Factors that Affect Spread of *Pseudomonas syringae* in the Phyllosphere

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ABSTRACT

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Successful spread of an organism to a new habitat requires both immigration to and growth on that habitat. Field experiments were conducted to determine the relative roles of dispersal (i.e., immigration) and bacterial multiplication in spread of *Pseudomonas syringae* pv. *syringae* in the phyllosphere. To study spread, individual plots consisted of three nested concentric squares with the inner 6 m² planted to snap beans serving as the sink. Each sink, in turn, was surrounded by a barrier zone, usually 6 m wide, which was surrounded by a 6-m-wide source area. The source areas were planted with snap bean seeds inoculated with doubly marked strains derived from wild-type *P. syringae* pv. *syringae* B728a. The treatments were designed to test the effects of the nature and width of the barrier zone and suitability of the habitat in the sinks on spread of *P. syringae* pv. *syringae*. The marked strains introduced into the source areas at the time of planting were consistently detected in sink areas within a day or two after emergence of bean seedlings in the sources as

assessed by leaf imprinting and dilution plating. The amounts of spread (population sizes of the marked strain in sinks) across barrier zones planted to snap bean (a suitable habitat for growth of *P. syringae* pv. *syringae*), soybean (not a favorable habitat for *P. syringae* pv. *syringae*), and bare ground were not significantly different. Thus, the nature of the barrier had no measurable effect on spread. Similarly, spread across bare-ground barriers 20 m wide was not significantly different from that across barriers 6 m wide, indicating that distance on this scale was not a major factor in determining the amount of spread. The suitability of the sink for colonization by *P. syringae* pv. *syringae* had a measurable effect on spread. Spread to sinks planted to clean seed was greater than that to sinks planted with bean seeds inoculated with a slurry of pulverized brown spot diseased bean leaves, sinks planted 3 weeks before sources, and sinks planted to a snap bean cultivar that does not support large numbers of *P. syringae* pv. *syringae*. Based of these results, we conclude that the small amount of dispersal that occurred on the scale studied was sufficient to support extensive spread, and suitability of the habitat for multiplication of *P. syringae* pv. *syringae* strongly influenced the amount of spread.

For an organism to spread to a new habitat, it must disperse to that habitat and then multiply on that habitat. If either of these steps does not occur, i.e., the organism does not disperse to the habitat or the organism fails to reproduce in the new habitat, spread to that habitat is not successful. When describing the epidemiology of plant diseases, we often say that a disease has spread when new disease occurs. The occurrence of new disease implies that the pathogen has successfully dispersed to a new habitat, a susceptible host, and that growth on the host has occurred, leading to disease. Much of the classical epidemiology of plant diseases has been developed for diseases in which disease development can be equated to pathogen reproduction (22). The theories developed for such cases often provide excellent models for diseases in which all or nearly all pathogen multiplication occurs in lesions, a process exemplified by numerous fungal diseases.

Many phytopathogenic bacteria multiply quite successfully in association with susceptible tissue without causing lesions (7). Attempts to fit classical epidemiological models to such diseases are often less successful and clearly have less biological relevance. Bacterial brown spot of snap beans (*Phaseolus vulgaris* L.), caused by *Pseudomonas syringae* pv. *syringae*, is an example of a disease in which pathogen multiplication is often independent of disease causation (14,21). In this system, the likelihood of dis-

ease occurring on a given leaf is a function of pathogen population size on that leaf (21). As pathogen population sizes increase, so does the probability of disease development. When pathogen populations remain small to modest in size, host and pathogen coexist without disease.

Dispersal and multiplication—spread of the pathogen—often occur independently from lesion occurrence. Lesions, on the other hand, occur when extensive pathogen multiplication occurs and population sizes become quite high. Raindrop momentum during intense rain events often triggers onset of extensive multiplication of *P. syringae* pv. *syringae* (3). Growth of the pathogen, already present on leaves, in response to rains results in population sizes that increase the probability of brown spot disease occurrence. Because pathogen spread requires dispersal to leaves to initiate populations followed by multiplication to increase pathogen population sizes, intense rains increase the amount of spread of *P. syringae* pv. *syringae* through its effects on pathogen growth.

Plant canopies are unusually open systems. In addition to splash dispersal by rain or irrigation (1,23), movement of phyllosphere bacteria occurs by aerosols generated when leaves are dry and wind speeds are greater than 1 m s⁻¹ (15,16). Epiphytic bacteria may also be dispersed by insects (2,11). When leaves of snap bean plants are wet, for example with dew, insects traversing leaf surfaces appear to become passively contaminated with bacteria that may be deposited on other leaves and plants as insects move about (2,6). Farm equipment, workers, and irrigation water are among the many other means by which bacteria may be dispersed (23). Hence, there are a number of ways that bacteria may immigrate to leaf habitats. The relative quantitative importance of the various

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dispersal mechanisms is not known and probably varies with conditions.

From the perspective of plant disease epidemiology, pathogen spread (dispersal plus multiplication) is important in assessing the likelihood of disease outbreaks. For the most part, previous research has focused on one or the other of the two components of spread. The objective of this research was to determine the effects of various factors on spread of *P. syringae* pv. *syringae*.

