Relative Humidity and the Survival of Epiphytic Bacteria with Buds and Leaves of Cucumber Plants

Curt Leben

Professor, Department of Plant Pathology, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster 44691.

Salaries and research support provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Journal Article No. 20-87.

Accepted for publication 10 August 1987 (submitted for electronic processing).

ABSTRACT

Leben, C. 1988. Relative humidity and the survival of epiphytic bacteria with buds and leaves of cucumber plants. Phytopathology 78:179-185.

Terminal buds of cucumber seedlings were inoculated with ice-nucleating-active isolates of *Pseudomonas syringae* pv. *lachrymans* (pathogenic to cucumber) or *P. s.* pv. *syringae* (pathogenic to bean), and the plants kept in a screened greenhouse at different levels of relative humidity (RH)—alternating (50–60% during the day and more than 90% at night), low (30–50%), or high (80 to more than 90%). The foliage was not wetted. Later, bacteria were detected with a selective medium. At low RH, no pathogens were detected by agar prints of healthy leaves; nor were they in terminal or axillary buds. At high RH, they were on nearly all of both bud types; at alternating RH, values were intermediate. Pathogen loci were distributed uniformly on the youngest, erratically on the intermediate, and sparsely on the oldest healthy leaves at high or alternating RH. Microscope observations of leaves showed that most bacteria were erratically distributed, even within 1–2 mm, on or near veins, parts that wetted best. Favored short-term survival sites were both types of buds and near the junction of the main veins and the petiole on the leaf lower surface. Field tests indicated that the terminal bud was colonized continuously by *P. s.* pv. *lachrymans*. Conclusions are that high RH favors continuous colonization of terminal buds, distribution of bacterial cells on unfolding plant parts, and survival of bacteria on exposed parts; low RH favors none of these events, which can be sequential.

Additional key words: Adherence, adhesion, ice-nucleating bacteria, INA bacteria, leaf wettability

During the last 20 years it has become widely recognized that a number of bacteria are capable of multiplying on the healthy plant shoot surface (1,11). Leaves have been studied the most and are thought to be the main habitats. The best-known of the epiphytic bacteria belong to the *Pseudomonas syringae* group. Some members are pathogens of plants, some have ice-nucleating activity, and some possess both characteristics (13,31,32). Consequently, the careful study of the life cycles of these organisms is warranted, not only because so many are pathogenic but also because isolates with ice-nucleating activity are receiving much attention for the control of plant frost damage.

Even though leaves have been studied extensively, very little is known about how epiphytic bacteria arrive on the leaf, where they multiply, and where and for how long they survive. Since most of these organisms are killed readily by drying and by ultraviolet light, it was suggested that they likely multiply and survive best for the short term (minutes or days) in "protected positions," i.e., places that are moist and subjected to little ultraviolet light (21). It also was suggested that shoot surface environments would be expected to change with weather conditions, especially with moisture changes, thereby causing perturbations in epiphyte populations. Indeed, several types of population changes have now been observed on leaves. These are seasonal variations (2,32), diurnal changes (12), and large variations in numbers of bacteria on individual leaves in the canopy of crop plants (9,10).

These variations suggest that additional study of the multiplication, survival, and protected survival sites of epiphytic bacteria would lead to a better understanding of their ecology. Our attention was drawn to the bud as a protected position, because earlier work, which has been reviewed (18,21), demonstrated that pathogenic and nonpathogenic bacteria could multiply in the bud, or *gemmisphere* (18), and that large numbers of bacteria are in the buds of some plants in the field (19). This is in contrast to the few bacteria seen on leaves (25). Moreover, recent studies suggest that buds may be a site of a portion of the life cycle of a number of pathogenic bacteria (3,6,8,14,15,34,35,37,43,44).

Relative humidity (RH) is believed to be important in the ecology of microbial epiphytes in general (17), but there are few studies of RH and bacteria in the absence of rain and arthropods, usually thought to be the most important transfer agents of bacteria on and among field plants. In the greenhouse part of the present work, rain and insects were excluded. The RH was maintained within ranges representing variations in nature.

The purpose of this paper is to describe the effects of RH on the distribution and survival of two epiphytic pathogens with buds and on leaves of cucumber plants (*Cucumis sativus* L.). The main intent was to examine the broad distribution of pathogen loci, and not to study total populations. The bacteria used, *P. s.* pv. *lachrymans* and *P. s.* pv. *syringae*, are pathogenic on cucumber and bean (*Phaseolus vulgaris* L.), respectively. Both pathovars possessed ice-nucleating activity as shown in the present studies. A short account of part of the work with *P. s.* pv. *lachrymans* appeared earlier (24).

MATERIALS AND METHODS

General procedure. Terminal buds of cucumber seedlings were inoculated with the pathogens. The plants were given a moist period, to establish bacteria, and then were taken to a greenhouse and kept at different RH levels. After further plant growth, parts were assayed to determine the location of the pathogen. In field tests, only the terminal buds of established, staked cucumber plants were inoculated with *P. s.* pv. *lachrymans*. After continued growth, symptoms on the entire plant were noted, and terminal buds were assayed.

Greenhouse tests. *Bacteria. P. s.* pv. *lachrymans* isolate 859, from a diseased cucumber leaf in Ohio, and *P. s.* pv. *syringae* isolate 622 (26), from a lima bean brown spot lesion, were used. In three tests, drops of water suspensions of either isolate, containing approximately 10^6 colony-forming units (CFU) per milliliter, froze when they were cooled to −4 C for 10 min (water controls did not freeze); thus both isolates had ice-nucleating activity. Stock cultures were stored on silica gel at −20 C (27). Working stock cultures were stored at 5 C on sucrose nutrient agar (SNA), containing nutrient agar (Difco), 23 g, and sucrose, 10 g/L. For plant inoculation, cell suspensions in water (approximately 10^7

CFU/ml) were made from SNA slants, usually seeded 7–10 days previously. Studies had indicated that cells this age survived better than younger ones (38).

