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Dynamics, Spread, and Persistence of a Single Genotype of *Pseudomonas syringae* Relative to Those of Its Conspecifics on Populations of Snap Bean Leaflets

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A rifampin-resistant strain of *Pseudomonas syringae* (R10) was introduced onto bean plants grown in field plots to examine the processes of growth, spread, and survival of a single genotype relative to the dynamics of its conspecifics on populations of individual leaflets. R10 was applied to four plots (400, 200, 100, and 50 m^2), each of which was centered in a quadrant of a bean field (90 by 90 m). Population sizes of the species *P. syringae* and of R10 were determined on each of 25 individual leaflets collected from the largest plot (A) at approximately weekly intervals during a 10-week period following application. The spread of R10 from all plots was monitored by leaf imprinting of individual leaflets collected at sites along four transects, each of which bisected two of the plots. The introduced strain was a dominant component of the species for about 5 weeks postinoculation on leaflets from plot A. Although the population sizes of R10 remained at about 5.0 to 5.5 $\log_{10}$ CFU per leaflet, the strain became a progressively minor component of the species as the population sizes of its conspecifics continued to increase during the latter part of the growing season. In general, a positive correlation was found for the population sizes of R10 and its conspecifics on individual leaflets collected throughout the growing season. This result suggests that large numbers of R10 early in the growing season did not exclude the colonization of bean leaflets by its conspecifics. It is apparent that the species pool comprised genotypes that were more fit than R10 under the conditions that prevailed during the latter part of the growing season. By 6 weeks postinoculation, R10 was detected at all sites sampled within the unsprayed areas of the field. However, it was present as a minor component of the species. The persistence of R10 throughout the winter and into the following growing season was monitored in plot A, which was plowed and replanted with wheat in the fall. R10 was detected on some of the samples (wheat seedlings or soil) taken at approximately monthly intervals from November to June of the following year. In June, the field was plowed and replanted with beans. We could not detect R10 on emerging bean seedlings in plot A. The results demonstrate that the successful spread and persistence of an introduced bacterium do not necessarily lead to the establishment of large populations of the bacterium in adjacent untreated areas or on its host plant in subsequent growing seasons.

Leaves of terrestrial plants provide favorable habitats for colonization by a diversity of bacterial species. We can infer from reports in the literature that many bacterial species present on leaves within a canopy at any given time are likely to contain numerous subpopulations (i.e., different genotypes) (2, 4, 8, 16, 17). There is evidence that temporal shifts among these naturally occurring subpopulations occur (2, 7, 15). Ercolani (2) isolated and characterized a large number of strains of *Pseudomonas syringae* pv. savastanoi that were isolated from olive leaves collected from 1974 to 1980. Each of the 1,050 isolates was characterized on the basis of 60 traits, including virulence, substrate utilization, antibiotic resistance, colony morphology, and growth characteristics in broth cultures. From the results obtained, Ercolani suggested that *P. syringae* pv. savastanoi exists on olive leaves as heterogeneous populations that fluctuate in response to the combined pressures of the host and the environment. Mew and Kennedy (15) observed shifts in subpopulations of *P. syringae* pv. glycinea on soybean leaves. The subpopulations in their study consisted of different races of *P. syringae* pv. glycinea. On the susceptible cultivar Acme, race 1 predominated early in the growing season; however, late in the season, race 2 was predominant. Given that a bacterial species present on leaf surfaces may contain subpopulations and that intraspecific shifts may occur in response to changes in the physical and/or biological environments, one of the questions posed in this study was the following: if a single genotype (i.e., subpopulation) of a species were introduced onto leaves, how would the dynamics of its populations compare with the dynamics of its naturally occurring conspecifics?

Several studies involving the introduction of a single bacterial genotype onto leaf surfaces have been reported (11–14, 19–21). Some were designed to evaluate the efficacy of a particular strain for biocontrol; others addressed questions related to the ecology of the species. Although the population sizes of the introduced strain and total bacteria were often measured, few studies have included a comparison of the dynamics of the introduced strain with those of its conspecifics. Notable exceptions are the experiments of Lindow and Panopoulos (13). Introduced strains of *P. syringae* that lacked a functional ice nucleation gene (Ice^-) maintained large population sizes on the treated leaves for several weeks. Negative correlations were found between population sizes of the naturally occurring ice nucleation-active (Ice^+) bacteria and the introduced Ice^- bacteria. Lindow attributed the phenomenon to competitive exclusion; that is, the establishment of introduced Ice^- bacteria on leaves before significant numbers of Ice^+ bacteria were

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present competitively excluded the subsequent colonization of the leaflets with Ice^+ bacteria.

Nearly all of the studies of the introduction of a single bacterial genotype onto leaves have relied on estimates of population sizes from bulk samples (i.e., several leaves were combined and processed as a single sample by dilution plating). However, bacterial population sizes among populations of leaves usually follow a highly skewed distribution that often can be approximated by a lognormal (3) or Weibull (5) distribution. Means estimated from bulk samples are overestimated in a way that depends upon variances; variances may be underestimated (3). Both the mean and the variance of the frequency distributions of bacterial population sizes vary with time (3). The considerable variability in population sizes of bacterial components (i.e., strain or species) on individual leaflets may be masked by estimates from bulk samples and will be completely missed if population size estimates are based on means alone. Thus, a second question addressed in this study was the following: to what extent will the population sizes of an introduced strain vary relative to those of its conspecifics on populations of individual leaves?

Because of the relatively open nature of leaf surface habitats, aerial dispersal is a significant route by which bacteria may immigrate to and emigrate from these habitats. Indeed, plant canopies have been shown to be a major source of airborne bacteria (9). Lindemann and Upper (10) reported that sunny days with wind speeds of greater than 1 m sec^{-1} were favorable for the dispersal of bacteria from leaves. The season-long net loss averaged 10^4 bacteria from a bean leaflet per day, atmospheric conditions permitting (10). Rain splash may also disperse leaf surface bacteria (10). This dispersal mechanism may be important for short-distance (i.e., leaf-to-leaf) movement of leaf surface bacteria.

The abundant, frequent aerial dispersal of bacteria from the phyllosphere is viewed as precluding the containment of bacteria on this habitat from the perspective of risk assessment, particularly in the context of the introduction of engineered microorganisms. Lindow et al. (12) examined the potential for the dispersal and survival of a marked strain of *P. syringae* introduced onto oat plants. Immediately after spraying was done, the introduced strain was detected on oat plants adjacent to the inoculated plots at densities of approximately 10^2 to 10^3 CFU/g (fresh weight) of leaf tissue, presumably because of drift of the inoculum. However, the strain failed to persist at population sizes above the limit of detection. In subsequent samples, most of the leaves taken from sites up to 33 m away from the treated plots had undetectable levels of the bacterium. When the bacterium was detected, the population sizes were smaller than 10^2 CFU/g. Thus, the spread of this strain was extremely limited. Recombinant Ice^- bacteria applied to plots of potato plants were not detected at any time on vegetation surrounding the experimental sites (13), a result indicating a complete lack of successful spread of the introduced strain.

