BIOLOGICAL CONTROL OF *PSEUDOMONAS AVENAE* WITH EPIPHYTIC BACTERIA ISOLATED FROM CORN PLANTS

I. FREQUENCY DISTRIBUTION OF BACTERIAL POPULATIONS FROM CORN WHORLS[1]

CARLOS ALBERTO LOPES[2] and ROBERT E. STALL[3]

ABSTRACT - Total populations of epiphytic bacteria and the populations of three groups of bacteria (fluorescent pseudomonads, *Erwinia herbicola,* and *Flavobacterium* spp.) were determined in the fluid collected in the whorls of sweet corn plants, susceptible and resistant to bacterial leaf blight and stalk rot. Total bacterial populations varied by a factor of less than 100 at each sampling date for each cultivar, whereas this factor was often above 10^4 for the three groups of bacteria. No qualitative or quantitative differences were observed on bacterial populations between the two genotypes. The bacterial populations in the whorls were lognormally distributed, as indicated by the normal probability plotting of residuals and by the Shapiro-Wilk test for normality. Bulked samples, obtained by mixing the first dilutions of individual samples, caused on overestimation of 1.71 6^2, indicating that bulk samples should not be used for quantitative surveys on bacterial populations in corn whorls.

Index terms: bacterial leaf blight and stalk rot, biological control, bacterial ecology.

CONTROLE BIOLÓGICO DE *PSEUDOMONAS AVENAE* COM BACTÉRIAS EPÍFITAS ISOLADAS DE PLANTAS DE MILHO

I. DISTRIBUIÇÃO DA FREQÜÊNCIA DE POPULAÇÕES BACTERIANAS EM CARTUCHOS DE PLANTAS DE MILHO DOCE

RESUMO - Populações totais de bactérias epífitas e populações de três grupos específicos de bactérias (Pseudomonas fluorescentes, *Erwinia herbicola* e *Flavobacterium* spp.) foram determinadas em fluido coletado em cartuchos de plantas de milho doce de cultivares suscetíveis e resistentes à queima-bacteriana-da-folha, causada por *Pseudomonas avenae.* A população bacteriana total variou menos de 100 vezes nas 15 amostras, para cada data de coleta e cada cultivar. Entretanto, para os três grupos de bactéria, este fator esteve normalmente acima de 10^4. Não se observaram diferenças significativas entre as populações bacterianas para as duas cultivares. As populações nos cartuchos das plantas apresentaram uma distribuição lognormal, de acordo com o teste de Shapiro-Wilk para normalidade e o teste gráfico de Montgomery. Isto pode ser comprovado quando se utilizaram amostras compostas obtidas pela mistura da primeira diluição das amostras individuais; esta mistura causou uma superestimação do número de bactérias por um fator de 1.71 6^2. Portanto, esta é mais uma indicação que amostras de populações bacterianas obtidas de plantas individuais não devem ser misturadas em levantamentos quantitativos.

Termos para indexação: queima-bacteriana-da-folha, controle biológico, ecologia bacteriana.

INTRODUCTION

Corn (*Zea mays* L.), as well as other upright grasses, has anatomical characteristic that make it very efficient for catching and retaining water. Rain, mist, dew, and guttation water run down the lamina, carrying with them leaf leachates, pollen, dust, and other

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[2] Eng. - Agr., Ph.D., EMBRAPA/Centro Nacional de Pesquisa de Hortaliças (CNPH), Caixa Postal 07.0218, CEP 70359 Brasília, DF, Brazil.

[3] Eng. - Agr., Ph.D., Dept. of Plant Pathology, University of Florida, Gainesville, FL 32611, EUA.

particles from the atmosphere deposited on the surface. Microorganisms, especially bacteria, which are known to form epiphytic populations on leaves of most, if not all, plant species, are also carried into the whorl (Garret & Crosse 1975, Hirano et al. 1982, Leben 1964, Ruinen 1971).

Part of this fluid is collected into the whorl and in the cisterns formed by the collar and sheath of older leaves (Ruinen 1971). According to Gitaitis (1976), the sites of water collection correspond to the areas where symptoms of bacterial leaf blight and stalk rot (BLBSR), incited by *Pseudomonas avenae*, occur in corn plants. Therefore, the composition of this fluid, especially the microbial populations, is an important factor in determining the suitability of the leaf environment to *P. avenae*.

