Lack of Survival of *Rhizomonas suberifaciens* on Lettuce and Barley Leaves

R. DOUGLAS O'BRIEN, KEN N. JOCHIMSEN, and ARIENA H. C. VAN BRUGGEN, Department of Plant Pathology, University of California, Davis 95616

ABSTRACT

O'Brien, R. D., Jochimsen, K. N., and van Bruggen, A. H.C. 1991. Lack of survival of *Rhizomonas suberifaciens* on lettuce and barley leaves. Plant Dis. 75:954-957.

Population sizes of strain CA1R of *Rhizomonas suberifaciens*, causal agent of corky root of lettuce, strain 714R of *Pseudomonas syringae*, a successful leaf colonizer, and strain EC1R of *Escherichia coli*, which is not a leaf epiphyte, were compared in a growth chamber. Equal densities of each strain were deposited onto lettuce (susceptible to corky root) and barley (immune to corky root) plants with an atomizer. After an initial incubation of 72 hr at 100% relative humidity, population sizes of all three strains were high, but those of *P. syringae* were slightly higher than those of either *E. coli* or *R. suberifaciens*. Cells of *P. syringae* and *E. coli* were more abundant on barley than on lettuce, whereas cells of *R. suberifaciens* were more abundant on lettuce than on barley. After a further 72-hr incubation at 50% relative humidity, populations of all strains declined, but those of *P. syringae* remained much larger than those of *E. coli* and *R. suberifaciens*, and those of *E. coli* were slightly larger than those of *R. suberifaciens*. Populations of *R. suberifaciens*, unlike those of *E. coli* and *P. syringae*, were larger on lettuce than on barley, indicating differential adaptivity of the bacteria to the two plant species. The epiphytic survival of *R. suberifaciens* on lettuce appears to be similar to that of *E. coli*, suggesting that leaves may not be a significant source of inoculum of *R. suberifaciens*.

Rhizomonas suberifaciens causes corky root disease of lettuce (17,18). Taproots and main laterals become ridged, brittle, and greenish brown as the disease develops. The only aboveground symptoms are wilting and yellowing of lower leaves. Disease distribution seems to be fairly uniform within a field, possibly as a result of airborne dispersal of the bacterium similar to that found for *Pseudomonas syringae* (6).

Very little is known about the epidemiology of corky root in general and dispersal mechanisms in particular. In field (12) and greenhouse (16) experiments, corky root spread rapidly to uncontaminated soil, despite precautions taken to prevent water splash and soil transfers. Insects may carry the pathogen, which was isolated from root aphids in a field experiment (17). However, use of insect-proof cages did not prevent spread of the pathogen to uninoculated plants in the greenhouse (16). An aboveground inoculum source, perhaps epiphytic populations on plant leaves, seemed possible. A closely related bacterium (17), *Sphingomonas paucimobilis* (19) (formerly *P. paucimobilis*), is a common epiphyte on rice leaves (4).

In one growth chamber experiment (A. H. C. van Bruggen, *unpublished*), a suspension of ground leaves of a lettuce plant inoculated with *R. suberifaciens* strain CA1 reacted positively with polyclonal antibodies to that strain in an enzyme-linked immunosorbent assay (ELISA). However, the bacterium could not be isolated from leaves of diseased lettuce plants grown under field and greenhouse conditions. Whether the isolation attempts failed because of the absence of *R. suberifaciens* or because of limitations in the isolation procedure was not clear. Even isolations from diseased root tissue are often unsuccessful (17).

Many bacteria colonize the leaf surfaces of a variety of plant species when humidity is near 100% and light levels are low (11). However, populations of species not commonly associated with aerial plant surfaces, such as *Escherichia coli, Salmonella typhimurium, Aeromonas hydrophila,* and *Rhizobium meliloti,* decline precipitously when humidity is low and light levels are high. In contrast, bacteria commonly associated with plant leaves, such as pathovars of *P. syringae* (11) and *Xanthomonas campestris* (14), maintain high population sizes even in dry environments.