The persistence of an introduced bacterium beyond the time frame of its intended application is a second concern in risk assessment. For leaf surface bacteria that are phytopathogens, persistence from one generation of the host plant to the next (i.e., overwintering) is of importance in evaluating inoculum sources.

Thus, the overall objective of this study was to examine several aspects of the ecology of *P. syringae*, including (i) the population dynamics of a specific introduced genotype relative to those of its conspecifics on bean leaflets, (ii) the spread of the introduced genotype from its sites of application and the likelihood of its growth on surrounding bean plants, and (iii) the persistence of the genotype from one generation of the host plant (i.e., bean) to the next. Of primary importance was to approach each aspect from the perspective that a plant canopy comprises populations of habitats. While knowledge of what occurs in the "average" habitat is useful, we were particularly interested in the variability associated with populations of individual habitats.

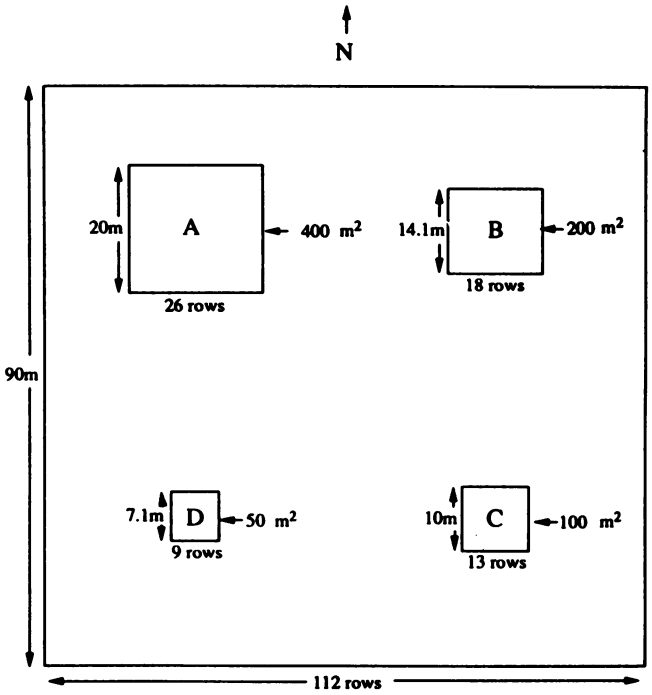

FIG. 1. Plot design. Strain R10 was applied to bean plants in plots A, B, C, and D. Bean plants in the areas surrounding the treated plots were used to monitor the dispersal of the introduced bacterium.

MATERIALS AND METHODS

Plot design. Seeds of the snap bean cultivar Eagle (Asgrow Seed Co., Kalamazoo, Mich.) were planted on 27 June in an area 90 by 90 m at the University of Wisconsin Arlington Experimental Farm. Rows, planted north to south, were 76 cm apart and contained approximately 10 plants per m. *P. syringae* R10 was applied to four plots within the 90- by 90-m area. Each plot was centered in one of the quadrants of the field (that is, the centers of the four plots were located at the corners of a square, 45 m on a side, symmetrically located within the field) (Fig. 1). The number of rows and length of each row within each plot were as follows: plot A, 26 rows of 20 m (ca. 400 m^2); plot B, 18 rows of 14.1 m (ca. 200 m^2); plot C, 13 rows of 10 m (ca. 100 m^2); and plot D, 9 rows of 7.1 m (ca. 50 m^2). The different plot sizes were used to assess the effect of source strength on the dispersal of the marked strain from the treated areas.

Preparation of the bacterial inoculum and application to bean plants. *P. syringae* K137 was selected for use in this experiment because it had originally been isolated from an aerosol over a soybean field and produced brown spot lesions on snap beans (1). The rationale for selecting strain K137 was based on the assumption that since K137 was

isolated from an aerosol fraction and was pathogenic to snap beans, it should have the potential to colonize bean plants and survive the dispersal process. A spontaneous rifampin-resistant mutant of K137 was obtained by spreading approximately 10^7 cells per plate on King's B medium (KMB) (6) containing 100 μg of rifampin per ml. The mutation rate for rifampin resistance was approximately 1.2×10^{-8}. The Rif^r isolate selected was designated R10. R10 was similar to parent strain K137 with respect to relative virulence in a pod inoculation assay and to the ability to grow on bean leaflets in a growth chamber assay.

The bacterial inoculum for application to field plots was prepared by growing strain R10 in nutrient broth on a rotary shaker at approximately 28°C. After 2 days of growth, the cultures were diluted sixfold with sterile distilled water to yield a final density of about 3×10^8 CFU/ml. A hand sprayer was used to apply the inoculum to the four plots. The volumes of the inoculum applied to plots A, B, C, and D were 40, 20, 10, and 5 liters, respectively. R10 was applied to the plots on 22 July (25 days after planting) starting at 0230 and ending at 0430. At the time of application, winds were calm and the leaves were wet with dew.

Sampling procedures for the estimation of bacterial population sizes within the treated areas. At each sampling time, 25 individual bean leaflets were collected at random from the top of the plant canopy from plot A. Plots B, C, and D were sampled similarly but less frequently than plot A. The samples were placed in paper bags and transported to the laboratory in a cooler for subsequent processing. At the laboratory, each leaflet was cut into approximately 1- to 2-cm^2 segments and placed in a flask with 25 ml of sterile potassium phosphate buffer (0.1 M, pH 7.0) supplemented with Bacto Peptone (0.1% [wt/vol]). The leaf segments were then washed for 2 h on a gyratory shaker set at 250 rpm. Portions (0.1 ml) from the original wash and appropriate 10-fold serial dilutions thereof, prepared in 0.01 M potassium phosphate buffer (pH 7.0), were plated on KMB to estimate total populations of *P. syringae* and on KMB supplemented with rifampin (50 μg/ml) (KMB-rif) to monitor population sizes of R10. In the absence of R10, there was no growth of other *P. syringae* strains and a very low background level of growth of other bacteria on KMB-rif. Cycloheximide (100 μg/ml for KMB; 200 μg/ml for KMB-rif) was added to all media to inhibit the growth of fungi. *P. syringae* colonies were counted after 3 days of incubation at ambient temperature (about 24°C). Oxidase-negative colonies with the appropriate colony morphology and fluorescence were designated *P. syringae*. Bacterial population sizes were expressed as $\log_{10}$ CFU per leaflet. The limit of detection was approximately 250 CFU per leaflet.

