Growth and Survival of Xanthomonads Under Conditions Nonconducive to Disease Development

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ABSTRACT

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Detached leaves or entire plants of host and nonhost species were sprayed with suspensions (10^3–10^4 cfu/cm^2 of leaf) of xanthomonads and held under different humidities. Leaf surface populations were determined by washing and dilution plating onto a semiselective Tween medium. Populations of *Xanthomonas campestris* pv. *vesicatoria* (*X. c.* pv. *vesicatoria*) increased 10- to 100-fold on tomato leaves at high relative humidity (RH) (>90%), fluctuated erratically at moderate RH (50–65%), and declined to low or nondetectable levels at low RH (10–25%). Bacteria did not survive well on tomato plants sprayed with *X. c.* pv. *vesicatoria* suspensions and placed immediately under conditions unfavorable for disease development. However, if plants were held under conditions of high moisture for 48 hr and then placed under adverse conditions, surface populations removable by washing declined slowly over several weeks and populations in leaf homogenates remained high. *X. c.* pv. *alfalfae*, *X. c.* pv. *campestris*, *X. c.* pv. *translucens*, *X. c.* pv. *pruni*, and a saprophytic isolate of *X. campestris* multiplied as well on detached tomato leaves at high RH as did *X. c.* pv. *vesicatoria*. At high RH, *X. c.* pv. *vesicatoria* populations remained constant or increased on leaves of almond, plum, peach, walnut, and sweet orange. When *X. c.* pv. *translucens* was applied to tomato leaves and plants were maintained at high RH for 48 hr, surface populations declined slowly over several weeks and populations in leaf homogenates remained as high as they did for *X. c.* pv. *vesicatoria* on tomato. Scanning electron microscopy revealed that xanthomonads survived on tomato leaf surfaces in small aggregates in an extracellular matrix. Xanthomonads are capable of multiplication and survival for at least several weeks on the surfaces of host species in the absence of disease and can also survive and multiply on nonhost plants under certain conditions.

Lesions or other diseased tissue have been traditionally considered the primary source of inoculum with most bacterial diseases including those caused by xanthomonads (5). However, several pathovars of *Xanthomonas campestris* (Doidge) Dye are known or suspected to have potential for epiphytic growth or survival (6). For example, Cafati and Saettler (2) recovered up to 10^5 colony-forming units (cfu) of *X. c.* pv. *phaseoli* from the surfaces of healthy bean leaves in field studies in a disease situation. Similarly, Daniel and Boher (3) found high populations of *X. c.* pv. *manihotis* on the aerial parts of cassava in the rainy season and low populations during the dry season in diseased fields, but bacteria were completely absent from healthy fields. *X. c.* pv. *juglandis* survived on dormant buds and catkins of walnuts and probably served as an inoculum source for foliage the following spring (11). *X. c.* pv. *malvacearum* also survived on buds and other plant surfaces in the absence of symptoms (15). In all of the above cases, epiphytic populations may have been derived from bacteria produced in diseased tissue, indicating that xanthomonads can survive as epiphytes for a short time but not necessarily that they can multiply on healthy leaf surfaces. Leben (9), however, demonstrated the multiplication of *X. c.* pv. *vesicatoria* on nondiseased tomatoes grown from infested seed.

Some research indicates that xanthomonads can survive and multiply as epiphytes on nonhost plant species. *X. c.* pv. *phaseoli* grew epiphytically on nonhost crop plants and weeds and was capable of reciprocal spread between beans and nonhosts (2). However, weeds were not considered an important source of primary inoculum. *X. c.* pv. *campestris* survived epiphytically on cruciferous weeds and was considered a source of inoculum for subsequent infection of crop plants (8). *X. c.* pv. *citri* has been reported to survive on the leaves and even on the roots of some grasses in Japan and Brazil (4,12).

Although there is a great deal of information indicating the presence of xanthomonads on nondiseased host tissue or on nonhost leaves, little quantitative data exist on the ability of these bacteria to grow and multiply on host plants under conditions that do not permit symptom development or on their development and survival on nonhost crops or weeds. This study was designed to investigate the multiplication and survival of xanthomonads under conditions nonconducive to disease development and on nonhost plants.