MATERIALS AND METHODS

Experimental design. Field experiments to study spread of *P. syringae* pv. *syringae* were conducted at the University of Wisconsin Experiment Station near Arlington, Columbia County, WI, during the growing seasons spanning 1994 to 1999. The experiments described in detail here were conducted in 1994 and 1997 to 1999. The standard individual plot design consisted of three nested concentric squares (Fig. 1). The outer 6 m of each plot served as the “source” and was planted with snap bean seeds inoculated with a derivative of *P. syringae* pv. *syringae* strain B728a. *P. syringae* pv. *syringae* B728a is a very good colonizer and pathogen of snap bean plants in the field (9). The inner 6 m² was the “sink” to which spread from the source was measured. The sink was surrounded on all sides by the source to eliminate directional biases (e.g., the effect of wind direction). Placement of the sink in the center of each plot also minimized cross-contamination of sinks from other sources because the nearest source to any given sink was the one surrounding that sink. The space between the source and sink was termed the “barrier.” Barriers were 6 m wide for most treatments. Barriers 20 m wide were included in some experiments to examine the effect of barrier width on spread. The overall size of each plot was 30 m² (6-m barrier) or 58 m² (20-m barrier). The plots were separated from each other by 5 m (1994) or 6 m (all other years) of bare ground.

To ensure reliable monitoring of spread of *P. syringae* pv. *syringae* B728a from source areas to sinks, we used doubly marked derivatives of the wild-type strain. *P. syringae* pv. *syringae* B728a is resistant to rifampicin. In 1994, we used a spontaneous nalidixic acid-resistant derivative of *P. syringae* pv. *syringae* B728a (B728aNal). Although B728aNal colonized bean leaves and caused disease in the field, we subsequently found that the derivative is slightly less fit than the wild-type strain B728a (9). In 1997, 1998, and 1999, we replaced B728aNal with either B728aCm (resistant to chloramphenicol) or B728aGm (resistant to gentamycin). The latter doubly marked derivatives were constructed by site-directed insertion of antibiotic resistance marker cassettes in an intergenic region in the genome of the bacterium (9). The derivatives were indistinguishable from *P. syringae* pv. *syringae* B728a in fitness in the field (9). All three *P. syringae* pv. *syringae* B728a derivatives are generically referred to as *P. syringae* pv. *syringae* B728a* in the remainder of this article.

Treatments. The treatments were designed to examine the effects on spread of the barriers and of the suitability of the habitat in sinks for multiplication of *P. syringae* pv. *syringae* B728a* (Table 1). Barrier treatments included comparisons of spread across bare ground, snap beans, and soybeans, all 6 m wide. The effect of barrier width was examined by comparing spread across 6- and 20-m bare-ground barriers.

Sink areas were altered in several ways to determine the effect of the suitability of the habitat for growth of *P. syringae* pv. *syringae* on spread of the bacterium. In the first, sinks were planted to either snap bean cv. Eagle or Hystyle or to soybean (cv. Lemke). The cv. Eagle generally supports larger population sizes of *P. syringae* pv. *syringae* and, therefore, is more susceptible to bacterial brown spot than cv. Hystyle (S. S. Hirano and C. D. Upper, unpublished data). *P. syringae* pv. *syringae* grows poorly on soybean and does not cause disease on that crop. Sinks were additionally altered by planting seeds of cv. Eagle or Hystyle that

were inoculated with either a slurry of pulverized dried bean leaves containing a mixture of phyllosphere bacteria, including naturally occurring *P. syringae* pv. *syringae* or with a single mutant (BSAL1) derived from *P. syringae* pv. *syringae* B728a. BSAL1 carries a mutation in *sala* (for syringomycin and lesion formation)—a regulatory gene required for brown spot lesion formation and syringomycin production in laboratory assays (13). The BSAL1 mutant is able to colonize bean plants in the field, although less well than the wild-type strain (S. S. Hirano and C. D. Upper, unpublished data). Finally, untreated cv. Eagle or Hystyle seeds were planted 3 weeks prior to planting the sources. Treatments in which sinks were planted with inoculated seeds or planted 3 weeks before the sources were designed to alter resident bacterial communities on sink plants and determine the effects of such altered communities on spread. For both treatments, the aim was to increase the sizes of resident bacterial populations, particularly naturally occurring *P. syringae* pv. *syringae*. Treatment of sinks with the BSAL1 mutant was intended to determine the effect of precolonization of leaves with a lesion minus mutant of *P. syringae* pv. *syringae* B728a on spread.

An additional treatment included in the 1994 experiment examined spread of a mutant (NPS3136) derived from *P. syringae* pv. *syringae* B728a that carries a mutation in the global regulatory gene, *gacS* (for global activator sensor kinase) (10,13). The *gacS* mutant NPS3136 (*gacS*::Tn5) is impaired in several phenotypes, including brown spot lesion formation, production of syringomycin, protease, alginate, and homoserine lactone, and a swarming behavior on soft agar media (cf. 8). The purpose of this additional treatment was to determine the effect of bacterial genotype on spread. Because the *gacS* mutant is able to establish population sizes similar to those of *P. syringae* pv. *syringae* B728a* on germinating seeds (5) and is less fit than the wild-type strain only on above-ground plant parts, we expected early immigration of the mutant strain to be similar to *P. syringae* pv. *syringae* B728a*, but subsequent multiplication of the *gacS* mutant in the sinks to which it immigrated to be reduced.

In all experiments, the treatments were arranged in a randomized complete block design, with three (1994 and 1997) or four

Fig. 1. Plot design. Each plot consisted of three nested squares. The inner square (6 × 6 m) was the sink to which spread of *Pseudomonas syringae* pv. *syringae* B728a* from the source (outer 6 m) was measured. The source and sink were separated by a 6 m wide zone termed the barrier. Overall plot dimensions were 30 × 30 m. Plots were separated by 5 m bare-ground alleys. Entry to the sink area for sampling purposes was limited to a specific path through the source and barrier as shown.

(1998 and 1999) blocks. Because each experiment required a rather large area (the largest conducted in 1998 required over 8 ha, with seven treatments and four blocks) and large numbers of samples were collected and processed, it was not logistically feasible to include all possible treatments in all experiments. Hence, test of the treatments and reproducibility of their effects were distributed across several years, as summarized in Table 1.

