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# Effect of Aerosolization on Subsequent Bacterial Survival

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**To determine whether aerosolization could impair bacterial survival, *Pseudomonas syringae* and *Erwinia herbicola* were aerosolized in a greenhouse, the aerosol was sampled at various distances from the site of release by using all-glass impingers, and bacterial survival was followed in the impingers for 6 h. Bacterial survival subsequent to aerosolization of *P. syringae* and *E. herbicola* was not impaired 1 m from the site of release. *P. syringae* aerosolized at 3 to 15 m from the site of release at a temperature of 12°C and a relative humidity of 80% survived 35- to 65-fold better than *P. syringae* released at 27°C and a relative humidity of 40%. No difference was observed in the survival of *P. syringae* and *E. herbicola* following aerosolization at the same temperature and relative humidity. Bacteria sprayed directly onto bean and oat plants established stable populations at comparable numbers on both plants over an 8-day period following inoculation. Bacteria that inoculated adjacent plants by drifting downwind up to 5 m were detectable at an initial population of $10^2$ CFU/g on oats and $10^5$ CFU/g on beans 2 h after the spray. However, bacterial populations on both plants were undetectable within 48 h.**

The use of biological agents in industrial and agricultural settings has caused an interest in understanding what effects released organisms may have on the natural environment. This interest has centered upon release of genetically engineered microorganisms (GEMs), a number of which have been, or are being, considered for release in small-scale field experiments (6). Most planned releases are for application of microbial pest control agents in agricultural environments, many of which can be expected to be applied in the form of aerosols (6). The use of aerosols as a means of applying GEMs to plant surfaces has raised concern regarding the dispersal to, and subsequent survival of GEMs in unintended locations (6). Specific research to answer these concerns include developing methods to predict the number of organisms entering the atmosphere, estimating downwind populations, and determining growth and survival of GEMs following aerosolization (6).

Much of what is known about the effect of aerosolization on bacteria has been elucidated in enclosed aerosol chambers. Webb (11) reported that a portion of *Escherichia coli* populations aerosolized in distilled water died during the first second of aerosolization, while the remainder died at a slower rate. He suggested that bacterial death was the result of water movement into and out of the cell, resulting in the collapse of cellular protein. Loss of viability in aerosolized bacteria has also been linked to breakdown of RNA (2, 3) and inhibition of energy production (3, 4), possibly due to oxygen free-radical activity damaging flavin-linked enzymes (2). Other influencing factors include the physiological state of the cell (5) and the presence of UV radiation (1, 9). These findings suggest that vegetative bacterial cells are stressed during aerosolization. Therefore, it is possible that aerosolization can impair the survival of vegetative cells following aerosolization.

The objective of this study was to determine whether aerosolization impairs the subsequent survival of bacteria by (i) developing a method to measure survival of bacteria following aerosolization, (ii) testing this method by measuring the effects of different temperatures and relative humidities (RHs) during aerosolization on bacterial survival subsequent to aerosolization, and (iii) comparing survival of bacteria sprayed directly onto plants with survival of bacteria that inoculated adjacent plants by drifting downwind.

## MATERIALS AND METHODS

**Cultures and media.** The microorganisms used in this study were a spontaneous mutant strain of *Pseudomonas syringae* (supplied by Steve Lindow, University of California, Berkeley), resistant to 100 μg of rifampin per ml and a spontaneous mutant strain of *Erwinia herbicola* (supplied by Steve Beer, Cornell University, Ithaca, N.Y.), resistant to 500 μg of nalidixic acid per ml. The organisms were grown in Luria-Bertani (LB; Difco Laboratories, Detroit, Mich.) broth amended with the appropriate antibiotic at 30°C with shaking at 200 rpm for approximately 18 h. Cell densities were adjusted to an optical density at 600 nm of 2.0, which corresponded to a population of about $10^9$ CFU/ml. The cells were washed twice with cold, sterile 10 mM phosphate buffer (pH 7.2) and suspended in sterile distilled water at room temperature. The cell suspension was diluted with sterile distilled water to $2 \times 10^8$ CFU/ml in the sprayer prior to aerosolization.