MATERIALS AND METHODS

Bacterial isolates and inoculum production. Isolates of *X. c.* pv. *vesicatoria* (originally isolated from tomato in Florida), *X. c.* pv. *alfalfae* (12D51), *X. c.* pv. *campestris* (2D511), *X. c.* pv. *translucens* (10D5), and *X. c.* pv. *pruni* (6D51) were obtained from C. I. Kado, University of California, Davis. Isolate 817, a saprophytic isolate of *X. campestris* recovered from leaf washings of *Bidens pilosa* L. in Florida was obtained from R. M. Sonoda, University of Florida, Ft. Pierce.

Bacterial inocula were produced on medium 523 containing per liter: sucrose, 10 g; enzymatic casein hydrolysate, 8 g; K_2HPO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; and agar, 15 g. Cultures were grown for 36–48 hr at room temperature (21–23 C), washed from the plates, and washed twice by centrifugation in Na-K phosphate buffer (Na_2HPO_4, 5.8 g/L, and KH_2PO_4, 3.5 g/L; pH 7.0). Suspensions were adjusted to 0.6 optical density units (about 3×10^8 cfu/ml) with a Bausch & Lomb Spectronic 20 and then diluted 1:100 or 1:1,000 in buffer for use as inocula.

Plant materials. Tomato plants (*Lycopersicon esculentum* Mill. 'Early Pak') were grown from seed in pots in the greenhouse until they were 30–40 cm tall and had several fully expanded complete leaves. Plums (*Prunus cerasifera* Ehrh. 'Myrobalan'), almonds (*P. amygdalus* Batsch 'Texas'), black walnuts (*Juglans nigra* L.), peaches (*P. persica* (L.) Batsch 'Lovell'), and sweet orange (*Citrus sinensis* (L.) Osb. 'Pineapple') were grown from seed until they were 50–80 cm tall. For detached leaf studies, single leaflets that had recently reached full expansion were selected.

Inoculation and incubation. Detached leaves or entire plants were sprayed with bacterial suspensions with a Gelman Chromist chromatography sprayer (Gelman, Ann Arbor, MI). Plants were

sprayed as uniformly as possible until all surfaces were covered with fine droplets.

Petioles of inoculated detached leaves were inserted into 5-ml, water-filled vials, and the vials were arranged randomly in a rack. Racks were then placed in plastic chambers to achieve the following conditions: chambers covered and the bottom of the chamber filled with water (90–95% relative humidity [RH]), chamber closed without water (50–65% RH), and chamber open without water (10–25% RH). Intact plants were covered with plastic bags to maintain free moisture on the leaf surface when needed. The RH was 90–95% inside the plastic bags, and the temperature was 2–4 C higher than room temperature. When detached leaves and intact plants were maintained in the laboratory, they were held at room temperature with 16 hr per day of artificial light (93 $\mu E/m^2/sec$). When plants were placed outdoors, they were located in a sheltered area that received full sun part of the day. No rainfall occurred during the experiments, and there was never free moisture from dew or other sources noted on the plant surfaces. Temperatures and relative humidities were monitored outdoors and in the laboratory by a Campbell 21X micrologger (Campbell Scientific, Inc., Logan, UT) with a model 207 temperature and relative humidity probe. Environmental data presented are the overall averages of data collected each minute between sampling times during the experiment.

Sampling and plating. In detached leaf experiments, single leaves served as the experimental unit and the sampling unit. In experiments with intact plants, individual plants served as the experimental units and a single leaf from each replicate was assayed at each sample time. Experiments in Table 1 and Figures 1–3 were replicated four times, and those in Figures 4–6 were replicated eight times. Data presented are from single experiments that were representative of results of two or more similar tests.

Single leaves were placed in 50 ml of sterile Na-K phosphate buffer plus 0.1% peptone and shaken vigorously on a rotary shaker for 15 min. The wash solution was diluted as needed in sterile Na-K phosphate buffer, and 0.1 ml of each dilution was spread on each of two plates of the Tween medium A (10). Where populations were low, the wash solution was centrifuged at 10,000 × *g* for 15 min, resuspended in 0.5 ml of buffer, and plated as above. In some experiments, the population of xanthomonads was determined in leaf homogenates after the leaves had been washed. Leaves were placed in 25 ml of buffer and triturated with a Polytron motorized tissue grinder. Homogenates were diluted as necessary and plated on the Tween medium. All populations were expressed as colony-forming units per square centimeter of leaf surface and presented graphically as means ± standard error.