Planting and inoculation. Seeds were planted with a tractor-mounted planter with rows spaced 76 cm apart. All sinks in an experiment were planted before any of the sources to avoid contamination of the sinks with *P. syringae* pv. *syringae* B728a* (or the *gacS* mutant NPS3136 in 1994) during planting. Further, sinks planted to clean seed were planted before those planted with seed inoculated with a mixture of phyllosphere bacteria from dried bean leaves or with the *salA* mutant BSAL1. In 1994, heavy rains occurred after planting of the sinks which delayed planting of the sources until 2 days later. In 1997, the sources were planted the day after the sinks. In 1998 and 1999, the sinks and sources were planted on the same day. The sources were planted to cv. Eagle in 1994 and 1997 and cv. Labrador in 1998 and 1999 (Asgrow Seed Co., Kalamazoo, MI). The change in cultivar was necessitated by the cessation of commercial production of cv. Eagle seeds.

Bacterial inocula (i.e., *P. syringae* pv. *syringae* B728a*, *gacS* mutant NPS3136, and *salA* mutant BSAL1) were prepared as described previously (5). Briefly, 2-day-old lawns of bacterial growth were harvested from plates containing King's medium B (KB) (12) supplemented with the appropriate antibiotics. The dense suspensions were diluted to 10^7 CFU/ml with water. Seeds planted to the sources were immersed in the diluted inoculum (B728a* or NPS3136) at a ratio of 4 kg of seeds in 2 liters of inoculum. After 1 min, excess inoculum was drained through a piece of cheesecloth, and the seeds were immediately placed into the hoppers of the planter and planted. For the largest experiment conducted in 1998, roughly 23 batches of seeds were sequentially treated during the 10 to 12 h required to plant all of the sources in the experiment. Seeds planted to BSAL1-treated sinks were inoculated by treating 1 kg of cv. Eagle seed in 1 liter of bacterial suspension at 10^7 CFU/ml.

Seeds for the mixed phyllosphere bacteria-treated sinks were inoculated by immersing 1 kg of cv. Eagle or Hystyle seeds in 1 liter of water containing dried bean leaf powder (500 mg) mixed with

Talc (2 g) as a carrier. The leaf powder was prepared by pulverizing dried brown spot diseased bean leaves of cv. Eagle. After 1 min, the seeds were drained, placed in the hoppers, and planted immediately. All parts of the planter that came in contact with seeds (hoppers, seed plates, plate bases, seed tubes, and packing wheels) were disinfected with 95% ethanol between seed treatments. Over the course of more than a decade, we have found no clear evidence for any contamination among seed treatments due to failure of disinfestation by this procedure.

Sampling protocol. Because the sinks were located within the sources, the following measures were used to avoid movement of the marked strain from source to sink during sampling or other human activity. Sinks were planted before sources and personnel were not allowed to enter the plots between completion of planting and the first sampling of the plots. A specific diagonal path was designated from the outer corner of a source to the corner of its sink (Fig. 1). Samplers were required to follow this path through the source (and barrier for plots in which the barrier was not bare ground) at all times. At the time of the first sampling, the plants in the sources (and barriers) were sufficiently small that samplers could walk through the path without touching any of these plants. After the first sample was taken in 1994, plants in the designated paths were removed to ensure that samplers would not come in contact with source plants as the plants developed. In the other experiments, the paths were left unplanted. Sampling was initiated in the morning after the dew had evaporated, usually by 0900 h. Samples were never taken when leaves were wet. When possible, different individuals were assigned to sample the sinks and sources. When sufficient numbers of samplers were not available, all sinks were sampled before any of the barriers and sources were sampled. All hands were sterilized with 95% ethanol before entry into the plots.

The first set of samples was taken from the sinks the day after most of the source plants had emerged. Because source plant emergence did not occur at the same time, a few source plants had been above ground for as long as 2 days at the time the first sample was taken from some of these experiments. The frequency and timing of subsequent samplings varied with experiment but generally followed a schedule in which samples were collected twice weekly for 2 to 3 weeks and then weekly for an additional 2 to 3 weeks. Individual leaflets from the top of the canopy were col-

TABLE 1. Summary of treatments tested^a

Treatment		Experiment			
Sink	Barrier	1994	1997	1998	1999
Control common to all experiments					
cv. Eagle (untreated)	6 m of bare ground ^b	+	+	+	+
Barrier treatments					
cv. Eagle	6 m of snap beans ^b	+	—	+	+
cv. Eagle	6 m of soybeans ^b	+	...	...	...
cv. Eagle	20 m of bare ground	+	+	...	...
Sink treatments					
cv. Hystyle (untreated)	6 m of bare ground	...	+	+	...
Soybean (untreated)	6 m of bare ground	...	+	+	...
cv. Eagle inoculated with mixed phyllosphere bacteria ^c	6 m of bare ground	...	+	—	+
cv. Hystyle inoculated with mixed phyllosphere bacteria ^c	6 m of bare ground	...	+	...	...
cv. Eagle inoculated with <i>salA</i> mutant BSAL1	6 m of bare ground	...	...	—	+
cv. Eagle planted 3 weeks before sources	6 m of bare ground	...	+	+	+
cv. Hystyle planted 3 weeks before sources	6 m of bare ground	...	+	...	...
Total number of treatments ^d		6	7	7	7
Number of blocks per treatment		3	3	4	4

^a + Indicates treatment included in experiment.

^b In 1994, these three treatments were done both with sources inoculated with *Pseudomonas syringae* pv. *syringae* B728aNaI and NPS3136, a *gacS* mutant of *P. syringae* pv. *syringae* B728a that is known to be less fit in the field than B728a (5).

