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Influence of Immigration on Epiphytic Bacterial Populations on Navel Orange Leaves

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Factors that influenced the increase in epiphytic bacterial population size on navel orange leaves during winter months were investigated to test the assumption that such populations were the result of multiplication on orange leaves. The population sizes of bacteria of different kinds, including ice nucleation-active (Ice^+) bacteria, were from 6- to 30-fold larger on leaves of navel orange trees adjacent to other plant species than on trees growing near other citrus species. Total and Ice^+ bacterial population sizes on other plant species growing near navel orange trees were from 18- to 60-fold and 2- to 18,000-fold larger, respectively, than on navel orange trees. About twice the number of bacterial cells of a given type were deposited onto petri dishes opened simultaneously in navel orange orchards with other plant species nearby as in orchards surrounded by citrus trees. Epiphytic bacteria and airborne bacteria were more numerous near the upwind edge of orchards bordering on other plant species, but not in orchards adjacent to other citrus trees, and decreased with distance from other plant species. Navel orange leaves also exhibited progressive increases in the ability to supercool as a function of increasing distance from the upwind edge of orchards adjacent to other plant species but not in orchards adjacent to other citrus trees. While the population size of three different bacterial strains remained nearly constant for 60 days after inoculation, total bacterial populations increased more than 50-fold during this period. These results suggest that immigration of bacteria from plants having high epiphytic bacterial populations could account for most, if not all, of the seasonal increase in bacterial populations on navel orange leaves and have important implications for procedures to modify bacterial communities on leaves.

All plant species in natural habitats have an associated epiphytic bacterial microflora. The aboveground parts of healthy plants frequently support epiphytic populations of phytopathogenic bacteria (13, 15). Plant-pathogenic bacteria commonly exist as epiphytes in a commensal relationship with plants without inciting disease. The probability of disease in several pathosystems is proportional to the epiphytic population size of the phytopathogen (13, 41). Ice nucleation-active (Ice^+) bacteria are also common inhabitants of plant surfaces. Nearly all plant species support epiphytic populations of Ice^+ strains of *Pseudomonas syringae*, *Erwinia herbicola*, or *Pseudomonas fluorescens* (1, 5, 6, 9–11, 14, 20, 22, 23, 25–29, 32–37). Epiphytic Ice^+ bacteria limit the ability of frost-sensitive plants to supercool and avoid damaging ice formation (27, 32, 35). The temperature to which leaves or flowers of a sensitive plant will supercool before freezing is inversely proportional to the logarithm of the Ice^+ bacterial population size (11, 31).

Considerable spatial and temporal variability is observed in epiphytic bacterial populations. Epiphytic populations frequently vary greatly on a given plant species at different times of the growing season, often being relatively low on immature plant parts and increasing with plant age (8–10, 12, 14, 16, 26, 33). The epiphytic population size of strains of *P. syringae* have been shown to vary greatly from day to day, with large variations occurring in as little as a few hours (12, 14). Epiphytic bacterial populations on different plant species, even those growing in close geographical proximity, can also vary greatly (22, 33). As a rule, glabrous plants or those having highly wettable leaf surfaces support much higher populations than do glaucous plant species (33). It therefore appears that epiphytic bacterial populations are strongly influenced by the plant on which they live and that these differences may be mediated by differences in the physical environment on leaf surfaces or by plant-derived resources such as nutrients. The large and rapid increases in epiphytic bacterial population sizes observed on species such bean or pear (10, 12, 14, 45) are suggestive of the rapid multiplication of cells on leaves. Multiplication has generally been assumed to be the major factor influencing bacterial population sizes on plant surfaces; this assumption may not always be true. For example, immigration was shown to be a major factor influencing changes in numbers of fungal propagules on apple leaves (18).

In this study, factors which influence the variation in epiphytic bacterial population size on navel orange leaves were carefully assessed to identify those associated with large bacterial population sizes. These studies were undertaken to test the assumption that bacterial multiplication is primarily responsible for increasing bacterial population sizes on this plant species and to better devise strategies to minimize epiphytic bacteria, particularly potentially detrimental bacteria, on navel orange trees; the results of this study have important implications for procedures to mitigate hazards such as frost damage and diseases incited by Ice^+ epiphytic bacteria.

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MATERIALS AND METHODS

Bacterial strains and culture media. The source and characteristics of *P. fluorescens* A510, *E. herbicola* B7, and *P. syringae* Cit7, which were applied to citrus trees in this experiment, have been described previously (28, 29). The source and characteristics of *P. fluorescens* A506, which was applied to vegetation near citrus orchards in this study, has also been described (45). All of these

strains are spontaneous mutants resistant to 100 μg of rifampin per ml selected like those in a previous study (40). Recovery of bacteria of all types was made on King's medium B (17) containing 100 μg of cycloheximide per ml (KB). Recovery of inoculated bacterial strains was on KB containing 100 μg of rifampin per ml (KBR). Inoculum of bacterial strains sprayed onto plants was recovered from the surface of KBR plates after 2 days of growth at 24°C. No bacterial colonies that did not match the distinctive colony morphologies of the rifampin-resistant introduced strains were detected on KBR plates.

Field plot design. Measurements of indigenous bacterial populations were made on eight occasions from leaves of Washington navel orange leaves collected from within a 10-ha orchard of this species grown at the University of California Lindcove Research and Extension Center located near Exeter, Calif. The insecticides formetanate (Carzol SP, 1.2 g/liter; Schering AG) and chlorpyrifos (Lorsban 4E, 6.0 g/liter; Dow Chemical Company) were applied in 1984 on 2 May and 12 May, respectively. In addition, a mixture of zinc sulfate (1.2 g/liter) and urea (6.0 g/liter) were applied on 18 April 1984. No pesticides were applied during the period of September to May. At each sampling time, four replicate samples of 20 leaves each were collected randomly from trees within this orchard (two leaves per tree). All samples were collected at least 100 m southeast of the western edge of this orchard, which bordered on a field of mixed grass and broadleaf plant species.

The population dynamics of bacteria applied to navel orange leaves was studied in a group of trees located in the southwest corner of the navel orange orchard described above. The trees were organized in a randomized complete block design with four replications. Each replicate block consisted of two trees; a single tree separated treated trees within a block. Bacteria were applied to trees on 25 October 1984 with a backpack mist blower to near runoff (about 3 liters per tree) as they were in other studies (1). Cells of strains A510, B7, and Cit7 were scraped from the surface of KBR plates and suspended in tap water. The cell concentration in the suspensions was estimated by measurements of turbidity made with a spectrophotometer, and the suspensions were diluted with tap water to a concentration of approximately 10^8 cells per ml.

