APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1997, p. 3211–3217 Vol. 63, No. 8
0099-2240/97/$04.00+0

Spatial Patterns of Rhizoplane Populations of *Pseudomonas fluorescens*†

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Received 3 April 1997/Accepted 9 June 1997

Geostatistical analysis was used to compare rhizoplane colonization patterns of an antibiotic-producing biological control bacterium versus a non-antibiotic-producing mutant strain. Pea seeds were inoculated with *Pseudomonas fluorescens* 2-79RN$_{10}$ or *P. fluorescens* 2-79-B46 (a phenazine-deficient Tn*5* mutant of *P. fluorescens* 2-79RN$_{10}$) (10^8 CFU/pea), planted in sterile sand, and incubated at 20°C. After 3 days, seedlings were prepared for scanning electron microscopy. Photomicrographs (×1,000) of the root surface were taken at the seed-root junction and at 0.5-cm intervals to the root tip. Bacterial counts on the root surface were made in 5- by 5-μm sample units over an area which was 105 by 80 μm. Coordinates and number of bacteria were recorded for each sample unit. Spatial statistics were calculated by covariance for the following directions: omnidirectional, 0, 45, 90, and 135°. The ranges of spatial influence and nugget (estimator of spatially dependent variation) were determined. For both *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46, spatial structure was evident along the entire root, particularly in the 0° direction (along the root length) (e.g., range = 24 μm, nugget = 0.52). The degree of spatial dependence observed indicated aggregation of bacterial cells. No differences were detected in the spatial patterns of colonies of *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46, indicating that the lack of phenazine production did not influence spatial patterns on the rhizoplane.

Successful manipulation of rhizoplane microorganisms to enhance biological disease control depends on a knowledge of their ecological associations (18, 32). Although the published literature contains considerable information on microbial colonization of roots, and general observations that rhizosphere microbes appear to be located in aggregates (8–10, 20, 24), there has been relatively little quantitative statistical investigation of colonization patterns. Energy input to the rhizoplane is largely due to root exudates, and certain zones of roots produce more exudate than others (26); thus, certain sites may be preferentially colonized by rhizoplane microbes (25), resulting in nonuniform patterns. Rhizoplane surfaces may provide structured habitats where the formation of microcolonies could permit accumulation of significant levels of antibiotics (4). *Pseudomonas fluorescens* 2-79RN$_{10}$, which produces the phenazine antibiotic phenazine-1-carboxylic acid (11), controlled take-all disease of wheat (36) and *Aphanomyces* root rot of pea (6), whereas the phenazine-deficient (Phe$^-$) Tn*5* mutant, *P. fluorescens* 2-79-B46, did not. In addition to biological control activity, antibiotic production may aid in initial colonization of a substrate (1) and antibiotics and other compounds may stimulate exudation which favors rhizobacteria (35). Although no differences were detected in populations of Phe$^-$ derivatives of *P. fluorescens* during initial stages of plant growth (36), the ability to produce phenazine was shown to contribute to long-term rhizosphere survival (19). However, it has not yet been determined whether spatial patterns of the Phe$^-$ mutant of *P. fluorescens* differ from those of the wild type on the rhizoplane.

The objectives of this study were to quantify spatial patterns of the biological control agent *P. fluorescens* 2-79RN$_{10}$ on the rhizoplanes of pea seedlings and to determine whether spatial patterns of *P. fluorescens* 2-79-B46, a phenazine-deficient Tn*5* mutant, differed from those of *P. fluorescens* 2-79RN$_{10}$.

The tendency for rhizosphere microbial populations to conform to lognormal or similar frequency distributions has been noted (2, 17, 20). However, frequency distribution analyses are of limited value for the description of spatially arranged biological phenomena, in part because they assume independence of observations (15). The existence of spatial structure implies that the assumption of independence is not met, because any ecological phenomenon located at a given sampling point may have an influence on other points close by or some distance away (15). Thus, there remains a need for appropriate quantitative descriptors of spatial patterns of microbes in the rhizosphere.

One statistical methodology, geostatistics, quantifies spatial patterns while maintaining the spatial integrity of data by analyzing the degree of association (autocorrelation) based on direction and distance (lag) between samples. Results can be plotted on a covariogram, the key aspects of which are as follows: sill, the point at which the sample covariance no longer increases with increasing lag; nugget or *y*-intercept, an estimate of the proportion of the total variation that is not spatially dependent (the proportion of variation that is spatially dependent is estimated by subtracting the value for the nugget from the total variation, which in this case is close to 1); and range, the range of spatial dependence, i.e., distance to the sill.

Spatial dependence is defined as the degree of association (covariance) of any two points at a given separation distance. Modeling of a covariogram is concerned with the response between the nugget and the sill and helps reveal the spatial structure of the investigated phenomenon.