For scanning electron microscopy (SEM), 5-mm-diameter disks were cut from the tomato leaves with a cork borer. These were fixed in 3% glutaraldehyde in Na-K phosphate buffer for 24 hr at room temperature and then washed in buffer and stored in the refrigerator. Leaf disks were then dehydrated in an ethanol series and critical-point-dried with CO_2 in a Ladd critical-point dryer. They were mounted and coated with gold for 2 min in a Ladd sputter coater. Samples were viewed on a Hitachi S-530 at 20 kV and 70 uA current. The numbers of bacterial cells per square centimeter of leaf surface were estimated by counting total bacteria in 50 microscope fields (23 × 30 μm) on each of three disks for each pathovar. The clumps of bacteria also were counted in the same fields. Individual bacterial cells were counted as different "clumps" if they were clearly separated from one another but as a single clump if they were aggregated and covered by an extracellular matrix.

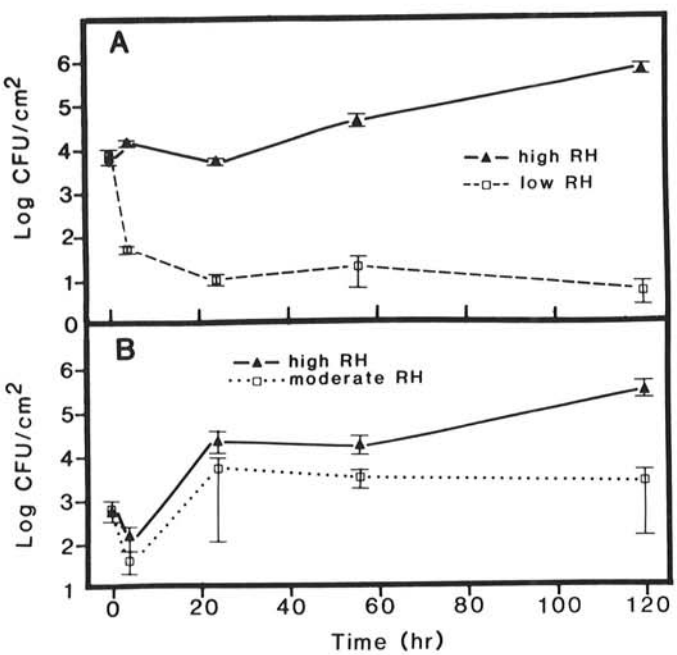

Fig. 1. Recovery of *Xanthomonas campestris* pv. *vesicatoria* from detached leaves of tomato spray-inoculated with bacterial suspensions and maintained in chambers at **A,** high (90–95%) and low (10–25%) relative humidity (RH) or **B,** at high and moderate (50–65%) RH.

RESULTS

Recovery of bacteria from compatible hosts. On detached leaves, *X. c.* pv. *vesicatoria* populations increased about 100-fold within 120 hr at 90–95% RH (Fig. 1A,B). Under moderate humidity (50–65%), populations were lower than under high humidity and were more variable as indicated by the error bars (Fig. 1B). Under dry conditions (10–25% RH), populations declined rapidly after 4 hr on detached leaves but were still detectable in leaf washes after 5 days (Fig. 1A).

With intact plants sprayed with *X. c.* pv. *vesicatoria* and held under dry conditions, surface populations again declined rapidly after 4 hr and were undetectable at 24 hr (Table 1). However, when leaves from these plants were removed and incubated in a moist chamber for 48 hr before washing and assay, *X. c.* pv. *vesicatoria* was still detectable at 24 but not at 48 hr. When plants were bagged to maintain high humidity, *X. c.* pv. *vesicatoria* increased rapidly and increased further when detached leaves were incubated for 48 hr in a moist chamber (Table 1). In a similar experiment where plants were placed outdoors under dry conditions immediately after applying the bacteria, *X. c.* pv. *vesicatoria* was detected once on a single leaf 12 days after application but not at other sampling times from 5 to 18 days after application (data not shown).