On 14 November 1984, when prevailing winds were gentle and from the East, a field of about 3 ha in size containing a mixture of grasses and broadleaf plant species adjacent to the northwest portion of the navel orange orchard at the Lindcove Field Station was sprayed with a suspension of approximately 10^8 cells of *P. fluorescens* A506 per ml. Bacterial cells were prepared as described above and applied at a rate of approximately 40 liters/ha. Leaf samples were then collected randomly at about 2-week intervals from navel orange trees within about 10 m of the western edge of the block (immediately opposite from the area to which strain A506 had been applied) for a period of 60 days.

In the winters of 1983-1984 and 1984-1985, surveys were conducted in commercial navel orange orchards having different types and amounts of plant species located nearby. All test orchards were located within about 35 km of Exeter, Calif., and had similar soil fertilities. As a result of extensive herbicide use, few if any plant species other than navel orange were found inside any of the orchards. In most orchards, trees were planted about 5 m apart within a row, were mature, and therefore were often touching each other within a row. Rows were about 9 m apart; about 3 m of bare soil separated trees in different rows. While insecticides and growth regulators had been applied to citrus trees during the summer months, no pesticide applications were made to any of these orchards during the sampling period of November to April. No copper bactericides were applied within 100 m of the trees sampled in these studies. The prevailing wind in this region was from the West during the winter months. In the winter of 1983-1984, five orchards in which rows of navel orange trees were oriented perpendicular to upwind areas planted to other citrus species were selected, while five other orchards in which upwind areas were planted to plant species other than citrus or olive were identified. In the winter of 1984-1985, 13 navel orange orchards which were downwind of large blocks planted to other citrus species or olive were identified, while 13 orchards were downwind of large blocks (at least 5 ha) of plant species other than citrus or olive. The upwind plantings consisted of fields of alfalfa, wheat, barley, mixed-grass pastures, or mixtures of grasses and broadleaf weeds. Leaf samples from navel orange trees were made at four different predetermined locations, each about 5 m from the western edge of an orchard. In the winter of 1983-1984, leaves were sampled on five dates in the period from mid-November until mid-April. In 1984-1985, leaf samples were made only on 12 December and 5 February. In January 1985, representative plant species other than citrus located in the vicinity of the test orchards were sampled. Four replicate samples of each plant species were collected randomly within about 20 m of the upwind edge of test orchards; each plant species was sampled at more than one site.

In the winter of 1985-1986, four commercial navel orange orchards that had rows perpendicular to upwind blocks of plant species other than citrus were identified; three of these upwind blocks consisted of mixed-grass pastures, while another one was a commercial alfalfa field. As a contrast, four commercial navel orange orchards which had rows perpendicular to upwind blocks of navel orange were also identified. For each of these eight commercial orchards, samples were collected from within a block of trees about 100 m wide that was perpendicular to the western edge of the commercial orchard; this block was not sprayed with copper hydroxide in October as were other trees in each orchard. Locations at the immediate edge of the unsprayed block in each citrus orchard and at a distance of 7, 14, 21, and 28 trees towards the center of the citrus grove (0 to 140 m) were predetermined; four random samples of leaves were collected at each sampling location at each sampling time. Concentrations of airborne bacteria were measured on gravity sedimentation plates (as described below) at each location in each orchard nearly simultaneously as above.

Enumeration of bacterial populations on plants. Epiphytic bacterial populations were evaluated in bulked leaf samples having a mass of about 20 g. Each sample therefore consisted of about 15 navel orange leaves or from 10 to 200 leaves of other plant species, depending on the average mass per leaf of the species. Population sizes were estimated on four replicate samples for each treatment. Leaves were placed in 200 ml of washing buffer (40), submerged, and sonicated for 7 min in an ultrasonic cleaning bath (Bransonic 52) to dislodge bacteria from the leaves as in other studies (40). Flasks containing leaves were also shaken for 30 s after sonication to disperse the bacterial cells prior to enumeration. Appropriate 10-fold serial dilutions of leaf washings were dilution plated onto KB and/or KBR as appropriate. Because of the low numbers of Ice^+ and occasionally fluorescent pigment-producing bacterial strains on leaves, leaf washings were concentrated 100-fold prior to dilution plating. Forty milliliters of leaf washings was centrifuged for 15 min at 30,000 × *g*, the supernatants were discarded, the pellets, containing bacterial cells and small amounts of plant debris, were resuspended in 0.4 ml of washing buffer, and aliquots of the suspended pellets were plated onto KB and/or KBR as appropriate. The theoretical limit of detection was therefore approximately 1 cell per g of plant tissue. After growth for 3 days at 24°C, bacteria producing fluorescent diffusible pigments were enumerated by viewing dilution plates while irradiating them with long-wavelength (λ = 360 nm) UV light. Bacteria of all types and distinctly yellow-pigmented bacterial colonies, typical of *E. herbicola* strains (34), were counted after 4 days of growth at 24°C. Ice^+ bacteria were quantified after 6 days of growth at 24°C by a replica freezing technique similar to that described elsewhere (33). Cells from colonies on dilution plates were transferred onto the surface of a paraffin-coated aluminum foil sheet with a sterile velvet pad. The sheet was floated on the surface of a refrigerated bath maintained at −9°C and sprayed with a fine mist of sterile distilled water. After about 30 s, Ice^+ bacterial colonies were readily apparent as frosty spots where the microdroplets of water had frozen.

Measurement of supercooling temperatures of leaves. The supercooling ability of individual navel orange leaves was assessed by a tube freezing method similar to that reported previously (31). Individual navel orange leaves were immersed into test tubes containing 10 ml of ice-nucleus-free water. Each sample consisted of 40 individual leaves. The test tubes were immersed in a circulating bath containing refrigerated ethanol held at −2°C. At 30-min intervals, the temperature of the bath was decreased by 0.5°C. After 20 min, when tubes had come to temperature equilibrium with the bath, the cumulative number of tubes which had frozen at that temperature was visually assessed. The tubes were exposed stepwise to progressively colder temperatures until all of the tubes had frozen. The mean freezing temperature was determined by extrapolation from the cumulative percentage of tubes which had frozen at each of several progressively colder temperatures.

Measurements of airborne bacteria. Concentrations of airborne bacteria were estimated from measurements of the numbers of cells deposited on gravity sedimentation petri plates similar to those of other studies (4, 24). Petri dishes were filled to within 3 mm of the top with KB to prevent turbulent deposition of bacteria by air passing over the surface of the plate. Plastic tarps (2 by 2 m) were spread on the ground, at locations 7 m apart within the space between the rows of trees, to prevent contamination from soil or vegetation during the sampling procedure. Ten petri dishes (10-cm diameter) containing KB were placed near the center of each tarp. Estimates of airborne bacterial populations were made nearly simultaneously at all sites; participants in the experiment removed the lids of a subset of the petri dishes within 5 min of 12:00 noon, and each plate was left open for exactly 1 h. Total bacterial colonies were enumerated, and Ice^+ bacterial colonies were estimated by a replica freezing procedure after 6 days of growth at 24°C. Bacterial colonies producing diffusible fluorescent pigments were enumerated after 3 days of growth at 24°C as described above.