Geostatistics has proven highly applicable to biological systems; for example, geostatistics has been used to evaluate insect spatial patterns (30), a spatial simulation model (29), and spatial patterns of zoospores on roots (7). In this study, we used geostatistical analysis to compare rhizoplane patterns of *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 to deter-

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† Published as research paper 97726 of the Idaho Agricultural Experiment Station, Moscow.

mine whether loss of phenazine production through mutagenesis was accompanied by changes in spatial patterns of root colonization.

MATERIALS AND METHODS

Organisms used. *P. fluorescens* 2-79RN$_{10}$ and 2-79-B46 were provided by D. M. Weller and L. S. Thomashow (USDA Agricultural Research Service, Pullman, Wash.). Strain 2-79RN$_{10}$ is a spontaneous mutant of strain 2-79 (NRRL B-15132) and is resistant to rifampin and nalidixic acid antibiotics (38). *P. fluorescens* 2-79-B46 is a Tn*5* mutant of *P. fluorescens* 2-79RN$_{10}$ and is deficient in production of a phenazine antibiotic (36). The strains were maintained at −80°C in 15% glycerol and were grown on King's medium B (14) agar with rifampin and nalidixic acid, each at 100 μg/ml (KMB-RN). Cell concentrations for experiments were estimated by using spectrophotometer readings at 600 nm compared to a standard curve.

Plant growth conditions. Peas (*Pisum sativum* L. 'Columbia') were surface sterilized in 5% sodium hypochlorite for 3 min, followed by repeated rinses in sterile distilled water. Peas were then coated with either *P. fluorescens* 2-79RN$_{10}$ or *P. fluorescens* 2-79-B46 by rolling in a bacterial suspension (48-h cultures, 10^9 CFU/ml) and allowed to dry in a laminar flow hood. Controls were peas that were coated with K_2HPO_4 buffer and allowed to dry as above. Initial inoculum levels were determined by dilution plating suspensions from three peas from each treatment onto KMB-RN and incubating the plates at 25°C for 2 days.

Ninety grams of commercially obtained silica sand (20/30 mesh size; Lane Mountain Silica Co., Valley, Wash.) were placed in 9-cm-diameter Pyrex petri dishes and sterilized by autoclaving for 90 min on two consecutive days. The matric potential of the sand was adjusted to −30 kPa with sterile distilled water. Treated peas (one per dish) were planted approximately 1 cm deep in the autoclaved sand. Dishes were then sealed with plastic film and placed in plastic bags to maintain constant moisture and incubated at 20°C for 3 days with 12 h of fluorescent light. Six replicate peas were planted for each treatment.

Enumeration of bacteria on seedlings. After incubation, half of the seedlings were removed, gently washed in sterile distilled water, and placed in K_2HPO_4 buffer. Mean population densities were determined by cutting the roots into 0.5-cm segments and dilution plating them onto KMB-RN. Control seedlings were processed as above and dilution plated onto KMB-RN and tryptic soy agar.

The remaining seedlings were prepared for scanning electron microscopy (SEM) by fixing with glutaraldehyde (1.5%) buffered with 0.2 M sodium cacodylate (pH 7.2) and adjusted to 370 mosmol. The samples were fixed overnight and then rinsed with 0.2 M sodium cacodylate (pH 7.2) followed by two rinses with distilled water. Samples were dehydrated by passing them through a graded series of ethanol (40%, 50%, 70%, 80%, 90%, 95%, 100%, and 100%), each for 30 min. Following dehydration, samples were critical-point dried in liquid carbon dioxide, mounted, and coated with a 60/40 Au/Pd ratio layer. The specimens were viewed with an Amray 1830 SEM. Photomicrographs (×1,000) of the root surface were taken at the seed-root junction and at 0.5-cm intervals along the root until the root tip was reached.

Image files were acquired with Hewlett-Packard DeskScan II software and a Hewlett-Packard ScanJet II scanner as grayscale images. Balance and contrast were optimized on the image portion of the photomicrograph. Enumeration of bacteria was accomplished by using SigmaScan/Image software by Jandel Scientific on manual measurement mode. Spatial calibration was set to the 10-μm bar on the photomicrograph image. A visual grid was overlaid on the image, from which the *x* and *y* coordinates were derived. Bacterial counts on the root surface were made in 5- by 5-μm sample units over an area which was 105 by 80 μm. Coordinates and number of bacteria were recorded for each sample unit.

Statistical analysis. Differences in population densities of the transformed data (log10) between populations on the pea and the first 0.5-cm root segment, the pea and the last 0.5-cm root segment, and the first and the last root segments were determined by paired *t* test.