When plants were bagged and held in the laboratory before placement outdoors, populations increased about 100-fold within the first 48 hr (Fig. 4). The plants were then placed outside at cool temperature (17–22 C) and moderate to high humidity (70–85%)

TABLE 1. Recovery of *Xanthomonas campestris* pv. *vesicatoria* from leaves of tomato plants spray-inoculated with a bacterial suspension and maintained under low relative humidity or under moist conditions (covered with plastic bags)

Relative humidity	Sampling (hr post-inoculation)	Bacteria recovered (cfu/cm²)	
		Attached leaves[a]	Detached leaves[b]
Low (10–25%)	0	935	...
	4	3	162
	24	0	17
	48	0	0
High (90–95%)	0	935	...
	4	4,220	4,261
	24	3,569	1.37×10^5
	48	6.72×10^4	1.40×10^5

[a] Bacteria recovered from washings of leaves from plants maintained under the indicated conditions and sample times.

[b] Bacteria recovered from washings of leaves from the same plants and collected at the same times but incubated for 48 hr in a plastic chamber at 90–95% RH before assay.

but without free water on the leaves. Under these conditions, surface populations of *X. c.* pv. *vesicatoria* and of the saprophytic isolate of *X. campestris* declined to 10^2–10^3 cfu/cm^2 and remained at that level through day 9. No disease symptoms developed on these plants during the experiment.

In a similar experiment in which plants were held longer under lower RH, surface populations of *X. c.* pv. *vesicatoria* and populations in leaf homogenates of the same leaves were monitored (Fig. 5). Surface populations rose to 10^6 cfu/cm^2 in the 48 hr that the plants were bagged in the laboratory. After the plants were placed outdoors, numbers dropped to about 10^3 cfu/cm^2, remained at that level until about day 20, and then declined to near 0 by day 43. Populations in homogenates were about 5×10^4 cfu/cm^2 initially and declined only slightly during the experiment.

Recovery of bacteria from nonhosts. On detached tomato leaves at 90–95% RH, *X. c.* pv. *alfalfae, X. c.* pv. *campestris, and X. c.* pv. *translucens* multiplied on the leaf surfaces as well (or in some cases slightly better) as did *X. c.* pv. *vesicatoria* (Fig. 2A). In another experiment under the same conditions, the saprophytic isolate of *X. campestris* increased more rapidly than did *X. c.* pv. *vesicatoria* or *X. c.* pv. *pruni* on tomato leaves (Fig. 2B).

X. c. pv. *vesicatoria* multiplied on detached leaves of almond and walnut under high humidity and survived on plum at about the same levels at which it was applied (Fig. 3A). On intact plants with continuous free moisture, populations of *X. c.* pv. *vesicatoria* increased on tomato, did not increase substantially on almond, and rose again after decreasing to low levels on peach and plum after the first 24 hr (Fig. 3B). On detached leaves under high humidity, *X. c.* pv. *vesicatoria* and the saprophytic isolate of *X. campestris* multiplied well on tomato and on sweet orange. Both increased more rapidly on tomato than on citrus, and the saprophytic isolate increased more rapidly than *X. c.* pv. *vesicatoria* on both hosts (Fig. 3C).

To determine the fate of a xanthomonad on a nonhost over a longer period, *X. c.* pv. *translucens* was applied to tomato plants, which were bagged for 2 days in the laboratory and then placed outdoors under warm, dry conditions. Surface populations determined by leaf washing increased to 10^6 cfu/cm^2 over the first 2 days, then declined to 10^2 cfu/cm^2 after being placed outdoors (Fig. 6). *X. c.* pv. *translucens* was still detected in leaf washes after more than 1 mo under adverse conditions on a nonhost. Populations in the homogenates of these leaves increased to day 15 and then remained uniform for the remainder of the experiment.

Localization of bacteria. Leaves of tomato inoculated with *X. c.* pv. *translucens* from the experiment in Figure 6 were collected on day 29, dipped in 70% ethanol for 1 min, and rinsed in sterile distilled water, then washed in the usual manner and extracted. Ethanol treatment reduced the populations in leaf washes to 0 and reduced populations in homogenates from 14,700 to 400 cfu/cm^2.