Statistical methods. The goodness-of-fit of the normal distribution of log-transformed bacterial population sizes of individual bulk samples was tested by the Shapiro-Wilk W statistic and calculated by use of SAS (release 6.04; SAS Institute, Cary, N.C.). Since most distributions were adequately described by a log-normal distribution, all estimates of bacterial population sizes were log-transformed prior to analysis. Samples in which population sizes of either fluorescent or Ice^+ bacteria were not detected were assigned the log-transformed value of 0 (which represented the approximate limit of detection). For a given type of bacteria and within a given year, the bacterial population sizes were combined for analysis; five different samplings in 1983-1984 and two samplings in 1984-1985 were so combined. Analysis of variance of log-transformed epiphytic bacterial population sizes, of the number of cells recovered on gravity sedimentation plates, or of the influence of orchard site, condition, or location on population sizes was computed by the General Linear Models procedure of SAS. The contribution of factors such as the presence or absence of plant species such as citrus on bacterial abundance was determined from the significance of F tests. Separation of treatment means was by Fisher's unprotected least significant difference (LSD) test; this method controls the comparison-wise error rate. Spacial gradients of epiphytic and airborne bacterial populations were assessed by regression of the logarithm of bacterial population size per gram (fresh

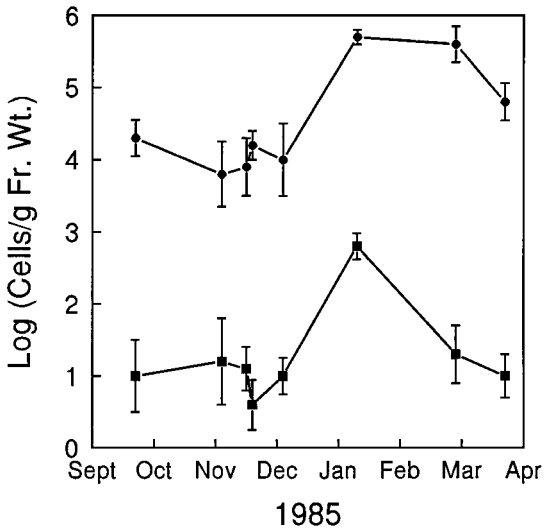

FIG. 1. Total bacterial populations (circles) and populations of Ice$^+$ bacteria (squares) recovered from the surface of untreated leaves of navel orange trees collected at various times during the year near Exeter, Calif. The vertical bars represent the standard error of the determination of mean log populations of bacteria per gram (fresh weight) of plant tissue.

weight) of leaf or the number of bacterial cells deposited on gravity sedimentation plates against either distance into the grove or log-transformed distance into the grove, by using the regression procedure of SAS.

RESULTS

Large seasonal variations in the population sizes of bacteria of different types on navel orange leaves, such as that shown for the 1984-1985 growing season (Fig. 1), were observed in all years of this study. Relatively low total and Ice$^+$ bacterial populations were observed during the summer and fall months in California, but these populations increased substantially in size during the winter and spring months (Fig. 1). A typical weather pattern of little or no rainfall and daily temperatures of as high as 40°C prevailed at this location from May through October; only three rainfall events totalling 4.3 mm occurred during this period in 1984. Rainfall increased in intensity and frequency during the period of November to April; 24 rainfall events totalling 263 mm occurred during this period in 1984-1985. The time when increases of population sizes of bacteria of different types on navel orange leaves occurred coincided with the time of maximum frequency of rainfall at this site (Fig. 1). From December through February, the daytime temperatures at the field plots were consistently low (from 2 to 10°C) and frequent periods of ground fog occurred. The Ice$^+$ bacterial strains recovered from navel orange trees were a nearly equal mixture of *P. syringae* and *E. herbicola*; no other Ice$^+$ bacterial species were observed. While at least some Ice$^+$ bacterial strains were detected on most samples at all times, population sizes remained relatively low, even during midwinter, compared with that on many other plant species nearby. Population sizes of Ice$^+$ bacteria, however, increased about 100-fold between mid-November and mid-January (Fig. 1). The increase of Ice$^+$ bacterial populations coincided with an increase of a similar magnitude of the total bacterial populations during this period (Fig. 1).

The total bacterial population size as well as that of Ice$^+$ bacteria varied substantially among the navel orange orchards sampled (over 100-fold), even though all orchards were of the same cultivar and under similar management (data not shown).

TABLE 1. Population sizes of bacteria of various types on navel orange leaves and on leaves of other plant species growing in close proximity to navel orange trees

Plant species	Bacterial populations (log cells/g [fresh wt])[a]			
	Total[b]	Fluorescent[c]	Yellow[d]	Ice$^+$
Henbit	8.09 a	6.79 a	5.53 a	4.74 a
Annual bluegrass	7.36 ab	6.04 a	5.73 a	3.01 bc
Chickweed	7.01 b	5.97 a	5.56 a	0.65 de
Malva	6.82 b	5.15 a	5.68 a	4.43 a
Alfalfa	6.75 b	5.83 a	4.69 a	3.42 ab
Mustard	6.57 b	5.09 a	5.29 a	1.86 cd
Navel orange	5.31 c	1.58 b	1.48 b	0.49 de
Olive	3.86 d	1.69 b	1.17 b	0.00 e

[a] Mean populations in each column followed by the same letter do not differ significantly ($P = 0.05$) by Fisher's unprotected LSD test.
[b] Total, total bacteria recovered on KB.
[c] Fluorescent, bacteria that produced diffusible fluorescent pigment on KB.
[d] Yellow, bacteria with distinctly yellow pigmentation on KB.

The only apparent difference between the test orchards was the presence of plant species other than citrus in the vicinity of all orchards having relatively high bacterial populations.

The population sizes of different groups of bacteria on the plant species growing in the vicinity of the test orchards were estimated (Table 1). All plant species except olive harbored much larger epiphytic bacterial populations than did navel orange; from 18- to 60-fold-larger total bacterial populations, from 2- to 18,000-fold larger Ice$^+$ bacterial populations, and from 3,200- to 160,000-fold-larger populations of fluorescent bacteria were found on these plant species. Even though these plant species were growing in close proximity to each other and to navel orange trees (often in contact with one another), each species consistently supported epiphytic bacterial populations that often differed from those on each other and on navel orange trees. The relatively high epiphytic bacterial population sizes observed on these plant species suggested that they could serve as a source of immigrant bacteria to navel orange trees.

