that time, no field research could be conducted in Florida using *X. c.* pv. *citri* group E.

MATERIALS AND METHODS

Plant material, site selection, and field plot design. Greenhouse-propagated seedlings (*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf. 'Swingle' citrumelo) were bud-grafted with either Valencia sweet orange (*Citrus sinensis* (L.) Osb.) or Duncan grapefruit (*Citrus paradisi* Macf.) and the scions were grown to approximately 10–20 cm and used to simulate two nursery plantings. Larger 3-yr-old potted plants, approximately 1 m in height, of Valencia and Duncan on Carrizo citrange (*Poncirus trifoliata* × *C. sinensis*) rootstock (191 of each) were also used for simulated new grove plots. All experiments were conducted at the U.S. Department of Agriculture, ARS, Foreign Disease and Weed Science Laboratory located on Fort Detrick, a U.S. Army installation near Frederick, MD, under strict security.

Two nursery plots, one of Duncan grapefruit and the other of Valencia sweet orange, were established to investigate the disease under citrus nursery conditions. Each nursery plot consisted of 11 rows with 45 plants per row. Plants were planted 15.24 cm (6 in.) apart within rows and 76.2 cm (30 in.) between rows. A single inoculated central focal plant was located at the 23rd plant in row 6 of each plot.

Two new grove plots, one each of the same two cultivars consisting of approximately 1-m-tall trees, were established to investigate the spread of *X. c.* pv. *citri* group E under new grove conditions. Trees were planted in three concentric circles of 4.6, 9.2, and 13.8 m (15, 30, and 45 ft) radii from a central focal tree infected with *X. c.* pv. *citri* group E as described below. Trees were approximately 76 cm (30 in.) apart within each circle. The concentric circle design was intended to mimic the 4.6-m tree spacing common in Florida and to maximize the availability of susceptible host tissue at each interval from the focal point of disease, regardless of wind or rain splash direction.

Inoculum and focal plant preparation. Pathogenic *X. c.* pv. *citri* group E isolates from CBS outbreaks in Florida were grown in liquid nutrient glucose broth shake cultures, pooled, and centrifuged to pellet the bacteria. The combined bacterial cell pellets were resuspended and adjusted to a final concentration of $3–6 \times 10^8$ cfu/ml spectrophotometrically. The cell suspension was atomized onto leaf surfaces until runoff and the plants were covered with plastic bags to retain foliar moisture for 3 days. Lesions were allowed to develop on inoculated plants for 30 days before use as source plants.

Sampling design and procedure. Within each nursery plot, sampling locations were established at 0.2, 0.7, 1.0, 2.0, and 3.0 m from the focal plants along six equidistant radiating transect lines that intersected at the focus. Two leaves each were collected from the 0.2- and 0.7-m sampling points and one leaf each was collected from the 1.0-, 2.0-, and 3.0-m sampling points on each date. Samples were pooled by distance from the focal plant.

In the two concentric circle new grove plots, four lines were established radiating from the focal plant to the northwest, northeast, southeast, and southwest. Where the lines passed through each concentric circle of trees, trees closest to the lines were designated as sampling points. Two additional trees were sampled in each concentric circle on the downwind (southeast) side of the plot only, to ensure detection of any bacterial spread in that direction. Samples consisted of four mature leaves per tree taken at equidistant points around the circumference of the tree.

Soil samples for population assay were collected from the top 0.5 cm of soil within 5 cm from the trunk around each focal tree. Soil samples were assayed by suspending 1.0 g of soil in 100 ml of sterile phosphate buffer, pH 7.0, plus 1.0 g/L of peptone, and shaken on a rotary shaker for about 30 min. Soil was allowed to settle and the supernatant was plated on semiselective medium (20). Resulting *X. campestris*-like colonies were tested for xanthomonadin pigment production (13) and for pathogenicity by infiltrating leaves of Duncan grapefruit seedlings with a bacterial suspension of approximately 10^6 cfu/ml of the suspect colony. Nonpathogenic isolates from suspect colonies were identified as *X. campestris* using diagnostic tests (24) and were infiltrated into tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* Mill.) leaves to test for a hypersensitive response.