Leaf disks from tomato plants that had been sprayed with *X. c.* pv. *vesicatoria* and *X. c.* pv. *translucens*, incubated at high

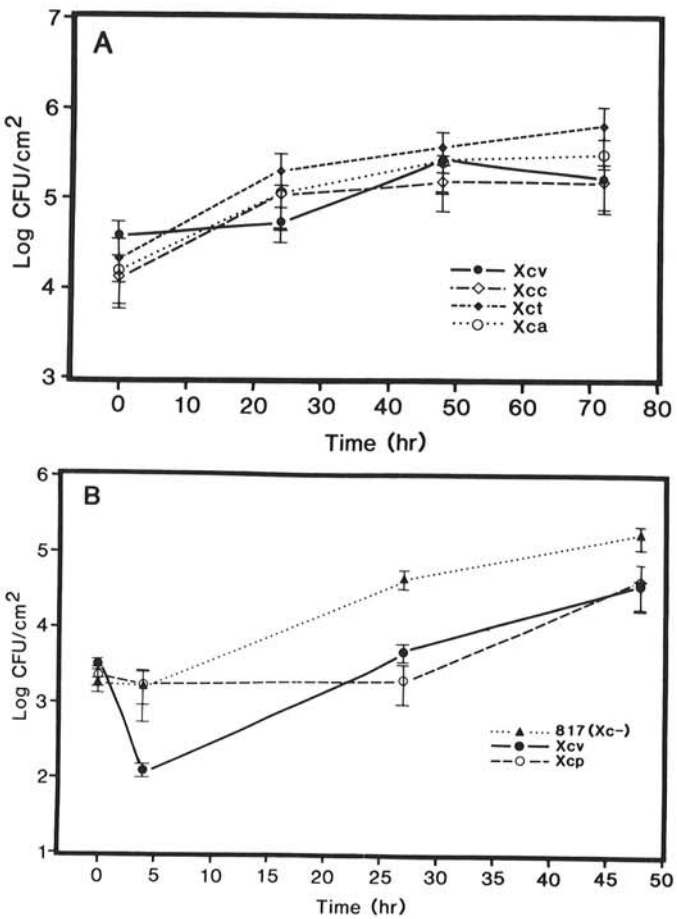

Fig. 2. Recovery of **A,** a compatible pathovar, *Xanthomonas campestris* pv. *vesicatoria* (Xcv), and noncompatible pathovars, *X. c.* pv. *campestris* (Xcc), *X. c.* pv. *translucens* (Xct), and *X. c.* pv. *alfalfae* (Xca), and of **B,** a compatible pathovar, *X. c.* pv. *vesicatoria*, a saprophytic isolate of *X. campestris* (817), and a noncompatible pathovar, *X. c.* pv. *pruni* (Xcp) from detached leaves of tomato spray-inoculated with bacterial suspensions and placed in chambers at high relative humidity (90–95%).