^c Seed inoculated with a slurry of pulverized brown spot diseased bean leaves (cv. Eagle). This powder is a good source of many bacteria common to the bean phyllosphere, including *P. syringae* pv. *syringae*.

^d Three additional treatments in 1994 and one additional treatment in each of the 1997 and 1999 experiments are not presented.

lected at all times from the sinks planted 3 weeks before the sources. For all other treatments, the sampling unit varied as the plants developed. The sampling unit at the first sampling time consisted of cotyledons and emerging primary leaves. The next few samples were individual primary leaves, after which leaflets from trifoliolate leaves were taken from the top of the canopy. All samples were taken randomly within sinks.

Detection and quantitation of spread. To rapidly assess the frequency with which spread occurred while the experiments were ongoing, individual samples collected from the sinks (*n* = 15 per sink in 1994, 1998, and 1999 and 17 in 1997) were imprinted on KB supplemented with rifampicin (50 µg/ml) (KBR) and either nalidixic acid (50 µg/ml) (KBRNal), chloramphenicol (30 µg/ml)

(KBRCm), or gentamycin (2 µg/ml) (KBRGm) for B728a* and KBR with kanamycin (30 µg/ml) (KBRKan) for the *gacS* mutant NPS3136. To minimize fungal growth, cycloheximide (50 µg/ml) and Dacnil 2787 (50 µg/ml; 75% chlorothalonil active ingredient) were added to all media. Leaf imprinting was done by placing the sample on the surface of the medium. A piece of filter paper (9-cm diameter, Fisher Brand filter paper P8, Fisher Scientific, Chicago) was placed over the sample and gently pressed with a rubber stopper (9-cm diameter). The sample and filter paper were removed after about 4 to 5 h. The plates were scored for the presence or absence of *P. syringae* pv. *syringae* B728a* (and the *gacS* mutant NPS3136 in 1994) after 2 to 3 days of incubation at room temperature.

Fig. 2. Frequencies with which *Pseudomonas syringae* pv. *syringae* B728a* and the *gacS* mutant NPS3136 (1994 only) were detected in sink areas: **A**, within 1 to 2 days after emergence of source plants, and **B**, about a week after emergence of source plants for field experiments conducted in 1994, 1997, 1998, and 1999. Detection frequencies are based on leaf imprinting and dilution plating. Each bar represents the mean frequency of detection (%) and standard error based on three (1994 and 1997) or four (1998 and 1999) replicate plots, with the following number of samples per plot: 25 in 1994; 34 at 7 days after planting (DAP) and 25 at 24 DAP in 1997; 30 in 1998; 30 at 7 DAP and 15 at 15 DAP in 1999.

A second set of samples was collected at random from each sink for enumeration of population sizes of the marked strain by dilution plating (number of samples per sink: 10 in 1994; 17 in 1997; and 15 in 1998 and 1999). Each sample was placed in a test tube (16 mm) containing 9 ml of sterile phosphate buffer (0.01 M, pH 7.0). The samples were stored frozen at -20°C for subsequent processing by dilution plating as described previously (4). At the time of processing, the samples were thawed, homogenized, and dilution plated onto KBRNal, KBRCm, or KBRGm for *P. syringae* pv. *syringae* B728a*; KBRKan for the *gacS* mutant NPS3136; and KBRSpC (spectinomycin at 50 $\mu\text{g}/\text{ml}$) for the *salA* mutant BSAL1. A semiselective medium for *P. syringae* pv. *syringae* (medium P of Mohan and Schaad [20]) was used to enumerate population sizes of all *P. syringae* pv. *syringae*, and 10% tryptic soy agar (TSA) was used for total culturable bacteria. Cycloheximide (50 $\mu\text{g}/\text{ml}$) was added to the media to prevent fungal growth. In 1997, the actual number of samples processed by dilution plating was initially 17 per sink but decreased to 10 or 8 per sink when population sizes of the marked strain were relatively large on all of the samples. Additional samples were taken from the barriers ($n = 8$ per plot in the 1994 experiment) and from the sources ($n = 5$ per source) for all experiments.

Weather. Parameters of the physical environment were recorded automatically with CR-21X data loggers (Campbell Scientific, Logan, UT). The time of event for accumulation of each millimeter or 0.254 mm of rainfall was sensed with a Sierra-Misco and TE525 tipping-bucket rain gauges, respectively, and used to determine the volume, duration, and intensity of each rain event. The following parameters were recorded each half hour: air temperature sensed with 75- μm evanohm/constantan thermocouples and thermistors, relative humidity with Model HMP35C temperature and relative humidity probe, solar radiation with Li-Cor silicon pyranometers, wind speed and direction with cup anemometers and wind vanes (MET-ONE wind speed and direction sensors and R. M. Young 03001 Wind Sentry unit). All sensors, except the thermocouples, were obtained from Campbell Scientific.

Data analyses. Bacterial counts per individual sample were $\log_{10}$ -transformed before calculation of population statistics. Sam-

ples with no detectable colonies were assigned the limit of sensitivity of the plating assay (2.57 log CFU per sample). Data management and routine calculations of population statistics were done with Microsoft Excel and MINITAB (Minitab Inc., State College, PA). Treatment effects and treatment-time interactions were determined over all sampling time points within an experiment using repeated measures analysis of variance tests (19) implemented with the Mixed Procedure of SAS (SAS Institute, Cary, NC).

RESULTS

Population sizes on source plants at emergence. At the time of seedling emergence (5 to 7 days after planting [DAP]), mean population sizes of *P. syringae* pv. *syringae* B728a* pooled across all of the source plots within an experiment were roughly 5.2 (1994, 9 plots, SE 0.29), 7.0 (1997, 18 plots, SE 0.17), 3.0 (1998, 28 plots, SE 0.08), and 5.9 (1999, 20 plots, SE 0.02) log CFU per sample. The weak source strength in 1998 was likely due to the relatively dry and cloddy soil conditions at the time of planting.