Determination of epiphytic bacteria. Three assays used for the detection of *X. c.* pv. *citri* group E from leaves were 1) immunoflourescence microscopy, 2) isolation on semiselective media, and 3) hybridization of sample bacterial DNA with radiolabeled *X. c.* pv. *citri* group E DNA as a probe.

For immunofluorescence microscopy tests, two 5-mm leaf disks from each sample leaf were pooled by sample, fixed in 3% glutaraldehyde in 0.066 M phosphate buffer, pH 6.8, for at least 48 hr, and rinsed twice in phosphate-buffered saline containing 0.2% sodium azide. Disks were minced in one or two drops of sterile distilled water with a razor blade, diluted with 2 ml of sterile distilled water, and mixed vigorously for about 15 sec. After coarse material settled, the supernatant was forced through a double filtration apparatus consisting of a 5.0-μm nitrocellulose prefilter, to remove large cell debris, followed by a 0.2-μm black polycarbonate membrane filter to trap bacteria. The polycarbonate filter was incubated with fluorescent-labeled IgG to *X. c.* pv. *citri* group E for 1–2 min (2). Excess stain was removed and the polycarbonate filter was mounted on a glass microscope slide and viewed at 1,000× under oil with epifluorescence (546–590 nm wavelength) for the presence of *X. c.* pv. *citri* group E bacteria.

For bacterial isolation, leaves were cut into four pieces each, placed in a 500-ml flask with 10 ml of distilled water per leaf, and shaken for 30 min on a rotary shaker. A 0.1-ml sample was spread onto semiselective media and incubated for 48 hr at 25 C to determine viable *X. c.* pv. *citri* group E cell concentration (20). Plate readings from semiselective media were then adjusted to cfu/cm^2 of leaf surface area.

DNA probe hybridizations. The remaining leaf wash suspensions were used for DNA probe hybridization. Each was transferred to sterile 250-ml microfiltration devices and drawn through sterile 0.22-μm nitrocellulose filter disks via vacuum microfiltration. The nitrocellulose filter disks were then aseptically tranferred to 50 × 10 mm nutrient agar petri dishes and placed on top of the agar surface with the trapping surface up. Plates were incubated for approximately 12–18 hr to enhance detection by allowing *X. c.* pv. *citri* group E microcolony formation on the filter surface. Filters were floated on about 10 ml of lysing solution (0.5 m of NaOH, 1.5 m of NaCl) for 15 min to kill all bacteria and denature the bacteria DNA. Filters were neutralized with 3 m of NaCl, 0.5 m

of Tris-HCl, pH 7.1–7.5, for 15 min, then soaked in 0.3 m of NaCl containing 0.03 m of sodium citrate in water, and baked for 2 hr at 55 C. Denatured and neutralized samples on filters were either stored or immediately hybridized against the ^{32}P-labeled DNA probe XCT11-85, as previously described (19). Probe XCT11-85 was specific for *X. campestris* as a species and did not hybridize, even under low-stringency hybridization conditions, to yellow *Erwinia herbicola* (Lohnis) Dye or to yellow pseudomonad strains.

Confirmation of disease. Before termination of the experiment, suspicious foliar lesions were assayed via a squash-blot version of the DNA probe technique. Individual lesions were tested for presence of *X. c.* pv. *citri* group E by placing the suspect leaf on top of a single sterile 100-mm-diameter Whatman No. 1 filter paper and crushing the lesion into the paper with the rounded end of a sterile glass rod. Diseased leaves from greenhouse source plants were used as a control. Individual filter papers were labeled with plot and spatial location designation and placed in a 50 C oven for 30 min to kill the pathogen.

The squash-blot samples were similarly denatured and hybridized, with the following modifications. DNA from heat-killed bacteria was denatured using the same solutions, but filters were placed on top of a stack of three Whatman No. 3 filters and soaked. This "wicking" procedure was repeated at each step. During hybridization, care was taken to avoid disturbing the squashed area. Filters were rinsed at room temperature three times, dried, and autoradiographed using Kodak X-Omat AR film at −90 C with intensifying screens. Individual dark to medium-dark circular spots on the film were scored as positive for *X. campestris*.