Plants. Seedlings of the cucumber cultivar National Pickling were grown in an autoclaved peat-soil mixture in 10-cm-diameter pots supplied with a small amount of slow-release fertilizer (Osmocote, Sierra Chemical Co., Milpitas, CA). Individual plants were tied to a pole. The soil was watered automatically, and the foliage was not wetted. Few plants produced axillary shoots. No fruit was formed, because no pollinating insects were present.

Temperature and RH. Tests were made in a shaded, screened greenhouse. The temperature was 27 C in the day and 21 C at night (±2 C). When the upper limit was exceeded in the summer, the house was cooled by passing outside air through water-saturated pads. The summer temperature variation was ± 5 C, except on some warm days when the high was 34 C. Fans provided a constant, slow movement of air, either by recirculation or by exhausting outside when cooling was required. Supplemental fluorescent light was provided 12–14 hr during the day.

Tests with plants at continuously low or high RH were completed in the winter, when the influence of the weather outside the greenhouse was least. Plants at low RH (30–50%, except during occasional periods of high outside RH) were in the open air of the greenhouse. Those at high RH were in a chamber (1.3 × 2.6 × 0.8 m high) covered with polyethylene film, within the same greenhouse. Daytime RH in the chamber (usually more than 80%) was provided by a continuously wet polyester mat in the chamber bottom; at night, when the temperature was lower and the lights were off, the RH was increased to more than 90% with an ultrasonic water vaporizer (Robeson Co., Castile, NY) operating periodically. The humidity was recorded with hygrothermographs. No drops of water were observed on the plants at any time. The light level on the plants inside the chamber was essentially the same as on those outside.

Tests with alternating RH were completed in the same greenhouse, during the summer and early autumn months. Day RH was 50–60% (occasionally higher when the outside RH was higher). Night RH was greater than 80% (often greater than 90%).

Inoculation. In the cell mass method, used for the low- and high-RH studies, a 0.5-mm^3 mass of bacterial cells (approximately 10^7 CFU) was taken from a slant culture with a small, flat needle and gently placed on one side of a terminal bud of a seedling with one or two expanding leaves. This was followed by a 0.05-ml drop of water. For plants at alternating RH, a terminal bud was inoculated by inserting a small piece of sterile blotting paper containing 0.01 ml of bacterial suspension behind the outermost leaf of the bud (less than 1 cm long) or by misting about 0.01 ml of bacterial suspension onto the bud with a plastic household water mister (expanding leaves below were shielded from the mist).

Moist period. Prior to inoculation, plants were placed in a dew chamber (Percival Co., Boone, IA) for 5–10 hr with air at 22–24 C. After inoculation, they were returned to the dew chamber for 1 day and then placed at a given RH.

Assay of bacteria associated with plant organs. Colonies of *P. s.* pv. *lachrymans* and *P. s.* pv. *syringae* were identified on a selective agar medium described for another *P. syringae* pathovar (20). It was modified to consist of 2 g instead of 1 g of boric acid per liter and is designated M72. After 3 days of incubation at 24 C, *P. s.* pv. *lachrymans* colonies are about 1 mm in diameter (see the color photograph in reference 22). Colonies of *P. s.* pv. *syringae* are similar, but are 1 mm in diameter in 2 days.

Confirmation of presumed *P. s.* pv. *lachrymans* and *P. s.* pv. *syringae* colonies was by a pathogenicity test. One or more representative colonies were taken up with a moist cotton swab and rubbed on a cucumber seedling leaf previously dusted with an abrasive. Typical water-soaked chlorotic lesions were produced on the lower side of the leaf, 5–7 days after inoculation with *P. s.* pv. *lachrymans*, and enlarged with time. Under the same conditions, *P. s.* pv. *syringae* produced chlorosis and a collapse of leaf tissue; there was little or no water soaking, and the lesions did not expand. Confirmation of both pathogens from terminal buds and from most leaves and axillary buds was determined by this method in all early tests. The confirmation rate was greater than 98%.

Assay of plant parts. Terminal buds were removed with sterile forceps and ground (with a sterile mortar and pestle) in 1 ml of water, and 0.05 ml was "run-streaked" (23) on M72 in a petri dish.

Axillary buds were likewise removed, rubbed in a line for 3–5 cm on the medium, and then inserted into and left in the medium.

The two youngest leaves (less than 3 cm long) below the terminal bud were designated *expanding leaves*. The lower and upper leaf surfaces were pressed to M72 and then removed.

The remaining leaves (L1, L2, etc., with L1 the oldest) were designated *expanded leaves*. The lower leaf surface was pressed firmly on M72, and the leaf was left on the medium for 2–4 hr, to ensure transfer of bacteria, and then removed. The area near the vein-petiole junction always was printed if a leaf was larger than a petri dish. To avoid artifacts when printing, it is essential to use a medium with a dry surface. Quantitative population studies cannot be made with printing methods, because one or many bacterial cells may produce a colony locus on agar. However, colony loci may be estimated or counted. Their location on the agar indicates the position of a living bacterium or bacteria on the printed part.

Observation of bacteria on the plant surface. The part to be observed was bleached by enclosing in a gas-tight chamber (0.5 L) containing 1 g of trichloroisocyanuric acid (97% purity) plus 10 ml of water. The specimen was elevated above the mixture so that it was bleached by evolving gas. Satisfactory removal of chlorophyll took place in 4–24 hr, depending on age, thickness, etc. The bleached part was adhered to a microscope slide with double-stick tape, stained with carbol fuchsin, and gently rinsed several times in large volumes of water. The specimen was examined without a cover slip under oil at 1,000×. The basic method was described elsewhere (4).