Geostatistical analysis of bacterial patterns was done by covariance. Sample covariance C_D of a defined spatial integral (D) was graphed as a function of the separation distance (h) between points. The calculation of $C_D(h)$ at each separation distance in a data set was:

$$C_D(h) = 1/n(h) \sum_{i=1}^{n(h)} z(x_i) \times z(x_i + h) - m_D(h) \times m_D(-h)$$

where $z(x_i)$ is the measured sample value at point x_i, $z(x_i + h)$ is the value at point $x_i + h$, $m_D(h)$ is the mean of all values which appear as $z(x_i + h)$, $m_D(-h)$ is the mean of all values that appear as $z(x_i)$, and $n(h)$ is the total number of sample pairs for any separation distance. The resulting plot of $C_D(h)$ versus the distance separating points is referred to as the covariogram (13). The shape of this plot defines the type of spatial structure and the range of spatial dependence. Covariance values were calculated with a C language program (Agricultural Software Development Group, University of Idaho, Moscow). The presence or absence of anisotropic patterns was determined by examination of the covariograms for the 0, 45, 90, and 135° directions, where 0° represents the direction along the length of the root. A spherical model was fitted to each data set by using the least squares approach of Cressie (5). Fitted models provided estimates of the size of the spatial influence (range) in each treatment, the random or measurement error (nugget), and the value (sill) around which the covariogram became stable. For our purposes, computed $C_D(h)$ values were divided by the sample covariance to provide a common scale for comparing standardized covariograms. This treatment of spatial estimators is a common geostatistical procedure where comparisons of spatial statistics between different sampling units or locations are important (13). This creates a sill that should be close to 1, irrespective of the sample variation.

TABLE 1. Population densities of *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 on 3-day-old pea seedlings as determined by dilution plate assay

Location	CFU/pea or segment	
	2-79RN$_{10}$	2-79-B46
Pea	5.2×10^7	8.0×10^7
First root segment	6.9×10^5	1.7×10^6
Last root segment	2.1×10^5	2.2×10^5

RESULTS

Enumeration of bacteria on seedlings by plate assay. Population densities from the pea, the first 0.5-cm root segment, and the last 0.5-cm root segment for both *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 are shown in Table 1. Population densities of *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 on seeds were 1.0×10^8 and 1.3×10^8 CFU/pea, respectively, at planting time, and after 3 days, population densities were 5.2×10^7 and 8.1×10^7 CFU/pea, respectively. There was no significant difference between population densities of *P. fluorescens* 2-79RN$_{10}$ and those of *P. fluorescens* 2-79-B46 on the pea seedlings as determined by analysis of variance. A paired *t* test indicated that population densities of both *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 were higher on the pea seed than on either the first or last root segment and higher on the first segment than on the last.

Spatial patterns of bacteria on seedlings. Results of spatial analysis of bacterial colonization patterns for *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 are shown in Tables 2 and 3, respectively. The parameters shown include covariance at the first and lowest lag distance (i.e., 5 μm) and, when a spherical model was fitted to the covariance values, the nugget (estimator of the total variation that is not spatially dependent), the range of spatial dependence, the sill, and the model coefficient of determination (R^2). Representative photomicrographs of colonization patterns and corresponding covariograms from two pea seedlings (replicate 2 from Tables 2 and 3) inoculated with *P. fluorescens* 2-79RN$_{10}$ or *P. fluorescens* 2-79-B46 are shown in Fig. 1 and 2, respectively.

Bacterial colonies of both *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 were found throughout the rhizoplane. However, with the exception of one root, bacteria were not present at the root tip. When the covariance value at the first lag is below the population covariance, spatial structure is evident. Spatial structure was evident for both *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46, indicating that bacterial cells were aggregated along the entire length of the root. Although bacterial density decreased towards the root tip, there was no consistent change in spatial structure along the root whether colonized by *P. fluorescens* 2-79RN$_{10}$ or *P. fluorescens* 2-79-B46 (Table 2 and Fig. 1 and 2). Residual analysis indicated that the density of bacteria did not affect the amount of variation that was spatially structured. Aggregation varied from area to area along the root. Covariograms generally revealed an anisotropic pattern of bacterial colonization. Spatial dependence for both *P. fluorescens* 2-79RN$_{10}$ and 2-79-B46, as

TABLE 2. Spatial statistics from geostatistical analysis of *P. fluorescens* 2-79RN$_{10}$ on 3-day-old pea seedlings at 0.5-cm increments from the pea seed

