Atmospheric Dispersal of Ice Nucleation-Active Bacteria: The Role of Rain

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ABSTRACT

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A strong net downward flux of bacteria was measured in a soybean field during two different rain events. The ice nucleation-activity and phytopathogenic host range of strains of *Pseudomonas syringae* isolated from rainwater and aerosols present during rain differed from those of the bacteria present on the soybeans. Bacteria that produced fluorescent pigments on an iron-deficient medium were 3–8%, 5–9%, and 81% of those isolated from aerosols during rain, from rain, and from the soybean leaves, respectively. These two observations—net downward flux and bacteria in the rain and air being different from those on the leaves—were interpreted to mean that the bacteria being delivered to the canopy came from a source outside the soybean field.

The net upward flux of viable bacteria over plant canopies during dry, sunny weather (18,19) has been interpreted to mean that plants are important sources of bacteria in the troposphere. Net movement of bacteria during rain is less well understood. Large drops, generated by impaction of raindrops on leaves, have been implicated in movement of bacteria during rain (3). Concentrations of viable phytopathogenic bacteria in aerosols increased during rain or irrigation (7,26). Although the plant canopy on which the rain or irrigation was falling has been assumed to be the source of these bacteria, neither the source nor the net movement (to or from plants) during rain has been clearly established.

Wind-driven rain has long been known to precede bacterial diseases (2–5,27). The usual explanation for this association has been that rain-splash disseminates the bacterial pathogens

from lesions to uninfected leaves. However, abundant epiphytic phytopathogenic bacteria frequently exist on healthy plants (10,15). These bacteria, growing in association with healthy leaves, are probably the immediate source of inoculum for at least some diseases, for example, bacterial brownspot of snap bean, caused by *Pseudomonas syringae* pv. *syringae* (10). This argument has been supported by the observation that the frequency with which individual leaves bear large populations of pathogenic bacteria is predictive of disease about a week later (8,17,22). If bacteria are already abundantly distributed among the leaves of a given canopy, is dispersal by rain necessary for disease development?

The present study was undertaken to determine the direction of net fluxes of bacteria during rain and to assess the potential importance of rain in dispersal of bacteria, particularly phytopathogenic and ice nucleation active (INA) species.

MATERIALS AND METHODS

Experiments were conducted in an 8-ha soybean field located on the University of Wisconsin Arlington Experimental Farm. All samples were taken and measurements made within 10 m of the downwind edge of the field. The anemometer mast was approximately 10 m NW of the sampling sites. During the experiments reported, wind direction ranged from SSW to WSW. Fetch was 300–400 m over soybeans. Samples were taken at 3-hr intervals from 0905 hours 16 August until 1530 hours 17 August, except that the sample that would have begun at 0905 hours 17 August was replaced by two samples, beginning at 0851 and 0927 hours in order to sample during a rain event that began at about 0850 hours. During the night of 12–13 September, samples were taken four times, beginning at 1925 and 2240 hours before rain, at 2325 hours during rain, and at 0048 hours after rain. The soybean plants were 0.73 m high during both sampling periods.

Meteorological measurements. Horizontal wind speeds were monitored with three totalizing, four-cup anemometers 2.05, 3.45, and 6.39 m above the soil surface. Air temperatures at the soil surface, inside the plant canopy, and at canopy level were measured with thermocouples. Wind and temperature data were recorded every 5 min with a CR5 data logger (Campbell Scientific, Inc., Logan, Utah). Rain was collected volumetrically with funnel traps (3) to assess amounts and intensities of rainfall. Other meteorological conditions recorded visually during sampling included wind direction, cloud cover, and presence or absence of rain.

Bacteriological methods. Plates for aerosol sampling (27 ml per plate) and for bacterial deposition (filled to the top) contained nutrient glycerol agar (20) supplemented with 200 mg/ml cycloheximide (NGA+C). Population sizes of bacteria on the soybean leaflets were estimated by dilution plating of leaf washings onto NGA and King's medium B (13). Both media were supplemented with 100 mg/ml cycloheximide. Leaflets were washed in 0.1 M potassium phosphate buffer, pH 7.0, supplemented with 0.1% Bacto-peptone, on a gyratory shaker (200 rpm) for 2 hr (18,19). All plates were incubated for 3–4 days at 22–24 C before bacterial colonies were enumerated.