RESULTS

Both nursery and replant trees grew well during the duration of the experiment, doubling to tripling their original size. At least two or three flushes of new growth occurred on most plants during the duration of the test. Average daily temperatures in Maryland from planting in early June to mid-October were similar to those occurring in Florida, usually varying no more than 1–4 C between the two localities. Using immunofluorescence microscopy, detection of epiphytic *X. c.* pv. *citri* group E from large numbers of samples over time was the least labor intensive. Moreover, the IgG polyclonal antisera used in the immunofluorescence method was sensitive and specific for *X. c.* pv. *citri* group E strains. Therefore, the immunofluorescence microscopy assay was generally used for repeated samplings in both nursery and new grove plots, and was occasionally confirmed by isolation on semiselective media and leaf wash assay via DNA probe (Tables 1 and 2). Viable epiphytic bacteria were detected by isolation on semiselective media at the focus, and at 0.2- and 0.7-m sampling distances in the grapefruit nursery and at the 2.0-m sampling distance in the orange nursery (Table 1).

Nursery plots. Initial disease incidence (number of infected leaves per total number of leaves per plant) was 0.56 and 0.58 for grapefruit and orange nursery focal plants, respectively. Disease incidence increased during the season on the grapefruit nursery focal plant only, and decreased on the orange nursery focal plant due to new flushes of healthy leaves with no new infection. *X. c.* pv. *citri* group E bacteria were detected in samples taken at the edge of the grapefruit nursery plot on day 50 via DNA probe, but were not detected by immunofluorescence microscopy (Table 1). The pathogen was only detected at the focus, and at the 1.0-, 2.0-, and 3.0-m sampling locations, and was not detected at the 0.2- and 0.7-m sampling distances closer to the focus. The pathogen was not detected by immunofluorescence microscopy at day 50, except at the focus. Bacterial spread in the orange nursery plot reached 2.0 m from the focus by day 50 of the epidemic, as determined by DNA probe, and was widespread and reached the 3.0-m limit of the plot by day 79, as evidenced by both immunofluorescence microscopy and DNA

Table 1. Immunofluorescence microscopy and DNA hybridization probe assay for detection of epiphytic bacteria of *Xanthomonas campestris*

Days post-inoculum introduction	Date (1984)	Detection method[a]	Distance from focus (m)[b]					
			Focus	0.2	0.7	1.0	2.0	3.0
			Duncan grapefruit nursery					
0	10 June	IF	+	−	−	−	−	−
		DNA	+	−	−	−	−	−
50	29 July	IF	+	−	−	−	−	−
		DNA	+	−	−	+	+	+
		SSM	13.0	0.0	5.63	0.45	0.46	4.0
79	27 August	IF	+	+	+	+	+	+
		DNA	−	+	+	+	+	+
102	19 September	IF	+	+	+	+	+	+
	27 October (frost)							
141	28 October	IF	+	−	+	+	−	+
		DNA	+	−	−	+	+	+
		SSM	32.5	0.0	0.6	0.001	0.02	0.31
	30 October (frost)							
144	31 October	IF	−	−	−	−	−	−
	23 and 25 November (frost)							
171	27 November	IF	+	−	+	−	−	+
	2–9 December (freezes)							
183	9 December	IF	+	−	−	−	−	−
			Valencia orange nursery					
0	10 June	IF	+	−	−	−	−	−
		DNA	+	−	−	−	−	−
50	29 July	IF	+	−	−	+	−	−
		DNA	+	−	+	+	+	−
		SSM	14.63	2.80	6.0	4.0	0.0	0.0
79	27 August	IF	+	+	+	−	+	+
		DNA	+	−	+	+	+	+
102	19 September	IF	+	+	+	−	−	+
	27 October (frost)							
141	28 October	IF	+	−	−	−	−	−
		DNA	−	−	+	+	+	+
		SSM	7.35	0.32	0.04	0.04	0.001	0.03
	30 October (frost)							
144	31 October	IF	−	−	−	−	−	−
	23 and 25 November (frost)							
171	27 November	IF	+	−	−	−	−	−
	2–9 December (freezes)							
183	9 December	IF	+	+	−	+	+	+

[a] Detection method: IF = immunofluorescence microscopy specific for *X. c.* pv. *citri* group E, DNA = DNA-DNA hybridization probe specific for *Xanthomonas* sp., SSM = semiselective media expressed as cfu/cm^2 of leaf surface area semispecific for *Xanthomonas* sp.