Detection of early spread. *P. syringae* pv. *syringae* B728a* (and the *gacS* mutant NPS3136 in 1994) was consistently detected in sinks by both leaf imprinting and dilution plating within a day or two following emergence of bean seedlings in the sources (Fig. 2A). This was the case even in 1998 when the population sizes of *P. syringae* pv. *syringae* B728a* on source plants were quite small (and thus, presumably, source strength was weak). Detection frequencies were usually low and often quite variable among replicate plots for a given treatment at this time. In one extreme case (1997 experiment), detection frequencies were 50, 5.8, and 0% ($n = 34$ per sink) for replicate cv. Eagle sinks surrounded by 6-m bare-ground barriers. Pooled over all treatments within an experiment, the frequencies with which *P. syringae* pv. *syringae* B728a* was detected in the sinks within a day or two after emergence of source plants were 4% in 1994 ($n = 225$), 5.8% in 1997 ($n = 612$), 3.8% in 1998 ($n = 840$), and 1.5% in 1999 ($n = 600$). In five other similar experiments (data not shown), spread was always detected at this time. In one case, *P. syringae* pv. *syringae* B728a* was detected in sinks a day or two after source plant emergence despite the total lack of rainfall between emergence of any source plants and collection of the first samples (data not shown). There were no consistent trends in detection frequencies within and across experiments to suggest that spread was affected by treatment or fitness of the spreading strain during the first day or two after source plant emergence. Thus, immigration of *P. syringae* pv. *syringae* B728a* and the *gacS* mutant NPS3136 from newly emerged bean plants in the sources to sink areas appeared to occur rapidly regardless of source strength and environmental conditions.

By 14 to 15 DAP (about a week after emergence of source plants), *P. syringae* pv. *syringae* B728a* was present at very high frequencies in the sinks in 1994 and 1997 (Fig. 2B). This was also true for NPS3136 in 1994. Detection frequencies, however, remained low at this time in 1998 and 1999. During the first 2 weeks of the experiments, weather conditions (i.e., timely rainfalls) were more favorable for multiplication of *P. syringae* pv. *syringae* in 1994 and 1997 than in 1998 and 1999. Hence, the higher detection frequencies in 1994 and 1997 compared with 1998 and 1999 were attributed to multiplication of early *P. syringae* pv. *syringae* B728a* immigrants probably coupled with local (within-sink) dispersal. When weather conditions were not suitable for multiplication of *P. syringae* pv. *syringae*, B728a* was detected in sinks but the frequency of detection of leaves bearing the marked strain remained low, suggesting that although immigration occurred during such conditions, the lack of subsequent growth limited spread.

Variability in the extent of spread across experiments. Spread of *P. syringae* pv. *syringae* B728a* to cv. Eagle sinks with 6-m bare-ground barriers was measured in all experiments and

Fig. 3. Spread of *Pseudomonas syringae* pv. *syringae* B728a* across 6 m bare-ground barriers to sinks planted to snap bean cv. Eagle in 1994, 1997, 1998, and 1999 field experiments. Population sizes of *P. syringae* pv. *syringae* B728a* in the sink areas were determined by dilution plating. The data are the mean log CFU/sample and standard error based on three (1994 and 1997) or four (1998 and 1999) replicate plots, with the following number of individual samples per plot: 10 in 1994; 17, 10, or 8 in 1997; and 15 in 1998 and 1999.

provided a way to compare experiments conducted under different environmental conditions. Population sizes of *P. syringae* pv. *syringae* B728a* in the control sinks increased more rapidly and were larger in 1994 and 1997 than in 1998 and 1999 (Fig. 3). The overall extent of spread based on bacterial population sizes was much greater in 1994 and 1997 than in 1998 and 1999, a pattern similar to that found by examining detection frequencies. Conditions were sufficiently favorable for multiplication of *P. syringae* pv. *syringae* in 1994 and 1997 that population sizes of the marked strain in the control sinks were similar to those in the sources by roughly 1 (1997) to 2 weeks (1994) after emergence of the source plants.

Effects of altering the barrier on spread. With one exception, altering the nature of the barrier between source and sink areas had no significant effect on population sizes of *P. syringae* pv. *syringae* B728a* and the less fit *gacS* mutant NPS3136 in their respective sinks (Figs. 4A, B, and C and 5; Table 2). The one exception was in the 1994 experiment, in which spread of NPS3136 across snap bean barriers was significantly less than across bare ground (Fig. 5E and F; Table 2). Spread of *P. syringae* pv. *syringae* B728a* and NPS3136 across snap bean and soybean barriers to their respective sinks was not always significantly different despite the large differences in population sizes of the marked strains that developed on snap bean and soybean plants in the barriers (Fig. 5). Thus, for distances of 6 m, the substrate across which dispersal occurred had little effect on spread, regardless of

whether it was highly conducive (snap beans) or unfavorable (soybeans and bare ground) for multiplication of the marked strains.

