Epiphytic Populations of *Pseudomonas syringae* on Susceptible and Resistant Bean Lines

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Sincere appreciation to A. Kelman for providing bacterial cultures, to S. A. Vicen for figure preparation, and to R. E. Rand for technical assistance.

Research supported by the College of Agriculture and Life Sciences, University of Wisconsin-Madison as Project 232.

Accepted for publication 4 November 1980.

ABSTRACT

Daub, M. E., and Hagedorn, D. J. 1981. Epiphytic populations of *Pseudomonas syringae* on susceptible and resistant bean lines. Phytopathology 71:547-550.

Large differences were found in populations of virulent *Pseudomonas syringae* (cause of bacterial brown spot of bean) on resistant and susceptible bean lines in the field. About 10^6 cells per gram fresh weight were isolated from leaves of the susceptible cultivar Eagle compared to about 10^3 cells per gram fresh weight from leaves of the resistant plant introduction, WBR 133. Epiphytic populations on resistant breeding lines were intermediate to those on cultivars Eagle and WBR 133. These lines were also intermediate in reaction to the brown spot pathogen. There was, however, no direct correlation between populations of *P. syringae* and brown spot disease severity on the breeding lines. *P. syringae* was identified by morphology on Crosse's medium and by production of typical brown spot symptoms on bean pods in two kinds of pod inoculation tests. Lower populations on the resistant lines did not appear to be due to the presence of antagonistic epiphytic microorganisms.

Bacterial brown spot of snap beans (*Phaseolus vulgaris* L.) caused by *Pseudomonas syringae* van Hall is a serious disease in Wisconsin. In 1974, Ercolani et al (6) reported that strains of *P. syringae* virulent on beans survived as epiphytes on hairy vetch (*Vicia villosa* L.) a common leguminous weed in bean-growing areas in central Wisconsin. Strains of *P. syringae* highly virulent on beans were the dominant components of the gram-negative epiphytic microflora on hairy vetch throughout most of the year. Populations as high as 10^6–10^7 cells per gram fresh weight (GFW) were isolated during the fall, winter, and spring. *P. syringae* could rarely be isolated in July and August, however. The presence of high populations of epiphytic *P. syringae* on hairy vetch in June was correlated with subsequent outbreaks of brown spot on beans in nearby fields.

P. syringae is an ubiquitous epiphyte. Lindow et al (12) found *P. syringae*-type bacteria (fluorescent, oxidase-negative, and arginine dihydrolase-negative) on apparently healthy plants of 74 of 95 species sampled in California, Colorado, Florida, Louisiana, and Wisconsin. Only conifers as a group did not harbor epiphytic populations of *P. syringae*. None of these isolates were tested to determine if they were potentially pathogenic to their plant associate, but *P. syringae* has been found to exist as an epiphyte on several of its host plants, namely peach (5), pear (14), tomato (16), and wheat (7). Leben et al (11) found that *P. syringae* could exist as an epiphyte on buds, young leaves, and stipules of healthy bean seedlings if the seedling buds were inoculated with the bacterium and placed in a humid atmosphere.

0031-949X/81/05054704/$03.00/0

Differences in epiphytic populations of bacterial pathogens on susceptible and resistant hosts have been noted. Crosse (3) found that epiphytic populations of *Pseudomonas mors-prunorum* on cherry were three times greater on the susceptible cultivar Napoleon than on the resistant cultivar Roundel. Lawrence and Kennedy (10) found that significantly larger populations of *Pseudomonas glycinea* developed on germinating seeds of a susceptible, as compared to a resistant, soybean cultivar. We conducted this study to determine whether *P. syringae* existed as an epiphyte on beans during the summer (when it cannot be isolated from hairy vetch), and whether epiphytic populations were different on different bean lines. This paper reports results of the isolation of *P. syringae* from resistant and susceptible bean lines in the field in 1976, 1977, and 1978.