We used the apparent correlation between the natural epiphytic habitat of a bacterium and its population dynamics in an epiphytic bioassay to evaluate the epiphytic potential of *R. suberifaciens* on susceptible and immune plants. Preliminary findings of this study have been reported (10).

MATERIALS AND METHODS

We compared leaf surface populations of strain CA1R of *R. suberifaciens* with those of strain 714R of *P. syringae,* which is known for its epiphytic survival under dry conditions (11), and strain EC1R of *E. coli,* which is known to die rapidly when exposed to unfavorable leaf surface conditions (11). All strains were applied to plants of lettuce cultivar Salinas (susceptible to corky root) and barley cultivar Kombar (a nonhost) (16). The plants were then incubated first at high humidity with dim light and then at low humidity with bright light.

Bacterial strains. Spontaneous mutants of strain CA1 of *R. suberifaciens* (17), strain 714 of *P. syringae,* and strain EC1 of *E. coli* (11) resistant to rifampicin at 100 ppm were selected from colonies of survivors of 10^9 cells on either King's medium B or S-medium (5.0 g of enzymatic casein hydrolysate [Sigma Chemical Co., St. Louis, MO], 2.5 g of glucose, 1.3 g of $K_2HPO_4 \cdot 3H_2O$, 0.5 g of KNO_3, 0.5 g of $MgSO_4 \cdot 7H_2O$, 60 mg of $Ca(NO_3)_2 \cdot 4H_2O$, and 11.0 g of Agar Noble per liter, pH 7.2) containing rifampicin at 100 μg/ml. All strains were stored in 15% glycerol at −80 C.

Rifampicin-resistant strains were compared with the parent strains for appearance in culture, growth in vitro, and pathogenicity to lettuce and barley. Strain CA1R grew to equal numbers in S-broth and was as pathogenic on lettuce as strain CA1 (2,9); neither strain was pathogenic on barley (16). Strain 714R of *P. syringae* was not pathogenic on lettuce (R. D. O'Brien, *unpublished*) or barley (11) and was similar to the parent strain in growth rate, appearance on unamended King's medium B, and pathogenicity on a range of host plants (11).

Plant inoculations. Barley and lettuce plants were grown in 10-cm-diam pots on saucers in a growth chamber with 14 hr of light (300 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 27 C and 10 hr of darkness at 18 C each day. Lettuce pots contained about six plants and barley pots about 10 plants. When they were 3 wk old, plants were spray-inoculated to runoff with an atomizer. Plants were watered from below to avoid introducing waterborne bacteria onto the leaves and to prevent periods of leaf wetness.

Bacterial strains were grown in S-broth at 28 C before inoculation. Strains EC1R and 714R were grown for 48 hr and strain CA1R for 96 hr, which corresponds to log-phase growth. Concentrations were determined spectrophotometrically and were adjusted to 10^9 and 10^8 cfu/ml for strain CA1R on barley and lettuce, respectively, 5×10^7 and 5

Accepted for publication 20 March 1991 (submitted for electronic processing).

© 1991 The American Phytopathological Society

$\times 10^6$ cfu/ml for strain 714R on barley and lettuce, respectively, and 10^6 and 10^5 cfu/ml for strain EC1R on barley and lettuce, respectively. These concentrations were chosen to achieve equal initial populations of the three strains on leaf surfaces and were based on data from preliminary experiments on initial cell retention and survival rates in sonicated samples. Standard dilution plate techniques with S-medium plus rifampicin were used to confirm cell concentrations.

Plant incubation. All inoculated plants were exposed to high humidity and low light levels for 72 hr, followed by low humidity and high light levels for 72 hr. The sprayed plants were enclosed in clear plastic bags to maintain high humidity and were held in a growth chamber at 27 C with a 10-hr light period (150 $\mu E \cdot m^{-2} \cdot s^{-1}$). After 72 hr, the plants were unbagged and allowed to dry at 50% relative humidity and a constant temperature of 27 C with a 14-hr daily exposure to light at 300 $\mu E \cdot m^{-2} \cdot s^{-1}$. A Campbell 21 micrologger (Campbell Scientific, Inc., Logan, UT) equipped with model 207 temperature and humidity probes monitored temperatures and relative humidities.