[b] Samples were collected from inoculated focus plants and from plants at different distances from the focus, + = *X. c.* pv. *citri* group E bacteria detected, − = no bacteria detected.

probe assays (Table 1). Fewer epiphytic *X. c.* pv. *citri* group E bacteria were detected via immunofluorescence microscopy, even on samples from the focal plants taken on 28 and 31 October, immediately following two evening frosts (Table 1). Following these light frosts, the weather became warmer through late November. Two more frosts occurred on 23 and 25 November. Epiphytic bacteria were detected by immunofluorescence microscopy in samples taken on 27 November at 0.7 and 3.0 m from the focus of infection in the grapefruit nursery only. However, viable populations were detected via semiselective media isolation on all but the 0.7-m sample in the grapefruit nursery, and at the focus and 1.0-m samples in the orange nursery. Several severe freezes of 6–20 hr duration occurred between 2 and 9 December. No epiphytic bacteria were detected from 9 December samples from the grapefruit nursery other than the focal plant, whereas bacteria were detected on all but the 0.7-m sample in the orange nursery, via immunofluorescence microscopy.

Leaf lesions were first detected in the grapefruit nursery on 12 September (day 94) on three plants, the farthest being 2.37 m from the focal point. Subsequent disease development was detected on 27 October (day 139), when three more diseased plants were found; the farthest was 5.41 m from the source. A preliminary estimation of spread of the disease within the grapefruit nursery was calculated by linear regression to be 0.029 m/day ($r^2 = 0.837$). However, the disease incidence was very low, therefore the rate represents that achieved at the beginning of an epidemic only. Because of extremely low disease incidence (1–2 lesions per plant) and the relatively few plants infected, no disease gradient was calculated. No disease symptoms were detected in the orange nursery throughout the duration of the experiment.

New grove simulations. Initial disease incidence was 0.50 and 0.30 on grapefruit and orange new grove focal plants, respectively. The disease incidence remained relatively constant on both focal trees, due to a few new infections on new flushes. *X. c.* pv. *citri* group E bacteria were detected at the 13.8-m limits of both plots by day 50 of the experiment by both immunofluorescence microscopy and DNA probe. Epiphytic bacterial detection frequency fluctuated moderately throughout the season, but declined in late October when temperatures decreased (Fig. 1). Frosts on 29 and 30 October caused further reductions in foliar epiphytic bacteria detection in both plots, as indicated by both immunofluorescence microscopy and DNA probe assays. Isolations on semiselective media on 1 November did not detect any epiphytic populations within either of the plots, including focal plants. A brief warming trend occurred during early and mid-November. Isolations of foliar leaf washings on semiselective media and the immunofluorescence microscopy assay demonstrated a resurgence of epiphytic *X. c.* pv. *citri* group E populations. A series of hard freezes in early December had little effect on the resurging bacterial population that continued to increase slightly (Fig. 1). Directionality of bacterial spread was examined by immunofluorescence microscopy and DNA probe within new grove plots (Fig. 2). Although prevailing winds were predominantly from northwest to southeast, spread in both plots appeared to be independent of wind direction.

Disease symptoms were detected on two grapefruit trees, 4.58 m from the focal tree in the grapefruit new grove plot on 28 October (day 141) and subsequently confirmed by DNA hybridization probe squash-blot assay. No disease was detected in the orange new grove plot.