Sampling procedures for assessment of the spread of R10 to untreated bean plants. The spread of R10 to untreated bean plants was monitored by imprinting leaflets collected along each of four transects. Two transects started near the north edge of the planting and ended near the south edge of the planting. One ran through the center of plots A and D (transect A-D), and the other ran through the center of plots B and C (transect B-C). Two transects started near the west edge of the planting and continued to the east edge. One ran through the center of plots A and B (transect A-B), and the other ran through the center of plots C and D (transect C-D). Sampling sites along the east-west transects were located at every fifth row of beans (approximately every 3.8 m). Leaflets were collected from sites located approximately every 4.5 m along the north-south transects. Samples were taken at 1 week postinoculation from transect A-D, at 2 and 3 weeks from transects A-D and A-B, and at 6 weeks from all four transects. At each sampling time, five leaflets were collected from each site and imprinted onto KMB-rif by gently pressing each sample onto the surface of the medium. At selected sampling times, an additional set of three or five leaflets was collected from each site between plots A and D for processing by dilution plating for the estimation of population sizes of R10 and conspecifics on leaflets within the untreated areas.

Sampling procedures for detection of the persistence of R10. For assessment of the persistence of R10 over the winter, bean plants in the 90- by 90-m area were disked on 9 October and wheat was planted as a cover crop on 10 October. The area occupied by plot A was redelineated by a survey. At approximately monthly intervals starting in November, 30 wheat seedlings were collected from plot A. Each wheat plant collected on 4 November, 9 December, 12 January, and 1 March was treated as a single sample consisting of leaves, roots, and adhering soil particles. The leaves were processed separately from the roots for wheat plants collected on 18 November and 12 April. In addition, soil samples were taken on 18 November. For increasing the mass of plant material sampled on 22 May and 26 June, leaves and roots from several wheat plants were bulked prior to processing by dilution plating. Although the bulked samples would not provide reliable estimates of the mean and variance of bacterial population sizes, some information on the relative frequency of occurrence of R10 could be obtained.

The wheat was green-chopped on 23 June, and the entire area was planted with beans (cultivar Eagle) on 1 July. Bean seedlings were collected from plot A on 14 and 27 July. On 14 July, each of the 60 samples processed by dilution plating consisted of approximately 3 g of leaves and roots combined. On 27 July, each of the 60 samples consisted of approximately 10 g of leaf tissue. All samples were washed and dilution plated as described above.

RESULTS AND DISCUSSION

Population sizes of R10 on bean leaflets immediately following inoculation. At the first sampling time, 1.5 h after the application of R10, the range of population sizes of R10 on a set of 25 individual leaflets was about 10-fold and the variance was 0.070, indicative of relatively little leaf-to-leaf variability (Fig. 2 and 3). The distribution of population sizes of R10 at 1.5 h postinoculation could be adequately fit by the lognormal and was better fit by a lognormal than a normal distribution, on the basis of the Shapiro-Wilk test for normality (18). This test generates a statistic (Shapiro-Wilk W statistic) that is a correlation between the spacing of the ordered data and that of the ordered expected values for a normal distribution. The value of W approaches one the more nearly normally distributed the data set is. W statistic values were 0.905 ($0.02 < P < 0.05$) and 0.955 ($0.1 < P < 0.5$) for untransformed and log-transformed population sizes of R10 at 1.5 h postinoculation, respectively. The hypothesis of normality would be narrowly rejected for the untransformed data.

In the following few hours, three things happened: population sizes of R10 decreased rapidly, variability among population sizes increased significantly, and the frequency distributions of population sizes shifted strongly toward a lognormal (Fig. 2 and 3). Between 1.5 and 13 h postapplication, mean population sizes of R10 declined nearly 100-fold, with little change in the following 13 h. The range of

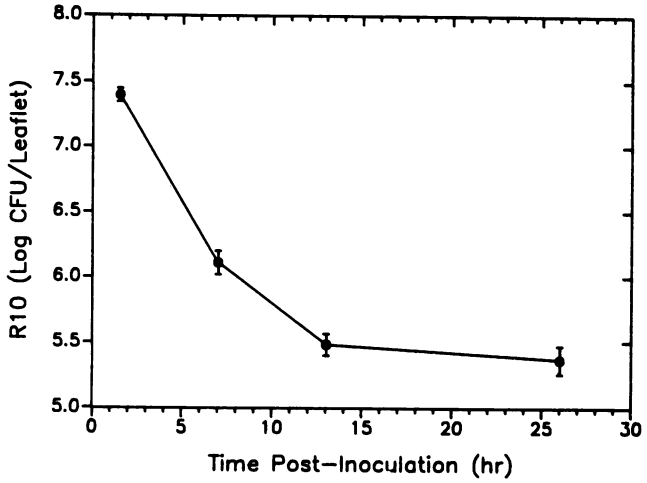

FIG. 2. Survival of R10 during the 26 h following application to plants in plot A. R10 population sizes were estimated for each of 25 individual bean leaflets per sampling time. Each datum point represents the mean R10 population size expressed as $\log_{10}$ CFU per leaflet. Error bars represent the standard error of the mean.

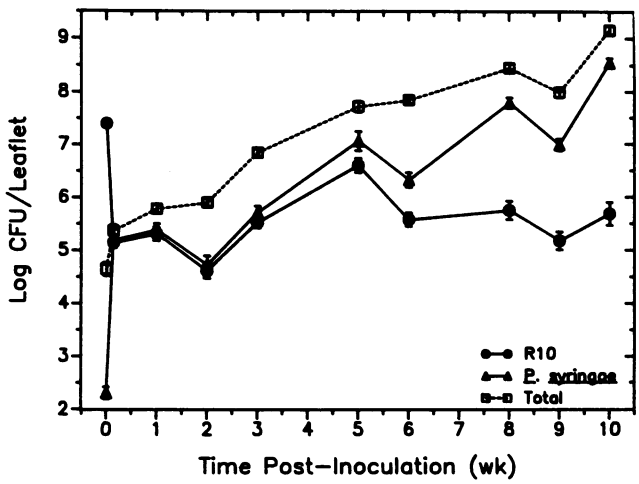

FIG. 4. Seasonal changes in mean population sizes of R10, total *P. syringae*, and total bacteria culturable on KMB. The mean population sizes of total *P. syringae* and total bacteria plotted at time zero were estimated from leaflets collected immediately before the application of R10. All leaflets were collected from plot A. Bacterial population sizes were estimated for each of 25 individual bean leaflets per sampling time. The mean $\log_{10}$ CFU per leaflet and standard error of the mean are shown.

population sizes 7 h postinoculation had increased to 150-fold (Fig. 3) and the variance had increased to 0.216, values typical for field samples. If the distribution at 1.5 h was better fit by a lognormal than a normal, then this distinction was much clearer a few hours later (W = 0.359 at 7 h, 0.700 at 13 h, and 0.488 at 26 h postinoculation for untransformed data, but W = 0.928 at 7 h, 0.990 at 13 h, and 0.943 at 26 h for log-transformed data). The lognormal provided a better fit than the Weibull distribution for data sets collected at these sampling times.