Field tests. There were four field tests, separated by more than 300 m, each consisting of 10–13 cucumber plants, half of which were inoculated with *P. s.* pv. *lachrymans*. Seedlings of the cultivar National Pickling were transplanted in the field on 29 May and 5 June 1986, with the systemic insecticide carbofuran in the planting hole. Plants were staked, 2.6 m apart, in one row. Control plants (not inoculated) were planted toward the direction of the prevailing wind. Plants were sprayed individually with the insecticide carbaryl every 7–10 days (leaves were barely wetted, so that runoff would not take place).

After the plants were well established, a terminal bud, consisting of a mass of leaves (less than 4 cm long), tendrils, and flower and shoot buds, was inoculated with approximately 0.5 ml of a suspension of *P. s.* pv. *lachrymans* (approximately 0.5×10^7 CFU), applied as a mist from a household mister. Lower leaves were screened from the inoculum with a circular plastic shield slit to receive the stem below the bud. Inoculations were made in the evening, so that the buds would remain moist with dew for more than 12 hr. Plots were inoculated between 2 and 15 July, with the earlier plantings inoculated first. The experiments were completed by 4 August, when the terminal buds of some plants stopped growth naturally.

Terminal buds were assayed twice. The first assay was nondestructive: the terminal bud structure, consisting of a mass of small leaves, tendrils, and buds 1–2 cm wide, was pressed several times against the surface of M72 medium in a petri dish. In the final assay, terminal buds were ground and run-streaked as described above.

RESULTS

Greenhouse tests. *Influence of alternating RH on the distribution of P. s. pv. lachrymans.* RH and temperatures in the greenhouse tests approximated conditions in our fields in the summer, except for dew, i.e., mostly warm and fairly humid in the day and cooler and highly humid at night. All terminal buds, about 700 axillary buds or bud parts, and about 800 leaves were assayed for *P. s.* pv. *lachrymans* 19–28 days after the terminal buds were inoculated. At the end of the experiments, 62% of the plants carried this ice-nucleating-active (INA) pathogen in the terminal portion, i.e., in the terminal bud and on the two expanding leaves below the

TABLE 1. Influence of relative humidity (RH) on distribution of *Pseudomonas syringae* pv. *lachrymans* with time on cucumber plants after inoculation of terminal buds of seedlings

Day	Plant development and plant assays[a]	Number of plants with *P. s.* pv. *lachrymans*[b]		
		Alternating RH (day, 50–60%; night, >80%)[c]	Low RH (30–50%)[d]	High RH (80 to >90%)[d]
0	Terminal bud (<1 cm long) inoculated with pathogen; plants with one or two leaves (L1, L2), 8–10 cm high	+[e]	+	+
7–8	Pathogen isolated from a small leaf (<1.5 cm long, L5 or L6) removed from the terminal bud	—	11/21	21/21
21–23	Pathogen isolated from terminal bud (<1 cm long) and from the two youngest expanding leaves (L11–L13); plants 50–70 cm high	—	0/20[f]	18/18[f]
19–28	Pathogen isolated from terminal bud (<1 cm long) and from the two youngest expanding leaves (L14–L17); plants 70–110 cm high	68/110	—	—

[a] L1 = oldest leaf on a plant, L2 = next oldest, etc. See text and Figures 1 and 2 for the distribution of the pathogen on older leaves and axillary buds. Plants were staked and grown in the greenhouse.
[b] The numerator is the number of plants with positive isolation; the denominator is the number of plants.
[c] Totals from 12 tests at different times.
[d] Totals from three tests at different times.
[e] Plus indicates that all plants were inoculated. Control plants were not inoculated, and the pathogen was not isolated from them (see text).
[f] The total is less than 21 plants because the terminal buds of some plants were killed, probably by the pathogen entering the wound made when leaves were taken from buds for assay at 7–8 days.

bud (Table 1). With those plants carrying the pathogen in the terminal portion, there were progressively fewer *P. s.* pv. *lachrymans* loci on older than on younger leaves. On the two expanding leaves below the bud, the pathogen appeared to be distributed uniformly (Fig. 1A and B). The bacterium was on nearly all of the two next youngest expanded leaves (Fig. 1C and D), often in a fan pattern near the vein-petiole junction (Fig. 1C). If the fan pattern was on one leaf, it usually would be smaller on the next older leaf below. On the oldest leaves, the pathogen often was confined to an area a few millimeters from the vein-petiole junction (Fig. 1E, near junction). Except in the fan pattern, however, the distribution on expanded leaves varied. Sometimes *P. s.* pv. *lachrymans* was more or less uniform on the whole leaf or a portion of it (Fig. 1D and E and Fig. 2). Most often, however, the bacterium was associated with short, long, or interrupted portions of the main veins (Fig. 1E and F). On many of the oldest leaves, the pathogen was not detected at all, and on those with lesions (see below) it often was associated only with lesions. Inspection of the print area of the agar surface showed that pathogen colonies were associated primarily with veins and trichomes on veins. The interveinal regions usually appeared to be devoid of bacteria.

The distribution of *P. s.* pv. *lachrymans* on parts of a given plant also varied. For example, some plants carried the pathogen only in the terminal portion. With other plants, it was found in this portion and on some of the lower leaves but not on the intervening leaves. Generally, however, loci were less prevalent on older leaves, as described above. Also, early tests demonstrated that there were more loci on the lower than on the upper sides of leaves, which may in part be due to topography. Since both leaf surfaces could not be printed without disturbing one to them, except as noted in Materials and Methods, only the lower leaf surface was used. This compromise seemed reasonable because the lower side likely would be more protected than the upper.

If *P. s.* pv. *lachrymans* was not found in the terminal portion of a plant, it was not present on other plant parts, except as noted below.