Numbers of INA bacteria were estimated by the plate replica-freezing method (20), and ice nucleation activity of purified isolates was verified by a droplet freezing test (21). Isolates that produced fluorescent pigments on King's medium B (13) gave negative reactions in the cytochrome oxidase (14), and arginine dihydrolase (24) tests were assumed to be strains of *P. syringae*.

Concentrations and flux of airborne bacteria and bacteria in rain. Concentrations of viable airborne bacteria were determined with Andersen six-stage microbial impaction samplers (1) (Andersen 2000 Inc., Atlanta, GA). Numbers of viable bacteria detected on stage 6 in samples taken over plant canopies are usually a very small fraction of the total number of particles bearing viable bacteria (19). Thus, stage 6 was omitted (for logistical reasons) during the experiment on 16–17 August. All six stages were used in the experiment on 12–13 September. Andersen samplers were deployed at two (1.27 and 2.18 m) or three (1.27, 3.76, and 10.21 m) heights above ground. Samplers at 1.27 and 2.18 m were supported on horizontal platforms mounted on tripods. Samplers at 3.76 and 10.21 m were suspended from the windward side of an 11-m scaffolding. The samplers were operated simultaneously for 15 min. During rain, alcohol-cleaned metal shields (33 × 57 cm) were suspended horizontally $\approx$ 0.5 cm above the inlets of the samplers to assure that only bacteria in aerosol-sized particles would be collected, and that those suspended in raindrops would be excluded (19). To determine if particles bearing viable bacteria remained airborne after rainfall, air samples were also taken 1–2.5 hr after rain ceased. Counts for each Andersen stage were corrected for coincidence (1), summed to determine total counts per air sample, and expressed as colony-forming units (cfu) m^{-3} of ambient air.

Flux (F) of viable airborne bacteria suspended in aerosols, expressed as cfu $m^{-2}s^{-1}$, was estimated according to:

$$F = -0.16 \quad \frac{([\Delta z_u \Delta u])}{([\ln (z_2/z_1)]_u^2)} \quad \frac{(\Delta c)}{(\Delta z_c)}$$

(6,18) where windspeeds u (in m s^{-1}) were measured at heights z_u (m), differences in windspeed Δu ($u_2 - u_1$) occurred at heights separated by Δz_u [$(z_2 - z_1)_u$], bacterial concentrations c (cfu m^{-3}) were measured at heights z_c (m), and differences in bacterial concentration Δc ($c_2 - c_1$) occurred at heights separated by Δz_c [$(z_2 - z_1)_c$]. Flux is positive if net movement is upward. Calculations assume a logarithmic wind speed vertical profile which was verified at all sampling times with a coefficient of determination (r^2) between 66 and 98% ($P < 0.01$). Net vertical movement of bacteria from the canopy is estimated as the difference between upward flux and deposition.

Deposition of airborne bacteria. Petri plates, filled to the top with NGA+C (19), were positioned horizontally at heights similar to those reported for the Andersen samplers. Plates were exposed sequentially for 5 min each, except when rain rates required shorter exposures to prevent flooding the plates with rainwater. To provide continuous sampling simultaneous with the aerosol samples, three (or more) sequential deposition samples were summed to estimate deposition corresponding to each aerosol sample. The shorter sampling duration prevented overloading of the deposition plates with bacteria. Deposition of particles bearing bacteria was expressed as cfu $m^{-2}s^{-1}$.