The selective medium for *P. syringae* was LB agar containing rifampin at 100 μg/ml. *E. herbicola* was selected on LB agar containing nalidixic acid at 500 μg/ml. In addition, all media contained 100 μg of cycloheximide per ml to inhibit growth of fungi. These antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to molten LB agar cooled to 58°C just prior to pouring into plates.

**Aerosol generation and sampling.** For all experiments, bacteria were aerosolized in a greenhouse (30 by 9 m). Target organisms were sprayed for 2 min at a pressure of 36 lb/in$^2$, using either a "solo" backpack (Cenex Corp., Tangent, Ore.) or a $CO_2$ backpack (R & D Sprayers, Inc., Opelousas, La.) sprayer. Both sprayers were equipped with a Teejet nozzle, no. 8004-SS (Spraying Systems Co., Wheaton, Ill.), which released approximately 2.5 liters with a median droplet diameter of 450 μm. Air temperature, RH,

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incidental light, and wind speed and direction were monitored during spray releases with a 21XL micrologger (Campbell Scientific, Logan, Utah).

Aerosol samples were collected during and for an additional 18 min after the spray, using all-glass impingers (AGIs; Ace Glass, Inc., Vineland, N.J.) that contained 20 ml of phosphate buffer (pH 7.2) and were calibrated to draw air at a rate of 15 liters/min. Samples were collected 1, 3, 5, 10 and 15 m from the spray source. Two AGIs were located in a row 1 m from the spray source, and rows of six AGIs were located at 3, 5, 10, and 15 m downwind from the spray source; samplers at each distance were located approximately 10 cm apart.

Prior to the start of all experiments, two additional AGIs were inoculated with bacteria directly from the spray tank, using a pipette, to serve as nonaerosolized controls. These samplers were placed 3 m upwind of the spray site to prevent them from being inoculated by aerosolized bacteria and were operated simultaneously with samplers located downwind of the spray source.

Five sets of experiments were conducted, each set at different temperatures and RHs. The temperature was increased by using two gas heaters located in the greenhouse. The RH was increased by using a mist irrigation system which was run in the upwind portion of the greenhouse until the desired RH was achieved and then deactivated prior to spray events. Experimental conditions tested were as follows: 19°C/45% RH, 12°C/80% RH, 27°C/76% RH, 27°C/54% RH, and 27°C/40% RH.

Experiments were repeated two or three times depending on the feasibility of achieving desired conditions in the greenhouse. While temperature and RH could be increased, it was not possible to obtain temperatures and RHs below ambient conditions.

**Measurement of post-aerosol survival.** AGIs were returned to the laboratory and initial populations were sampled by spread plating onto selective media or filtration, using 0.45-μm, 37-mm-diameter membrane filters (Millipore Corp., Bedford, Mass.). AGIs were placed a water bath at 30°C, and each was sampled at 2-h intervals over a 6-h period. All plates were incubated at 30°C for 48 h and then counted.

**Bacterial survival on plant surfaces.** Experiments designed to compare the survival of bacteria on plant surfaces following aerosol application were conducted in the greenhouse, using bean (*Phaseolus vulgaris humilis* cv. Bush Blue Lake) and oat (*Avena sativa* L. cv. Cayuse) plants. Oats were planted in three beds, and beans were planted in two beds. Each bed was 10 m long and divided into two 5-m sections. Prior to the inoculation of the plants, the exhaust fans located downwind of the beds were activated to create an air flow in the direction of the plant beds. The upwind section of each bed was sprayed until runoff with *P. syringae*, using the $CO_2$ sprayer at a pressure of 36 lb/in$^2$. Plants in the adjacent 5-m section were inoculated by bacteria that drifted downwind.