Direction	Root[a]	Segment	Nugget	Sill	Range[b] (μm)	R^{2c}	COV[d]	LCV[e]	LAG[f]
Omni	1	0.0	0.81	1.01	21.5	0.44	0.89	0.89	1
	1	0.5	0.28	1.06	31.2	0.98	0.50	0.50	1
	1	1.0	0.72	1.01	12.6	0.81	0.91	0.91	1
	1	1.5	0.77	1.01	31.4	0.90	0.82	0.82	1
	2	0.0	0.66	1.06	27.4	0.96	0.79	0.79	1
	2	0.5	0.53	0.94	19.8	0.84	0.70	0.70	1
	2	1.0	0.83	1.02	23.7	0.86	0.91	0.91	1
	2	1.5	0.81	1.02	36.4	0.95	0.85	0.85	1
	2	2.0	0.63	1.07	37.7	0.91	0.70	0.70	1
	2	2.5	0.90	1.01	27.8	0.50	0.93	0.93	1
	3	0.0	0.61	1.08	36.2	0.82	0.64	0.64	1
	3	0.5	0.68	1.01	14.8	0.78	0.87	0.87	1
	3	1.0	0.75	1.01	17.7	0.96	0.88	0.88	1
	3	1.5	0.53	1.05	32.8	0.97	0.66	0.66	1
	3	2.0	0.95	1.01	62.4	0.52	0.94	0.94	1
	3	2.5	0.80	1.02	29.6	0.94	0.86	0.86	1
90°	1	0.0	NA[g]	NA	NA	NA	1.02	0.92	5
	1	0.5	0.28	1.05	20.8	0.91	0.56	0.56	1
	1	1.0	0.87	0.98	39.5	0.66	0.88	0.88	1
	1	1.5	0.70	0.93	9.0	0.30	0.88	0.88	1
	2	0.0	0.66	1.07	20.9	0.89	0.79	0.79	1
	2	0.5	0.67	0.96	36.9	0.86	0.70	0.70	1
	2	1.0	0.80	1.02	19.4	0.59	0.90	0.90	1
	2	1.5	0.81	1.03	43.9	0.88	0.82	0.82	1
	2	2.0	0.74	1.15	38.2	0.65	0.78	0.78	1
	2	2.5	NA	NA	NA	NA	0.95	0.87	2
	3	0.0	0.59	1.06	34.2	0.78	0.60	0.60	1
	3	0.5	0.32	0.95	9.9	0.85	0.76	0.76	1
	3	1.0	0.82	1.00	18.0	0.69	0.91	0.91	1
	3	1.5	0.48	1.06	23.8	0.78	0.64	0.64	1
	3	2.0	NA	NA	NA	NA	0.97	0.97	1
	3	2.5	NA	NA	NA	NA	0.95	0.82	2
0°	1	0.0	0.43	1.01	23.8	0.93	0.62	0.62	1
	1	0.5	0.18	0.93	46.0	0.99	0.30	0.30	1
	1	1.0	0.74	1.04	16.5	0.83	0.87	0.87	1
	1	1.5	0.40	0.99	16.5	0.94	0.66	0.66	1
	2	0.0	0.52	1.02	28.5	0.96	0.67	0.67	1
	2	0.5	0.11	0.85	10.5	0.89	0.60	0.60	1
	2	1.0	0.79	1.07	41.0	0.99	0.85	0.85	1
	2	1.5	0.62	1.00	12.0	0.76	0.84	0.84	1
	2	2.0	0.28	0.88	29.8	0.98	0.43	0.43	1
	2	2.5	0.77	0.98	18.3	0.91	0.86	0.86	1
	3	0.0	0.08	0.99	14.4	0.89	0.54	0.54	1
	3	0.5	0.78	1.05	18.6	0.65	0.88	0.88	1
	3	1.0	0.68	1.03	30.9	0.91	0.74	0.74	1
	3	1.5	0.54	0.95	34.6	0.95	0.60	0.60	1
	3	2.0	0.92	0.95	16.9	0.06	0.94	0.94	1
	3	2.5	0.43	1.01	9.9	0.95	0.83	0.83	1

[a] Three replicate peas were inoculated with 10^8 CFU/pea prior to planting.
[b] Range of spatial dependence.
[c] Model R^2.
[d] COV, covariance at the first lag.
[e] LCV, lowest covariance value.
[f] LAG, lag position of the lowest covariance value.
[g] NA, not applicable.

determined by the nugget, was greatest along the length of the root (0°). For *P. fluorescens* 2-79RN$_{10}$, spatial structure along the root length (0°) varied from little aggregation to highly aggregated populations. For root replicate 1 (0° direction), 48% of the variation (as determined by subtracting the nugget, which estimates the non-spatially dependent variation, from the population covariance, which is close to 1) at the seed-root junction was spatially structured (Table 2 and Fig. 1), indicating that the bacteria were aggregated. At 0.5 cm away from the seed-root junction, 89% of the variation was found to be spatially structured, indicating a highly aggregated population. The bacterial population 1 cm down the root was less aggregated, as only 21% of the variation was spatially structured. An increase in aggregation was observed at the 1.5- and 2.0-cm positions, as 38 and 72%, respectively, of the variation was found to be spatially structured. On the root segment which was 2.5 cm from the seed, bacteria were less aggregated: only 23% of the variation was found to be spatially structured. The

TABLE 3. Spatial statistics from geostatistical analysis of *P. fluorescens* 2-79-B46 on 3-day-old pea seedlings at 0.5-cm increments from the pea seed