The decrease in mean population sizes indicated that death was the dominant process following inoculation. The increase in population variance suggested that the rate at which death occurred and/or the duration of the period during which death was likely to occur differed among the individual leaflets. Because a spray application of bacteria should generate a random distribution of sizes of the applied bacterium on leaflets, we expected that there would be relatively little variability immediately after application and that the bacteria would be normally distributed among leaves. Although the variability was still relatively small 1.5 h postinoculation, even at this early time, population distributions were becoming lognormal, suggesting that death had influenced the populations substantially by this time. Because the inoculum consisted of cells that had been grown under conditions (i.e., in broth cultures) very unlike those on leaf surfaces, it is perhaps not surprising that large proportions of the introduced populations died. Wilson and Lindow (22) examined the effect of culture conditions on the survivability of *P. syringae* introduced onto plants. Cells grown on solid media or those harvested from inoculated plants were better able to survive on plants immediately following inoculation than cells grown in liquid media (22). Nonetheless, the rapid development of the lognormal distribution and the increase in leaf-to-leaf variability suggest strongly that the likelihood of death was affected by the leaf habitat upon which the bacteria arrived.

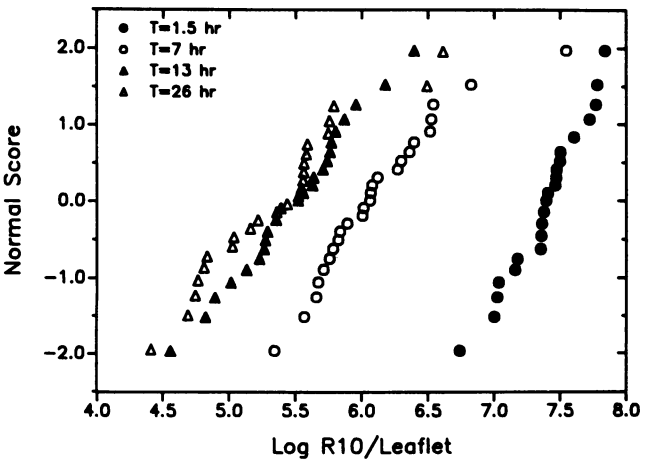

FIG. 3. Distributions of population sizes of R10 on individual bean leaflets collected at 1.5, 7, 13, and 26 h postinoculation.

Seasonal dynamics of population sizes of R10 on bean leaflets. Changes in mean population sizes of R10 relative to those of the species *P. syringae* (i.e., R10 plus naturally occurring conspecifics) and total bacteria culturable on KMB are shown in Fig. 4. Population sizes of *P. syringae* and total bacteria plotted at time zero were estimated from bean leaflets collected immediately prior to the application of R10. At the time of inoculation, very small numbers of *P. syringae* were present on the bean plants. Indeed, naturally occurring *P. syringae* was detected on only 7 of the 25 leaflets sampled. Within 26 h postinoculation, population sizes of R10 had decreased to levels approximating those of total bacterial populations present at the time of inoculation. Thus, 1 day after application of a >100-fold excess of R10, total bacterial numbers were only slightly larger than they had been immediately before application. The application of large numbers of bacteria had little effect on the apparent sizes of populations that the leaves could support.

R10 remained the dominant *P. syringae* strain for at least

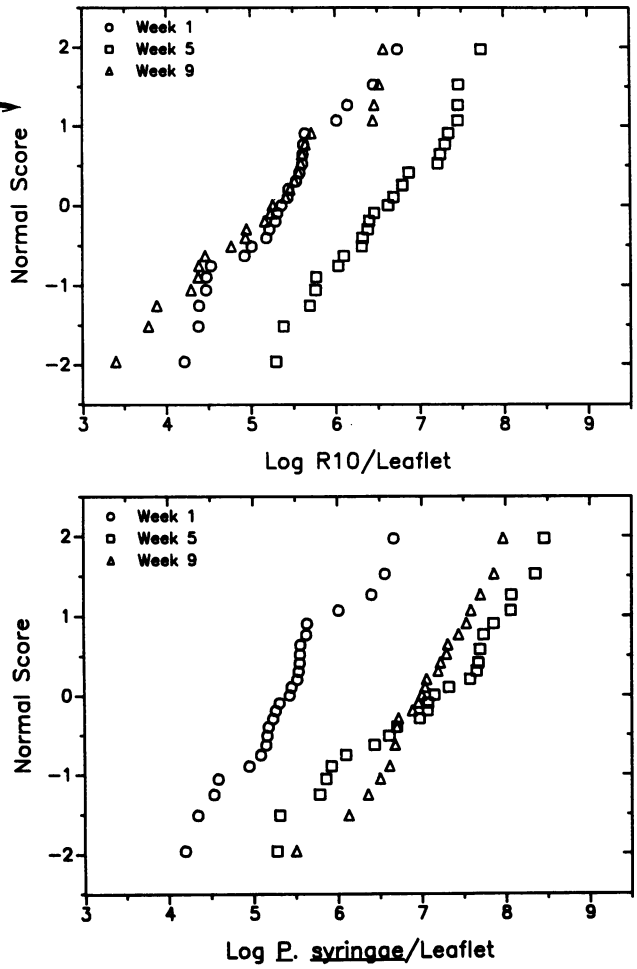

FIG. 5. Distributions of population sizes of R10 and total *P. syringae* on individual leaflets collected from plot A at 1, 5, and 9 weeks postinoculation.

5 weeks postinoculation. During this period, conditions were favorable for the growth of R10 as reflected in the almost 100-fold increase from 2 to 5 weeks postinoculation. Conditions during this period were also favorable for growth of the species *P. syringae*. From 6 to 10 weeks postinoculation, the mean population sizes of R10 persisted at about 10^5 CFU per leaflet. This single genotype became an increasingly smaller component of the species as the population sizes of *P. syringae* increased about 100-fold during this period.

Plots of normal scores versus population sizes of R10 and the species on individual leaflets for selected sampling times are shown in Fig. 5. Although population sizes of R10 and *P. syringae* varied by 100- to 1,000-fold among individual leaflets at a given sampling time, the data indicate that when conditions were favorable for population increases, bacterial populations increased on all leaflets. Similarly, when decreases in mean population densities occurred, R10 and *P. syringae* populations on all leaflets responded similarly. This is evidenced by the shifts in distributions of populations of R10 from week 1 to 5 (increase) and from week 5 to 9 (decrease).