Leaf populations were determined by bulk sampling five oat leaves or three bean leaves selected at random from each section. A total of six replicate samples were collected for each sample day. Samples were placed in sterile stomacher bags (Tekmar Scientific, Cincinnati, Ohio), diluted in 20 ml of 10 mM phosphate buffer, and blended for 1 min in a stomacher blender (Tekmar). Dilutions were plated onto LB agar containing rifampin. All plates were incubated for 48 h and colonies were counted. Plate counts were standardized on a per-gram basis. After sampling, plants were misted for 5 min to simulate dew. Populations were sampled periodically over a period of 8 days.

**Statistical design and analysis.** The effects of aerosolization under the five sets of environmental conditions on the subsequent survival of bacteria over time were compared by using linear regression (SAS Institute Inc., Cary, N.C.). It was recognized that measurements from each distance may be correlated with measurements obtained at adjacent distances. To help account for this, data from each distance were analyzed separately. The linear regression model used the ln-transformed CFUs observed as the dependent variable and included time in the buffer and indicator variables for different environmental conditions as independent variables (7). The rates of change of the ln CFUs over time, as measured by the coefficients of time in the regression model for different environmental conditions, were compared by using a $t$ test. $P$ values of 0.05 or less were considered significant.

Comparisons of log-transformed CFUs observed on each sample day between the directly inoculated and drift-inoculated plants were carried out by using simple $t$ tests when the levels of CFU were above detectable limits. $P$ values of 0.05 or less were considered significant.

## RESULTS

**Survival subsequent to aerosolization.** The slope of regression lines generated from bacterial survival in nonaerosolized control samplers and samplers located 1 m from the site of release were not significantly different from 0. Therefore, no significant change in populations occurred over the 6-h period following aerosolization by bacteria contained in nonaerosolized control AGIs or in AGIs located 1 m from the site of release under all conditions tested.

The effect of aerosolization on subsequent survival of bacteria recovered in samplers 3 to 15 m from the site of release depended on the conditions present when the bacteria were suspended in the aerosol. The slopes of regression lines generated from survival of bacteria following aerosolization at a temperature of 19°C and 45% RH declined at rates that ranged from −0.360 ln CFU/h at 3 m to −0.582 ln CFU/h at 10 m. These rates of decline were significantly different from 0. Bacteria aerosolized at 12°C and 80% RH declined at the same rate for 3 and 10 m (−0.010 ln CFU/h); these rates were not significantly different from 0. Bacteria aerosolized at 19°C and 45% RH declined 7-fold faster at 3 m and 68-fold faster at 10 m than bacteria aerosolized at 12°C and 80% RH. These differences were significant at the $P < 0.05$ level. At 15 m, survival of bacteria differed by eightfold: −0.498 ln CFU/h compared with −0.058 ln CFU/h for bacteria aerosolized at 19°C and 45% RH and 12°C and 80% RH, respectively. These differences were also significant.

**Effect of temperature.** Linear regressions generated from survival of bacteria following aerosolization at an RH of 76 to 80% but at different temperatures (12 versus 27°C) are illustrated in Fig. 1. The slopes of regression lines from releases conducted at either temperature did not differ significantly from 0 and were not significantly different from each other. In fact, the slopes generated from linear regressions at 15 m were identical for the two temperatures tested.

**Effect of RH.** Linear regressions generated from survival of bacteria after aerosolization at the same temperature (27°C) but different RHs (40 versus 80%) are illustrated in Fig. 2. The rate of decline of bacteria following aerosolization at 40% RH was significantly higher than that of bacteria aerosolized at 80% RH. At 3 m, the slope of regression lines