Direction	Root[a]	Segment	Nugget	Sill	Range[b] (μm)	R^{2c}	COV[d]	LCV[e]	LAG[f]
Omni	1	0.0	0.72	1.30	58.9	0.92	0.86	0.86	1
	1	0.5	0.78	1.03	50.6	0.95	0.82	0.82	1
	1	1.0	0.77	1.06	40.9	0.90	0.79	0.79	1
	1	1.5	0.92	1.01	27.9	0.71	0.96	0.95	2
	1	2.0	0.81	1.03	27.8	0.81	0.88	0.88	1
	2	0.0	0.85	1.03	54.8	0.58	0.83	0.83	1
	2	0.5	0.06	1.01	14.5	0.82	0.62	0.62	1
	2	1.0	0.46	1.03	20.5	0.98	0.70	0.70	1
	2	1.5	0.70	1.06	41.5	0.90	0.75	0.75	1
	3	0.0	0.58	1.06	38.0	0.88	0.63	0.63	1
	3	0.5	0.61	1.00	11.4	0.76	0.89	0.89	1
	3	1.0	0.68	1.06	35.9	0.96	0.76	0.76	1
	3	1.5	0.67	1.03	26.8	0.94	0.80	0.80	1
	3	2.0	0.93	1.00	17.6	0.21	0.97	0.97	1
90°	1	0.0	NA[g]	NA	NA	NA	0.88	0.83	4
	1	0.5	0.74	1.05	51.3	0.95	0.76	0.76	1
	1	1.0	NA	NA	NA	NA	0.83	0.80	6
	1	1.5	0.97	1.03	27.7	0.49	0.98	0.98	1
	1	2.0	0.66	1.04	15.1	0.81	0.84	0.84	1
	2	0.0	NA	NA	NA	NA	0.89	0.78	3
	2	0.5	NA	NA	NA	NA	0.72	0.72	1
	2	1.0	0.22	1.05	10.9	0.84	0.75	0.75	1
	2	1.5	0.82	1.12	51.0	0.82	0.86	0.86	1
	3	0.0	0.67	1.10	39.5	0.60	0.67	0.67	1
	3	0.5	NA	NA	NA	NA	0.93	0.83	10
	3	1.0	0.68	1.10	25.0	0.82	0.84	0.84	1
	3	1.5	0.65	1.05	20.0	0.86	0.82	0.82	1
	3	2.0	NA	NA	NA	0.00	0.94	0.92	11
0°	1	0.0	0.78	1.02	19.7	0.56	0.88	0.88	1
	1	0.5	0.67	0.98	32.6	0.97	0.72	0.72	1
	1	1.0	0.64	1.03	42.1	0.95	0.68	0.68	1
	1	1.5	0.77	1.01	25.2	0.87	0.86	0.86	1
	1	2.0	0.63	0.99	10.0	0.81	0.88	0.88	1
	2	0.0	0.22	0.99	16.5	0.97	0.56	0.56	1
	2	0.5	0.0	1.02	29.6	0.98	0.26	0.26	1
	2	1.0	0.34	0.98	33.2	0.99	0.50	0.50	1
	2	1.5	0.38	0.98	24.1	0.92	0.55	0.55	1
	3	0.0	0.22	1.07	35.2	0.99	0.39	0.39	1
	3	0.5	0.66	0.96	18.7	0.96	0.77	0.77	1
	3	1.0	0.24	0.87	17.4	0.96	0.50	0.50	1
	3	1.5	0.61	0.96	36.4	0.92	0.68	0.68	1
	3	2.0	0.95	1.05	35.1	0.74	0.96	0.96	1

[a] Three replicate peas were inoculated with 10^8 CFU/pea prior to planting.
[b] Range of spatial dependence.
[c] Model R^2.
[d] COV, covariance at the first lag.
[e] LCV, lowest covariance value.
[f] LAG, lag position of the lowest covariance value.
[g] NA, not applicable.