Although population sizes of R10 did not increase from 5 to 10 weeks postinoculation, population sizes of the species *P. syringae* did. In Fig. 6, the population size of R10 on a given leaflet is plotted against the population size of *P. syringae* on that same leaflet. Since *P. syringae* population sizes include R10 as well as naturally occurring conspecifics, datum points on or near the broken line (*P. syringae* = R10) indicate that R10 was the dominant genotype within the *P. syringae* pool on those leaflets. Thus, at weeks 1 and 3 postinoculation, R10 was the dominant *P. syringae* genotype on almost all of the leaflets. By weeks 5 and 6, R10 was still dominant on some of the leaflets; however, large numbers of conspecifics were present on others. By weeks 8 and 9, R10 was clearly a minor *P. syringae* component on all of the leaflets.

From 6 to 9 weeks after application, when conspecifics became more frequent than R10 on almost all of the leaflets, there appeared to be no correlation (week 6: $r^2 = 0$) or a small positive correlation (week 8: $r^2 = 0.31$, $P < 0.01$, with the exclusion of an extreme outlier plotted at R10 = 3 and *P. syringae* = 9 in Fig. 6; week 9: $r^2 = 0.51$, $P < 0.01$) between the population sizes of R10 and *P. syringae* on individual leaflets (Fig. 6). The slopes of the regression lines of *P. syringae* versus R10 were much less than one during this period (week 6: m = 0.147; week 8: m = 0.370, with the exclusion of the outlier described above; week 9: m = 0.467) (Fig. 6). Thus, although leaflets with larger population sizes of R10 tended to support larger population sizes of conspecifics, the proportion of R10 within the *P. syringae* population increased as the population of conspecifics increased.

From 6 to 10 weeks after introduction, population sizes of R10 changed little, while those of the species *P. syringae* increased more than 100-fold, resulting in a comparable diminution in the proportion of the total *P. syringae* population that was R10. It is not likely that the increase in population sizes of conspecifics relative to the population sizes of R10 was due to a selective increase in a subpopulation of R10 cells that had reverted to rifampin susceptibility. A large number of conspecifics were isolated in pure cultures and assayed for their ability to cause symptoms of bacterial brown spot in a pod inoculation assay. The strains tested were more virulent than R10 and hence differed phenotypically with regard to pathogenicity. If rates of change in relative abundance are an indication of relative fitness, then R10 was less fit than its conspecifics during the period when population sizes of R10 varied little while those of the species increased substantially. Strains of *P. syringae* are known to be highly variable genotypically and phenotypically (4). Perhaps different subpopulations of *P. syringae* (i.e., different genotypic pools within the species) are dominant under different physical or biological environmental conditions, as has been reported for *P. syringae* pv. savastanoi on olive leaves (2).

It is interesting to note that a single genotype was able to grow and persist on bean leaflets as the host plants developed through the flowering stage (ca. weeks 2 to 4 postinoculation), pod set and maturity (pod harvest would have occurred at weeks 5 to 6 postinoculation), and senescence (plants were dead by week 10 because of frost). R10 persisted for almost 1 month (weeks 6 to 10) as a minor component on all of the leaflets sampled. Whatever the processes that led to its relative diminution as a proportion of the total species, R10 was driven to extinction on none of the sampled leaflets. During this time, the bacterium successfully maintained its presence in the gene pool of the species. We do not know whether this was achieved through dormancy or whether growth was balanced by death. Some dispersal and growth must have occurred, because the emergence and growth of new leaves continued throughout

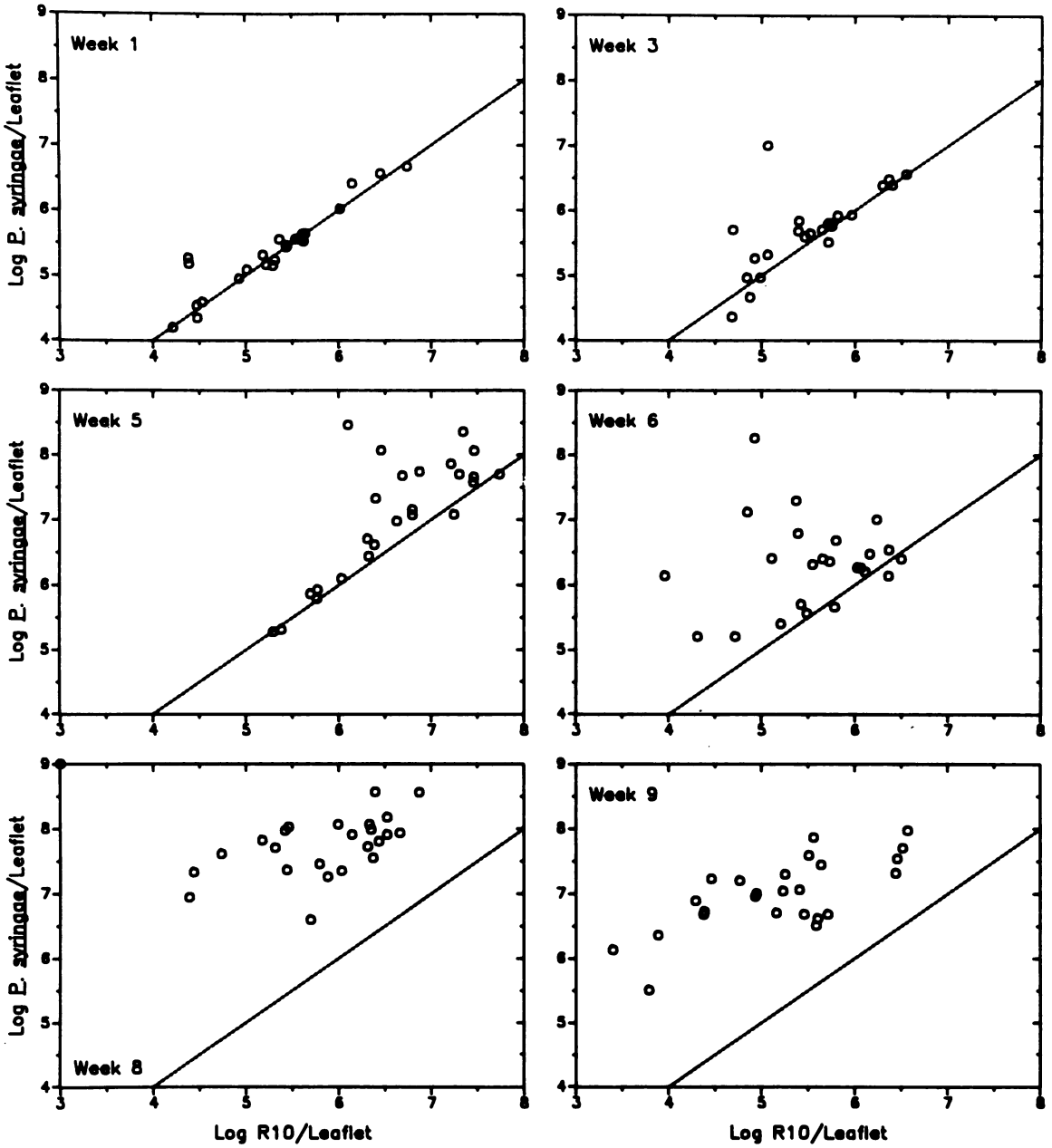

FIG. 6. Population sizes of R10 and total *P. syringae* on individual leaflets. Population sizes of R10 were enumerated on KMB-rif. Population sizes of total *P. syringae* were enumerated on KMB and include R10. The lines represent the hypothetical case in which all *P. syringae* are R10 and are provided as a reference for comparing the densities of R10 and total *P. syringae* over time.

this period. Nonetheless, if the ability to transmit its genotype to progeny is used as a definition of fitness, R10 successfully demonstrated its fitness to maintain itself within the community on every leaf habitat within the area to which it was applied.