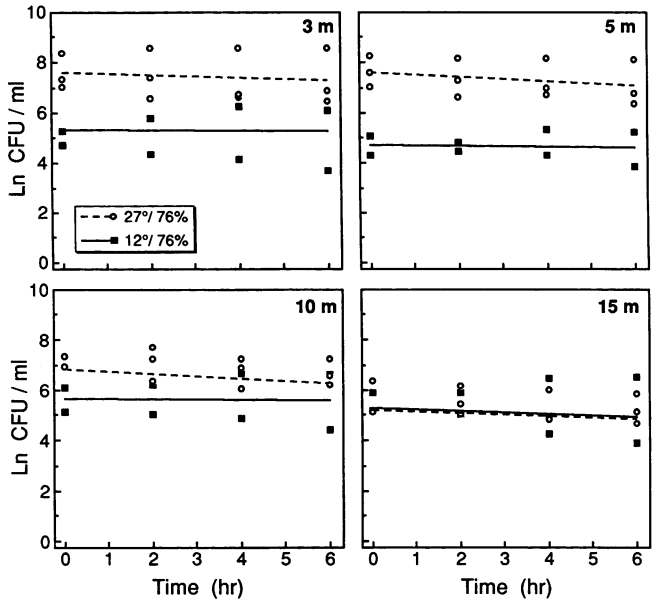


FIG. 1. Regression lines generated from survival of *P. syringae* following aerosolization at different temperatures, collected in samplers at increasing distances from the site of release. Each point denotes the mean ln CFU of six samplers. Correlation coefficients for each distance were as follows: 1 m, 0.67; 3 m, 0.59; 5 m, 0.52; 10 m, 0.59; 15 m, 0.62.

of bacteria following aerosolization at 80% RH was 11-fold higher than the slope of bacteria aerosolized at 40% RH: −0.047 and −0.537 ln CFU/h, respectively. At 10 m, the difference decreased to sixfold: −0.099 and −0.545 ln CFU/h for 80 and 40%, respectively. At 15 m, the difference was eightfold: −0.059 and −0.492 ln CFU/h for 80 and 40%, respectively.

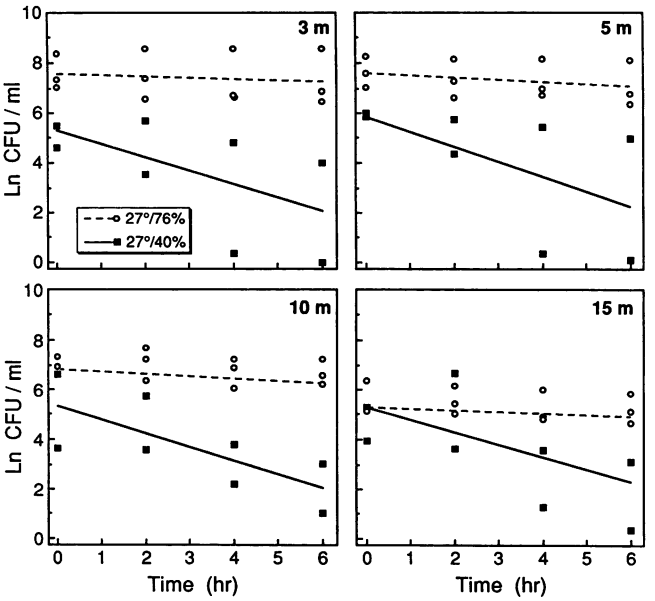


FIG. 2. Regression lines generated from survival of *P. syringae* following aerosolization at different relative humidities, collected in samplers located at increasing distances from the site of release. Each point denotes the mean ln CFU of six samplers. $r^2$ values are given in the legend to Fig. 1.