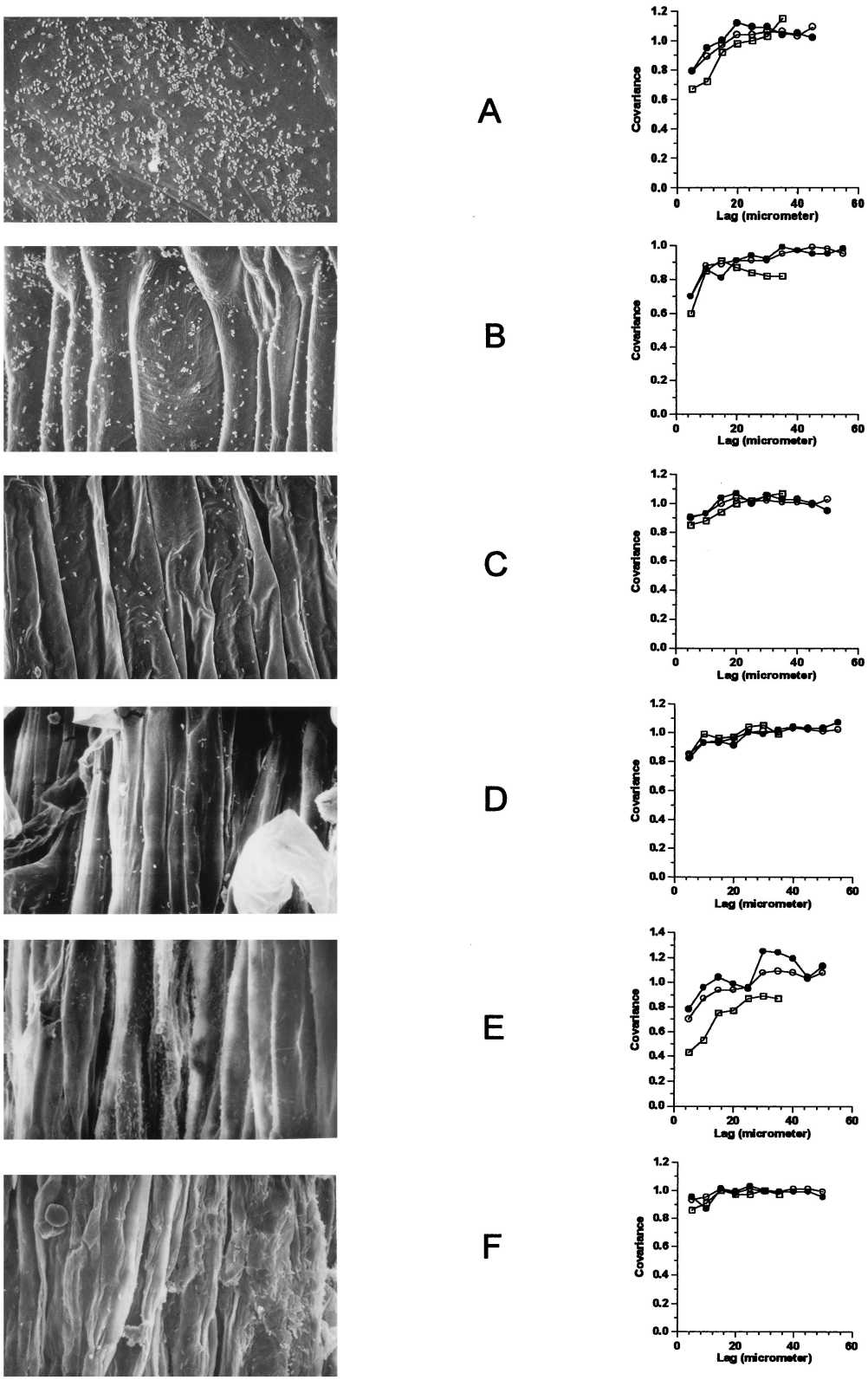

FIG. 1. Photomicrographs taken at 0.5-cm intervals from the pea seed toward the root tip showing colonization of *P. fluorescens* 2-79RN$_{10}$ on pea roots with corresponding covariograms. (A) At seed-root junction; (B) 0.5 cm from seed; (C) 1.0 cm from seed; (D) 1.5 cm from seed; (E) 2.0 cm from seed; (F) 2.5 cm from seed. Symbols: ○, omnidirectional; ●, 90° direction; □, 0° direction.