Within the growth chamber, the experiment consisted of four blocks in a randomized complete block factorial design of two plant species $\times$ three bacteria $\times$ two humidity regimes. The experiment was performed twice.

Sampling procedure. Fifteen to 30 individual leaves (15–20 g fresh weight) were collected from each pot (replicate) of plants at each harvest date and tested for epiphytic bacterial populations. Samples were collected before inoculation, immediately after inoculation, and after wet and dry incubations in the growth chamber. Conversion factors of 0.75 for lettuce and 0.95 for barley, based on measured water retention, were used to convert wet leaf surface weights to air-dry weights.

Bacteria were washed from leaves, and population sizes were determined as described previously (11). The harvested leaves were submerged in 200 ml of sterile distilled water plus 0.2 ml of Tween 20 and sonicated for 7 min in an ultrasonic cleaner (Bransonic 5200, Branson Ultrasonics Corp., Danbury, CT) to remove cells from the leaves. Tween 20 was used instead of phosphate buffer (7) because the latter was toxic to CA1R in earlier tests (9). Precautions were taken to exclude soil and roots from samples.

To determine total background bacterial contamination, serial dilutions of sonicated leaves of plants from four pots each of uninoculated lettuce and barley were plated on S-medium containing cycloheximide at 100 μg/ml plus benomyl at 50 μg/ml but no rifampicin. The plates were incubated at 28 C, and bacterial colonies were counted after 2–10 days.

Appropriate 10-fold serial dilutions from all the postinoculation samples were plated on S-medium containing rifampicin at 100 μg/ml, cycloheximide at 100 μg/ml, and benomyl at 50 μg/ml. The fungicides were included to inhibit fungi and did not affect recovery of any of the three bacterial strains (9). Colonies of *E. coli* and *P. syringae* were counted after 2–3 days and those of *R. suberifaciens* after 7–10 days. Representative colonies presumed to be *R. suberifaciens* were tested for affinity to monoclonal antibodies by ELISA (15). All 240 colonies reacted with the antibody, a positive test for the pathogen.

We expressed bacterial population sizes as log cfu per gram of fresh weight of leaf tissue, even though lettuce and barley had slightly different specific leaf areas (0.022 and 0.033 cm^2/g, respectively). However, when populations were expressed as log cfu per square centimeter of leaf area, the results were essentially the same as those expressed as log cfu per gram of fresh weight (*data not shown*).

To correct for possible differences in retention of the bacteria by the two plant species, we washed three replicate leaf samples per treatment twice for 10 sec in 200 ml of distilled water and then ground them for 1 min at high speed in a Waring Blendor. The wash water was discarded and replaced by 200 ml of sterile distilled water plus 0.2 ml of Tween 20. The suspensions were filtered through one layer of cheesecloth and dilution-plated as outlined above.

The data were analyzed with software from Statistical Analysis Systems (SAS) (release 6.03, SAS Institute Inc., Cary, NC). The SAS general linear models procedure was used to perform analysis of variance and calculate orthogonal contrasts on log-transformed populations. The experimental design was a randomized complete block with incubation environment, bacterial species, and plant species as treatments and pot position within the incubator as a block. Contrasts were estimated with the estimate procedure.

RESULTS

The second experiment supported the first, except for initial population densities deposited on the leaves. For simplicity, only the results from the second experiment are presented in detail.

The percentage of bacterial cells retained did not differ significantly between plant species or among bacterial species, and there were no significant interactions ($P = 0.68$). On average, sonication removed 81% of the bacteria on the leaves.

Populations of background bacteria and of test bacteria immediately after inoculation. Before inoculation, bacterial population size on leaves averaged 2.8 log cfu per gram of fresh weight (standard deviation 0.4). Populations on lettuce and barley were not significantly different.