Studies on biological control of foliar diseases with bacterial generally involve measurement of initial populations of the antagonist at the infection unit or the ability of these populations to survive on plant surfaces (Chakravarti et al. 1972, Crosse 1965, Lindow et al. 1983, McIntyre et al. 1973, Rao & Devadath 1978, Riggle & Klos 1972, Scherff 1973, Teliz-Ortiz & Burkholder 1960, Thompson et al. 1976, Verma et al. 1978). Measurements of populations of epiphytic bacteria have usually been made through the bulk-sampling procedure (Hirano et al. 1982), with the exception of Crosse (1959), who accounted for the high variability in the populations of *Pseudomonas syringae* pv. *mors-prunorum* among individual cherry leaves. Hirano et al. (1982) examined the varibility and distribution of epiphytic bacterial populations on individual leaves of various plant species and found that these populations follow a lognormal distribution, i.e., a distribution in which the logarithm of the original variate is distributed normally. It meant that estimation of population sizes based on bulked samples overestimates the population mean by a factor of approximately 1.15 6^2. A lognormal distribution was also found for the total and specific bacterial populations in the rhizosphere of greenhouse and field plants of five species (Loper et al. 1984).

The objective of this study was to determine the variability and the distribution of populations of epiphytic bacteria in the fluid accumulated in the whorls of two cultivars of sweet corn, as related to the biological control of BLBSR of corn incited by *P. avenae*.

MATERIALS AND METHODS

Eighteen rows of sweet corn were planted at the Horticultural Unit, University of Florida, Gainesville, on April 1985. Three-row plots were planted alternately with cultivars Merit and Silver Queen, which are susceptible and resistant to BLBSR, respectively (Summer & Schaad 1977). Plants were about 20 cm apart in 100 m rows; rows were 0,8 m apart. Cultural practices recommended for the crop in Florida were followed throughout the season (Showalter 1984).

Water in corn plant whorls was collected every five or six days beginning 30 days after planting (eight-leaf stage) and continuing until tasseling. Approximately 0.5 ml of fluid was withdrawn with sterile disposable pipets early in the morning from whorls of eight randomly selected plants in the center row of each cultivar (24 plants per cultivar on each of five dates). Periodic collections were obtained from the same individual plants. Rain and temperature data during the period were taken from a local meteorological unit.

Pipets containing fluid were sealed and transferred to the laboratory on ice. Each sample was tenfold diluted five times in sterile tap water, and 0,05 ml of the three last dilutions were spread on Trypticase Soy Agar (TSA) (BBL Microbiology systems, Cockeysville, MD) and on King's Medium B (KBM) with a sterile glass rod. A bulk sample made by mixing the first dilution of all individual samples of a given treatment (for example, from cultivar Merit, at sampling date one) also was diluted and cultured. Bacterial colonies were counted after three days of incubation at 30ºC. For each cultivar and sampling date, the total bacterial population and

populations of three groups of bacteria (fluorescent pseudomonads, *Erwinia herbicola,* and *Flavobacterium* spp.) were counted, which totaled 40 data sets. These three groups were chosen due to their prevalence on the plates and to the ease of separation based on their colony characteristics. Their identity was confirmed through quick tests such as shape of the cells, motility, Gram stain, anaerobic growth, and production of catalase and oxidase.

Colonies of fluorescent pseudomonads were counted on KMB, whereas the other two groups and the total number of colonies were counted on TSA and KMB.

In order to prevent an understimation of the number of colonies due to presumable in vitro antibiosis, counts were made on plates with less than 50 colonies whenever the total count did not decrease proportionally with the dilution. Counts on duplicate plates accounted for the lack of accuracy of utilizing a low number of colonies for estimation of populations, which were expressed in colony--forming units per milliliter (cfu/ml).

Two statistical procedures were used to determine if bacterial populations in individual whorls were normally distributed. First, a normal probability plot of the residuals was constructed according to Montgomery (1984). The residuals were found by subtracting the data set average from each observation of the data set. The normal probability plots were constructed by arranging the residuals in increasing order and the Kth value of these ordered residuals was plotted against the cumulative probability point Pk = (K - 1/2) N, where N is the number of observations. If the underlying error distribution is normal, this plot will resemble a straight line. To illustrate the results, residuals on the the graph were substituted by their respective original values, i.e., the bacterial populations or the logarithm of the bacterial populations.