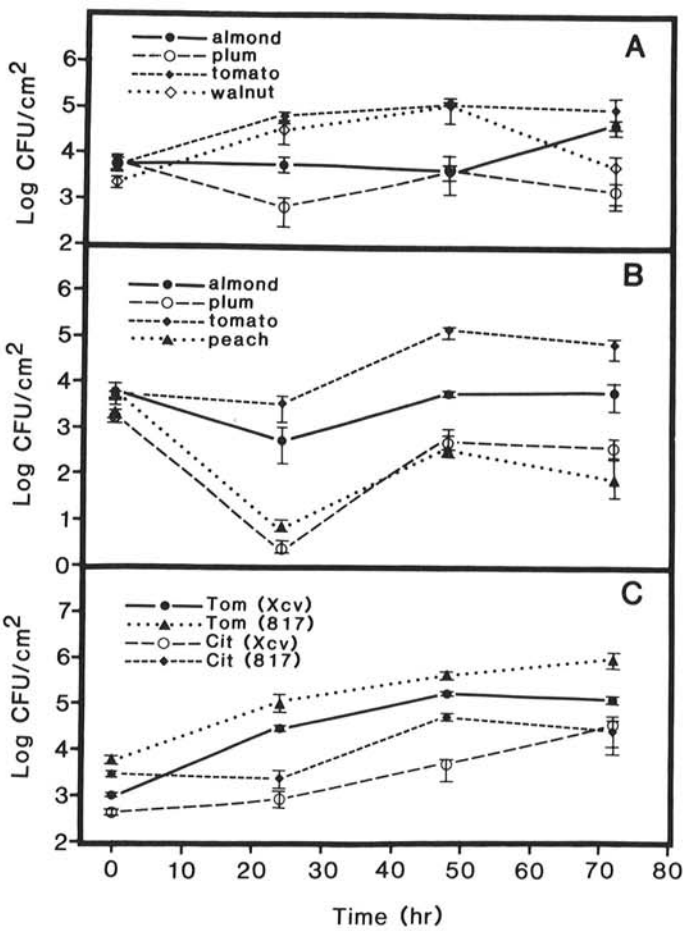

Fig. 3. Recovery of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) and a saprophytic isolate of *X. campestris* (817) from host and nonhost plants. **A,** Recovery of Xcv from detached leaves of almond, plum, walnut, and tomato spray-inoculated with bacterial suspensions and maintained at high (90–95%) relative humidity (RH) in a chamber. **B,** Recovery of *X. c.* pv. *vesicatoria* from leaves of almond, peach, plum, and tomato plants spray-inoculated with bacterial suspensions, covered with plastic bags, and maintained under the lights in the laboratory. **C,** Recovery of Xcv from detached leaves of tomato and sweet orange and of a saprophytic isolate of *X. campestris* from detached tomato and sweet orange leaves spray-inoculated with a bacterial suspension and maintained at high RH in a chamber.

humidity for 48 hr, and then placed outdoors under warm, dry conditions for 20 days were examined by SEM. At the time leaf disks were collected, populations of both pathovars were less than 10 cfu/cm^2 in the leaf washes and were 10,300 cfu/cm^2 for *X. c.* pv. *translucens* and 46,300 cfu/cm^2 for *X. c.* pv. *vesicatoria* in homogenates.

Most of the bacteria observed on the leaf surfaces were in clumps and appeared to be firmly affixed to the leaf and embedded in an extracellular matrix (Fig. 7A,B). Some clumps were found at the bases of trichomes and surrounding stomata, but many were not associated with any leaf structure. In addition, single bacteria were also found scattered over the leaf surface. Many more bacteria were found on the adaxial than on the abaxial surface. No differences were found in the behavior of the two pathovars on the tomato leaf surfaces.

Total numbers of bacterial cells were in the range of 10^5–10^6/cm^2 of leaf surface. However, if clumps were counted rather than individual cells, and assuming that they would not be disrupted in the extraction process, it was estimated that $4–5 \times 10^4$ cfu/cm^2 would be obtained. Assuming that some bacteria in each aggregate were alive, these results were consistent with the $1–4 \times 10^4$ cfu/cm^2 obtained by plating.

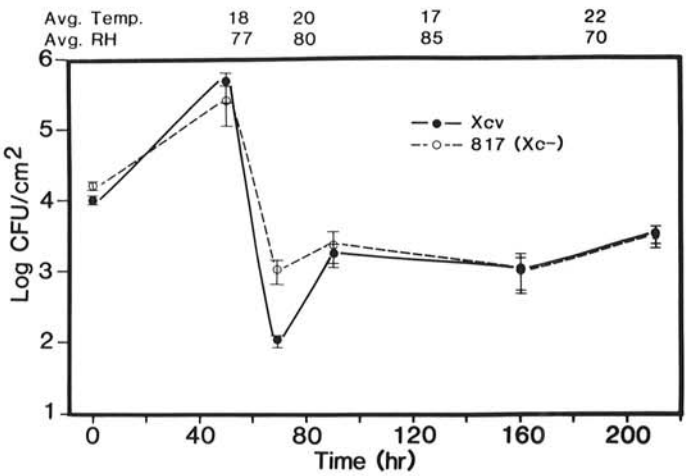

Fig. 4. Recovery of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) and a saprophytic isolate of *X. campestris* (817) from leaves of tomato plants spray-inoculated with bacterial suspensions. Plants were covered with plastic bags for the first 48 hr in the laboratory before placement outdoors under the indicated conditions. Temperature and RH data are the means between sample times.

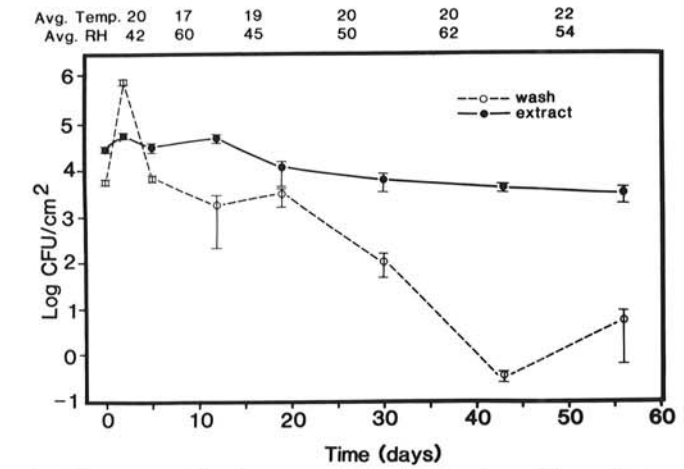

Fig. 5. Recovery of *Xanthomonas campestris* pv. *vesicatoria* by washing or by extracting tomato leaves of plants spray-inoculated with bacterial suspensions. Plants were covered with plastic bags for 48 hr in the laboratory before placement outdoors under the indicated conditions. Temperature and RH data are the means between sample times.