Although our intent was to deposit equal densities of the three bacteria on both plant species, more cells of *R. suberifaciens* were recovered from lettuce than from barley immediately after inoculation in the first experiment. In the second experiment, populations of the three strains on the two plant species were not significantly different. The average initial bacterial density was 4.8 log cfu per gram of fresh weight (standard deviation 0.2).

Bacterial populations after wet and dry incubation. Incubation environment and bacterial and plant species had significant effects on the size of epiphytic bacterial populations (Table 1). In addition, there were significant interactions between environment and bacterial strains, between bacterial strains and plant species, and among all three of these factors.

During the high-humidity incubation, plant leaves remained wet. Populations of all bacterial strains were larger after wet incubation than after dry incubation (Table 2). However, the strains responded differently to the change from wet to dry incubation: The decline in populations of *P. syringae* was much less than those of the other strains (1.1 vs. 3.2 log cfu per gram of fresh weight, respectively). The declines in cell densities of *E. coli* and *R. suberifaciens* after the change in incubation environment were not significantly different (3.1 vs. 3.3 log cfu per gram of fresh weight, respectively).

We used contrast analysis (Table 3) to further investigate the significant interaction between bacterial strain and plant species (Table 1). After both wet and dry incubations, cell densities of *R.*

Table 1. Summary analysis of variance of the effects of incubation environment, bacterial strain, and plant species on the logarithm of population sizes of *Rhizomonas suberifaciens* strain CA1R, *Escherichia coli* strain EC1R, and *Pseudomonas syringae* strain 714R on leaves of Salinas lettuce and Kombar barley

Source of variation	Degrees of freedom	Sum of squares[a]
Experiment	1	0.2
Incubation environment (E)	1	76.3***
Bacterial strain (B)	2	75.6***
E × B	2	12.0***
Plant species (P)	1	7.5***
E × P	1	0.7
B × P	2	18.7***
E × B × P	2	3.5***
Block	3	0.2
Error	75	5.4

[a] Three asterisks indicate significance at $P = 0.001$. Sums of squares without asterisks are not significant at $P = 0.05$.

suberifaciens were higher on lettuce (susceptible to corky root) than on barley (a nonhost), whereas cell densities of both *P. syringae* and *E. coli* were lower on lettuce than on barley (Table 2). After the wet incubation, populations of *E. coli* and *P. syringae* were similar on both plant species. After the dry incubation, the much higher populations of *P. syringae* on barley relative to those of *E. coli* resulted in a significant interaction between plant species and these two bacterial strains (Table 3).

DISCUSSION

R. suberifaciens was not a successful epiphyte on lettuce or barley leaves under the conditions of the study reported here. Our data also suggest that *R. suberifaciens* may not have a significant epiphytic phase under natural conditions, either, for the following reasons. Populations of *R. suberifaciens* were equal to or less than those of *E. coli* and much less than those of *P. syringae* in both humid and dry environments. And the epiphytic performance of *R. suberifaciens* resembled that of *E. coli,* which is not commonly found on plant leaves (5), much more than it resembled that of *P. syringae,* which is commonly found on plant leaves (7).

The very small populations of both *R. suberifaciens* and *E. coli* compared to those of *P. syringae* during the dry, high-light incubation indicate that the former two species would have difficulty surviving in sunny, dry areas (11). Such conditions are common in the coastal valleys of California where corky root disease is widespread. In those areas, however, periods of bright sunshine are interspersed with periods of maritime fog or dew in the summer, and with sporadic rain in the spring and fall growing seasons. We did not include an additional period of high humidity after the dry period in our study, and we did not assess the possible recovery of bacterial populations. Because our study did not address dew formation or the possible colonization of plant wounds, we cannot draw a definite conclusion. We also did not address variations in temperature; however, 27 C is near the optimum temperature for growth of *R. suberifaciens* (18).