Plants bearing *P. s.* pv. *lachrymans* in the terminal portion also carried the pathogen in axillary buds (Fig. 2). These buds evidently were short-term survival sites, because there were many examples where the pathogen was not on the leaf but was in the associated axillary bud. *P. s.* pv. *lachrymans* was detected on each axillary bud component (shoot terminal buds, staminate or carpellate flower buds, and coiled tendrils). It also was found on the distal portion of young, uncoiled tendrils. A few plants produced shoots 10–15 cm long from axillary buds of lower leaves; the pathogen was detected in terminal and axillary buds and on leaves of these shoots.

About one fourth of the plants had leaf spots incited by *P. s.* pv.

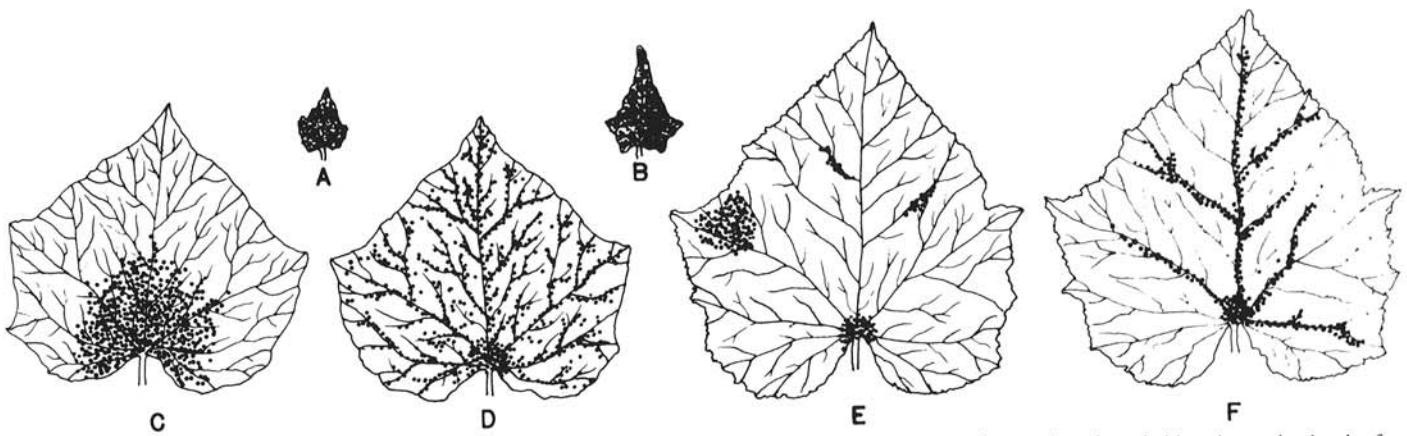

Fig. 1. Examples of distribution patterns of two *Pseudomonas syringae* pathovars on differently aged leaves of cucumber plants held at alternating levels of relative humidity (RH), low in the day and high at night. The terminal buds of small seedlings were inoculated previously. Plants were protected from insects, and foliage was not wetted. **A and B,** Closely set bacterial loci on youngest leaves. **C and D,** Distribution patterns commonly seen on the next or the two next older expanding leaves: **C,** fan pattern (see text); **D,** general distribution, associated with veins. **E and F,** Examples of erratic distribution on the next older expanded leaves. The oldest leaves (not shown) usually had fewer erratically distributed or no detectable bacteria. The location of bacteria was determined by printing leaves on a selective agar medium. At continuously high RH, patterns were similar but usually were more dense; at continuously low RH, no bacteria were detected.

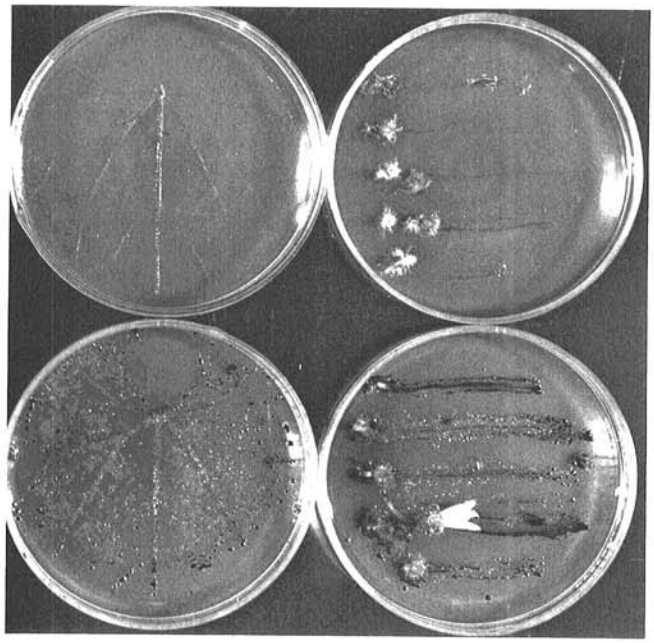

Fig. 2. The influence of relative humidity on the distribution of *Pseudomonas syringae* pv. *lachrymans* from the terminal buds of cucumber plants. Terminal buds were inoculated 28 days previously. In the top row, from a plant grown at low relative humidity, no bacteria are present. In the bottom row, from a plant grown at high relative humidity, only colonies of the pathogen are present; a dye in the selective agar medium produces characteristic red colonies (which appear black in the photograph). Expanded leaves (left) were printed in the agar medium. Axillary bud structures (right) from five successive leaf nodes were rubbed on the agar surface horizontally and left embedded.

lachrymans on the leaves that were outermost when the bud was inoculated. There also were isolated lesions on a few of the younger leaves.