Phytopathogenicity tests. A total of 104 strains of *P. syringae* isolated from soybean leaves and from rainwater and aerosols collected during rainfall were tested for pathogenicity to soybean seedlings and to bean pods. Bacterial inoculum grown on NGA+C at 24 C for 24 hr was suspended in buffer (0.01 M K_2PO_4, pH 7.0) and adjusted turbidimetrically either to $\approx 10^8$ cells/ml for the bean pod inoculation assay (16) or to $\approx 10^5$ cells/ml for inoculation of soybean leaves. Soybean seedlings (four seedlings per isolate) were inoculated by spraying the abaxial surface of partially expanded primary or trifoliolate leaves with an airbrush until watersoaking became visible and then incubated at 20–26 C for 9 days.

RESULTS AND DISCUSSION

Concentrations, deposition, and flux of airborne bacteria were measured before, during, and after two rain events (Table 1). The first event occurred on the morning of 17 August. Rain fell from approximately 0850 until 0945 hours with an intensity of 19 mm hr^{-1}. Throughout the 23.5-hr overcast period before rain began, concentrations of airborne bacteria were low to very low (19–150 cfu m^{-3}), and little, if any, upward movement of bacteria was detected. For example, from 0610 until 0625 hours concentrations of bacteria in aerosols were only 33 cfu m^{-3} (at 1.27 m), and upward flux was <10 cfu $m^{-2}s^{-1}$ (Table 1). For comparison, flux values on sunny days averaged 195 cfu $m^{-2}s^{-1}$ over snap bean throughout the 1980 growing season (19). When rain occurred (0850–0945 hours 17 August), bacterial aerosol concentrations increased $\approx$ 30-fold. This increase might be due

either to bacteria removed from leaves by impaction of rain drops or to bacteria in the falling rain becoming suspended in aerosol-size particles by evaporation or fracturing of raindrops. Had the plant canopy been an important source of bacterial aerosols during rain, a large increase in the vertical concentration gradient of these aerosols would have been expected above the canopy. However, measurable increases in the difference between bacterial concentrations at 1.3 and 2.2 m were not observed (Table 1).

Similarly, during the second rain event from 2318 until 2339 hours on the night of 12–13 September (intensity 10 mm h^{-1}), aerosol concentrations of bacteria increased ≈ twofold when rain began to fall (Table 2). However, there was virtually no difference in gradients in bacterial aerosol concentrations from before to during rain, nor was there a sizable increase in the estimated aerosol flux. Hence we did not detect a major upward flux of bacterial aerosols during two separate rain events (Table 1).

The low values of upward flux were not due to lack of epiphytic bacteria on the leaves. For example, the median total epiphytic bacterial populations ranged from 4.0 to 6.3 × 10^6 cfu g^{-1} fresh weight on 16 and 17 August.

During both rain events, deposition of bacteria was about 30- to 80-fold greater than that during the preceding dry periods (Table 1). Thus, although concentrations of bacterial aerosols increased during rain, in agreement with previous reports (7,26), the net flux—that is, aerosol flux less deposition—of airborne bacteria was strongly downward (60–175 cfu $m^{-2}s^{-1}$). Although our estimation of flux is relatively imprecise and is influenced both by imperfections in the physical model and errors associated with bacterial measurements, the size of deposition due to rainfall was so much greater than aerosol flux, there is little question that net movement of bacteria during these two events was downward. Few viable bacteria remained suspended in the air 1–2.5 hr after the rain had stopped, and upward fluxes of bacteria were negligible at those times. Thus, scrubbing of the atmosphere by rain was quite efficient, particularly on 12 September.

The proportion of fluorescent bacteria during the rain event of 17 August ranged from 3 to 8% in the aerosol samples and from 5 to 9% of the deposition samples taken between 0851 and 0942 hours. However, fluorescent colonies represented ≈ 81% of the bacteria recovered by plating from leaves harvested at 0900 hours on 17 August. The similarity of the relative abundance of fluorescent bacteria in the air and rain suggests that these samples arose from a common source and that the source was probably not the soybean canopy. Had the soybean canopy been the source, nonfluorescent bacteria would have to have been dispersed from the canopy about 80 times as frequently as fluorescent bacteria to account for these differences in relative abundance.