In 1997, increasing the width of bare-ground barriers from 6 to 20 m delayed the time at which *P. syringae* pv. *syringae* B728a* was first detected in the sinks from roughly 1 to 4 days after emergence of source plants. By 10 DAP in this experiment, population sizes of *P. syringae* pv. *syringae* B728a* were approximately 100-fold larger in sinks with 6-m barriers than in sinks with 20-m barriers (Fig. 4D). The differences in bacterial population sizes, however, decreased with time. When analyzed over all sampling times (1997 experiment), population sizes of *P. syringae* pv. *syringae* B728a* in sinks with 6- versus 20-m bare-ground barriers were not significantly different (Fig. 4D; Table 2). In 1998, *P. syringae* pv. *syringae* B728a* was detected in sinks with 20-m barriers within a day or two following emergence of plants in the sources (Fig. 2A). Season-long changes in population sizes of the marked strain were not significantly different in sinks surrounded by 6- and 20-m barriers (Fig. 4E; Table 2). Thus, increasing the width of bare-ground barriers from 6 to 20 m may have delayed spread slightly at times but did not have a season-long impact on spread of *P. syringae* pv. *syringae* B728a* from source to sink areas that was large enough to be measured in these experiments.

Effects of altering the nature of the sink on spread. The times at which *P. syringae* pv. *syringae* B728a* was first detected

Fig. 4. Effects of the barrier zone on spread of *Pseudomonas syringae* pv. *syringae* B728a*. **A to C.** The barrier between source and sink was either snap bean, soybean, or bare ground. **D and E.** The width of bare-ground barrier zones was either 6 or 20 m. The sinks were planted to snap bean cv. Eagle in all experiments. Population sizes of *P. syringae* pv. *syringae* B728a* in the sink areas were determined by dilution plating. The data are the mean log CFU/sample and standard error based on three (1994 and 1997) or four (1998 and 1999) replicate plots, with the following number of individual samples per plot: 10 in 1994; 17, 10, or 8 in 1997; and 15 in 1998 and 1999.

in sinks planted to cv. Eagle, cv. Hystyle, or soybeans relative to days after planting of the sources were: 1997, 7 DAP for cvs. Eagle and Hystyle; 1998, 5 DAP for cv. Eagle and 7 DAP for cv. Hystyle and soybeans; and 1999, 7 DAP for cv. Eagle and 9 DAP for soybeans (some of the data are presented in Figure 2A). By 14 DAP in 1997, *P. syringae* pv. *syringae* B728a* was detected on all (cv. Eagle) or nearly all (cv. Hystyle, 92%) of the samples from sinks planted to the snap bean cultivars. Thus, early dispersal of *P. syringae* pv. *syringae* B728a* was not measurably affected by the nature of the sink. If there were differences in early dispersal, they were too small to be measured in these experiments. In contrast, overall population sizes of *P. syringae* pv. *syringae* B728a* were significantly larger in sinks planted to cv. Eagle than to cv. Hystyle or soybean (Fig. 6A, B, and C; Table 2). In the 2 years in which soybean sinks were tested, weather conditions were less than optimal for growth of *P. syringae* pv. *syringae* and little multiplication occurred on either snap bean or soybean. However, even under highly favorable environmental conditions, population sizes of *P. syringae* pv. *syringae* B728a* remained small on soybean. This is clearly illustrated by population sizes of the bacterium in association with soybean plants in barrier zones in 1994 (Fig. 5C). In this experiment, the soybeans in the barrier zones were in a sense sinks for spread of *P. syringae* pv. *syringae* B728a* from snap beans in the sources and inner “true” sinks once *P. syringae* pv. *syringae* B728a* had colonized the latter.

Similar to uninoculated sinks, *P. syringae* pv. *syringae* B728a* was detected at low frequencies in the inoculated sinks roughly 2 to 7 days after emergence of source plants. However, significantly smaller population sizes of *P. syringae* pv. *syringae* B728a* developed on cv. Eagle plants in inoculated sinks than in uninoculated sinks (Fig. 6D and F; Table 2). Population sizes of *P. syringae* pv.

TABLE 2. Summary of statistical significance of selected pair-wise comparisons of barrier and sink treatments on spread of *Pseudomonas syringae* pv. *syringae* B728a* from repeated measures analyses of variance^a

Treatment comparisons	Experiment	P value
Barrier treatment		
6 m of bare ground vs. 6 m of snap beans	1994	0.2138
	1994	0.0161 ^b
	1998	0.3385
	1999	0.3277
6 m of bare ground vs. 6 m of soybeans	1994	0.5343
	1994	0.5342 ^b
6 m of bare ground vs. 20 m of bare ground	1997	0.0800
	1998	0.6446
Sink treatment		
cv. Eagle vs. cv. Hystyle	1997	0.0057
	1998	0.0061
cv. Eagle vs. soybeans	1998	0.0014
	1999	0.0028
cv. Eagle vs. cv. Eagle + mixed phyllosphere bacteria	1997	0.0206
	1999	0.0030
cv. Eagle vs. cv. Eagle + <i>salA</i> mutant BSAL1	1999	0.0035
cv. Hystyle vs. cv. Hystyle + mixed phyllosphere bacteria	1997	0.1021
	1997	0.0002
cv. Eagle vs. 3-week cv. Eagle	1998	0.0032
	1999	0.1896
cv. Hystyle vs. 3-week cv. Hystyle	1998	0.5262

^a P values for overall treatment and treatment–time effects were 0.001 and <0.0001 in 1994 with six treatments, three blocks, and four sampling times; 0.0018 and 0.0001 in 1997 with six treatments, three blocks, and eight sampling times; 0.002 and <0.0001 in 1998 with seven treatments, four blocks, and eight sampling times; and 0.007 and <0.0001 in 1999 with seven treatments, four blocks, and seven sampling times.

^b Sources were inoculated with NPS3136 (*gacS*::Tn5); all others were inoculated with *P. syringae* pv. *syringae* B728a*.