While the population sizes of different bacterial groups generally increased during the period from November to February, little increase in numbers of strains of *P. fluorescens*, *P. syringae*, or *E. herbicola* was observed after they were spray inoculated onto navel orange trees (Fig. 2). While inoculated leaves supported about 10^7 cells per g (fresh weight) immediately following spray inoculation (data not shown), population sizes of the inoculated strains dropped to about 10^3 cells per g within 1 week following inoculation (Fig. 2). Populations of these three strains changed little after the first week following spray inoculation; populations decreased slightly and then maintained a size of about 10^3 cells per g (Fig. 2). The population sizes of the three inoculated strains were similar at a given time after inoculation (Fig. 2). While the population sizes of these strains remained rather constant in the period from November through mid-January, the total bacterial population size on the inoculated trees increased about 50-fold (Fig. 2).

To determine if the presence of plant species other than citrus affected the population size of bacteria on citrus species, the population sizes of different groups of bacteria were measured on navel orange leaves and in the air in navel orange orchards which had different types and amounts of vegetation nearby (Tables 2 and 3). The population sizes of different groups of bacteria were always larger on the leaves of navel orange trees in orchards with plant species other than citrus nearby (Table 2). Except for yellow-pigmented bacteria in test

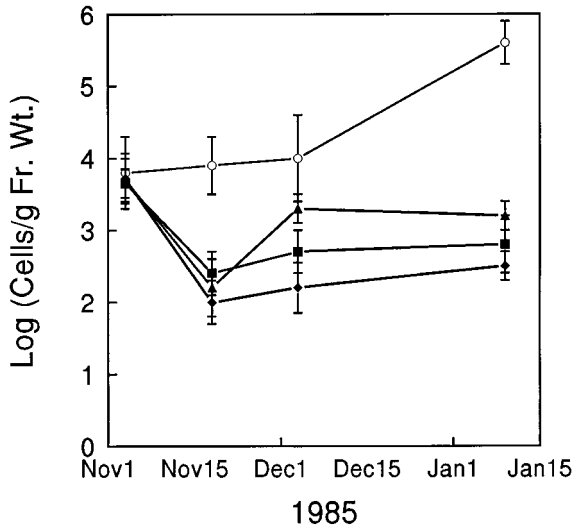

FIG. 2. Total bacterial populations recovered from the surface of uninoculated navel orange leaves (circles) or from the leaves of trees treated on 15 October with a suspension of about 10^8 cells per ml of *E. herbicola* B7 (squares), *P. syringae* Cit7 (triangles), or *P. fluorescens* A510 (diamonds). The vertical bars represent the standard error of the determination of mean log population size per gram (fresh weight) from four replicate samples collected for each treatment at each sampling time shown on the abscissa.

orchards in 1984, bacteria of all groups were always significantly more abundant on trees near other plant species than on trees surrounded only by other citrus species (Table 2). The population size of bacteria of all types was from 6.9- to 7.2-fold higher on orange leaves in orchards having other plant species nearby compared with that of orchards without such plant species, while the numbers of fluorescent and Ice^+ bacteria were 5.9- to 30.9-fold higher and from 1.7- to 3.3-fold higher, respectively (Table 2).

The supercooling temperature of navel orange leaves, the lowest temperature to which leaves could be cooled before ice formation occurred, was rather low in leaves collected both from trees surrounded by other citrus trees (−5.46°C) and from trees near other plant species (−4.94°C), reflecting the relatively small number of Ice^+ bacteria recovered from citrus (Table 2). Importantly, leaves of trees in orchards surrounded only by other citrus species supercooled significantly ($P = 0.03$) more than did leaves from trees surrounded by other plant species. The reduced supercooling ability of leaves from trees near other plant species was generally associated with higher

TABLE 2. Population sizes of bacteria of different types on navel orange leaves from trees grown near other navel orange trees or other plant species

Bacterial group[a]	Bacterial population (log cells/g [fresh wt])[b]			
	1984		1985	
	Navel orange	Other species	Navel orange	Other species
Total	4.18 a	5.04 b	4.53 a	5.37 b
Fluorescent	1.40 a	2.17 b	1.07 a	2.56 b
Yellow pigmented	2.92 a	3.68 a	1.26 a	1.68 b
Ice^+	0.44 a	0.96 b	0.39 a	0.62 b

[a] See Table 1, footnotes *b*, *c*, and *d* for description of bacterial groups.
[b] Means in each row for a given year that are followed by the same letter do not differ significantly ($P = 0.05$) by Fisher's unprotected LSD test.

TABLE 3. Air deposition of different types of bacteria near navel orange trees grown near citrus species or different plant species in 1985

Bacterial group[a]	No. of cells deposited/plate[b]	
	Navel orange	Other plant species
Total	34.1 a	73.6 b
Fluorescent	0.84 a	2.45 b
Yellow pigmented	1.10 a	3.27 b
Ice^+	0.46 a	0.71 b

[a] See Table 1, footnotes *b*, *c*, and *d* for descriptions of bacterial groups.
[b] Means in each row for a given year that are followed by the same letter do not differ significantly ($P = 0.05$) by Fisher's unprotected LSD test. Air deposition of the bacteria was measured near navel orange trees grown near other navel orange trees or other plant species.

Ice^+ bacterial populations on these trees compared with that on trees surrounded only by citrus species.

Assuming that plant species other than citrus were the source of at least some of the bacteria found on nearby navel orange leaves, we measured bacterial abundance in the air in test orchards having various plant species nearby. Airborne bacterial populations were estimated indirectly by measuring the rates of sedimentation of bacteria onto open petri dishes. Petri dishes were opened nearly simultaneously for 1 h in all test orchards to ensure that similar meteorological conditions prevailed and to better enable direct comparisons of airborne bacterial concentrations at these sites. The numbers of cells of different groups of bacteria that were deposited onto petri dishes varied substantially from one site to another at a given sampling time (Table 3). Bacterial deposition nearly always was highest in orchards with plant species other than citrus growing nearby (Table 3). On average, about twice the number of bacterial cells of a given type were deposited onto petri dishes in orchards with other plant species nearby than in orchards surrounded only by citrus trees (Table 3). While the number of Ice^+ and fluorescent bacterial strains were a relatively small percentage of the total bacteria collected on gravity sedimentation plates, significantly higher numbers of cells of these groups were deposited in orchards with other plant species nearby as compared with orchards surrounded only by navel orange trees (Table 3).

To determine whether particular bacterial strains could be transferred from other plant species to nearby navel orange trees, a large area of mixed grasses and broadleaf weed species growing upwind from a navel orange orchard was inoculated with rifampin-resistant *P. fluorescens* A506 when winds were blowing away from the test orchard. Populations of this strain subsequently averaged about 10^6 cells per g on most plant species, constituting about 10% of the total bacteria (data not shown). Strain A506 was not detected on gravity sedimentation plates placed in the orchard at the time of inoculation. However, within 1 week following inoculation of the other plant species, rifampin-resistant cells resembling strain A506 were detected on navel orange trees. Within 1 month, populations of strain A506 subsequently increased to approximately 10^2 cells per g on navel orange leaves (data not shown). These results indicate that particular bacterial strains can immigrate from nearby plant species onto navel orange trees. While the method of this immigration was considered to be via aerosols, this study could not rule out other modes of transport such as insect vectoring.