INA bacteria comprised approximately 5.5% of the total bacteria present in rain and in aerosols sampled during rain as estimated by the replica-freezing method (20). Most (92% of 375 isolates) of the INA bacteria were fluorescent on King's medium B, oxidase and arginine dihydrolase negative, and were thus identified as putative *P. syringae*. Concentrations of INA bacteria in aerosols sampled during rain on 17 August ranged from 45 to 78 cfu m^{-3}, a 27-fold increase over that found before rain. Numbers of INA bacteria were too small to provide reliable estimates of vertical gradients, and hence flux. However, the proportion of the aerosol bacteria that were INA was approximately the same as the proportion of INA bacteria isolated from the deposition plates (≈ 3% from 0851 until 0906 hours and ≈ 6% from 0927 until 0942 hours on 17 August). Thus, by comparison to the total bacterial flux, we infer that a net downward flux during rain for INA bacteria also occurred.

The ice nucleation activity and phytopathogenic host range of the putative strains of *P. syringae* isolated from aerosols during rain and from rainwater were compared to those isolated from soybean leaves. For each bacterial isolate, ice nucleation activity was determined at −5 and −10 C by the droplet freezing test (21). Although all strains of *P. syringae* from soybean leaves were active as ice nuclei at −10 C (Table 2), only 17% were active at −5 C. In contrast, 80% of the strains of *P. syringae* from aerosols and rainwater produced ice nuclei active at −5 C, 10% were active only at −10 C, and the rest were not active as ice nuclei at either temperature. Assessment of phytopathogenicity to soybean leaves and snap bean pods (Table 2) revealed that a large proportion (≈ 80%) of the putative strains of *P. syringae* from soybean leaves caused bacterial blight to soybeans (*P. s.* pv. *glycinea*), while none were pathogenic to snap beans. In contrast, only 6% of the 50 isolates from rain aerosols and rainwater were pathogenic to soybeans, 10% caused bacterial brown spot symptoms on snap beans (*P. s. syringae*), and 84% were not pathogenic to either host. These differences in ice-nucleation activity and pathogenicity indicate that not more than about 6–12% of the *P. syringae* isolates in rain aerosols and rainwater could have originated from the local soybean canopy.

Thus, by two criteria (overall proportion of bacteria that produced fluorescent pigments and characteristics of INA bacteria), the bacteria in aerosols and in rain can be judged similar to each other, and different from those on the soybean canopy. This, with the lack of a substantial upward flux of bacterial aerosol and the strongly downward net bacterial fluxes during rain, may be viewed as evidence that the immediate source of the bacteria in the rain was the column of air through which the rain had fallen. The bacteria present in aerosols generated during rain were probably scrubbed from the atmosphere by rain and resuspended

TABLE 1. Concentration, vertical flux, and deposition of airborne bacteria over a soybean canopy before, during, and after rain

Time of day		Bacteria in aerosols: Concentration[a] (cfu/m^3)	Bacteria in aerosols: Difference[b] (cfu/m^3)	Flux (cfu/m^2/sec)	Deposition[c] (cfu/m^2/sec)	Net[d] (cfu/m^2/sec)	Wind speed: Height[e] 2.05 (m/sec)	Wind speed: Height[e] 6.39 (m/sec)
17 August								
0610–0625	No rain	33	−12	6.7	2.5	4.2	1.8	2.8
0851–0906	Rain	1,029	−26	20.6	194.8	−174.2	2.7	4.0
0927–0942	Rain	1,111	−7	5.0	123.9	−118.9	2.7	3.9
1205–1220	No rain	73	−19	12.9	...	...	2.3	3.4
12–13 September								
1925–1940	No rain	299	−85	5.8	4.9	0.9	1.7	2.8
2240–2255	No rain	271	−76	3.3	2.6	0.7	1.3	2.1
2325–2340	Rain	586	−96	8.1	68.9	−60.8	3.1	4.5
0048–0103	No rain	27	−12	0.9	...	...	2.7	3.9

[a] Concentrations measured 1.27 m above ground.
[b] Differences in bacterial concentrations with height, $c_2 - c_1$, where c_2 represents concentrations at 2.18 m (17 August) or 10.21 m (12–13 September) above ground and c_1 represents concentrations at 1.27 m above ground.
[c] Deposition was estimated as the number of particles bearing bacteria impacted on petri plates filled with NGA+C and positioned horizontally at 2.18 (17 August) or 10.21 m (12 September) above ground.
[d] Net = Flux − Deposition. Negative values indicate net downward bacterial movement.
[e] Height above ground (m).