DISCUSSION

When *X. c.* pv. *vesicatoria* was sprayed on tomato plants or detached leaves and immediately transferred to adverse conditions, most of the bacteria died within a short time. Thus, bacteria spread from lesions to healthy tissue in wet weather probably would not persist if the leaves dried quickly. However, if bacteria-sprayed plants were exposed to high RH for a day or two before incurring adverse conditions, surface populations would persist for long periods. Even after those bacteria removable by washing had essentially disappeared, 10^4–10^5 cfu/cm^2 of leaf were recovered by homogenizing leaves in buffer. In SEM studies, xanthomonads on tomato leaf surfaces appeared firmly aggregated and affixed to the leaf in an extracellular matrix. The number of clumps observed on the surface of the leaves was comparable to the number of colony-forming units obtained on selective media, indicating that the bacteria were xanthomonads and that at least some cells in the clumps were alive. The fact that these bacteria were killed by a 1-min dip in ethanol also indicates that they were present on the leaf surface and were not internal. Because *X. c.* pv. *vesicatoria* and *X. c.* pv. *translucens* behaved similarly on tomato leaves, bacterial survival did not appear to depend on infection of a host plant.

Most studies of the epiphytic potential of xanthomonads do not clearly distinguish between resident and casual epiphytes. In most, disease symptoms were present, and many epiphytic bacteria were probably the result of multiplication of the pathogen in diseased tissue. Xanthomonads surviving in buds of plants (11,15), while important in persistence of the bacterium during unfavorable conditions, do not represent a resident epiphytic stage. The current study and that of Leben (9) indicate that *X. c.* pv. *vesicatoria* and perhaps other xanthomonads may multiply and persist as epiphytes for long periods without producing symptoms.

Most pathovars of *X. campestris* multiply on tomato leaves as well as does *X. c.* pv. *vesicatoria* if free moisture is present on leaves. *X. c.* pv. *vesicatoria* did not multiply and persist on other

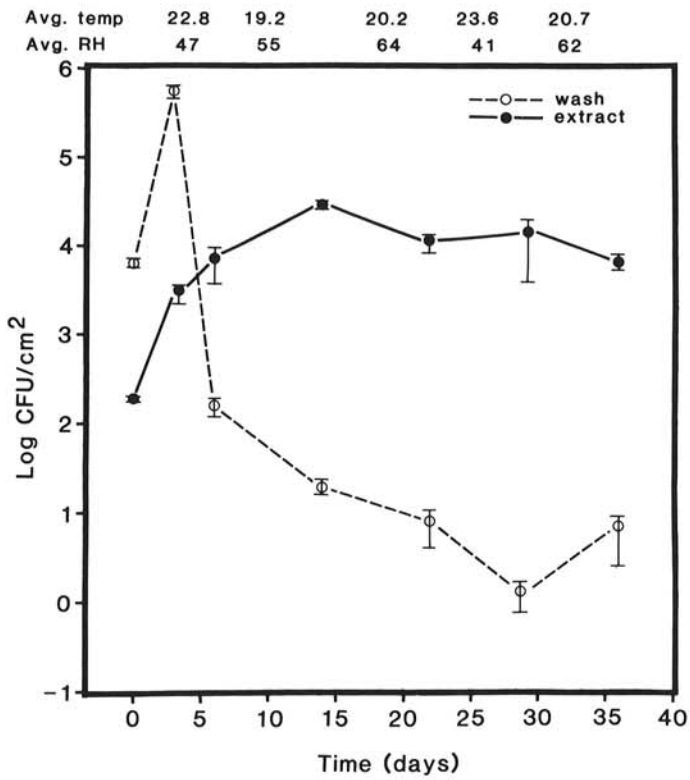

Fig. 6. Recovery of *Xanthomonas campestris* pv. *translucens* by washing or by extracting leaves of tomato plants spray-inoculated with bacterial suspensions. Plants were covered with plastic bags for 48 hr in the laboratory before placement outdoors under the indicated conditions. Temperature and RH data are the means between sample times.