Influence of continuously low or high RH on the distribution of P. s. pv. lachrymans. Three tests demonstrated clearly that this isolate of *P. s.* pv. *lachrymans* was able to survive in terminal buds at high RH but not at low RH (Table 1).

At continuously low RH, the pathogen was not isolated from terminal buds (Table 1), axillary buds (Fig. 2), or leaves (Fig. 2), except the one or two leaves that were outermost when the bud was inoculated. These leaves sometimes bore lesions. Apparently *P. s.* pv. *lachrymans* died out of terminal buds at low RH, as indicated by assay results at 7–8 days compared with those at 21–23 days (Table 1).

At continuously high RH, the distribution pattern on various parts of the plant was similar to that described above for alternating RH. However, since more *P. s.* pv. *lachrymans* loci were present, the patterns on leaves were not so distinct as those shown for alternating RH in Fig. 1, and the distribution was erratic on most expanded leaves. Survival of the pathogen in terminal buds at continuously high RH was greater than at alternating RH, 100% instead of 62% (Table 1). There were more lesions on leaves that were outermost when the bud was inoculated and more scattered lesions on younger leaves; lesions on leaves of bud-inoculated plants at high RH had been noted previously (5,6).

At high RH there was a limited "systemic invasion" (36) by the pathogen. Thus, on 12 of the 21 plants, water-soaked areas were formed on or near veins in the fan region of lower leaves. Subsequently drops containing high numbers of *P. s.* pv. *lachrymans* cells formed on the petiole, which usually became flaccid and died. Water-soaked lesions extended downward a short distance on the stem from the petiole-stem junction of some of these leaves (36).

Microscope observation of P. s. pv. lachrymans on the leaf. Microscope observations of the lower surface of leaves from plants at high RH verified the distribution of bacteria as determined by leaf prints. At 1,000× bacteria were seen on short, long, and interrupted portions of veins. They were not on all veins of a leaf. Large numbers of bacteria were observed in depressions between vein epidermal cells as well as in depressions between basal cells of vein trichomes. They were not seen on the distal third of trichomes or within them, and there were comparatively few on the terminal cells of glandular trichomes. Even within 1–2 mm along epidermal vein cells, bacterial cells varied from none to enormous numbers that could be seen at 100×. In interveinal regions, bacteria occasionally were seen in depressions between base cells of trichomes. They were rare between epidermal cells or near stomata.

The distribution of bacterial cells on the upper surface of leaves was also examined. As with the lower surface, they were associated irregularly with vein epidermal cells and base cells of vein trichomes and were uncommon on interveinal areas. In addition, they were in narrow bands on epidermal cells 1–2 mm away from the center of depressions over larger veins.

In previous tests it was demonstrated that *P. s.* pv. *lachrymans* cells adhered primarily to veins when cucumber leaves were immersed in suspensions (28). Additional adherence tests using the same methods were completed, and leaf surfaces examined microscopically. These observations showed that the adherence patterns were similar to the distribution patterns described above.

Multiplication of P. s. pv. lachrymans. The total number of cells produced by *P. s.* pv. *lachrymans* on a plant could not be determined by assaying plant parts at the end of an experiment, because cells appeared to die on exposed leaves. However, numbers applied to the seedling terminal bud and found in the terminal bud at the end of an experiment could be compared. In four tests at alternating RH, the numbers in terminal buds of six plants were determined 21–28 days after the buds were inoculated with approximately 10^5 CFU. There was an average of 7.6×10^6 CFU per bud (with a range of $2–20 \times 10^6$), a 70-fold increase. This does not take into account pathogen cells that had died or were still living on leaves and other structures that unfolded since inoculation.

Influence of RH on the distribution of P. s. pv. syringae. In tests parallel with those described for *P. s.* pv. *lachrymans*, the bean pathogen was examined with cucumber plants at the different RH levels. This ice-nucleation-active isolate produced a hypersensitive reaction and a chlorosis and drying of leaf tissue when cucumber leaves were inoculated with an abrasive to test for pathogenicity. These symptoms were not observed in the RH tests.

At continuously low RH, *P. s.* pv. *syringae* was found in the terminal buds of five of the 21 plants when a small leaf was assayed 7–8 days after inoculation; at the final assay, 17–21 days after inoculation, it was found in none of the terminal buds. The pathogen was not found on any leaf at low RH, except the two that were outermost when the bud was inoculated; nor was it in axillary buds. In contrast, at continuously high RH, 19 of the 21 plants carried *P. s.* pv. *syringae* in the terminal bud or on the two youngest leaves at the final assay. At alternating RH, there were 65 plants in seven tests. The bacterium was in 37% of the terminal buds. The distribution on leaves and in axillary buds at high or alternating RH was similar to that described above for *P. s.* pv. *lachrymans* (Figs. 1 and 2).

Other bacteria. Bacteria other than the inoculated *P. s.* pv. *lachrymans* and *P. s.* pv. *syringae* were isolated from less than 10% of the plants at alternating or high RH. Usually "contaminant" colonies were small or low in number and did not appear to inhibit the pathogen. A few, however, were numerous or grew quickly, so that pathogen colonies could only be detected when well separated from the others. Thus, when a pathogen was not found, it could have been present but inhibited in culture. Usually there was only one contaminant on a plant, but different plants in a given experiment often carried different contaminants. The distribution of contaminants was similar to that of the pathogens (Figs. 1 and 2). In contrast to these results with plants at alternating or high RH levels, terminal buds of only a few plants at low RH carried contaminants, even when isolations were made on the nonselective medium SNA (plus cycloheximide, 50 mg/L, to inhibit fungi).