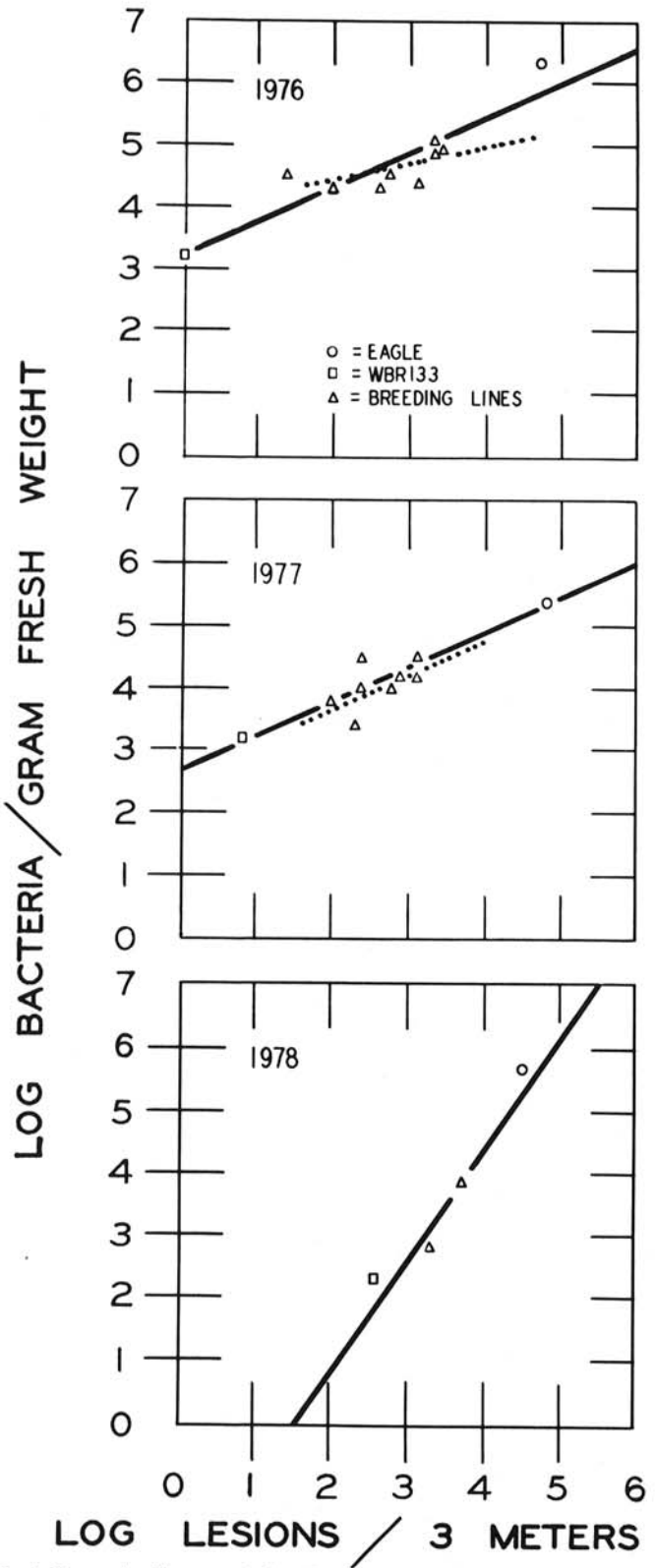

Fig. 1. Regression lines correlating disease susceptibility (number of lesions per 3 m of row) with epiphytic populations of *Pseudomonas syringae* on bean cultivars Eagle, WBR 133, and eight breeding lines in 1976, 1977, and 1978: ———— regression line for all 10 bean lines; • • • • • regression line for breeding lines only.

MATERIALS AND METHODS

Studies were conducted in a bean bacterial brown spot resistance-breeding nursery at the Hancock Experimental Farm in central Wisconsin. "Test" rows were planted with six of the bean lines to be tested and four control plantings (susceptible cultivar Eagle) so that each test line bordered on a control planting. These test rows alternated with "spreader" rows, which were planted with Eagle seed mixed with leaf powder inoculum. This inoculum was prepared from leaves naturally infected with *P. syringae* that had been collected during the previous season. After being dried rapidly at 20 C, the leaves were ground in a hammer mill and stored in plastic bags at 8 C. Test lines were randomized within each of three to four replicates.

Studies were carried out during the summers of 1976, 1977, and 1978. Plots were planted about the second week in June. Bean lines were sampled for epiphytic bacteria from the first to the second trifoliolate leaf stage (3–4 wk from planting date) until leaf senescence.

Disease severity was determined after pods developed by estimating the number of lesions on a particular test line per 3 m of row. The lesions on a representative sample of plants from at least two replications were counted, and the average number of lesions per 3 m of row was calculated based on these counts.