Fig. 5. Population sizes of *Pseudomonas syringae* pv. *syringae* B728a* and the *gacS* mutant NPS3136 in association with samples collected from source, barrier, and sink areas at **A and D**, 7 days after planting (DAP); **B and E**, 14 DAP; and **C and F**, 21 DAP, in the 1994 field experiment. The data are the mean log CFU/sample and standard error based on three replicate plots, with 10 individual samples per plot.

syringae B728a* tended to be smaller in sinks planted to inoculated cv. Hystyle than in untreated Hystyle sinks (Fig. 6E). The differences, however, were not statistically significant (Table 2).

The effect of planting the sinks 3 weeks before the sources was variable across experiments (Fig. 6G, H, and I; Table 2). Spread of *P. syringae* pv. *syringae* B728a* to 3-week cv. Eagle sinks was significantly reduced relative to cv. Eagle sinks planted on the same day or a day before the sources in 1997 (Fig. 6G) and 1998 (Fig. 6H) but not in 1999 (Fig. 6I). In 1998, spread of *P. syringae* pv. *syringae* B728a* to cv. Hystyle sinks was similar regardless of when the sinks were planted relative to the sources (Fig. 6H;

Table 2). In both treatments, the extent of spread was minimal and at or nearly at the limit of detection of the plating assay.

Population sizes of *P. syringae* pv. *syringae* B728a*, total *P. syringae* (i.e., B728a* plus conspecifics based on colony forming units enumerated on P medium), and total culturable bacteria (enumerated on 10% TSA) representative of uninoculated, mixed phyllosphere bacteria-inoculated sinks and 3-week cv. Eagle sinks are shown in Figure 7. Total *P. syringae* and B728a* population sizes in the uninoculated cv. Eagle sinks were similar, indicating that the marked strain was the dominant *P. syringae* in these sinks. In contrast, total *P. syringae* population sizes were much larger

Fig. 6. Effects of the suitability of the sink habitat on spread of *Pseudomonas syringae* pv. *syringae* B728a*. **A to C,** Sinks planted to snap bean cultivars that differ in population sizes of *P. syringae* that they will support (cv. Eagle or Hystyle) or to soybean. **D to F,** Sinks planted to uninoculated snap bean seeds or seeds inoculated with a slurry of dried brown spot diseased bean leaf powder or with BSAL1 (*salA* mutant of *P. syringae* pv. *syringae* B728a). **G to I,** Sinks planted at the time of planting of the sources or 3 weeks prior to planting of the sources. The data are the mean log CFU/sample and standard error based on three (1994 and 1997) or four (1998 and 1999) replicate plots, with the following number of individual samples per plot: 10 in 1994; 17, 10, or 8 in 1997; and 15 in 1998 and 1999.

than those of B728a* in the mixed phyllosphere bacteria-inoculated and 3-week sinks (Fig. 7B, C, E, and F). Although population sizes of naturally occurring *P. syringae* may not have been the only factor contributing to the reduction in spread of *P. syringae* pv. *syringae* B728a* to inoculated and 3-week sinks, the presence of large numbers of conspecifics was clearly associated with a decrease in spread of *P. syringae* pv. *syringae* B728a*.

Taken together, results of the various sink treatments strongly indicate that the suitability of the habitat for multiplication of *P. syringae* strongly influenced spread.

DISCUSSION

Successful spread requires dispersal of an organism to a new habitat and multiplication on that habitat. Dispersal of *P. syringae* across a barrier 6 m wide occurred within a day after source plant emergence. In each of nine experiments of this kind (data from four presented here) that we have conducted over six growing seasons, *P. syringae* pv. *syringae* B728a* was consistently detected at low frequencies in the sinks when samples were first taken.

When conditions were suitable for growth of *P. syringae* pv. *syringae*, as in the 1994 and 1997 experiments, population sizes of *P. syringae* pv. *syringae* B728a* on plants that originated from clean seed (i.e., in sinks of cv. Eagle surrounded by 6 m of bare ground) were indistinguishable from those on source plants (from seeds inoculated with *P. syringae* pv. *syringae* B728a*) a few weeks after emergence. On this basis, we can conclude that num-

bers of bacteria dispersing from source to sink were sufficient to give rise to *P. syringae* pv. *syringae* population sizes at or near the carrying capacity of the bean leaves within a few weeks under the conditions prevalent during those experiments. Although dispersal may have initially limited the rate at which spread occurred, dispersal could not have limited the final outcome under these conditions.

If dispersal does not limit spread during conditions conducive for growth of *P. syringae* pv. *syringae*, what is the case during rain-free periods when these bacteria do not grow? Lindow and Anderson (18) suggested that accumulation of immigrants may be sufficient to account for most, if not all, of total culturable bacterial and ice nucleation active bacterial populations on leaves of naval orange in the California Central Valley during the winter. In general, leaves of orange trees are not favorable habitats for growth of bacteria. If we compare frequencies with which *P. syringae* pv. *syringae* B728a* dispersed to sink leaves 1 and 8 days after source plant emergence in 1998 and 1999, we can rule out simple accumulation of immigrants as an important mechanism for spread of *P. syringae* pv. *syringae* in the bean phyllosphere. If immigrant accumulation is important, then the frequencies 8 days after source plant emergence should be four to eight times greater than frequencies 1 to 2 days after emergence, assuming a more or less constant immigration rate. In 1998, the frequencies of detection of *P. syringae* pv. *syringae* B728a* actually declined between 5 and 14 DAP, and in 1999, the frequencies of detection of *P. syringae* pv. *syringae* B728a* also declined between 7 and 14 DAP (Fig. 2). Thus, on habitats suitable for multiplication of *P.*

Fig. 7. Relative population sizes of *Pseudomonas syringae* pv. *syringae* B728a*, total culturable *P. syringae*, and total culturable bacteria in sinks planted to **A and D**, uninoculated snap bean cv. Eagle seeds; **B and E**, cv. Eagle (Egl) seeds inoculated with mixed phyllosphere bacteria from a slurry of dried brown spot diseased bean leaf powder; and **C and F**, cv. Eagle seeds planted 3 weeks prior to planting of the sources. The data are the mean log CFU/sample and standard error based on three (1994 and 1997) or four (1998 and 1999) replicate plots, with the following number of individual samples per plot: 10 in 1994; 17, 10, or 8 in 1997; and 15 in 1998 and 1999.

syringae pv. *syringae*, but under weather conditions not conducive to growth, not only did detectable *P. syringae* pv. *syringae* B728a* immigrants not accumulate, but their numbers declined.