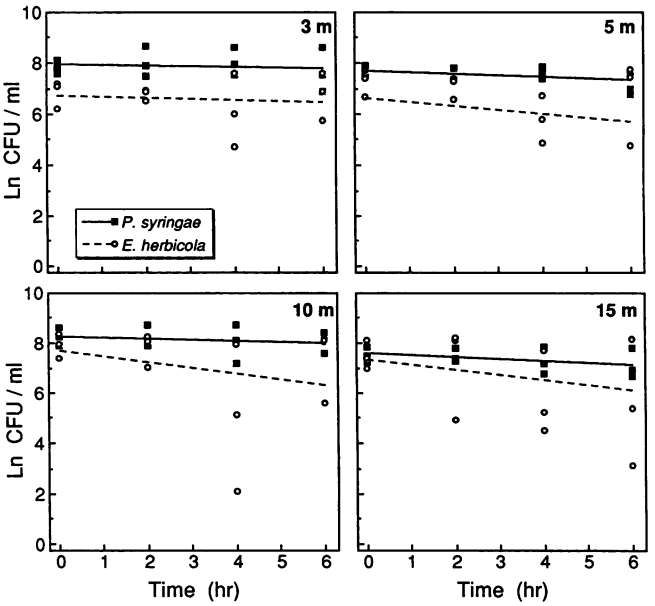


FIG. 3. Regression lines comparing the survival of *P. syringae* and *E. herbicola* following aerosolization over increasing distances from the site of release. Each point denotes the mean ln CFU of six samplers. $r^2$ values are given in the legend to Fig. 1.

**Comparison of genera.** Aerosol survival experiments indicated that *E. herbicola* survived significantly better in an aerosol than *P. syringae* (data not shown). Therefore, experiments were conducted comparing the survival of *P. syringae* and *E. herbicola* in the AGI buffer following aerosolization at 27°C and 54% RH. Regression lines from these experiments are illustrated in Fig. 3. There was no significant difference in the survival of either genus following aerosolization at any distance tested. Furthermore, the slopes of regression lines generated from these experiments did not differ significantly from 0 for either organism.

**Phyllosphere survival experiments.** Survival of *P. syringae* inoculated onto oats and beans by either direct spray or downwind drift is illustrated in Fig. 4. When applied by direct spray, the survival of the microbe was measurable on either plant species over the 8 days of the experiment. However, bacteria that drifted onto oats were detected 2 h after inoculation in low numbers ($10^2$/g), but declined below the level of detection within 24 h. Downwind inoculation of beans resulted in a relatively high population ($10^5$/g), but this population declined below the level of detection within 48 h.

## DISCUSSION

The release of GEMs into the environment as aerosols could lead to the dissemination of these organisms to unintended locations. However, if the organisms aerosolized are stressed or injured due to aerosolization and are unable to survive, then they would be ecologically insignificant. By calculating the slope of linear regressions generated over the 6-h period following aerosolization and comparing them with the slopes of bacteria that were not aerosolized, or were aerosolized under different environmental conditions, it is possible to determine whether aerosolization can impair subsequent bacterial survival.

Control populations of bacteria (not aerosolized) or bacteria aerosolized over a distance of 1 m did not demonstrate

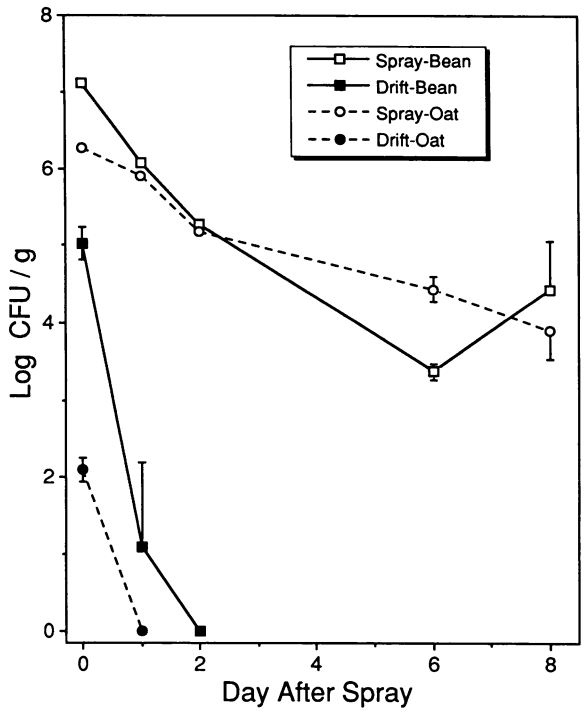


FIG. 4. Survival of *P. syringae* following aerosol application onto beans and oats either by direct spray or by organisms that drifted downwind up to 5 m from the site of release.

slopes that were significantly different from 0, indicating that the populations did not change over the 6-h sampling period. Therefore, survival of these organisms was not impaired or did not seem to be impaired in either the presence or the absence of aerosolization. Since this was observed for all conditions tested, it suggests that the survival of bacteria that are sprayed directly onto crops and travel less than 1 m would not be affected by aerosolization.