If it is assumed that plant species other than citrus are a significant source of immigrant bacteria that can then accumulate on navel orange leaves, we would expect that there would

TABLE 4. Population sizes of bacteria of different types on navel orange leaves from trees growing at different distances from various plant species

Tree location	Distance from edge of orchard (no. of trees)	Bacterial population (log cells/g [fresh wt])[a]		
		Total	Fluorescent	Ice^+
Adjacent to navel orange trees[b]	0	4.81 a	0.81 a	0.00 a
	7	4.68 a	0.00 a	0.00 a
	14	4.74 a	0.00 a	0.17 a
	21	4.86 a	0.51 a	0.11 a
	28	4.71 a	0.24 a	0.18 a
Adjacent to other plant species[c]	0	6.08 a	3.38 a	1.25 a
	7	5.75 a	3.26 a	0.57 ab
	14	5.85 a	1.93 ab	0.84 ab
	21	5.51 a	0.00 b	0.11 b
	28	5.39 a	0.62 ab	0.24 b

[a] Mean population sizes in a given column for trees grown near the same plant species that are followed by the same letter do not differ significantly ($P = 0.05$) by Fisher's unprotected LSD test. See Table 1, footnotes *b* and *c*, for description of bacterial groups.

[b] Leaves were collected from trees at different distances downwind from the upwind edge of an orchard adjacent to another navel orange orchard.

[c] Leaves were collected from trees at different distances downwind from the upwind edge of an orchard adjacent to a field of mixed grasses and broadleaf weeds.

be a gradient of abundance of both bacterial epiphytes and airborne bacteria away from such sources of inoculum. Therefore, in the 1985–1986 growing season, we identified four different navel orange orchards which had an abundance of other plant species upwind. We also identified four nearby test orchards in which only navel orange trees were immediately upwind. Populations of different groups of bacteria were then measured at locations at different distances away from the upwind edge of the orchard; the results from two representative orchards are shown in Table 4. Neither total bacterial populations nor populations of Ice^+ or fluorescent bacterial ice strains differed at sampling locations at various distances downwind from the edge of the navel orange orchard (Table 4). In contrast, the highest total bacterial populations as well as that of Ice^+ and fluorescent bacterial strains occurred near the upwind edge of orchards located near other plant species (Table 4). Fluorescent and Ice^+ bacterial populations were significantly higher on trees adjacent to other plant species than on trees in the interior of the orchard, up to 140 m away (Table 4). A consistent decrease in population sizes of all groups of bacteria was observed as the distance from other plant species increased (Table 4). While total bacteria were about 5-fold more numerous on navel orange trees near the upward edge of the orchard than on those in the interior, there was about a 500-fold-higher population size of fluorescent bacteria and about a 10-fold-higher population of Ice^+ bacteria near the other plant species (Table 4). Position within an orchard accounted for a significant fraction of the variation in population sizes of Ice^+ and fluorescent bacterial species when populations on all of the test orchards adjacent to other plant species were considered ($P < 0.10$ and $P < 0.07$, respectively). Because of the relatively large differences in total bacterial populations of these orchards, the position within an orchard did not account for a significant fraction of the variation in these populations ($P < 0.29$). It is noteworthy that position within an orchard did not account for a significant proportion of the variation in populations of any group of bacteria on navel orange leaves in orchards adjacent to other navel orange trees when all the orchards were considered ($P < 0.96$, $P < 0.26$, and $P < 0.72$ for total, Ice^+, and fluorescent bacteria, respectively). The largest decreases in epiphytic bacterial population sizes from the edge to the interior of navel orange orchards were observed in the three orchards downwind of grass fields; the gradient of epiphytic population sizes away from an upwind alfalfa field was substantially smaller (data not shown). Similar results to those depicted in Table 4 were also observed at other sampling times (data not shown).

While the epiphytic populations of different groups of bacteria were always highest on navel orange trees that were adjacent to other plant species and lowest at the greatest distance from such species, consistent gradients of such populations were not always observed. The logarithm of total and fluorescent bacterial strains was significantly linearly related ($P < 0.1$) to distance from the upwind edge of the orchard for two of four orchards. When all four navel orange orchards adjacent to other plant species were considered, a significant ($P < 0.008$) linear relationship was observed between the logarithm of Ice^+ population size and distance away from the upwind edge of the plot. However, because of variable total and fluorescent bacterial population sizes among orchards, no significant linear relationship was found between the logarithm of these populations and the distance from the upwind edge of the plot ($P < 0.12$ and $P < 0.35$, respectively). Not unexpectedly, epiphytic population sizes for the four citrus orchards which were downwind from other navel orange orchards were not significantly linearly related to position in the orchard (data not shown).

The numbers of bacterial cells of different types that were deposited onto gravity sedimentation plates located in different portions of navel orange orchards varied greatly. Even though petri plates were opened nearly simultaneously at all locations, the number of cells deposited varied substantially from site to site (Table 5) (unpublished data). More importantly, the numbers of bacterial cells deposited onto sedimentation plates varied substantially with location within test orchards adjacent to other plant species but not within test orchards bordering on other citrus trees (Table 5). Generally, much larger numbers of bacteria were deposited near the upwind edge of such orchards (Table 5); a progressive decrease in the numbers of deposited bacterial cells was generally observed as the distance from the upwind edge of the orchard increased (Table 5). No obvious differences in meteorological conditions such as wind speed or direction was apparent between sample sites within an orchard. Significant linear relationships between the total deposited bacteria and the distance from the upwind edge of orchards both downwind from citrus species and from other plant species were observed when considered for all test orchards ($P < 0.001$). In contrast, significant linear relationships between the numbers of deposited fluorescent, Ice^+, and yellow-pigmented bacteria and distance from the upwind edge of an orchard were observed only in those orchards located downwind from other plant species and not in orchards located downwind from other orange trees ($P < 0.001$ versus $P < 0.79$, $P < 0.05$ versus $P < 0.17$, and $P < 0.01$ versus $P < 0.96$, respectively). The significant positional influence on bacterial numbers in orchards downwind from navel orange trees was probably due to the occasional high abundance of cells near the edge of some orchards due to anthropogenic sources (such as dust generated by unanticipated vehicular traffic). The significance of the linear relationship between the numbers of bacterial cells deposited and distance into the orchard was not increased by log transformation of the deposited cells (data not shown). Similar gradients of decreasing bacterial deposition with distance away

TABLE 5. Deposition of bacteria of various types onto petri dishes placed at different locations within navel orange orchards adjacent to various plant species

