APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2001, p. 1945–1948 Vol. 67, No. 4
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.4.1945–1948.2001

Growth Interactions during Bacterial Colonization of Seedling Rootlets

P. DE BELLIS AND G. L. ERCOLANI*

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari, 70126 Bari, Italy

Received 1 November 2000/Accepted 26 January 2001

Rootlet elongation and bacterial growth on rootlets were determined after inoculation of cucumber and spinach seedlings with *Pseudomonas* strains differing in production of siderophores and HCN. Siderophore producers grew more profusely than nonproducers on both species and promoted rootlet elongation on cucumber. Coinoculation of siderophore producers and nonproducers resulted in restricted growth of the latter. The total populations of nonproducers of HCN in the presence of HCN producers were not decreased, but the tenacity of their association with the rootlet surface was altered.

Germinating seeds and growing plants influence the activities of soil microorganisms in the adjoining volumes of soil known as the spermosphere and the rhizosphere, respectively (24). Conversely, microorganisms in these settings condition the seeds and plants in a number of ways. Some of the microorganisms (e.g., the so-called plant-growth-promoting rhizobacteria) may enhance plant health and productivity by synthesizing phytohormones, increasing the local availability of nutrients, facilitating the uptake of nutrients by the plants, decreasing heavy metal toxicity in the plants, antagonizing plant pathogens, and inducing systemic resistance in the plants to pathogens (5, 8, 9). Detrimental effects are produced by other organisms, such as the so-called deleterious rhizosphere microorganisms, and these effects include release of toxic products of microbial metabolism, alteration of nutrient cycling, impairment of uptake of nutrients, competition for nutrients, and retardation of root growth (28). Among the factors involved in plant-microbe interactions, as well as in microbe-microbe interactions, in the rhizosphere, siderophores and hydrocyanic acid (HCN) have received special attention. Siderophores are high-affinity Fe^{3+} chelators that are synthesized and released extracellularly under iron limitation conditions, where they make otherwise inaccessible supplies of insoluble iron available to organisms with specific membrane-bound siderophore receptors (14, 17). Such receptors are present in the microorganisms that produce the corresponding siderophores but can also be found in plants. The iron nutrition of these plants is thus enhanced. In addition, since plant-growth-promoting rhizobacteria produce siderophores with higher Fe^{3+} affinity than the siderophores produced by deleterious rhizosphere microorganisms, the latter microorganisms are outcompeted in their quest for iron. HCN is released as product of secondary metabolism by several microorganisms and affects sensitive organisms by inhibiting the synthesis of ATP mediated by cytochrome oxidase (22). Therefore, depending on the target organisms, HCN-producing microorganisms are regarded as harmful when they impair plant health and beneficial when they suppress unwanted components of the microbial community (23, 29). The significance of siderophore and HCN generation in rhizosphere management and engineering has been studied and reviewed extensively (11, 13, 16, 21, 26, 36), but there is relatively little information on comparative population dynamics and interactions of producing and nonproducing microorganisms during the early stages of root colonization. These issues were specifically addressed in the present work.

Experimental procedures. The following strains were selected from a collection of isolates of *Pseudomonas* spp. established previously (7): CC13 and CC19 from nonrhizosphere soil close to an isolated plant of mahaleb cherry; CC148 from the rhizosphere of broccoli rab; CC209 from the rhizosphere of leaf beet; CC219/2 from the rhizosphere of artichoke; and CC295 from nonrhizosphere soil located between four blocks containing artichoke, cauliflower, leaf beet, and onion. These strains were identified as *Pseudomonas aeruginosa* (CC19), *Pseudomonas aureofaciens* (CC295), *Pseudomonas fluorescens* biovar 1 (CC13, CC209, and CC219/2), and *Pseudomonas putida* biovar A (CC148) on the basis of fatty acid methyl ester profiles as described by Stead (33). Table 1 shows the identifying characteristics that were used routinely in the present work to differentiate the strains with respect to production of siderophores with the chromeazurol S test (30), with respect to production of HCN with the test devised by Castric and Castric (6), and with respect to utilization of maltose, sorbitol, or methyl α-D-glucopyranoside at a concentration of 2 mg ml^{-1} as a sole C source for growth in the synthetic medium of Ayers et al. (3) solidified with 15 g of agar per liter. The strains were maintained on 2% (wt/vol) glycerol nutrient agar at 4°C.