The lack of significant epiphytic leaf populations makes it unlikely that lettuce roots are infected by epiphytic cells of *R. suberifaciens* washed from the leaves to the soil. Because a biotic cause for corky root disease has only recently been identified (18), little is known about how the pathogen is spread. Some alternative vehicles include infested, windblown dust, contaminated water (1), insects (17), aerosols arising from the soil surface similar to aerosols from plant leaves (6), and plant debris (13).

Grinding of sonicated leaves recovered about 20% more bacteria than sonication alone. However, neither plant nor bacterial species significantly affected the percentage of bacteria removed by sonication, confirming previous work (11). This result indicates that although grinding gives a better estimate of bacterial populations, it is not necessary for comparisons of plant or bacterial species.

Populations of *R. suberifaciens,* unlike those of the other two species, were larger on lettuce than on barley, which supports the hypothesis that a susceptible host provides a more hospitable epiphytic environment than a nonhost. The differences in initial inoculum concentrations in the first experiment (*data not shown*) were not relevant to this outcome, because the same result was seen in the second experiment, when initial inoculum concentrations were similar for all hosts and bacterial strains. Other work has shown that some bacteria grow and survive better on certain plants than on others (3,8,11). A strain of *P. syringae* pv. *morsprunorum* pathogenic to cherry and a strain of *P. syringae* pathogenic to pear persisted as epiphytes only on their respective susceptible hosts (3). O'Brien and Lindow (11) found that strains of *P. syringae* varied in their epiphytic ability on a range of plants, but this variation was not related to pathogenicity to particular hosts. A frost-sensitive cultivar of oats supported larger populations of ice nucleation-active bacteria than did hardier cultivars (8). Our finding that populations of *R. suberifaciens* were higher on lettuce leaves than on barley leaves resembles results obtained by Ercolani (3). The lack of foliar symptoms of corky root disease, both in this experiment and in the field, and the extremely low populations of *R. suberifaciens* found on lettuce leaves seem to preclude a foliar infection stage for corky root disease. Why populations of strain CA1R were higher on lettuce leaves than on barley leaves is not yet known. A larger experiment involving many strains and plant species would help to confirm this observation.

Table 2. Populations (log cfu per gram of fresh weight) of *Rhizomonas suberifaciens* strain CA1R, *Escherichia coli* strain EC1R, and *Pseudomonas syringae* strain 714R on leaves of Salinas lettuce and Kombar barley after a 72-hr incubation in a wet environment and after a further 72-hr incubation in a dry environment[a]

	Wet incubation			Dry incubation		
Strain	**Lettuce**	**Barley**	**Both plants**	**Lettuce**	**Barley**	**Both plants**
P. syringae	6.2	7.7	7.0	4.5	7.0	5.9
E. coli	5.5	7.3	6.4	2.9	3.6	3.3
R. suberifaciens	5.1	4.8	4.9	2.5	0.7	1.6

[a] Data are the means of four observations.

Table 3. Contrast analysis of interactions among bacterial strain (*Rhizomonas suberifaciens* [Rs], *Escherichia coli* [Ec], and *Pseudomonas syringae* [Ps]), plant species (lettuce and barley), and incubation environment (wet and dry)

	Orthogonal contrast estimate[a]		
Interaction	**Wet incubation**	**Dry incubation**	**Both plant species**
Plant × [Rs vs. (Ps + Ec)]	1.9***	3.4***	
Plant × (Ps vs. Ec)	−0.3	−1.8***	
Incubation × [Ps vs. (Ec + Rs)]			−2.1***
Incubation × (Ec vs. Rs)			−0.2

[a] Three asterisks indicate significance at $P = 0.001$. Estimates without asterisks are not significant at $P = 0.05$.

ACKNOWLEDGMENTS

This study was supported in part by the California Iceberg Lettuce Research Advisory Board, Salinas, CA. We thank J. J. Marois, J. M Duniway, and S. E. Lindow for manuscript suggestions.