Leaf wettability. The distribution patterns of pathogen and contaminant loci on leaves were positively related to the wettability of the cucumber leaf. Wettability (as defined here) was demonstrated by examining leaves that had been immersed briefly in water, sprayed with water, or taken from plants that had been in the dew chamber. After most of the water had drained or evaporated, surfaces were examined with reflected light and a stereo microscope (7–30×). On the upper surface, only the depressions over veins appeared to be wet, in the form of a narrow continuous ribbon. The location of bands of bacterial cells observed with the microscope was at the edges of these ribbons. On drying, the ribbons became narrower and then irregularly segmented. On the lower leaf surface, with its protruding veins, there were discontinuous areas of water on veins and on vein flanks. The interveinal areas of both surfaces appeared not be wetted, except for an occasional residual water drop.

Additional detail was obtained by immersing leaves briefly in India ink (Universal 3080F, Koh-i-noor Rapidograph, Bloomsbury, NJ), 0.1 ml in 20 ml of water. When the leaves had drained and dried, they were examined at 7–30×. The deposit patterns were similar to those described for water. In addition, discontinuous patterns between vein epidermal cells and vein trichome base cells on both sides of leaves were observed at 1,000×. These patterns were similar to those of bacterial cells on leaves from plants at high RH or on leaves in adherence tests with *P. s.* pv. *lachrymans.*

Plant-to-plant transfer. Lesions were never observed on control plants, and pathogens were not isolated from them. The same was true with uninoculated leftover plants growing in the same greenhouse, despite repeated isolation attempts, particularly from terminal buds. Thus, there appears to have been little transfer of bacteria, by the continuously moving air of the greenhouse or other means.

Field tests. The results of all four tests with *P. s.* pv. *lachrymans* were similar, with variation in detail, depending primarily on plant development when the plants were inoculated and when the experiments were terminated (Table 2). The first lesions on leaves were seen 4–8 days after bud inoculation. By 11–14 days after inoculation (10 cm of rain had fallen after inoculation), all of the 15–20 expanded leaves present before inoculation bore large numbers of typical angular lesions. Since these leaves had been shielded during the application of inoculum to buds, the pathogen presumably was transferred downward to them from buds by rain. There were also scattered small lesions on most of the four to seven leaves that expanded after inoculation. The experiments were terminated when some of the terminal buds naturally stopped growing. By the end of the experiments, most leaves expanding after bud inoculation bore lesions.

Control plants also became diseased during the course of the experiments (Table 2). Although aerosol and rain transfer cannot be excluded, inoculum probably was brought from inoculated plants by insects, which were observed despite the use of insecticides. However, symptoms on control plants were fewer and observed later than on inoculated plants (Table 2).

Terminal buds of plants were assayed twice, and the pathogen was found only in buds from inoculated plants (Table 2). The pathogenicity of *P. s.* pv. *lachrymans* was proved for representative isolates. Even though M72 medium is selective, there were many other kinds of bacteria in field buds, in contrast to comparatively few found in terminal buds of greenhouse plants. These may have inhibited the pathogen during assay, so the numbers in Table 2 may be understated.

The results in Table 2 do not prove without question that there was a continuous colonization of terminal buds of field plants by *P. s.* pv. *lachrymans.* Downward movement by rain early in the season clearly was responsible for the heavy infection on the lower leaves of inoculated plants. Later, when rainfall was low (Table 2), the upper leaves produced after bud inoculation also became infected, which is taken as further evidence for bud colonization.

DISCUSSION

The results of greenhouse tests help to explain the variations in populations of epiphytic bacteria on leaves observed in the field. Aside from the movement of these organisms by rain and insects, RH in the absence of these transport agencies was shown to have a profound effect on epiphyte activities and survival. As expected on the basis of previous observations and experiments, high RH was more favorable than low RH for bacterial activity. However, as explained below, these studies further suggest a general and sequential influence of RH in nature, probably in many bacterium-plant species combinations. For example, results with cucumber

TABLE 2. Distribution of *Pseudomonas syringae* pv. *lachrymans* with time from inoculated terminal buds of staked field cucumber plants

Day[a]	Plant development, plant assays, and rainfall[b]	Inoculated plants[c]	Control plants[c]
0	Plants 0.8–1.2 m high, with 15–20 expanded leaves	Pathogen suspension (about 0.5×10^7 colony-forming units) mist-inoculated on terminal buds; leaves below shielded from inoculum	Water misted on terminal buds
4–8	Small new leaves unfolding from terminal bud	Small lesions on largest new leaves	No lesions
6–13	Terminal buds assayed for pathogen[d]	Pathogen detected in 83% of plants	Pathogen not detected
11–14	Four to seven leaves expanded after day 0	Large, typical lesions on 15–20 leaves expanded before day 0; small lesions on most leaves expanded after day 0	Lesions on one leaf expanded before day 0
0–14	Rain: 10 cm	Rain: 10 cm	Rain: 10 cm
21–28	Plants 2–3 m high, with 15–27 leaves expanded after day 0	Large lesions on 15–20 leaves expanded before day 0; smaller lesions on most leaves expanded after day 0	Scattered lesions on most leaves expanded before day 0; a few small lesions on 10% of leaves expanded after day 0
21–28	Terminal buds assayed for pathogen	Pathogen detected in 39% of plants	Pathogen not detected
14–28	Rain: 3 mm	Rain: 3 mm	Rain: 3 mm

[a] A range is given because in the four tests the planting, inoculation, pathogen assay, and test termination times were necessarily different (see text).
[b] Axillary shoots and fruit were removed from plants throughout the tests. One stem was trained to stakes 3 m high.
[c] A total of 23 plants in four tests.
[d] This assay did not destroy terminal buds.

were similar with two INA *P. syringae* pathovars and with other unidentified epiphytic bacteria.