Soil recovery. *X. campestris* was repeatedly recovered from soil at populations of 10^3–10^4 cfu/g of soil from under the focal plant within the grapefruit nursery (Table 3). *X. campestris* was not detected in soil under asymptomatic plants adjacent to the focal plants in the plots. There was about a 10-fold decrease in soil population levels in response to decreasing temperatures in late October and November. However, soil population levels did not appear to decrease further immediately following the first early December freeze. Nonpathogenic *X. campestris* isolates were recovered from soil under focal plants in the orange nursery and orange new grove plots, but not in the grapefruit new grove plot. As temperatures decreased, there was a shift toward recovery of nonpathogenic *X. campestris* isolates (Table 3).

DISCUSSION

Although samples were taken from the same leaves from the same trees, detection of the presence or absence of epiphytic *X. c.* pv. *citri* group E by immunofluorescence microscopy, DNA probe, and semiselective media assay often differed among the three techniques. This may be related to variation in the sensitivity, specificity, and sampling area used in the respective assays. The immunofluorescence microscopy assay was the most sensitive, selective, and convenient method for field-sample processing. Single *X. c.* pv. *citri* group E bacteria could be detected with immunofluorescence, while the DNA probe and semiselective media assay methods required colony growth. The immunofluorescence microscopy assay consisted of directly screening two 5.0-mm leaf disks, one from each side of the midvein. Leaf tissues were macerated, thus releasing any endophytic as well as epiphytic bacteria for detection. DNA probe and semiselective media sampling consisted of washing entire leaves followed by overnight enhancement of bacterial growth on Millipore filters placed on growth media. Therefore, a much larger foliar surface area is sampled by the DNA probe and semiselective

Table 2. Immunofluorescence and DNA probe assay of *Xanthomonas campestris* pv. *citri* group E strain in concentric circle new grove simulation plots in Frederick, MD, during first two sampling dates following plot establishment

Distance (m) from focus	Percentage of positive trees by assay[a]																			
	10 June 1986 (day 0)[b]						29 July 1986 (day 50)[b]				27 August 1986 (day 78)[b]				28 October 1986 (day 141)[b]					
	GCCP[c]			OCCP[c]			GCCP		OCCP		GCCP		OCCP		GCCP			OCCP		
	IF	DNA	SSM	IF	DNA	SSM	IF	DNA	IF	DNA	IF	DNA	IF	DNA	IF	DNA	SSM	IF	DNA	SSM
Focus	1/1	1/1	32.50	1/1	1/1	37.5	1/1	1/1	1/1	0/1	1/1	1/1	1/1	1/1	1/1	2/3	32.50	1/1	0/2	7.2
4.6	0/6	0/6	0.0	0/0	0/0	0.0	4/6	3/6	2/5	5/7	4/6	6/6	0/6	5/6	2/6	4/4	0.32	2/6	3/4	0.005
9.2	0/6	0/6	0.0	0/0	0/0	0.0	4/6	3/6	6/6	2/6	4/6	6/6	4/6	6/6	2/6	4/4	0.15	4/6	4/4	0.003
13.8	0/6	0/6	0.0	0/0	0/0	0.0	3/6	0/6	3/6	1/6	4/6	5/6	2/6	5/6	3/6	6/6	0.06	2/6	3/7	0.001

[a] Data represent qualitative analysis (+ or −) for the presence of epiphytic *X. campestris* on individual trees expressed as the number of positive trees/total trees assayed. IF = immunofluorescent microscopy assay, DNA = DNA-DNA hybridization probe assay, SSM = isolation of *X. c.* pv. campestris group E bacteria from leaf washings in semiselective media.

[b] Focal plants placed in plots on 10 June 1985.

[c] GCCP = cv. Duncan grapefruit concentric circle plot, OCCP = cv. Valencia orange concentric circle replant plot. Plots were established with concentric circles of replant trees at distances of 4.6, 9.2, and 13.8 m from inoculated focal plants.

media techniques. However, these assays are laborious and not well suited to processing large numbers of field samples.