TABLE 2. Ice-nucleation activity and pathogenicity of strains of *Pseudomonas syringae* isolated from soybean leaves and from rainwater and aerosols during rainfall

Source	Strains tested (no.)	Ice nucleation activity			Pathogenicity	
		−5 C	−10 C	Inactive	Soybean[a]	Bean[b]
Leaves[c]	54	16.7%	83.3%	0	81.5%	0
Air & rain[d]	50	80.0%	12.0%	8.0%	6.0%	10.0%

[a]Incidence of bacterial blight (*P. syringae* pv. *glycinea*) was assessed 9 days after inoculation of soybean seedlings.
[b]Inoculated bean pods were scored for brown spot (*P. syringae* pv. *syringae*) 3 days after inoculation. Markedly sunken, water-soaked green lesions on pods represented a positive reaction.
[c]The strains tested were isolated from bulk leaf samples (noon, 16 August).
[d]Strains tested were isolated from rainwater and aerosols collected during rain (17 August).

as aerosols by evaporation of raindrops. Thus, the rain was the final stage in a net transport of bacteria, including INA bacteria, to the soybean field from some unknown and possibly distant source(s).

Only small numbers of INA or phytopathogenic bacteria, relative to the sizes of populations frequently present on leaves, are likely to be introduced onto a plant canopy during a given rain event. For example, the rain event on 17 August that is described in Table 1 lasted about 50 min and delivered $\approx$ 1–5 $\times 10^3$ INA bacteria per m^2. This corresponds to $\approx$ 1–10 cfu per soybean leaflet. The potential for bacteria to be introduced to fields, or even areas, where they are not currently resident on leaves deserves further consideration. The relative quantitative importance of this "long distance" transport of INA and phytopathogenic *P. syringae* in relation to the epidemiology of frost injury and disease remains to be determined.

Although short-range dispersal of bacteria, particularly in ballistic particles generated by rain-splash, undoubtedly does occur during rain (3,19), the data presented here are consistent with the conclusion (19) that bacterial dispersal within a field during rain is rather short-range in nature (one or a few meters) and quantitatively rather small. We did not find evidence for substantial aerosol dispersal of bacteria from soybean leaves during rain. Thus, dispersal of phytopathogenic bacteria by rain-splash or aerosols during rain does not appear to be sufficient to have given rise to the large population sizes of bacteria necessary to increase the probability of disease (8,17,22). On the other hand, intense rainfall triggers rapid growth of *P. syringae* on snap bean leaflets (9,11,12), and this rapid growth can lead to sudden, large increases in population sizes of *P. syringae* on bean leaflets (12). Thus, triggering of rapid growth of this pathogen, rather than its dispersal, is probably the reason for the association between rain and diseases caused by *P. syringae*.

INA *P. syringae* appear to be aerosol-stable microbes under a range of environmental conditions. They leave plant surfaces and enter the troposphere during dry, warm weather (18,19) and are transported and washed out by rain. It is tempting to speculate that INA bacteria may be active participants in this cycle, since ice nucleation of supercooled cloud droplets may initiate precipitation. Naturally occurring ice nuclei active at temperatures above about −10 C are frequently of biogenic origin, and may be of bacterial origin (23,25). If initiating formation of a raindrop can increase the probability that a bacterial cell will escape the cloud and return to a leaf, then this may have evolutionary significance to *P. syringae*. This advantage to INA bacteria may be profound, even if the proportion of rain produced by bacterial ice nucleation in a given storm is negligible.

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