Tree location	Distance from edge of orchard	No. of bacteria recovered/plate/h[a]		
		Total	Fluorescent	Ice$^+$
Adjacent to navel orange trees[b]	0 trees	33 a	0.4 b	0.0 b
	7 trees	29 a	1.2 b	0.1 ab
	14 trees	37 a	2.1 ab	0.4 a
	21 trees	37 a	1.0 b	0.0 b
	28 trees	37 a	3.2 a	0.1 ab
Adjacent to other plant species[c]	10 m upwind[d]	126 a	19 a	0.6 a
	0 trees	97 a	7.6 b	0.9 a
	7 trees	51 b	3.0 bc	0.2 a
	14 trees	55 b	2.8 bc	0.2 a
	21 trees	46 b	1.2 c	0.0 a
	28 trees	34 b	1.4 c	0.1 a

[a] Mean estimates of deposition onto petri plates in a given column for trees grown near the same plant species that are followed by the same letter do not differ significantly ($P = 0.05$) by Fisher's unprotected LSD test. See Table 1, footnotes *b* and *c*, for description of bacterial groups.

[b] Gravity deposition plates were placed at different distances downwind from the upwind edge of an orchard adjacent to another navel orange orchard.

[c] Gravity deposition plates were placed at different distances downwind from the upwind edge of an orchard adjacent to a field of mixed grasses and broadleaf weeds.

[d] Gravity deposition plates were placed on a tarp in a field of mixed weed species approximately 10 m immediately upwind of the navel orange orchard where deposition was also simultaneously measured.

from the edge of navel orange orchards adjacent to other plant species were commonly observed in samplings conducted over a 2-year period; such gradients were never observed in test orchards adjacent to navel orange trees.

Since the population size of Ice$^+$ bacteria can influence the supercooling ability of plants, and since positional variations in population sizes of Ice$^+$ bacteria were observed in navel orange orchards located downwind from other plant species, the supercooling temperature of individual navel orange leaves was determined for trees located at different positions within test orchards as another measure of the effect of bacterial immigration. Little variation in the mean supercooling temperature of navel orange leaves from different locations in navel orange orchards adjacent to navel orange trees was observed (Fig. 3). While the supercooling temperature of one orchard which had abundant decaying vegetation on the orchard floor was considerably higher than those of the other two orchards, no obvious difference in the supercooling temperature of leaves from different locations within a given orchard was observed (Fig. 3). In contrast, the supercooling temperature of leaves from three orchards adjacent to other plant species was highest at the upwind edge of the orchards and progressively decreased with distance into the orchards (Fig. 3). For most orchards, the mean supercooling temperature of leaves was about 1°C warmer on trees located at the upwind edge of an orchard than from those up to 140 m further downwind (Fig. 3).

The severity of frost injury to navel orange fruit was influenced by the distance of the trees from the upwind edge of orchards adjacent to other plant species but not in orchards adjacent to navel orange trees. Radiative frosts occurred on at least two occasions in the first 2 weeks of January 1986 in three plot locations in this study. The minimum air temperatures during these events ranged from about −5°C to about −6°C.

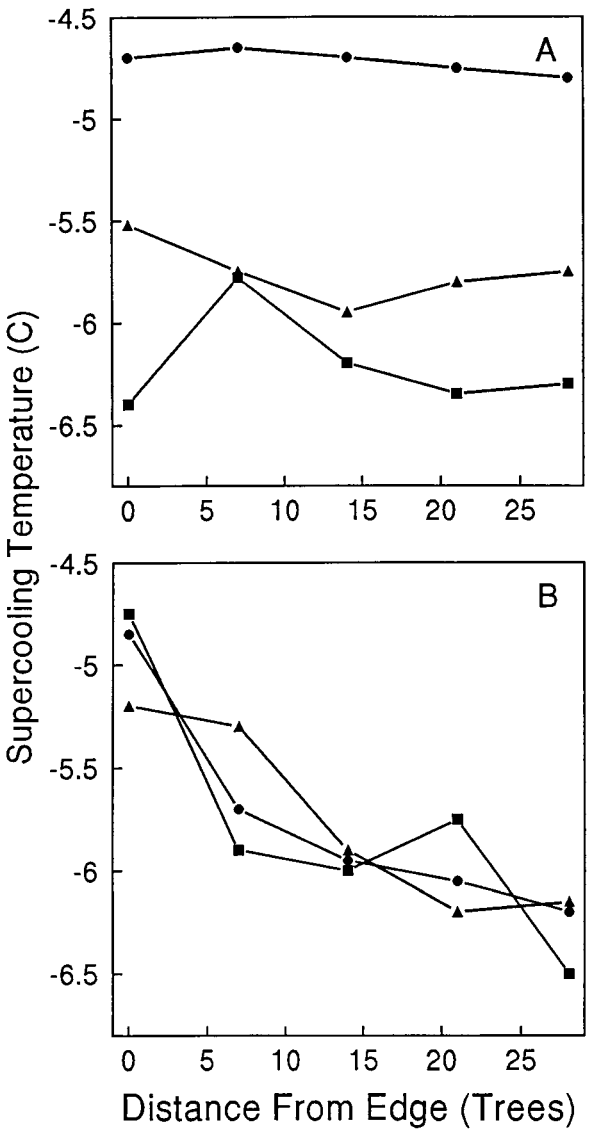

FIG. 3. Mean supercooling temperature of navel orange leaves from trees in three different orchards located immediately downwind from other navel orange trees (A) or on trees in 3 orchards adjacent to plant species other than citrus (B). The mean temperature to which 40 leaves collected at each of the distances from the upwind edge of the orchard (shown on the abscissa) could be cooled before freezing is reported.

Freezing temperatures were encountered in one orchard adjacent to other plant species and in two orchards adjacent to navel orange trees. Two of the orchards which experienced freezing temperatures had been divided into two equal-sized blocks oriented perpendicular to the upwind edge of the orchard; one block received copper hydroxide sprays applied by cooperating growers in October, while the other block was left untreated. The severity of frost injury was highest in the orchard located downwind from other plant species. The severity of frost injury at this site was greatest in those trees located near the edge of the orchard and decreased with distance away from the edge of the orchard (Fig. 4). At a given distance from the edge of an orchard, trees treated with copper hydroxide always had a lower severity of frost damage than unsprayed trees (Fig. 4). The severity of frost damage varied little with