The polyclonal antisera used in the immunofluorescence microscopy assay was specific for *X. c.* pv. *citri* group E, with little background reaction to any other bacteria tested. The immunofluorescence microscopy method was tested against eight other *X. campestris* pathovars, including 12 strains, and five *X. c.* pv. *citri* strains from groups A, B, and C. All these tests were negative, while tests against four *X. c.* pv. *citri* group E strains were all positive, indicating a high degree of specificity of the IgG polyclonal antisera used in the assay (Brlansky, *unpublished data*). The DNA probe used was much less specific than the antisera and could react to other xanthomonads capable of colonizing citrus plants. Since these studies were of short duration and since *Xanthomonas* species in general exhibit a high level of host specificity (10), we assumed that most xanthomonads detected within the plots using the DNA probe assay would be *X. c.* pv. *citri* group E from the source plants. Since the DNA probe method allows a larger surface area to be sampled, it might be expected to give an earlier indication of spread than the immunofluorescence microscopy assay. Initial assays, both with immunofluorescence microscopy and DNA probe, did not detect any other bacteria in any of the plots and comparisons of immunofluorescence microscopy, and DNA probe sampling methods were generally consistent (Tables 1 and 2). The DNA probe was most useful in the "leaf squash" sampling method, because it requires $>10^3$ cfu/mm^2 of lesion to give a positive response, and low numbers of other *Xanthomonas* epiphytes will not give a false-positive reaction. As expected, epiphytic populations were often detected with semiselective media, yet not with immunofluorescence microscopy or the DNA probe, which is probably an indication of the relative sensitivity of this method.

The CBS bacterium was detected at the 3.0-m plot limits more quickly in the grapefruit nursery than in the orange nursery (Table 1). No differences in spread were detected between grapefruit and orange new grove plots. This was because the bacteria were detected at the limits of both plots on the first sampling date. Had an earlier sample been taken, differences in spread might have been detected. More grapefruit trees sampled were found to have epiphytic *X. c.* pv. *citri* group E bacteria on the foliage than orange trees during early and midseason (Fig. 1). Grapefruit is more susceptible than orange to Asiatic citrus canker (29). Greenhouse inoculations of grapefruit and orange and inoculations of detached leaves have demonstrated that grapefruit leaves are more susceptible than orange leaves to *X. c.* pv. *citri* group E (Civerolo, *unpublished data*). Greater spread on grapefruit may be due to a more rapid increase of *X. c.* pv. *citri* group E populations on the phylloplane on the more susceptible Duncan grapefruit. Thus, more bacteria would be available

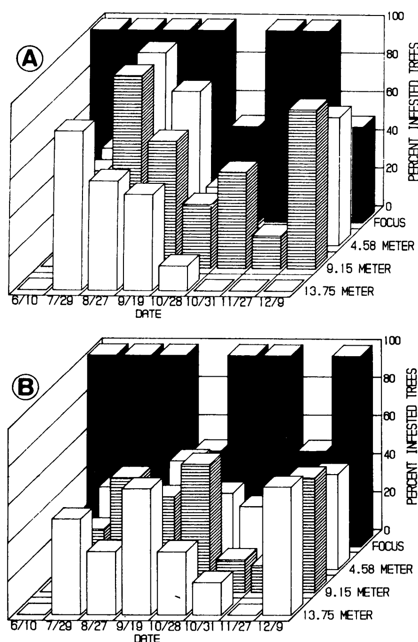

Fig. 1. Distribution of epiphytic phylloplane populations of the Florida isolate of *Xanthomonas campestris* pv. *citri* in concentric circle replant plots in Frederick, MD, over time as determined by immunofluorescence microscopy. Inoculated focal plants (FOCUS) were established in the plots on 10 June 1985. Spread of the bacterium to concentric rings of **(A)** cv. Duncan grapefruit and **(B)** cv. Valencia sweet orange was detected by immunofluorescence microscopy.