In the second method, the Shapiro-Wilk (Shapiro & Wilk 1965) test for normality was performed according to a univariate procedure provided by SAS (Statistical Analysis System, Release 5.08, SAS Institute Inc., Cary, NC). In this procedure, the test statistic W is interpreted as the correlation between the spacing of the ordered data and that of the ordered expected values. The W value will approach one if the observations in the data set are normally distributed; as the data depart from normality, W will become smaller than one. The P value describes the confidence level with which one can reject the null hypothesis of normality.

Tests for normality were performed for 15 data sets which had more than 15 observations. Data sets with 10 or more missing values (due to numbers of bacteria below the detection level) were excluded.

RESULTS

Populations of epiphytic bateria in whorls of sweet corn cultivars Merit and Silver Queen were variable from plant-to-plant and on the same plant at different sampling dates (Fig. 1). No qualitative or quantitative differences were detected between the two cultivars. Total bacterial populations in whorls varied by a factor of less than 100 at each sampling date for each cultivar, whereas for the three groups of bacteria this factor was often above 10^4, and ranged from undetectable to values close to the total population. The bulked samples, obtained by mixing the first dilution of individual samples for each sampling date and cultivar, caused an overestimation of bacterial numbers in individual whorls by a factor of $1.71\ 6^2$.

Distribution of bacterial populations estimated in individual whorls in cfu/ml was consistently positively skewed when plotted in a histogram (Fig. 2A). Therefore, a high number of individual whorls harbored a relatively low number of bacteria, while fewer whorls had unusually high populations. This pattern was more evident for the selected individual groups of bacteria as compared with total bacteria. When the total bacterial population values were transformed to $\log_{10}$ cfu/ml, a more symmetrical distribution was obtained (Fig. 2C). When normal probability of residuals was plotted against the cumulative probability point, a substantial curvature for the data was evident with the nontransformed values, whereas the log-transformed values approximated a straight line (Fig. 2B).

In the Shapiro-Wilk test, the null hypothesis was not rejected (P ≤0,05) for 10 of the 15 data sets when the population numbers were transformed logarithmically. When the nontransformed values were used, only four of 15 data sets were normally distributed (Table 1).

DISCUSSION

Hirano et al. (1982) found that the frequency distribution of sample estimates of ice nucleation-active bacteria on plants approximated a lognormal distribution. This study showed the same trend for bacterial populations in whorls of sweet corn. The largest sample populations contributed disproportionately to arithmetic population means. Additionally, an overestimation of means occurred when samples were bulked, since bulking involves an arithmetic averaging of the individual bacterial populations. This phenomenon was observed in this study when the numbers obtained by the dilution of bulked samples were compared with the mean of the logarithm of populations in individual samples. Bulking of samples caused an overestimation of bacterial numbers by a factor of $1.71\ 6^2$, as compared with the factor of $1.15\ 6^2$ found by Hirano et al. (1982) for leaf-surface bacteria.

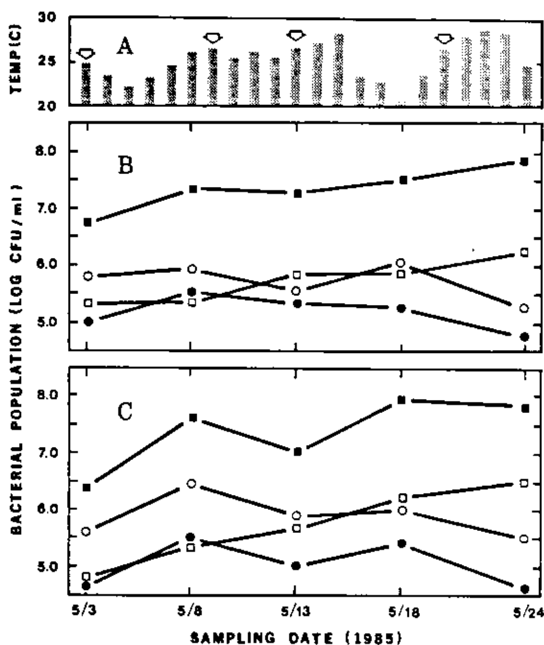

FIG. 1. A - Daily average temperature for the period. Arrows indicate when rain occurred. B and C - Populations of total bacteria (■) fluorescent pseudomonads (●), *Erwinia herbicola* (○), and *Flavobacterium* spp. (□) isolated from sweet corn cultivars Merit (B) and Silver Queen (C) on five sampling dates. Numbers represented are the average of the logarithm of the populations on 18 to 24 plants of each cultivar on each sampling date. Samples for which any of the three groups of bacteria were not detected were assigned the value of the detection limit for that date.