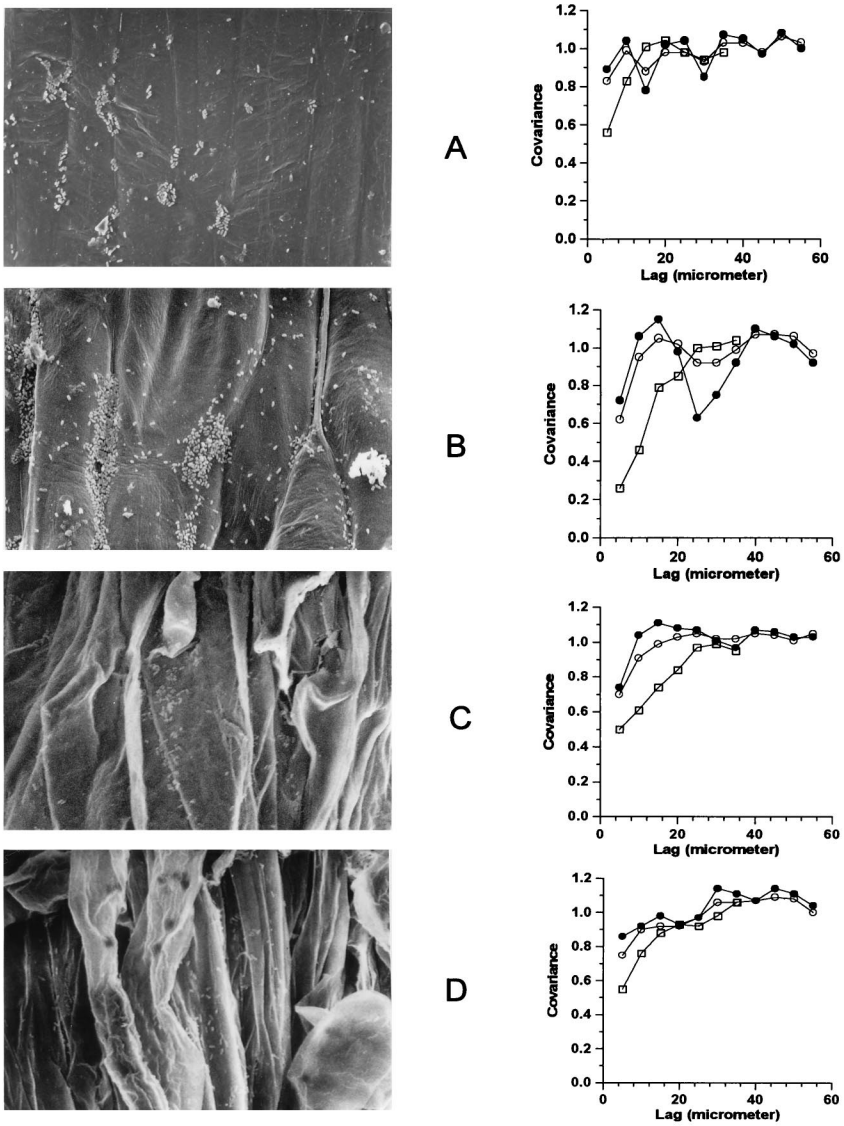

FIG. 2. Photomicrographs taken at 0.5-cm intervals from the pea seed toward the root tip showing colonization of *P. fluorescens* 2-79-B46 on pea roots with corresponding covariograms. (A) At seed-root junction; (B) 0.5 cm from seed; (C) 1.0 cm from seed; (D) 1.5 cm from seed. Symbols: ○, omnidirectional; ●, 90° direction; □, 0° direction.

range of influence of an aggregate of *P. fluorescens* 2-79RN$_{10}$ varied from 10.5 to 41.0 μm (Table 2).

For *P. fluorescens* 2-79-B46, bacteria at the seed-root junction were aggregated: 88% of the variation was spatially dependent in the 0° direction. At the 0.5-cm position along the root, the population of bacteria was highly aggregated. Almost all of the variation was estimated to be spatially dependent. At 1 cm and 1.5 cm, 66 and 62%, respectively, of the variation was spatially dependent, indicating that bacteria were also aggregated at these positions on the root surface. A similar range of influence (16.5 to 33 μm) was found for *P. fluorescens* 2-79-B46 (Table 3) as was found for *P. fluorescens* 2-79RN$_{10}$.

For the other two roots inoculated with either *P. fluorescens* 2-79RN$_{10}$ or *P. fluorescens* 2-79-B46, bacteria were also aggregated (Tables 2 and 3, respectively). However, as in the first root, the amount of variation that was spatially dependent varied from segment to segment. No consistent trend was apparent in the development of spatial structure along the length of any of these roots.

On some segments, bacteria were highly aggregated along cell walls, which generated cycling of the covariance in the 90° direction as the lag increased. This is particularly evident for *P. fluorescens* 2-79-B46 replicate 2 on the second root segment (1 cm from the pea) (Fig. 2B). In this example, the covariance at the first lag (5 μm) is 0.72; it increases to 1.15 at the third lag (15 μm) and decreases to 0.63 at the fifth lag (25 μm) before becoming stable at the sill. The lag distance from one trough to the next (25 μm) corresponds to the width of the plant cells (20 to 25 μm). A similar pattern is also evident for the first root segment of this root (Fig. 2A). Bacterial colonies are aligned along the cell walls, which again generated cycling of the covariance in the 90° direction as the lag increased. On this root segment, however, the width of the plant cells was narrower, ranging from 10 to 15 μm. The lag distance from one trough to the next corresponds to this distance (10 to 15 μm), indicating that colonies were 10 to 15 μm apart (in the 90° direction).

The scale at which the phenomena are observed is important. Initial counts were made on 10- by 10-μm squares for

each micrograph (data not shown). However, no spatial structure was observed at this scale in the 0° direction. Therefore, the spatial analysis was repeated by counting bacteria on each micrograph at a smaller scale (5 by 5 μm).