Population sizes of R10 were estimated less frequently on leaflets from plots B, C, and D than from plot A (Fig. 7). However, a comparison of population sizes of R10 in plots A and D at week 1 and plots A, B, C, and D at week 5 indicated that the numbers of R10 were quite similar in all plots.

Spread of R10. The spread of R10 from the source plots was monitored primarily by leaf imprinting (Fig. 8). At each sampling time, leaflets were collected from within the treated areas as well as the untreated areas along the selected transects. All leaflets from within the plot areas had detectable levels of R10 throughout the experiment (data not included in Fig. 8 for clarity of illustration). The first sampling, conducted at 1 week postinoculation, was along a row that bisected plots A and D. R10 was detected at only 4 of 15 sites in the unsprayed area. At 2 weeks postinoculation, two transects were sampled, along a row that bisected plots A and D (transect A-D) and across rows that bisected plots A and B (transect A-B). A larger number of sites along transect A-D had detectable levels of R10 at week 2 than at

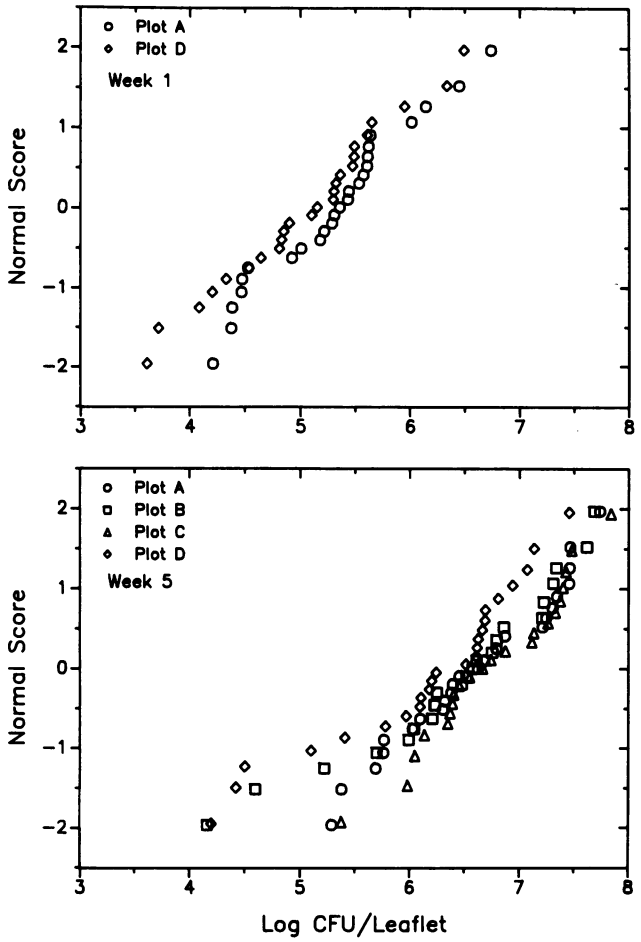

FIG. 7. Distributions of population sizes of R10 in plots A and D at week 1 postinoculation and in all four plots at 5 weeks postinoculation.

week 1. In addition, R10 was present at a larger number of sites along a row (transect A-D) than at sites across rows (transect A-B). The absence of a completely continuous canopy across rows versus along rows may have contributed to the latter observed difference. If this were the case, then zones of barren ground around an inoculated area should minimize the spread of bacteria introduced onto foliage. Alternatively, the physical environment may have favored north-south spread over east-west.

By 6 weeks postinoculation, R10 was detected by leaf imprinting at almost all sites along all transects in the field. The frequency with which R10 was found on individual leaflets appeared to decrease with increasing distance from the inoculated areas along rows (i.e., transects A-D and B-C). However, no obvious gradients were observed across rows (transects A-B and C-D). In addition, no obvious effect of source strength was detected, at least on the basis of the frequency with which leaflets harbored detectable levels of R10.

More precise measurements of R10 population sizes in the unsprayed areas were made by dilution plating of leaflets collected at sites along transect A-D (Table 1 and Fig. 9). Population sizes of the applied bacterium were, in general, relatively small on leaflets in the unsprayed area, on the basis of samples collected along transect A-D (Table 1 and Fig. 9). We compared the qualitative view of the extent to which R10 spread (Fig. 8) to the quantitative data for R10 population sizes in areas in which R10 was introduced in large numbers (Fig. 4) and in areas to which it spread (Table 1). We found reason to speculate as to what processes might have been important in the extensive but relatively slow spread of this single genotype. Dispersal must have occurred to most if not almost all of the leaves in the bean planting. However, dispersal of one or a few cells alone would not provide stable population sizes sufficiently large to be detected. The limit of detection by dilution plating was about 200 CFU per leaflet. Thus, for dispersal to provide population sizes sufficient to be detected by this method, more than 200 viable and platable immigrants must have been present on the leaflets on which the strain was found. Alternatively, if a single viable immigrant had arrived on each of such leaflets, it must have grown successfully (without cell death) for at least eight generations to reach a detectable population size. Thus, multiple immigration events, growth, or (more likely) some combination thereof must have occurred on each leaflet on which R10 was detected by dilution plating. Leaf imprinting was somewhat more sensitive than dilution plating, and fewer immigrants may have been detected by the latter method. However, the frequency with which leaf imprinting detected R10 in transect A-D was not greater than 1.5 times that for dilution plating (compare Table 1 and Fig. 8). Even detection by leaf imprinting probably was preceded by both immigration and growth.

Although the net growth of R10 inside plot A was minimal from day 1 to week 6, there was about a 100-fold increase in the median population size of this strain from weeks 2 to 5 (Fig. 4). The population sizes of R10 outside plot A at the end of week 6 (Table 1) were approximately those that would be expected if one or a few cells had immigrated to leaves and then grown at the level that occurred during the first 5 weeks in the inoculated area.