Seeds of cucumber cv. Miracross F1 hybrid (Blumen brand; Agritecnica, Bari, Italy) and spinach cv. Kent hybrid Cal 9 (Asgrow Seed Co., Kalamazoo, Mich.) were soaked in running tap water for 3 h, washed with several changes of sterile distilled water (SDW), and allowed to germinate in 15-cm-diameter petri dishes lined with filter paper saturated with SDW. After 3 days of incubation at 20°C, 30 seedlings were inoculated by dipping them in the appropriate bacterial suspension for 15 s. For single-strain inoculations, the strain to be tested

* Corresponding author. Mailing address: Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari, Via Amendola 165/a, 70126 Bari, Italy. Phone: 39 080 5442945. Fax: 39 080 5442911. E-mail: ercolani@agr.uniba.it.

TABLE 1. Identifying characteristics of bacterial strains used in this study

Strain	Production of siderophores[a]	Production of HCN[b]	Utilization as sole C source[b]		
			Maltose	Sorbitol	Methyl-α-D-glucopyranoside
CC13[c]	++	+	+	+	−
CC19[c]	+	+	−	−	+
CC148[c]	++	−	+	−	+
CC209[c]	+	−	+	+	+
CC219/2	−	−	+	+	−
CC295	−	+	−	−	+

[a] Data indicate the radii of the discolored halos around bacterial streaks on chromeazurol S agar plates (30), as follows: +, 4 to 5 mm; ++, 6 to 8 mm; −, no halo.
[b] +, positive; −, negative.
[c] A positive reaction for the pyoverdine type of siderophores was obtained with these strains when the test described by de Weger et al. (10) was used.

was grown on 2% (wt/vol) glycerol nutrient agar at 25°C for 24 h, and the resulting growth was suspended in SDW at a concentration of 10^8 CFU ml^{-1} and used as the inoculum. For two-strain inoculations, bacteria were grown as described above and strains were combined in preparations containing 10^8 CFU of each per ml as follows: CC148 plus CC219/2, CC13 plus CC295, CC295 plus CC219/2, and CC13 plus CC148. Inoculated seedlings were shaken to remove the excess liquid and returned to fresh petri dishes as described above. Each dish accommodated 10 seedlings. Thirty seedlings to be used as a control were treated as described above except that they were dipped in SDW instead of a bacterial suspension. Two, 48, and 96 h later, the length of the rootlet was measured for each of 10 inoculated and 10 control seedlings. At the same time, the seedlings were inspected visually for growth disorders. The rootlet was then removed from each of the inoculated seedlings and subjected to two washes with vortexing at 2,500 rpm for 15 s in 3 ml of SDW, followed by comminution in 1 ml of SDW with a manual tissue grinder (model 358103; Wheaton, Millville, N.J.). Following single-strain inoculation, the washes and final slurry were serially diluted in SDW and plated on medium B of King et al. (19) solidified with 15 g of agar $liter^{-1}$, and then colonies were counted with a precision of 3% (25) after 3 days of incubation at 25°C. Counts for coinoculated strains were obtained by the same procedure on two plates of the synthetic medium of Ayers et al. solidified with 15 g of agar per liter, each containing 2 mg of the C source specific for one strain per ml. In all experiments, the numbers of CFU in the first and second washes and in the slurry were considered indicators of the numbers of bacteria that were loosely adsorbed (fraction L), reversibly adherent (fraction R), and firmly anchored (fraction F) to the rootlet surface, respectively; the total number of bacteria in the root sample was calculated by adding these three numbers. Each measurement was obtained for three sets of 10 seedlings, and each experiment was carried out twice. The data were subjected to statistical analysis (analysis of variance and the multiple-range test by the Student-Newman-Keuls procedure for significance of inoculation treatments and time of sampling) as described by Zar (37). The numerical values presented below are means ± standard deviations based on the two experiments.