The effect of aerosolization at distances of 3 m and greater depends on environmental conditions at the time of aerosolization. Bacteria aerosolized in moderate RH (54%) were not affected by aerosolization over a 15-m distance, even at temperatures as high as 27°C. However, bacteria at similar temperatures, but at 40 to 45% RH, demonstrated impaired survival following aerosolization. This implies that, under the conditions we investigated, the RH was more influential in determining the detrimental effect of aerosolization on subsequent bacterial survival.

Another important aspect of bacterial survival, both during and following aerosolization, would be the size of the droplets containing the bacteria. Estimates of droplet size distributions from the teejet nozzle used in this experiment indicate that, of the $8 \times 10^6$ droplets formed per s, 40% are in the size range of 100 to 250 μm. With a mean temperature of 24.5°C, mean wind speeds of 0.6 m/s, and 47% RH, droplets of 100 μm would evaporate in about 10 s and droplets of 200-μm diameter would evaporate in 30 to 50 s (10). Bacteria contained in the larger droplets would presumably be better protected from adverse affects of aerosolization, such as desiccation and osmotic stress, for a longer period. Since all distances were sampled simultaneously, samplers located closer to the site of release received a more diverse range of droplet sizes than those located farther away. This would result in highly variable bacterial populations in these samplers. Bacteria contained in larger droplets would be less affected by aerosolization than those contained in smaller droplets due to differences in evaporation time. The bacteria contained in smaller droplets would have a greater chance of injury and, hence, impaired survival. Air speeds monitored during these experiments were approximately 0.5 m/s. Therefore, it would require a minimum of 30 s for a droplet to travel the 15-m-long sampling area. If the evaporation rate for all droplets was constant, then only certain sized droplets could be expected to travel the 15-m distance without evaporating. Bacteria contained in these droplets would be affected similarly since even the largest droplets to reach this distance would have experienced evaporation to some extent. This effect would be manifested in the variability of the data. Samplers located closest to the site of release would receive the most diverse droplet sizes. This would result in highly variable bacterial populations in these samplers relative to the amount of stress incurred during aerosolization.

Results of experiments that measured bacterial survival in the phyllosphere, following aerosol application, supported the results of survival experiments conducted in AGIs. In both cases, bacterial survival following aerosolization was better when bacteria were aerosolized over a distance of 1 m or less. The initial populations observed in oats inoculated by downwind drift may have been insufficient to establish stable populations. However, the initial population observed on bean was 1,000-fold higher, $10^5$ compared with $10^2$/g of leaf on oats, yet survival was roughly equivalent.

Results from these experiments are in agreement with results of field experiments of *P. syringae* following aerosolization. Lindow et al. reported that survival of bacteria on directly sprayed plants was 10-fold higher than that of bacteria that drifted 3 to 27 m downwind (6).

The survival of GEMs released into the environment can be expected to be influenced by numerous ecological factors. O'Brien and Lindow (8) demonstrated that survival of *P. syringae* was influenced by strain, plant species, and physical environment. They reported that waxy cuticle plants such as corn and oats tended to have lower bacterial populations than rough trichomatic leaved plants such as bean. Our results indicate that, under certain conditions, prior stress due to aerosolization can also influence bacterial survival.

Results from these experiments suggest that the dissemination of GEMs during aerosol release can be minimized under conditions of low RH. Such information affects not only monitoring strategies, but also application methods.

Future research will be directed towards comparing survival following aerosolization, as measured by the technique reported here, with survival in the phyllosphere in the field and investigating the initial population required to establish a stable population in the phyllosphere.

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