The observation that R10 did not establish large population sizes at outside the inoculated plots despite the presence of large numbers within the inoculated areas parallels the results of other experiments involving the introduction of bacteria to the phyllosphere (12, 13). Ice$^-$ strains of *P. syringae* that were applied to potato plants maintained large population sizes on potato leaves but were nondetectable or present at only very low numbers on vegetation surrounding the experimental plots (13). Lindow attributed the lack of survival of the applied strains to their inability to compete with the resident microflora on the uninoculated plants (11, 13). The results of our experiments indicated that the inability of R10 to multiply to large numbers following dispersal to untreated plants did not prevent the successful spread of this strain. It is reasonable to believe that the restraint placed on population sizes of R10 in the inoculated areas was also present outside those areas. Clearly, other genotypes of conspecifics present within the bean field were more fit (relative-abundance definition) than R10. If competition from these genotypes provided the restraint (one of several possibilities) that prevented the multiplication of R10, the same competition should have occurred outside the inoculated areas, the sites to which R10 spread quite successfully.

Although R10 became a minor subpopulation of the species with time, the applied bacterium was detected on all leaflets sampled from the inoculated plots. Hence, this particular genotype was successful in persisting on bean leaflets. Furthermore, R10 appeared to survive dispersal well, as evidenced by its presence, albeit in low numbers, throughout the field by 6 weeks postinoculation. Perhaps by

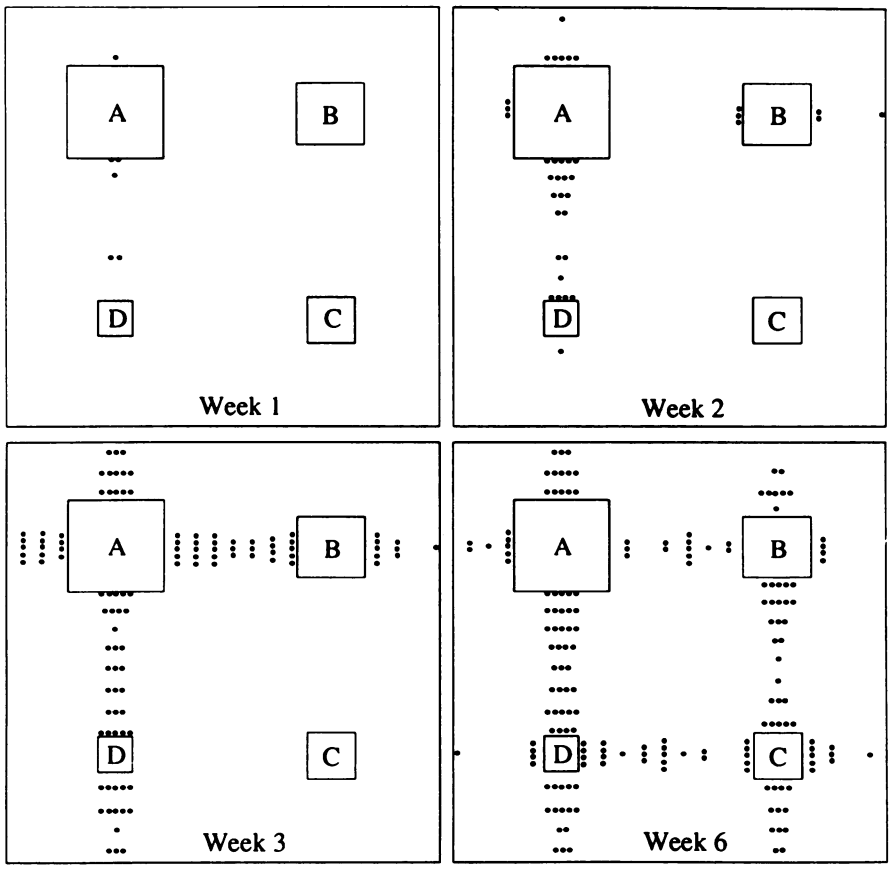

FIG. 8. Spread of R10 from plots A, B, C, and D. Five leaflets were collected every 4.5 m along transects that bisected plots A and D and plots B and C and every fifth row along transects that bisected plots A and B and plots C and D. Each leaflet was imprinted onto KMB-rif. Closed circles represent leaflets with R10. All transects were not sampled at each sampling time. Transect A-D was sampled at weeks 1, 2, 3, and 6, transect A-B was sampled at weeks 2, 3, and 6, and transects B-C and C-D were sampled at week 6 only. All leaflets collected at sites within the treated plots had detectable R10 at all sampling times.

choosing a strain for use in these experiments that was initially isolated from a natural aerosol, we inadvertently selected a genotype that derives its fitness (transmission-of-genotype-to-progeny definition) from adaptation to dispersal and persistence rather than from the achievement of large population sizes on bean leaflets.

Overwintering of R10. The entire field was disked on 9 October. By this time, the bean plants were dead because of frost. Thirty pods were collected on 9 October, just prior to disking of the field. R10 was present on 29 of the 30 pods sampled. Population statistics (expressed as $\log_{10}$ CFU per pod) for R10 and total *P. syringae* were as follows: for R10, mean = 5.58, s^2 = 1.54; for *P. syringae*, mean = 8.211, s^2 = 0.55. Thus, relatively large numbers of R10 were present on the leaves (Fig. 4; week 10 = 30 September) and pods at the time of disking. Wheat seeds were planted in the field on 10 October, and plot A was redelineated. Because the sampling unit and weight per sample were not the same for all samples taken during the following winter, spring, and early summer, we include this information in Table 2. R10 was detected on some of the samples at all times throughout the winter (Table 2). On three occasions when the leaves were processed

TABLE 1. Population sizes and frequencies with which total *P. syringae* and R10 were detected in the unsprayed area at sites between plots A and D along transect A-D

Wk postinoculation	R10			Total *P. syringae*		
	Population size[a]		% with detectable R10 (no. of leaflets sampled)	Population size[a]		% with detectable *P. syringae* (no. of leaflets sampled)
	Mean	SE		Mean	SE	
0				2.52	0.093	28.0 (25)
1	2.33	0.016	8.8 (34)	3.49	0.190	79.4 (34)
2	2.53	0.098	25.7 (35)	4.13	0.220	82.3 (35)
3	2.65	0.103	41.2 (34)	5.64	0.154	100.0 (30)
6	3.36	0.240	62.5 (24)	6.76	0.082	100.0 (23)

[a] Expressed as $\log_{10}$ CFU per leaflet. Leaflets with no detectable bacteria were assigned a limit of sensitivity of 2.3 $\log_{10}$ CFU per leaflet.