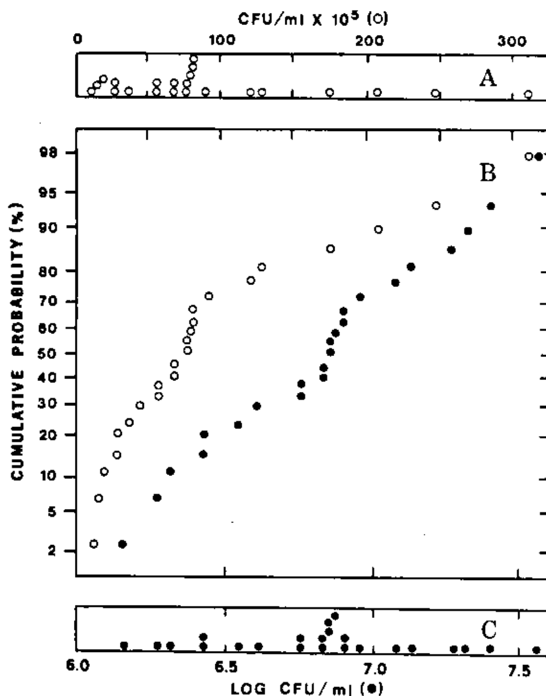

FIG. 2. Cumulative probability plots of total bacterial populations in the fluid accumulated in the whorl of plants of sweet corn cv. Merit in the field (B). Populations sizes are indicated in cfu/ml (A) or log_{10} cfu/ml (C).

TABLE 1. Test for normality of 15 data sets of bacterial populations from the whorls of sweet corn cultivars Merit (M) and Silver Queen (SQ).

Data set	Sample size	CFU/ml		Log 10 cfu/ml	
		W	P	W	P
Total M1	23	.80	.01	.97	.70
Total M2	24	.60	.01	.91	.05
Total M3	23	.63	.01	.97	.66
Total M4	23	.77	.01	.93	.16
Total M5	24	.93	.17	.91	.04
Total SQ1	16	.01	.13	.93	.34
Total SQ2	22	.96	.57	.86	.01
Total SQ3	19	.71	.01	.95	.47
Total SQ4	18	.95	.47	.90	.06
Total SQ5	23	.85	.01	.95	.30
Erwinia herbicola 1	20	.89	.04	.87	.01
Erwinia herbicola 2	20	.62	.01	.94	.33
Flavobacterium	15	.79	.01	.87	.05
Fluorescent	16	.58	.01	.89	.07
Fluorescent	16	.53	.01	.91	.20

Values W and P were calculated by the Shapiro-Wilk test for normality performed according to a univariate procedure provided by SAS. Values W approaching one indicate normality. Values of P describe the confidence level with which one can reject the null hypothesis of normality.

Variation in the density of bacterial populations occurred among plant whorls, especially when particular groups of bacteria were considered. The same trend has been observed for leaf-surface (Hirano et al. 1982) and rhizosphere (Loper et al. 1984) bacteria on several plant species. Because some plants tended to support specific groups of bacteria over time, the predominance of a given bacterial species may be determined by chance; whenever a competent bacterial epiphyte colonizes a favorable niche in a young plant, it predominates, and inhibits growth of other colonizers. If a threshold population of a biocontrol agent against *P. avenae* is determined, the frequency with which this threshold population is met or exceeded, either through application or through multiplication, may be utilized to predict the intensity of BLBSR of corn. The lack of significant qualitative and quantitative differences on bacterial populations, for the groups of bacteria tested, indicated that resistance of cultivar Silver Queen to BLBSR is not dependent on epiphytic bacteria present on their whorls.

The cumulative probability plots imply that sample collection for isolation of biocontrol agents in the whorls should be done on an individual plant basis rather than by bulking samples from an entire field or even sections of a field. The uniqueness of each whorl as an ecosystem suggests that the isolation of biocontrol agents should be made from symptomless plants. It has been observed for the *P. avenae*-corn pathosystem that scattered plants in the field are symptomless, whereas the majority of the plants are covered with lesions. This observation may be explained by developmental variation such as time of whorl formation, structural conformation of the whorl so that water is not

collected, or the presence of naturally occurring biocontrol agent(s) effective against *P. avenae*. This last factor is in accordance with Baker and Cook's assertion that biological control occurs at all times in nature (1).

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