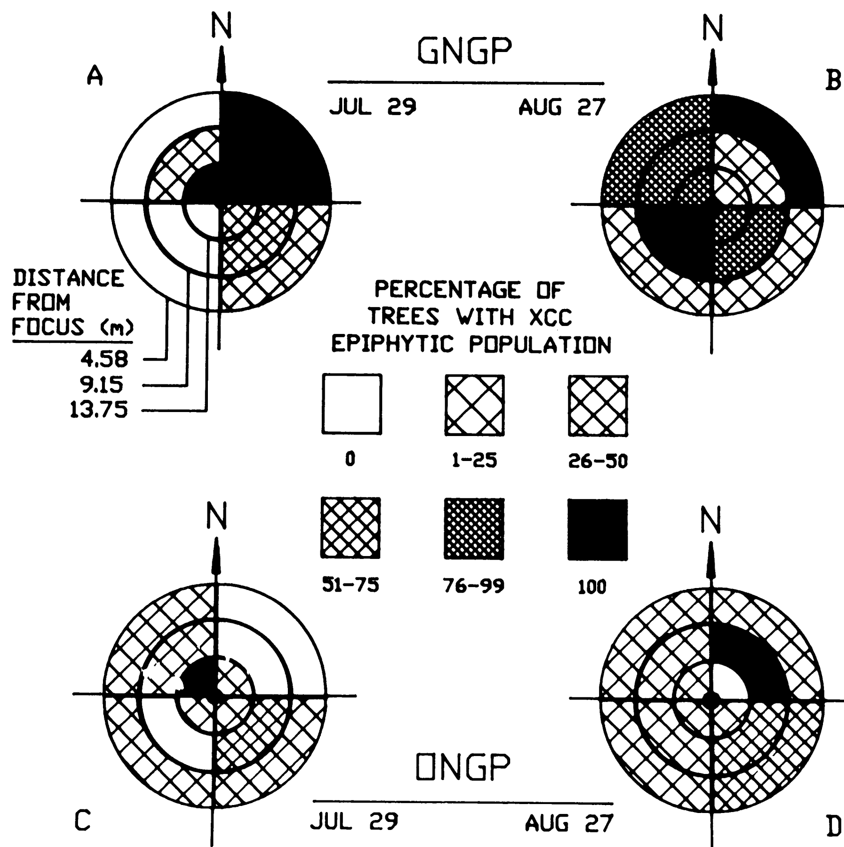

Fig. 2. Spatial distribution of epiphytic populations of *Xanthomonas campestris* pv. *citri* group E strains in concentric circle new grove simulation plots during the first two sampling dates after plot establishment. The plots were divided into four quadrants (NE, SE, SW, NW), and disease percentage was assigned on a distance by quadrant scheme. Immunofluorescence and DNA detection techniques were given equal weight in importance of detection. Therefore, percentage of epiphytic population expressed is the average of the number of trees detected with epiphytic populations of *X. campestris* via immunofluorescence or via DNA probe divided by the total number of trees assayed per quadrant and distance from the focal tree. Concentric bands represent epiphytic populations at those distances indicated. Note apparent directional spread to the northeast in the grapefruit new grove plot (GNGP) on first sampling date (day 50) versus nearly random spread in the orange new grove plot (ONGP) for the same date. By the 27 August sample date (day 79), epiphytic populations were spread throughout both plots.

for dissemination to nearby trees. Similar new grove plots in Argentina have demonstrated that higher epiphytic populations develop on grapefruit than orange, which led to a more rapid dissemination of Asiatic citrus canker (11).

In all the plots, bacterial spread appeared to be unrelated to wind direction or dissemination by windblown rain. Water splash and aerosol production by overhead sprinklers, such as those employed in the plots, would account for the general nondirectional spread observed (11). Taller trees, such as those in the new grove plots, would also provide superior dissemination of bacteria by originating inoculum and generating aerosols farther aboveground for longer-range dispersal.