Epiphytic populations were monitored on all lines in 1976 and 1977 and on cultivars Eagle and WBR 133, and two breeding lines

TABLE 1. Brown spot disease reaction of bean cultivars Eagle, WBR 133, and eight breeding lines used in epiphyte sampling studies

Bean line	Mean no. of lesions per 3 m of row		
	1976	1977	1978
Eagle	4.7×10^4	5.6×10^4	3.4×10^4
25	1.2×10^3	1.2×10^3	5.0×10^3
85	2.5×10^3	4.5×10^2	...
211	2.0×10^3	1.2×10^3	...
189	1.8×10^3	6.5×10^2	...
164	5.0×10^2	1.0×10^2	...
19-6	4.0×10^2	2.0×10^2	...
128	2.5×10^1	2.5×10^2	...
BBSR 130	1.0×10^2	2.5×10^2	1.7×10^3
WBR 133	1	6	3.9×10^2

TABLE 2. Mean epiphytic populations of *Pseudomonas syringae* isolated from bean cultivars Eagle, WBR 133, and eight breeding lines in the field in 1976, 1977, and 1978

Bean line	Log mean epiphytic population per gram fresh weight		
	1976[a]	1977[b]	1978[c]
Eagle	6.317	5.407	5.681
25	4.411	4.488	3.903
85	4.903	3.967	...
211	5.102	4.219	...
189	4.886	4.298	...
164	4.543	3.786	...
19-6	4.343	3.370	...
128	4.488	4.450	...
BBSR 130	4.273	4.030	2.824
WBR 133	3.216	3.240	2.287

[a] LSD ($P = 0.01$) = 0.638; LSD ($P = 0.05$) = 0.480.
[b] LSD ($P = 0.01$) = 0.719; LSD ($P = 0.05$) = 0.393.
[c] LSD ($P = 0.01$) = 1.043; LSD ($P = 0.05$) = 0.777.

(25 and BBSR 130) only in 1978. Samples were taken in the morning at weekly intervals until leaves started to senesce or until no control leaves could be found without lesions. All samples were taken from plants in the test rows. Samples were taken from two replications in the field. The lines were sampled by picking individual leaflets from trifoliolate leaves of the plants. Care was taken to pick a representative selection of leaves including old and young leaves from upper and lower parts of the plant and on both sides of the row; these were inspected carefully to make sure they had no visible lesions and were transported immediately to the laboratory and sampled.

Epiphytic populations of *P. syringae* were determined by using the methods of Ercolani et al (6). Approximately 10 g of whole leaflets without visible lesions were placed in 300 ml of sterile distilled water in a 1-L flask and shaken for 2 hr on a reciprocal shaker at room temperature. One-milliliter samples of 10-fold dilutions of the leaf washings were pipetted into petri dishes and mixed with 20 ml of the plating medium (molten Crosse's medium [2] mixed with 10 ml/L of a sterile 21% [w/v] solution of manganous sulfate). Plates were incubated at room temperature and colonies were counted under a dissecting microscope after 2–3 days. *P. syringae* forms a distinctively characteristic colony on this medium (2). The minimum detectable population of *P. syringae* using this method was 30 cells per gram fresh weight.

Each week a representative sample of colonies identified as *P. syringae* by colony morphology on Crosse's medium was tested for production of typical brown spot symptoms on bean pods in order to confirm that they were in fact virulent *P. syringae*.

Two different pod inoculation tests were used: Test A—Pods of the susceptible cultivar Tenderwhite were injected with three concentrations (10^8, 10^6, 10^4 cells per milliliter) of the test isolate. Virulent isolates produced green, sunken, water-soaked lesions that extended beyond the water-soaked area; avirulent isolates produced sunken, necrotic lesions that did not expand (6). Test B—Drops of suspension (10^9 cells per milliliter) of the test isolates were placed on pods, and the pods were pricked through the drops (15). Virulent isolates produced sunken, necrotic, and water-soaked lesions. Avirulent isolates produced small, necrotic spots at the point of inoculation.

Both tests could distinguish virulent isolates of *P. syringae* from isolates of *P. phaseolicola* as determined with tests using 16 isolates each of *P. syringae* and *P. phaseolicola* from bean obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, England. All epiphytic isolates tested in 1976–1978 were rated virulent in these tests.