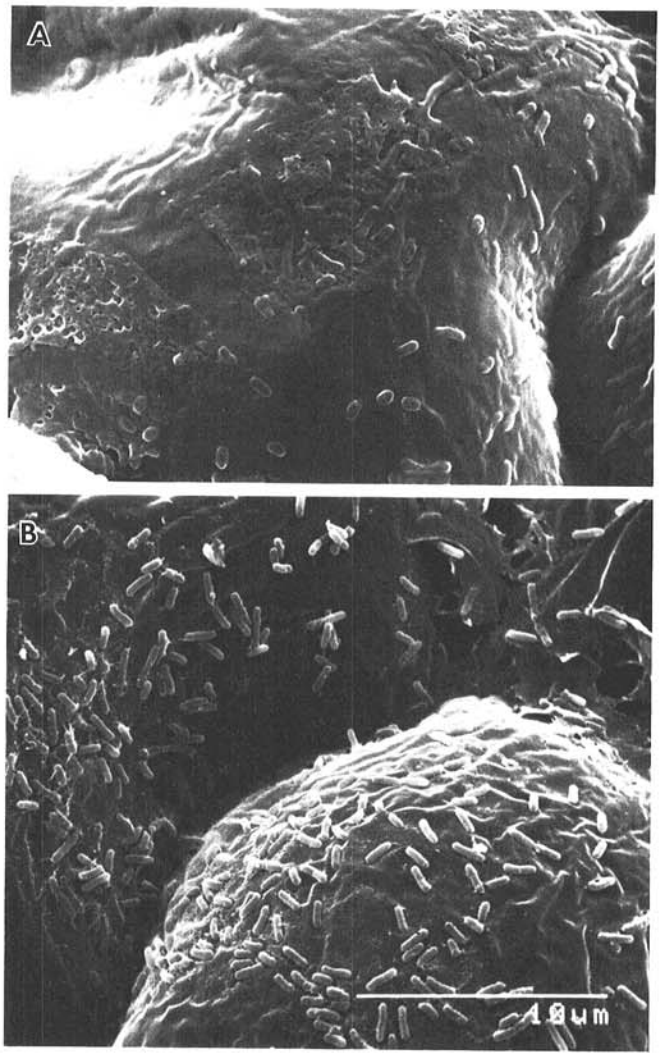

Fig. 7. Scanning electron micrographs of xanthomonads on the leaf surface of tomato: **A,** *Xanthomonas campestris* pv. *vesicatoria* and **B,** *X. c.* pv. *translucens*. Note the extracellular matrix that aggregates the bacteria and affixes them to the leaf surface.

plants as well as on tomatoes. However, this may be due to the fact that tomato leaves are pubescent and hold surface moisture better, whereas most of the other plants used have smooth or waxy leaves and retain less water. Thus, it appears that epiphytic growth is not host-specific. Some studies have found differences in populations between resistant and susceptible cultivars (2,13), but these were done under conditions where disease symptoms were present and probably resulted from differences in inoculum production in diseased tissue. Because *X. c.* pv. *translucens* multiplied and persisted for long periods on tomato leaves, it appears that xanthomonads may also be resident even on nonhost species. Cafati and Saettler (1) demonstrated that *X. c.* pv. *phaseoli* multiplied on weeds and spread to beans, causing disease.

Considering the ability of xanthomonads to survive and grow as epiphytes on hosts and nonhosts, it is perhaps surprising that most eradication programs for citrus canker caused by *X. c.* pv. *citri* have been successful (7,14). However, in all eradication programs, there have been repeated recurrences of the disease even after all symptomatic plants have been destroyed and eradication has been achieved only after many years. Such outbreaks probably occur because of buildup of bacterial populations and eventual lesion development during long periods favorable for symptom development. Current regulations call for destruction of all citrus within 40 m of symptomatic plants (14). Because *X. c.* pv. *citri* is capable of easily spreading that distance from a single infected plant (5), it may be necessary to increase that distance and to eliminate all plants in the area, especially where there are large amounts of inoculum present.

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