Disease eventually developed in both the grapefruit nursery and grapefruit new grove plots. No disease developed in either of the orange plots. Again, this is most likely related to the greater susceptibility of grapefruit to infection by *X. c.* pv. *citri* group E, as well as the potential for greater bacterial population proliferation on the phylloplane. Disease incidence in both grapefruit plots was very low, with only a few lesions developing on a few leaves. This is undoubtedly related to the short duration of environmental conditions conducive to population increase, bacterial spread, and disease development at Frederick, even though plants grew very well. Additional nursery plots at the same site of the highly susceptible rootstock, Swingle citrumelo, resulted in very limited spread of *X. c.* pv. *citri* group E and disease development in plants ≤ 30 cm from the focus (Gottwald, Civerolo, Garnsey, *unpublished data*). Such limited disease spread is likely the result of the necessity and difficulty of simulating subtropical disease epidemics in a northern temperate climate because of strict quarantine regulations. Had it been possible to conduct the experiment in a citrus-growing area, or had the duration of weather conducive for citrus growth and CBS development in Maryland been longer, the epidemic most likely would have progressed further and possibly more rapidly. Rapid disease buildup and bacterial dissemination occurred in similar nursery plots in Argentina, where citrus canker is endemic (11). We believe the major constraints in the experiment were short duration and early cold weather. However, in similar plots recently established in Florida, more than one season appears to be required for epiphytic populations of *X. c.* pv. *citri* group E bacteria to increase to a level high enough to cause a significant epidemic, even under Florida conditions (Gottwald, Civerolo, and Graham, *unpublished work in progress*).

Soil recovery of *X. c.* pv. *citri* group E was most often associated with grapefruit focal trees. This, again, was probably related to greater susceptibility of grapefruit and its support of greater bacterial populations. Higher epiphytic population levels would thus be washed off grapefruit foliar surfaces to the soil. Interestingly, there was a shift in soil-recovered *X. c.* pv. *citri* group E from pathogenic to nonpathogenic *X. campestris* as the season progressed and the weather turned cooler. *X. c.* pv. *citri* group E may be selected against by cool weather or frost-damaged foliage and saprophytic *X. campestris* populations begin to proliferate. The resurgence of epiphytic foliar populations of *X. c.* pv. *citri* group E in the new grove plots detected by immunofluorescence microscopy (Fig. 2) may likely be related to this shift toward nonpathogenic forms. However, nonpathogenic xanthomonads fluoresce poorly when reacted with *X. c.* pv. *citri* group E fluorescent-labeled IgG antibody.

Table 3. Recovery of *Xanthomonas campestris* pv. *citri* group E strain and nonpathogenic *X. campestris* from soil in Fort Detrick nursery and concentric circle plots in 1985[a]

Date	Plot[b]	Quadrant[c]	Cfu/g dry soil	Pathogenicity test remarks[d]
11 September 1985	GN	E	1.3×10^4	Pathogenicity not tested
	GN	S	2.6×10^4	
	ONGP	E	2.6×10^4	ID based on Xanthomonadin pigment
28 October 1985	GN	S	4.1×10^4	Citrus (+)
	ONGP	W	1.4×10^4	Citrus (−) HR (+) tobacco HR (+) tomato
4 November 1985	GN	N	4.4×10^3	Citrus (+)
	GN	W	8.9×10^3	Citrus (+)
2 December 1985	GN	N	9.3×10^3	Citrus (−) HR (−) tobacco HR (+) tomato
	GN	E	4.6×10^3	Citrus (−) HR (+) tobacco HR (+) tomato
	ON	W	4.6×10^3	Citrus (−) HR (+) tobacco HR (+) tomato

[a]The soil from under the focal plants in all four plots and all four quadrants under each focal plant was assayed on each date. However, only those where *X. campestris* was recovered are represented here. No viable bacteria were recovered from soil in all the rest of the plot/quadrant combinations on each date.

[b]GN = Duncan grapefruit nursery, ON = Valencia orange nursery, and ONGP = orange new grove plot.

[c]Samples were taken under source plants in N = north, E = east, S = south, and W = west quadrants under canopy and about 5 cm from the plant trunk.

[d]All citrus (−) are *X. campestris* types determined by nutritional and biochemical tests. ID = identification, HR = hypersensitive response on indicated host.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to numerous personnel at the USDA-ARS Foreign Disease Weed Science Research Laboratory, Frederick, MD, for providing the land and helping to establish and maintain the research plots. We also wish to express our gratitude to Jack Dibler, Beckey Huckle, Grace Ingegneri, Cita Helkie, Jane Lyons, Marc Bruce, Alice Dow, Connie Ferriola, Stephanie Burgess, Ray McGuire, and Steve Walters for technical assistance.

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