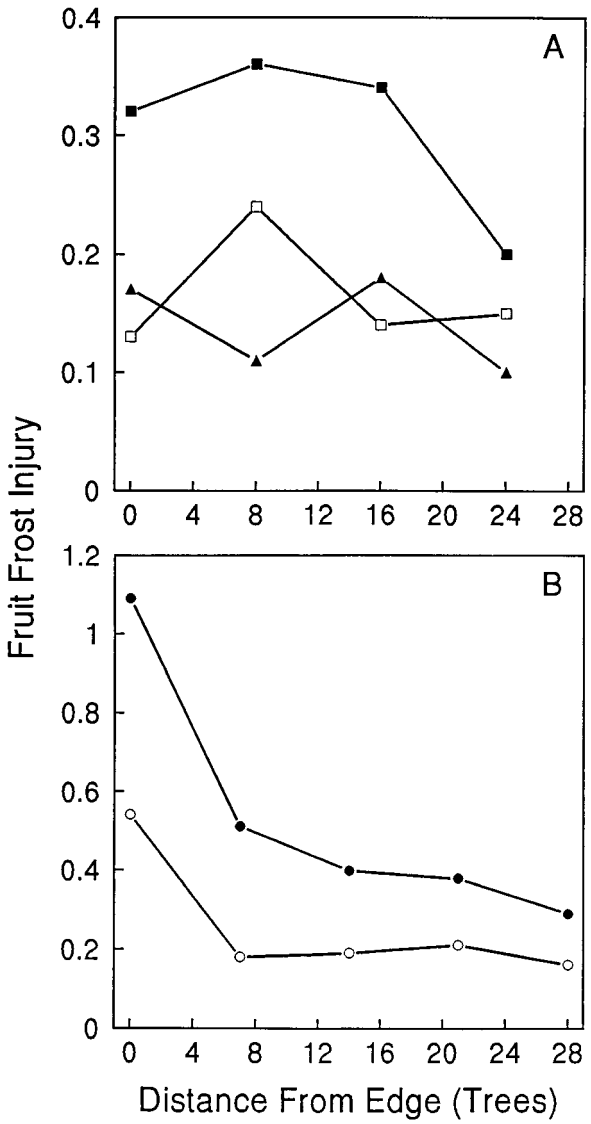

FIG. 4. The severity of frost damage to navel orange fruit collected at the distances shown on the abscissa from the upwind edge of two navel orange orchards (squares and triangles) located downwind from other navel orange orchards (A) or an orchard (circles) located downwind from a field of mixed grasses (B). Orchard blocks that had received foliar sprays of copper hydroxide (2 g/liter) (open symbols) or which did not receive any bactericide sprays (filled symbols) are compared. Internal frost injury was scored as follows: 0, no visible frost damage; 1, detectable frost damage involving <20% of the interior of the fruit; 2, frost damage involving >20% of the interior of the fruit. The mean fruit frost injury rating for 300 fruit collected from each location of each orchard block is reported.

distance from the upwind edge of test orchards adjacent to navel orange trees (Fig. 4).

DISCUSSION

The presence of nearby plant species other than citrus or olive was strongly associated with large epiphytic bacterial populations on navel orange leaves. It seems unlikely that the microclimate in navel orange orchards is appreciably different in orchards adjacent to different plant species since the orange trees themselves probably have the largest impact on environmental factors such as the relative humidity near leaves. During the winter months when most of these studies were conducted, the soil was moist and the air was usually cool (5 to 12°C) and rather humid (>85% relative humidity). Several findings indicated that bacterial populations were probably high on navel orange trees near other plant species because of immigration from these plants, which had much higher population sizes. The observation that *P. fluorescens* A506 could move from other plant species onto navel orange trees offers strong support for this conjecture. Some movement of genetically marked bacterial strains from the cover crop plant species underneath nursery trees into the trees has also been detected (38). The magnitude of the movement of those bacterial strains was less than in this study, perhaps because of the relatively small areas from which the applied bacterial strains could have emigrated in their study (ca. 1 m^2 versus 3 ha in this study). The process by which bacterial cells are liberated from the surface of plants is not well understood; if the efficiency of liberation is relatively low, as it is in other studies (42), then winds would need to pass over relatively large areas of plant canopy to entrain significant numbers of bacteria.

The apparent immigration of bacterial cells to navel orange trees from other plant species seems most likely to have occurred via aerosol particles. Air temperatures were relatively low (5 to 12°C) during these studies; at these temperatures, the mobility of most insect vectors would have been limited. It is also noteworthy that similar types and proportions of fluorescent, yellow-pigmented, and Ice^+ bacterial strains were observed on both navel orange and other plant species and were recovered from the air by deposition. Since the abundance of Ice^+ bacterial strains is very low in soil and in other habitats other than on plants (30, 37), the airborne Ice^+ bacteria were presumably liberated from plants. Lindemann and coworkers demonstrated that plants are a relatively good source of airborne bacteria compared with other surfaces such as soil (23). This group also demonstrated that an airborne inoculum of *P. syringae* was important in the initiating epiphytic populations on bean plants since remote plantings of this host harbored relatively low numbers of this pathogen compared with those closer to infected fields (22). The differences in bacterial populations on citrus in this study are in agreement with their assertion that airborne immigrant bacteria are important in establishing epiphytic populations on plants. Unlike on beans, epiphytic populations on navel orange trees remain relatively low; the role of an immigrant inoculum may be relatively more important on a plant such as the navel orange than on bean plants, which can support rapid and sustained multiplication of the immigrant inoculum. The rate of deposition of bacterial cells onto gravity sedimentation plates measured in this study was similar to that observed in other locations (4, 24). Since the concentration and deposition of airborne bacteria are highly correlated (24), we have found evidence that plants can affect the local concentration of airborne bacteria. The cells released from plants apparently can remain viable for time periods sufficient to travel more than 100 m, leading to the observed gradients of bacteria away from such sources (Table 4). We therefore suspect that immigrant bacteria arriving at navel orange leaves come from both a background source of bacteria released from plants or from other sources relatively far away and locally from the epiphytic populations on nearby plants. The background airborne population may be enriched for those bacterial strains that are most tolerant of transport as dry particles for prolonged periods of time in the atmosphere and that such strains are not representative of those bacterial strains most capable of exploiting leaves. In contrast, the airborne bacterial populations near plants harboring large numbers of epiphytic bacteria may be enriched in those strains

capable of growth or survival on leaves such as those of the navel orange. Thus, not only might the number of immigrant bacteria be greater in the vicinity of other plant species, but the characteristics of the immigrant cells might facilitate colonization of navel orange leaves after immigration had occurred.

Interestingly, the gradient of cells deposited onto petri plates found in this study was substantially less steep than that observed in earlier studies in which individual strains were spray inoculated onto plants, thereby generating aerosols (19, 36, 42). Several differences in these studies may account for the various apparent dispersal gradients. Cells of *P. syringae* recovered from plant surfaces are apparently more tolerant of environmental stress than cells recovered from common culture media (46). Bacteria released from plant surfaces might be relatively tolerant of the stresses encountered during their transport to navel orange leaves (7, 46). The air during these studies was relatively cold and humid, in contrast to that of studies in which recombinant *P. syringae* strains were released into the environment (42). Such conditions would increase the survival of cells while in the air. While wind speeds observed during this study were relatively low (less than 5 m/s), they were frequently higher than those during the release of recombinant bacterial strains (2, 42). Taken together, these results suggest that relatively stress-tolerant bacterial cells could be rapidly transported as particles from the surface of other plant species and deposited in navel orange groves before significant loss of viability had occurred; such a process would result in a rather shallow dispersal gradient.