It also was demonstrated that the cucumber terminal bud may be a site of continuous multiplication of the two pathogens and other epiphytic bacteria. Inoculum of *P. s.* pv. *lachrymans*, for example, was placed on terminal buds of young plants, and the pathogen was recovered later in the terminal buds of the same plants, with an increase of bacterial numbers, which also was reported previously (6). As this bacterium also was found on leaves and in axillary buds that developed in the interim, one must conclude that the bud also served to distribute the bacterium to these other plant parts as they unfolded, perhaps with additional multiplication on some young parts at high RH. The diminishing number of *P. s.* pv. *lachrymans* loci as the leaves grew older indicated that the bacterium died gradually, even at high RH. Distribution of epiphytic bacteria from the gemmisphere was suggested before (18), but extinction on leaves has received little attention.

The favorable influence of high humidity, at least for part of the day, on the life cycle of an epiphytic bacterium may be viewed as a series of events. First, there is bud colonization, then distribution of bacteria on plant parts as they unfold, and finally survival on these parts. The work suggests that with alternating RH in the absence of rain, as in arid regions with heavy dews and soil irrigation, there can be colonization of terminal buds with an attendant distribution of the pathogen on unfolding plant parts. Few or no lesions would be seen on these field plants, even though high numbers of living pathogen cells are present in buds and on some leaves. The increased death rate of epiphytic pathogens could explain decreased populations on leaves at low RH or during a period of dry weather (7,16,34,39,40).

The present work identified three protected positions where epiphytic bacteria may survive over the short term. These sheltered places are terminal buds, axillary buds, and leaf veins near the petiole junction, or fan region, of the lower leaf surface. When populations of *P. s.* pv. *lachrymans* are high in the fan region, the plant may become systemically invaded. In other work from this laboratory the fan pattern was noted with nonpathogenic epiphytic bacteria, but its significance was not recognized (16).

As noted by others (2,19,33) and in the present study, bacteria are usually associated with vein epidermal cells and with basal cells of trichomes on veins. However, there was a widely erratic distribution on a single leaf (Fig. 1). These irregularities appeared to be related to the wettability of the surface: bacteria were found in areas that best retained water. This subject deserves further study.

In these studies, both *P. syringae* pathovars had ice-nucleating activity, which indicates that for field studies of pathogens or INA bacteria, buds and the youngest expanding leaves as well as older leaves should be examined for bacterial populations. Many bacteria found on older leaves may be recent arrivals, having been washed downward from buds, as demonstrated by the appearance of *P. s.* pv. *lachrymans* lesions on leaves below inoculated buds in these field studies and as indicated by other work (41). There is evidence that the pathogen was also distributed by insects, despite the use of insecticides and as demonstrated by the the late appearance of lesions on control plants. There may also have been distribution in air (29,30,42), but this evidently did not take place in the greenhouse.

It is concluded that on plants in nature, where there are many interacting biological, chemical, and physical variables, details of the colonization, distribution, and survival of epiphytic bacteria will be found to be uneven and difficult to predict. Occasional erratic distribution of specifically added rhizosphere bacteria with roots similarly has been noted (David M. Weller, *personal communication*). Thus, patchy populations of bacteria on plant surfaces may be common.