Altering the nature of the barrier had no measurable effect on spread of *P. syringae* pv. *syringae* B728a*. Increasing the distance the bacteria were forced to disperse from 6 to 20 m may have had a transient effect on spread. At the time the sinks were first sampled, *P. syringae* pv. *syringae* B728a* was detected less frequently in sinks surrounded by 20-m compared with 6-m bare-ground barriers. However, the marked strain was consistently detected at both distances by the next sampling time, usually about 3 days later. Because of the high level of variability associated with the low frequency of detection of dispersal events 1 or 2 days after source plant emergence, our data are relatively insensitive for distinguishing between the possibilities that there may have been an early slowing of the rate of spread across the 20-m (as opposed to 6 m) barriers from random variability. Snap bean barriers might have been expected to increase spread by providing an opportunity for the bacteria to spread step-wise across the barrier, and by functioning as a larger, closer source. However, spread across snap bean barriers was not significantly different from that across bare soil or soybean barriers. Because these treatments are compared across experiments done under very different environmental conditions, our conclusions may average trends that differ with conditions. For example, there was a hint (significant difference for the *gacS* mutant NPS3136 but not for B728a*) that spread across snap bean barriers was decreased relative to spread across bare-ground barriers in the 1994 experiment, when there was rapid growth of *P. syringae* pv. *syringae*. On the other hand, the trend (also not significant) was exactly the opposite with greater spread across bean barriers than across bare ground in both 1998 and 1999, years with poor conditions for growth of *P. syringae* pv. *syringae*. Although the only tenable conclusion at this time is that we could identify no barrier effect, there remains the possibility that barrier effects may be real but highly dependent on conditions for growth of the bacteria.

Environmental conditions and suitability of the habitat for bacterial growth had a marked effect on the amount of spread of *P. syringae* pv. *syringae* B728a*. Comparison of amounts of spread of *P. syringae* pv. *syringae* B728a* to cv. Eagle sinks surrounded by 6-m bare-ground barriers across all nine experiments clearly demonstrates that the extent of spread was strongly influenced by environmental conditions that favored growth of *P. syringae* pv. *syringae*. Further, the treatments that decreased the suitability of the habitat for growth of *P. syringae* pv. *syringae* B728a* diminished the amount of spread to that habitat. Soybean plants do not support much growth of *P. syringae* pv. *syringae*, and correspondingly, there was little spread to soybean sinks. Snap bean cv. Eagle plants support more growth of *P. syringae* pv. *syringae* than cv. Hystyle. There were correspondingly greater amounts of spread of *P. syringae* pv. *syringae* B728a* to cv. Eagle sinks than to cv. Hystyle. Increasing numbers of bacteria on leaves of sink plants by either inoculation of seeds with a mixture of phyllosphere bacteria or a single strain (*salA* mutant BSAL1), or by planting sinks well before the sources (i.e., 3-week sinks) to allow ample time for bacterial colonization, all diminished spread. This is reminiscent of what has been called competitive exclusion (17).

Although source strength at the time of source plant emergence varied by 4 orders of magnitude among the experiments described here, we could identify no consistent effect of source strength on spread. In the 1998 experiment, population sizes of *P. syringae* pv. *syringae* B728a* on emerging source plants were unusually low. In general, weather conditions were not optimal for growth of *P. syringae* pv. *syringae*, and the amount of spread was not particularly different from that of other experiments in which conditions were not suitable for growth (data not shown).

A number of these experiments were done at times when weather conditions were unsuitable for growth of *P. syringae* pv.

syringae (e.g., 1998 and 1999 experiments). Early dispersal was detected in these experiments, but lack of subsequent growth limited the amount of successful spread. Our conclusions that there were no significant differences between some treatments in these experiments were probably influenced by the very low numbers of bacteria associated with samples with these treatments. However, our inability to distinguish between no real difference and no measurable differences between these treatments did not influence our overall conclusions.

A question we sought to address with these experiments was the relative roles of dispersal and growth in spread of *P. syringae* pv. *syringae* in the phyllosphere. The answer is quite clear. Dispersal did not limit spread of *P. syringae* pv. *syringae* in these experiments. The suitability of conditions for growth of *P. syringae* pv. *syringae* on the habitat to which the bacteria immigrated had a significant effect on spread of this bacterium. These conclusions of course must be qualified by the limitations of the scale on which the experiments were performed, the sizes of our data sets, and the various caveats associated with comparisons between experiments done at different times and under different conditions.

The amount of bacterial brown spot disease is predictable on the basis of population sizes of *P. syringae* pv. *syringae* in association with individual leaves (14,21). The results presented here make it quite clear that within-field spread of *P. syringae* pv. *syringae* is not limited by dispersal of the pathogen but by the suitability of the habitat for growth of the pathogen. If *P. syringae* pv. *syringae* is present, then epidemics of bacterial brown spot disease should be driven almost completely by the suitability of conditions for multiplication of the pathogen in association with leaves or pods in the field. We find this a compelling explanation of why epidemics of bacterial brown spot most often occur in fields following intense rain storms, events that create conditions favorable for pathogen multiplication.

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