In 1977 and 1978 numbers of epiphytic bacteria also were determined by the plate dilution frequency technique with most probable number (MPN) analysis (1,8). This technique consisted of inoculating eight replicate samples (0.01 ml) from four dilutions of a 10-fold dilution series of leaf washings onto areas delineated on agar plates and recording the presence or absence of growth in each area. The number of specific organisms present in the original sample was determined from three of these dilutions by reference to MPN tables (13). Leaf washings were plated on Crosse's medium, nutrient dextrose agar (NDA), and potato-dextrose agar (PDA) in order to observe epiphytic organisms other than *P. syringae*. On the latter two media, counts were made of total pigmented and nonpigmented bacteria (*P. syringae* is nonpigmented on NDA and PDA) and of any dominant colony types that appeared. Representative colonies were tested for cell type, motility, reaction to Gram's stain, and fluorescence on King's B medium (9). Isolates that produced fluorescent pigments on King's B were tested for virulence by using bean pod injection techniques A and B.

RESULTS

Brown spot disease reactions (mean number of lesions per 3 m of row) of the bean lines used in epiphyte sampling studies are shown in Table 1. Eagle always had the greatest number of lesions ($3–6 \times 10^4$ per 3 m of row) and WBR 133 always had the fewest lesions. Lesion numbers on the breeding lines were intermediate to Eagle and WBR 133 and spanned a wide range.

Mean numbers of *P. syringae* epiphytes isolated each year from Eagle, WBR 133, and the eight breeding lines are shown in Table 2. Each year the highest populations were isolated from Eagle and the lowest populations were isolated from WBR 133. Populations of *P. syringae* on Eagle differed significantly from those on the eight breeding lines. Populations on the breeding lines showed few significant differences among themselves. In 1976, *P. syringae* populations on WBR 133 were significantly lower than those on the breeding lines, but in 1977 and 1978 populations on WBR 133 differed significantly from those on some breeding lines, but not from those on the more resistant breeding lines.

Regression lines correlating the epiphytic populations of *P. syringae* isolated from a specific bean line to the number of lesions found on that line are shown in Fig. 1. In general, there was a high correlation between the susceptibility of the line, as determined by lesion number, and the size of the epiphytic population of *P. syringae* isolated from those lines. Correlation coefficients were 0.91, 0.90, and 0.98 for 1976, 1977, and 1978, respectively. However, comparisons between the two variables on the eight breeding lines for deviations of the points from regression indicated no statistically significant ($P = 0.05$) relationship between lesion number and the size of the *P. syringae* populations on the breeding lines in both 1976 and 1977.

The hypothesis that antagonistic epiphytic microorganisms were responsible for the differences in epiphytic populations of *P. syringae* on susceptible and resistant beans was tested by isolating saprophytic organisms and *P. syringae* from bean lines using the plate dilution frequency technique with MPN analysis. A comparison of the *P. syringae* populations obtained by the MPN and the traditional plate-count techniques is shown in Fig. 2. The

TABLE 3. Mean epiphytic population of *Pseudomonas syringae* and other organisms on bean cultivars Eagle, WBR 133, and breeding lines 25 and BBSR 130

	Log bacteria per gram fresh weight					
	1977		1978			
Bacteria	Eagle	WBR 133	Eagle	25	BBSR130	WBR133
P. syringae[a]	4.8	3.3	6.2	5.4	3.0	2.7
Total nonpigmented[b]	4.9	4.0	6.3	5.8	5.3	5.4
Total pigmented[b]	3.8	3.7	4.3	4.6	5.0	4.8

[a] Based on most probable number (MPN) counts on Crosse's medium.
[b] Mean of MPN counts on PDA and NDA.

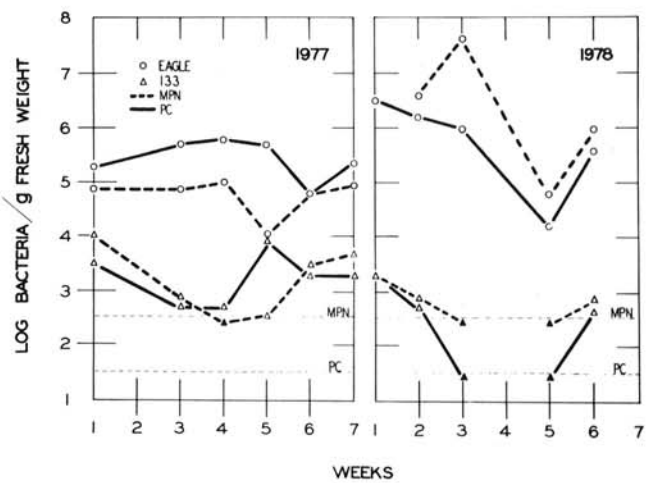

Fig. 2. Weekly epiphytic populations of *Pseudomonas syringae* on bean cultivars Eagle and WBR 133 as determined by plate count (PC) and most probable number analysis (MPN): ----- the minimum populations detectable (populations that will result in one colony per plate for the undiluted leaf washing) for each of the two methods. Populations below the minimum detectable limits (no colonies on the plates) are indicated by solid symbols. Time at week 1 on the graphs corresponds to the first sampling date, 3–4 wk after planting.