LITERATURE CITED

1. Blakeman, J. P. 1982. Phylloplane interactions. Pages 307-333 in: Phytopathogenic Prokaryotes, Vol. 1. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.
2. Blakeman, J. P. 1985. Ecological succession of leaf surface microorganisms in relation to biological control. Pages 6-30 in: Biological Control on the Phylloplane. C. E. Windels and S. E. Lindow, eds. American Phytopathological Society, St. Paul, MN.
3. Burr, T. J., and Katz, B. H. 1984. Overwintering and distribution pattern of *Pseudomonas syringae* pv. *papulans* and pv. *syringae* in apple buds. Plant Dis. 68:383-385.
4. Daft, G. C., and Leben, C. 1966. A method for bleaching leaves for microscope investigation of microflora on the leaf surface. Plant Dis. Rep. 50:493.
5. de Lange, A., and Leben, C. 1970. Colonization of cucumber buds by *Pseudomonas lachrymans* in relation to leaf symptoms. Phytopathology 60:1865-1866.
6. de Lange, A., and Leben, C. 1971. The cucumber bud as a possible factor in the pathogenesis of *Pseudomonas lachrymans*. Pages 391-393 in: Ecology of Leaf Surface Micro-organisms. T. F. Preece and C. H. Dickinson, eds. Academic Press, London.
7. Diab, S., Bashan, Y., Okon, Y., and Henis, Y. 1982. Effects of relative humidity on bacterial scab caused by *Xanthomonas campestris* pv. *vesicatoria* on pepper. Phytopathology 72:1257-1260.
8. Dueck, J., and Morand, J. B. 1975. Seasonal changes in the epiphytic population of *Erwinia amylovora* on apple and pear. Can. J. Plant Sci. 55:1007-1012.
9. Hirano, S. S., Baker, L. S., and Upper, C. D. 1985. Ice nucleation temperature of individual leaves in relation to population sizes of ice nucleation active bacteria and frost injury. Plant Physiol. 77:259-265.
10. Hirano, S. S., Nordheim, E. V., Arny, D. C., and Upper, C. D. 1982. Lognormal distribution of epiphytic bacterial populations on leaf surfaces. Appl. Environ. Microbiol. 44:695-700.
11. Hirano, S. S., and Upper, C. D. 1983. Ecology and epidemiology of foliar bacterial plant pathogens. Annu. Rev. Phytopathol. 21:243-269.
12. Hirano, S. S., and Upper, C. D. 1984. Diurnal changes in population sizes and ice nucleation activity of *Pseudomonas syringae* on snap bean (*Phaseolus vulgaris* L.) leaflets. (Abstr.) Phytopathology 74:825.
13. Hirano, S., and Upper, C. D. 1985. Ecology and physiology of *Pseudomonas syringae*. Biotechnology 3:1073-1078.
14. Latorre, B. A., González, J. A., Cox, J. E., and Vial, F. 1985. Isolation of *Pseudomonas syringae* pv. *syringae* from cankers and effect of free moisture on its epiphytic populations on sweet cherry trees. Plant Dis. 69:409-412.
15. Latorre, B. A., and Jones, A. L. 1979. *Pseudomonas morsprunorum*, the cause of bacterial canker of sour cherry in Michigan, and its epiphytic association with *P. syringae*. Phytopathology 69:335-339.
16. Leben, C. 1965. Influence of humidity on the migration of bacteria on cucumber seedlings. Can. J. Microbiol. 11:671-676.
17. Leben, C. 1965. Epiphytic microorganisms in relation to plant disease. Annu. Rev. Phytopathol. 3:209-230.
18. Leben, C. 1971. The bud in relation to the epiphytic microflora. Pages 117-127 in: Ecology of Leaf Surface Micro-organisms. T. F. Preece and C. H. Dickinson, eds. Academic Press, New York.
19. Leben, C. 1972. Micro-organisms associated with plant buds. J. Gen. Microbiol. 70:327-331.
20. Leben, C. 1972. The development of a selective medium for *Pseudomonas glycinea*. Phytopathology 62:674-676.
21. Leben, C. 1974. Survival of plant pathogenic bacteria. Ohio Agric. Res. Dev. Cent., Spec. Circ. 100. 21 pp.
22. Leben, C. 1981. How plant-pathogenic bacteria survive. Plant Dis. 65:633-637.
23. Leben, C. 1983. Association of *Pseudomonas syringae* pv. *lachrymans* and other bacterial pathogens with roots. Phytopathology 73:577-581.
24. Leben, C. 1986. Survival of a *Pseudomonas syringae* pv. *lachrymans* INA isolate in buds of cucumber seedlings. (Abstr.) Phytopathology 76:1086.
25. Leben, C., and Daft, G. C. 1967. Population variations of epiphytic bacteria. Can. J. Microbiol. 13:1151-1156.
26. Leben, C., Schroth, M. N., and Hildebrand, D. C. 1970. Colonization and movement of *Pseudomonas syringae* on healthy bean seedlings. Phytopathology 60:677-680.
27. Leben, C., and Sleesman, J. P. 1982. Preservation of plant-pathogenic bacteria on silica gel. Plant Dis. 66:327.
28. Leben, C., and Whitmoyer, R. E. 1979. Adherence of bacteria to leaves. Can. J. Microbiol. 25:896-901.
29. Lindemann, J., Constantinidou, H. A., Barchet, W. R., and Upper, C. D. 1982. Plants as sources of airborne bacteria, including ice nucleation-active bacteria. Appl. Environ. Microbiol. 44:1059-1063.
30. Lindemann, J., and Upper, C. D. 1985. Aerial dispersal of epiphytic bacteria over bean plants. Appl. Environ. Microbiol. 50:1229-1232.
31. Lindow, S. E. 1982. Epiphytic ice nucleation-active bacteria. Pages 335-362 in: Phytopathogenic Prokaryotes, Vol. 1. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.
32. Lindow, S. E. 1983. The role of bacterial ice nucleation in frost injury

to plants. Annu. Rev. Phytopathol. 21:363-384.
33. Mariano, R., and McCarter, S. M. 1985. Scanning electron microscopy observation of *Pseudomonas syringae* pv. *syringae* and *P. syringae* pv. *tomato* on tomato and epiphytic weed hosts. (Abstr.) Phytopathology 75:1381.
34. Moffett, M. L., and Wood, B. A. 1985. Resident population of *Xanthomonas campestris* pv. *malvacearum* on cotton leaves: A source of inoculum for bacterial blight. J. Appl. Bacteriol. 58:607-612.
35. Mulrean, E. N., and Schroth, M. N. 1982. Ecology of *Xanthomonas campestris* pv. *juglandis* on Persian (English) walnuts. Phytopathology 72:434-438.
36. Pohronezny, K., Leben, C., and Larsen, P. O. 1977. Systemic invasion of cucumber by *Pseudomonas lachrymans*. Phytopathology 67:730-734.
37. Roos, I. M. M., and Hattingh, M. J. 1986. Pathogenic *Pseudomonas* spp. in stone fruit buds. Phytophylactica 18:7-9.
38. Sleesman, J. P., and Leben, C. 1976. Bacterial desiccation: Effects of temperature, relative humidity, and culture age on survival. Phytopathology 66:1334-1338.
39. Smitley, D. R., and McCarter, S. M. 1982. Spread of *Pseudomonas syringae* pv. *tomato* and role of epiphytic populations and environmental conditions in disease development. Plant Dis. 66:713-717.
40. Timmer, L. W., and Marois, J. J. 1986. Effect of humidity and host plant on epiphytic growth and survival of xanthomonads. (Abstr.) Phytopathology 76:847.
41. Umekawa, M., Watanabe, Y., and Inomata, Y. 1981. Facilitative effect of rainfall on the transmission of the pathogen and the development of angular leaf spot of cucumber. Ann. Phytopathol. Soc. Jpn. 47:346-351.
42. Vennette, J. 1982. How bacteria find their hosts. Pages 3-30 in: Phytopathogenic Prokaryotes, Vol. 2. M. S. Mount and G. H. Lacey, eds. Academic Press, New York.
43. Wimalajeewa, D. L. S., and Flett, J. D. 1985. A study of populations of *Pseudomonas syringae* pv. *syringae* on stonefruits in Victoria. Plant Pathol. 34:248-254.
44. Wrather, J. A., Sappenfield, W. P., and Baldwin, C. H. 1986. Colonization of cotton buds by *Xanthomonas campestris* pv. *malvacearum*. Plant Dis. 70:551-552.