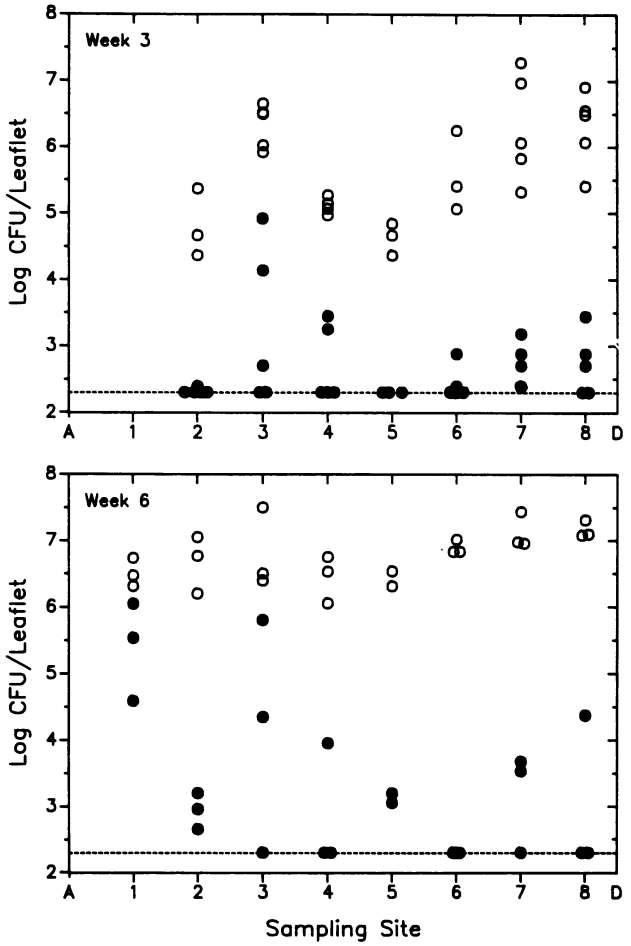

FIG. 9. Population sizes of R10 (●) and total *P. syringae* (○) at sites between plots A and D. Site 1 was located immediately south of plot A; site 8 was located immediately north of plot D. Values plotted at 2.3 $\log_{10}$ CFU per leaflet were at our limit of sensitivity (i.e., no R10 was detected on these leaflets).

separately from the roots of the wheat plants, the percentage of samples with R10 was higher for roots than for leaves. R10 was also present in samples that consisted of soil and plant debris. Representative examples of the distributions of measurable population sizes of R10 are given in Fig. 10. We do not know whether R10 was able to colonize to some extent the root systems of the wheat cover crop or was associated with the root systems as a hitchhiker in adhering soil and small bean debris particles.

Although R10 was detected on some of the samples taken throughout the winter and spring and even at a low frequency (18.3%; Table 2) on wheat roots collected on 26 June, the bacterium was not detected on bean root or foliage samples collected on 14 and 27 July. Thus, if R10 were present on the second generation of bean plants, population sizes of the bacterium were less than approximately 100 CFU/g, the limit of sensitivity of the method used.

Populations of leaf surface bacteria exist on populations of habitats within a plant canopy. Variability in population sizes of these bacteria among individual leaf habitats is an inherent characteristic of the system. An understanding of the factors that regulate the dynamics of leaf surface bacterial populations and communities must include an understanding of the mechanisms that underlie this quantitative variability. There is considerable variability within the gene pool of a species. It is of interest to understand those attributes (genes) that confer fitness to subpopulations within the species as a function of changes in the physical and biological environments.

Although the primary motivation of this study was to examine aspects related to the ecology (i.e., potential for growth, dispersal, and persistence) of a single genotype introduced onto leaf habitats, the information gathered may

TABLE 2. Persistence of R10 in plot A throughout the winter and subsequent growing season

Sampling date	Sampling unit[a]	Avg wt of sample ± SD (g)	No. of samples	% with R10
Wheat				
4 November	Leaves + roots	0.15 ± 0.05	30	60.0
18 November	Leaves	0.05 ± 0.01	30	0.0
	Roots	0.17 ± 0.09	30	43.3
9 December	Leaves + roots	0.13 ± 0.04	30	40.0
	Soil + debris	0.53 ± 0.03	30	60.0
12 January	Leaves + roots	0.14 ± 0.02	30	26.6
1 March	Leaves + roots	0.30 ± 0.14	30	46.6
12 April	Leaves	0.25 ± 0.09	30	13.3
	Roots	0.70 ± 0.26	30	46.6
22 May	Leaves[b]	9.34 ± 1.84	30	6.6
	Roots[b]	7.50 ± 3.27	30	46.6
26 June	Roots[b]	9.06 ± 1.65	60	18.3
Beans				
14 July	Leaves + roots	3.27 ± 0.67	60	0.0
27 July	Leaves[b]	9.97 ± 0.40	60	0.0

[a] Unless otherwise indicated, sampling units consisted of individual seedlings (i.e., leaves plus roots), all leaves from an individual plant (leaves), or all roots from an individual plant (roots). Root samples included adhering soil particles.

[b] Sampling units consisted of several leaves or root systems (i.e., bulk samples).

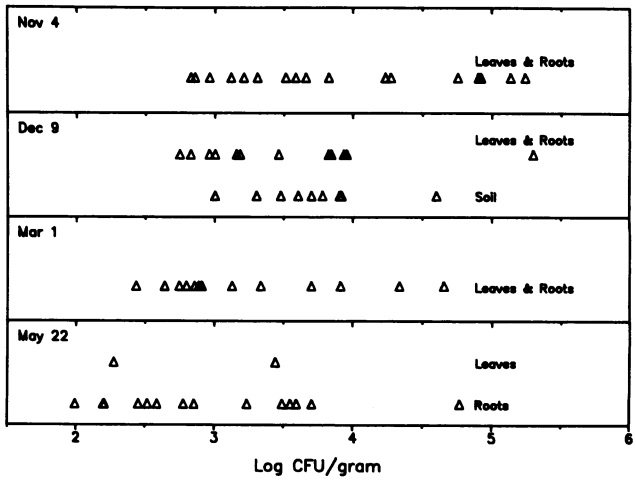

FIG. 10. Overwintering of R10. The sampling unit on 4 November, 9 December, and 1 March consisted of individual wheat seedlings (i.e., leaves plus roots). On 22 May, the leaves from individual wheat plants were processed separately from the roots. The soil samples (9 December) consisted of soil and plant debris. Population sizes of R10 are shown for samples with detectable numbers of R10. For each sampling time, the proportions of samples that had no detectable R10 are presented in Table 2.

be useful in a consideration of the risks associated with the introduction of recombinant bacteria onto these habitats. Our results emphasize the importance of evaluating the likelihood that an introduced bacterium will grow on leaf surfaces away from its site of introduction, not merely whether dispersal will occur or not. Similarly, the fact that an introduced bacterium may persist after the completion of an experiment (e.g., overwintering in the plot area) is of no consequence if it is not able to become reestablished on plants in the following growing seasons. In evaluating the potential for the growth of an introduced bacterium on leaves to which it is applied, on adjacent nontreated plants, and on subsequent generations of plants, it is useful to approach these issues from the perspective that we are concerned with populations of bacteria on populations of habitats.

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