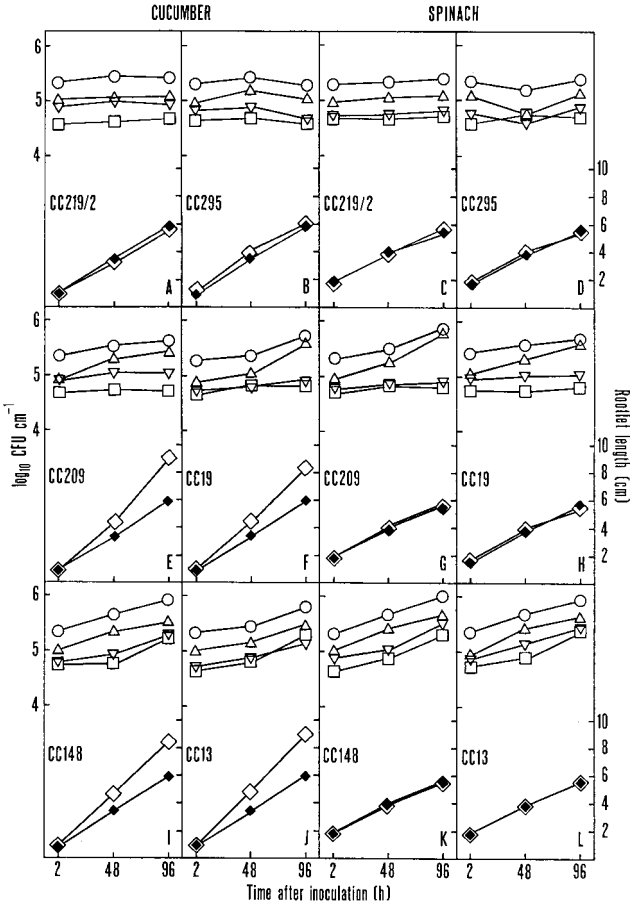

FIG. 1. Bacterial populations and rootlet elongation after inoculation of cucumber and spinach seedlings with the following bacterial strains: CC13, CC19, CC148, CC209, CC219/2, and CC295. Separate counts are given for total bacteria (○) and for the bacteria that were considered loosely adsorbed (△), reversibly adherent (▽), and firmly anchored (□) to the rootlet surface. The length of inoculated rootlets (◇) is shown along with the length of the controls (◆). Each value is the mean based on two independent experiments performed with triplicate sets of 10 seedlings. Distinguishing characteristics of bacterial strains are shown in Table 1.

Single-strain inoculations. The average total number of bacteria was $2.1 \times 10^5 \pm 0.19 \times 10^5$ CFU cm of rootlet^{-1} 2 h after inoculation of either cucumber or spinach seedlings with any strain (Fig. 1). Most of each count was contributed by fraction L ($9.1 \times 10^4 \pm 1.27 \times 10^4$ CFU cm^{-1}), followed by fractions R ($6.8 \times 10^4 \pm 0.75 \times 10^4$ CFU cm^{-1}) and F ($4.8 \times 10^4 \pm 0.34 \times 10^4$ CFU cm^{-1}). Later counts did not differ significantly ($P = 0.872$) from the initial values for any of the strains unable to synthesize siderophores except CC295 on spinach, where the values for fractions L and R were lower ($P < 0.05$) and the value for fraction F was higher ($P < 0.05$) at 48 h (Fig. 1 A through D). In contrast, the values for all fractions of strains that produce high levels of siderophores increased 2.5- to 3.8-fold on cucumber and 3.5- to 5.0-fold on spinach during the experiments (Fig. 1I through L). With strains that produce moderate levels of siderophores, the counts for fraction L and the counts for fractions R and F exhibited the same trends as the counts obtained for strains that produce high levels and for

strains that do not produce siderophores, respectively (Fig. 1E through H). The length of cucumber rootlets increased significantly from 3.3 ± 0.37 to 4.57 ± 0.59 cm at 48 h and from 5.74 ± 0.52 to 8.65 ± 0.69 cm at 96 h after inoculation with strains that produce moderate or high levels of siderophores, independent ($P = 0.747$) of production of HCN (Fig. 1E, F, I, and J). None of the strains affected elongation of spinach rootlets ($P = 0.901$), but dark necrotic strips of tissue were visible in the periderm 48 to 96 h after inoculation with HCN producers. The nature of the different responses of cucumber and spinach rootlets to HCN- and siderophore-producing strains was not investigated. When 3-day-old seedlings were dipped in SDW and then grown for 4 days on filter paper saturated with 10^{-4} M $FeCl_3$, the final length of cucumber rootlets increased significantly ($P < 0.05$) from 6.09 ± 0.58 to 8.60 ± 0.44 cm, but elongation of spinach rootlets was not affected ($P = 0.844$), suggesting that cucumber, but not spinach, was iron limited under the conditions used. Additional observations were made with seedlings that were raised initially for 3 days as usual and then dipped in SDW and kept for 4 additional days on SDW-impregnated filter paper in two opposite compartments of an X dish in which one of the HCN-producing strains was growing on 2% peptone agar in the other two compartments. In this setting, mild necrotic symptoms similar to those described above were seen on the rootlets of spinach but not on the rootlets of cucumber, supporting the hypothesis that there was etiologic involvement of a gaseous bacterial product.