values obtained by these two methods were occasionally the same or very similar, but often differed by at least 0.5 log units and occasionally differed by as much as 1.5 log units. Agreement between the two methods was generally better with isolations from WBR 133 at low population values.

For isolation of saprophytic organisms, leaf washings were plated on NDA and PDA in addition to Crosse's medium. Counts were made of total pigmented and nonpigmented bacteria and of any dominant colony types that appeared. Mean values for each of the lines sampled are shown in Table 3. *P. syringae* appeared to be the dominant component of the nonpigmented epiphytic microflora on the more susceptible lines, Eagle and 25, but not on the more resistant lines, BBSR 130 and WBR 133. Total populations of nonpigmented bacteria were higher on Eagle and 25, perhaps due to the increased populations of *P. syringae*. Populations of pigmented bacteria were similar on all lines and generally were lower than the populations of nonpigmented bacteria. In general, all lines had few pigmented bacteria early in the season, but the number of chromogens increased as the season progressed. There were specific colony types that were present at high population levels throughout the season, but none were consistently isolated from resistant lines that were not also isolated from susceptible lines or isolated from susceptible lines at lower populations. We were, therefore, unable to find any correlation between the populations of *P. syringae* and the populations of total pigmented or nonpigmented bacteria or of specific colony types isolated throughout the season.

DISCUSSION

Our studies have shown that *P. syringae*, virulent on bean, can exist as an epiphyte on susceptible and resistant bean lines in the field. Populations were much higher on susceptible than on resistant lines. About 10^6 cells per gram fresh weight were isolated from leaves of the susceptible cultivar Eagle compared to 10^4–10^5 cells on the breeding lines and 10^3 cells on WBR 133. In general, epiphytic populations were inversely correlated with the degree of brown spot resistance of the lines.

An unresolved question in these studies was whether the high *P. syringae* counts on the more susceptible lines was actually attributable to higher epiphytic populations, or whether bacteria were simply being washed out of developing lesions. Only apparently healthy leaves were used in sampling, but rapid multiplication of bacteria occurs for about 24 hr in leaves prior to lesion development (4). Data collected during this 3-yr study tend to indicate that the population counts actually reflect the presence of epiphytic bacteria. The bases for this conclusion are: (i) Even though epiphytic *P. syringae* populations generally were inversely correlated with the degree of resistance of the lines, the degree of resistance of the individual breeding lines was not correlated with their epiphytic *P. syringae* population. An analysis of the deviations from regression indicated no relationship ($P = 0.05$) between numbers of lesions and epiphytic *P. syringae* population on the breeding lines. (ii) Epiphytic population counts on all lines were lower in 1977 than in 1976, although numbers of lesions were about the same. (iii) Epiphytic population counts on BBSR 130 and WBR 133 were considerably lower in 1978 than in the previous years, but numbers of lesions were considerably higher.

The hypothesis that antagonistic epiphytic organisms were responsible for the differences in epiphytic populations of *P. syringae* on susceptible and resistant bean lines was tested. No evidence was found to indicate that this was the case. There were higher populations of nonpigmented bacteria on the susceptible lines, but these differences appeared to be due to increased numbers of *P. syringae* on these lines. Populations of pigmented bacteria were the same on susceptible and resistant lines, and differences in populations of bacteria with specific colony types isolated throughout the season did not correlate with the degree of susceptibility or resistance of the plant lines.

Two methods were used for isolating epiphytic organisms, the traditional plate count method and the plate dilution frequency technique combined with MPN analysis. Though values obtained by the MPN technique did not always agree with those obtained through the plate count method, often they were very close, particularly at lower population densities. The MPN technique offered a considerable time savings over the plate count method, and should, therefore, be investigated for use in ecological studies of this type. Precision of the method may be increased by using twofold or fourfold dilutions rather than the 10-fold series used in this study (1,8). This would be particularly useful for studies in which different samples do not span as wide a range of population densities.

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