DISCUSSION

Geostatistical analysis of bacteria on the rhizoplane of 3-day-old pea seedlings indicated that a high degree of bacterial aggregation occurred in the rhizoplane. Analysis of the patterns revealed anisotropy in the bacterial aggregates; the greatest spatial dependence was observed along the root length (0°), and aggregates were frequently found along the length of cell walls. The amount of variation that was spatially structured varied along the root. For a few roots, bacteria were highly aggregated close to the seed and became less aggregated toward the root tip. For most roots, however, the spatial pattern of bacteria varied from highly aggregated to random and the spatial pattern found did not correspond to a specific root region. *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 appeared to adhere to the root surface in a nonspecific manner. Fukui et al. (10) found that *Bacillus subtilis* colonized the basal pore of sugar beet seeds and that *Pseudomonas putida* 33-2 and *P. fluorescens-putida* ML5 colonized the indented surface of cells of the perianth and operculum. *Azospirillum brasilense* was found to colonize wheat mainly in the root hair zone and the sites of lateral root emergence during the first days of association (37). However, 5 to 7 days after inoculation, the bacteria became more widely spread over the root surface (37). Others have reported aggregated patterns of bacteria on the rhizoplane by use of electron microscopy (8, 10, 20, 24, 27). The application of geostatistics, however, has enabled us to quantify the spatial structure and the range of spatial dependence of rhizoplane bacteria. We found that the amount of variation that was spatially dependent ranged from as little as 6% to almost entirely spatially dependent. For *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46, the range of influence was between 10 and 41 μm.

No differences were detected in the spatial patterns of colonies of *P. fluorescens* 2-79RN$_{10}$ and those of *P. fluorescens* 2-79-B46, indicating that the lack of phenazine production did not influence spatial patterns on the rhizoplane. Initial colonization of wheat roots by *P. fluorescens* 2-79-B46 was found to be similar to that of *P. fluorescens* 2-79RN$_{10}$ (36), although long-term colonization differed in the two strains (19). Spatial patterns of the two strains may diverge over time.

In these experiments we found a high degree of variability in the spatial patterns of bacteria along the root. Large variability in rhizosphere bacterial populations along a root, as well as among root systems, has been widely reported (2, 16, 20). Roots typically consist of a few heavily colonized areas with the remaining areas colonized lightly or not at all. Although the cause of the variability in colonization patterns of bacteria on root surfaces remains unclear, quantitative data on spatial patterns may lead to predictions about the underlying causes for the observed patterns and may help to explain factors controlling the growth of bacteria on roots (20).

Nutrient availability has been widely reasoned to be the cause for nonuniform colonization of bacteria on root surfaces. Areas where exudation is greatest have been reported to be the root tips, the vicinity of the apical meristem, the region of elongation, and the region of developing root hairs of primary, lateral, and adventitious roots (21, 30, 31, 33). However, in our study, the greatest accumulation of *P. fluorescens* on the rhizoplane was not consistently found in sites where exudation is reported to be greatest. Thus, although nutrient availability undoubtedly is a factor in establishing spatial patterns of *P. fluorescens*, other factors, such as features of the root surface as well as intraspecific interactions between adhering bacteria, may be equally important in the establishment and development of bacterial spatial patterns. We found that *P. fluorescens* often aggregates along the junctures of root cells. Rovira (24) and Foster and Bowen (9) reported a similar accumulation of bacteria along the junctures of root cells and proposed that the observed alignment of the microorganisms along these junctures is in response to nutrients being exuded from them. However, the aggregation of bacteria in epidermal cell junctures on roots may be a response to the roughness of the surface, as was found for *Saccharomyces cerevisiae* (28). *S. cerevisiae* had high aggregation rates on rough surfaces and highly polished materials but low aggregation rates on surfaces of intermediate roughness (28). Quirynen et al. (23) have suggested that colonization of bacteria occurs preferentially in the niches of surface irregularities because microbes are protected against the mechanical shear that constantly occurs on a smooth surface. Spatial patterns have been analyzed to measure the effect of an attached bacterium on the adsorption of other bacteria nearby (3, 12, 34). Under positive cooperation, initially adhering bacteria would be arrayed in an aggregated pattern with many near neighbors (12). Under blocking, where cells inhibit adsorption of others, initial bacteria would be in a uniform pattern, with few near neighbors (12). We found that populations of *P. fluorescens* were aggregated on the rhizoplane, which suggests that the effect of an attached bacterium on the adsorption of other bacteria was positive. Phenazine antibiotic production in *Pseudomonas aureofaciens* 30-84 has been reported to be regulated by the production of a diffusible signal, which may be cell density dependent (22, 39). The aggregation of bacteria on the rhizoplane may be important to achieving a cell density required for sufficient amounts of diffusible signal to accumulate in order to induce phenazine production.

The evidence provided here suggests that the underlying surface (e.g., surface topography and nutrient source), as well as interactions among bacteria, may play a role in determining colonization patterns of rhizoplane bacteria. In these experiments, geostatistical analysis provided a quantitative evaluation of spatial variability of patterns of *P. fluorescens* on the rhizoplane. Further studies and the use of geostatistics could provide insight into how mixed populations of biocontrol agents partition resources and coexist in the rhizoplane.

ACKNOWLEDGMENTS

We thank Rachel Mosher for technical assistance and Franklin Bailey for excellent help with SEM.

This research was financially supported by NRICGP grant 95-37312-1637 from the U.S. Department of Agriculture.

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