LITERATURE CITED

1. Cappaert, M. R., Powelson, M. L., Franc, G. D., and Harrison, M. D. 1988. Irrigation water as a source of inoculum of soft rot erwinias for aerial stem rot of potatoes. Phytopathology 78:1668-1672.
2. Compeau, G., Al-achi, B. J., Platsouka, E., and Levy, S. B. 1988. Survival of rifampicin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl. Environ. Microbiol. 54:2432-2438.
3. Ercolani, G. L. 1969. Sopravvievenza epifitica di popolaxioni di *Pseudomonas morsprunorum* Wormald da ciliegio e di *P. syringae* van Hall da pero sulla pianta. Phytopathol. Mediterr. 8:197-206.
4. Jones, R. K., Barnes, L. W., Gonzalez, C. F., Leach, J. E., Alvarez, A. M., and Benedict, A. A. 1989. Identification of low-virulence strains of *Xanthomonas campestris* pv. *oryzae* from rice in the United States. Phytopathology 79:984-990.
5. Kreig, N. R. 1984. Bergey's Manual of Systematic Bacteriology, Vol. 1, 8th ed. Williams & Wilkins, Baltimore.
6. Lindemann, J., Constantinidou, H. A., Barchet, W. R., and Upper, C. D. 1982. Plants as sources of airborne bacteria, including ice nucleation-active bacteria. Appl. Environ. Microbiol. 44:1059-1063.
7. Lindow, S. E., Arny, D. C., and Upper, C. D. 1978. Distribution of ice nucleation active bacteria on plants in nature. Appl. Environ. Microbiol. 36:831-838.
8. Marshall, D. 1988. A relationship between ice-nucleation-active bacteria, freeze damage, and genotype in oats. Phytopathology 78:952-957.
9. O'Brien, R. D. 1991. Epidemiology of corky root

disease of lettuce and population dynamics of the causal agent, *Rhizomonas suberifaciens*. Ph.D. dissertation. University of California, Davis.
10. O'Brien, R. D., Jochimsen, K. N., and van Bruggen, A. H. C. 1990. Growth and survival of *Rhizomonas suberifaciens* on lettuce and barley leaves. (Abstr.) Phytopathology 80:891.
11. O'Brien, R. D., and Lindow, S. E. 1989. Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. Phytopathology 79:619-627.
12. O'Brien, R. D., and van Bruggen, A. H. C. 1990. Soil fumigation with dazomet and methyl bromide for control of corky root of iceberg lettuce. Plant Dis. 74:1022-1025.
13. Pérombelon, M. C. M., Fox, R. A., and Lowe, R. 1979. Dispersal of *Erwinia carotovora* in aerosols produced by the pulverization of potato haulm prior to harvest. Phytopathol. Z. 94:249-260.
14. Timmer, L. W., Marois, J. J., and Achor, D. 1987. Growth and survival of xanthomonads under conditions nonconducive to disease development. Phytopathology 77:1341-1345.
15. van Bruggen, A. H. C. 1990. Etiology, epidemiology and control of lettuce corky root. Pages 41-59 in: Annual Report 1989–1990. Iceberg Lettuce Research Program, Salinas, CA.
16. van Bruggen, A. H. C., Brown, P. R., and Jochimsen, K. N. 1990. Host range of *Rhizomonas suberifaciens,* the causal agent of corky root of lettuce. Plant Dis. 74:581-584.
17. van Bruggen, A. H. C., Grogan, R. G., Bogdanoff, C. P., and Waters, C. M. 1988. Corky root of lettuce in California caused by a gram-negative bacterium. Phytopathology 78:1139-1145.
18. van Bruggen, A. H. C., Jochimsen, K. N., and Brown, P. R. 1990. *Rhizomonas suberifaciens* gen. nov., sp. nov., the causal agent of corky root of lettuce. Int. J. Syst. Bacteriol. 40:175-188.
19. Yabuuchi, T. Y., Oyaizu, H., Hashimoto, Y., Ezaki, T., and Yamamoto, H. 1990. Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. Microbiol. Immunol. 34:99-119.