Even though citrus trees were rather massive compared with most of the other plant species near them in this study, little bacterial emigration apparently occurs from citrus trees because of the relatively low epiphytic populations that they harbor; this was evident from the lower rates of bacterial deposition observed within citrus groves than near other plant species (Table 5). Citrus trees therefore apparently constitute a relatively low-strength source for airborne bacteria. Since some plant species such as bluegrass harbored over 100,000-fold more bacteria per unit leaf mass (Table 1), even a low abundance of such plant species could be a significant source of airborne bacteria compared with navel orange trees. If there is a direct proportionality between the epiphytic population size of bacteria on a plant species and the numbers of bacterial cells immigrating from it (3), then it may be possible to identify those species that would act as the best sources of immigrant bacteria. Although this conjecture is not proven here, epiphytic bacterial population sizes were highest on navel orange trees proximal to plant species such as grasses, which had highest epiphytic population sizes; epiphytic population sizes and the observed bacterial gradients were lower in orchards adjacent to species such as alfalfa, which harbored lower numbers of bacteria (Tables 1 and 4).

Bacterial populations observed on navel orange trees in this study were relatively low compared with those observed on other plant species (9–12, 26, 33), even during winter months when moisture was present on leaves for prolonged periods. More importantly, the population sizes of bacteria of different types on navel orange leaves were much lower than on most other plant species located nearby (Table 1). Since bacterial population sizes are frequently measured at different times of the year or in different geographical locations, they often cannot be directly compared. Some studies, however, have reported differences in epiphytic populations in closely adjacent plant species at a given time (22, 33). Other studies have also shown that epiphytic bacterial populations on citrus species and olive trees are low relative to those on other plant species (6, 8, 26). The reason why citrus species and olive trees support relatively low population sizes is unclear. Low populations, however, have been loosely associated with the presence of a thick waxy cuticle, such as that seen on citrus and olive trees (33), which may limit nutrient diffusion onto the leaf (43). If the availability of nutrients on leaves limits bacterial populations on navel orange trees as it does on other plants (47, 48), thick cuticles could thus reduce the potential for epiphytic growth. If the availability of nutrients on navel orange leaves is relatively low, and if bacterial cells do not multiply extensively on navel orange leaves, the bacteria may be more quiescent than they are on plants such as beans, for which rapid bacterial growth and large population sizes have been described (10, 14, 45).

We found no evidence that bacteria exhibit net growth on the surface of navel orange leaves under California growing conditions. Since the inoculated strains of *P. syringae*, *P. fluorescens*, or *E. herbicola* did not increase in population size after application to leaves while total epiphytic populations did during the winter months (Fig. 2), it is tempting to speculate that most of the cells on navel orange leaves simply accumulate following airborne immigration and persist in a relatively quiescent state on leaf surfaces. While the apparent rate of deposition of bacterial cells onto leaf surfaces was measured only intermittently during winter months in this study, the measurements do allow an estimate of the cumulative number of cells which would have been deposited from airborne sources during the course of the winter. In this study, about 50 total bacteria were deposited on a 10-cm-diameter petri dish each hour. At this rate of immigration, about 1,000 total bacterial cells would have been deposited in a 78-cm^2 area each day. From early December to mid-January, total epiphytic populations increased on untreated navel orange trees from about 1×10^4 to about 3×10^5 cells per g; a net increase of about 300,000 cells per g (about 300,000 cells per 78 cm^2 of navel orange leaf) thus occurred. In this 40-day period, about 40,000 total bacterial cells would have been deposited on an area of about 78 cm^2 at the observed rate of immigration. This level of immigration, however, accounts for only about 15% of the increase in bacterial population size that occurred over this interval. It is possible that the rate of immigration estimated here is lower than the average over the season as a whole. Other studies had shown that the apparent rate of release of bacterial cells varies greatly with time of day and with other meteorological conditions such as strong winds and rain (4, 21, 23, 24, 39, 44). If the rate of immigration of bacterial cells to citrus is modestly higher than that estimated here, most, if not all, of the net increase in bacterial numbers might be due to immigration alone.

Bacterial ice nuclei appear important in limiting the supercooling ability of navel orange leaves. The numbers of Ice^+ bacteria on navel orange leaves were always quite low (Table 2). Because of the relatively low population size of Ice^+ bacteria, the supercooling temperature of navel orange leaves was rather low (Fig. 3). Navel orange leaves supercooled to a substantially lower temperature than that of other species harboring higher Ice^+ bacterial populations (5, 6, 11). Leaves from orchards adjacent to other navel orange trees or in the interior of all test orchards had the lowest Ice^+ bacterial populations and supercooled the most (Fig. 3). Similarly, the lowest incidence of frost damage was also observed on trees at these locations (Fig. 4). It is also noteworthy that frost damage was reduced substantially on trees on which Ice^+ populations on leaves were reduced by copper bactericide sprays (Fig. 4). These results all indicate that bacteria are important incitants of frost damage to navel orange trees in California and apparently represent the predominant ice-nucleating agents on this

species. These results are also consistent with our observations that freezing of navel orange fruit is most often initiated by propagation of ice from leaves in which they are in contact; enhancement of the supercooling of leaves should also reduce the likelihood that contiguous fruit avoid freezing. Nonbacterial sources of ice nuclei appear not to be important on navel orange trees in California as suggested for citrus species in Greece (5). The supercooling ability of navel orange leaves (about −5°C), which harbored about 10 to 100 Ice^+ bacterial cells per g of tissue, is close to that of bean leaves harboring similar numbers of Ice^+ cells (31). These results suggest that Ice^+ bacteria, even at the very low populations observed here, are still important in limiting the supercooling ability of navel orange leaves. These results support the conjecture that treatments that reduce the population size of Ice^+ bacteria on navel orange leaves should decrease the likelihood of frost damage by increasing their supercooling ability. The higher Ice^+ populations on navel orange trees in proximity to other species, however, indicates that greater decreases in Ice^+ populations would be needed on such trees to achieve the same degree of supercooling and hence a similar likelihood of avoiding frost damage as for trees with fewer immigrant Ice^+ bacteria. These results also suggest that management of vegetation in or near cultivated plant species such as the navel orange, whose epiphytic bacterial populations are strongly influenced by immigration, could be used to influence population sizes of Ice^+ bacteria and hence reduce the likelihood of frost damage.

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