Two-strain inoculations. The total and fractional counts for all strains in each pair up to 48 h after inoculation did not differ significantly ($P = 0.866$) from the counts obtained during the same period when the same strains were inoculated separately (Fig. 2). However, significant differences compared with the single-strain experiments emerged later. When a siderophore producer was coinoculated with a nonproducer, the counts for all fractions of the latter declined significantly ($P < 0.05$) by 1.3 to 1.8 log units between 48 and 96 h (Fig. 2A through D). Consequently, the total populations of these strains decreased by the corresponding values. This was true whether both strains in the inoculum did or did not produce HCN. For pairs in which the strains differed in production of HCN, the values for fractions R and F of the nonproducer declined by $4.5 \times 10^4 \pm 0.9 \times 10^4$ to $10 \times 10^4 \pm 2.2 \times 10^4$ and $3.9 \times 10^4 \pm 0.55 \times 10^4$ to $6.9 \times 10^4 \pm 1.2 \times 10^4$ CFU cm^{-1}, but the values for fraction L increased by approximately the same amounts (Fig. 2E through H). Therefore, the total populations reached the same levels ($P = 0.831$) as those in single-strain experiments whether both strains in the pair were HCN producers or nonproducers. For combinations in which a strain positive for production of siderophores or HCN was paired with a strain negative for the same characteristic, the total and fractional populations at 96 h did not differ significantly ($P = 0.838$) from those recorded at the same time after inoculation of the positive strain alone. Whenever one or both strains in the inoculum produced siderophores, elongation of cucumber rootlets at 48 and 96 h was significantly greater ($P < 0.05$) than elongation in the controls (Fig. 2A, B, and F). Necrotic symptoms similar to those described above for single-strain inoculations were seen on spinach rootlets beginning 48 h after inoculation

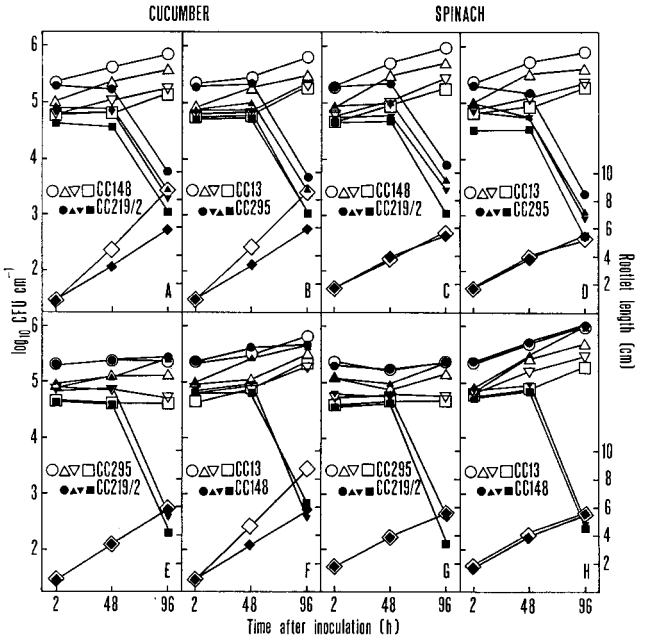

FIG. 2. Bacterial populations and rootlet elongation after inoculation of cucumber and spinach seedlings with the following pairs of bacterial strains: CC148 plus CC219/2, CC13 plus CC295, CC295 plus CC219/2, and CC13 plus CC148. Separate counts for each strain in every pair (open and solid symbols) are given for total bacteria (○ and ●) and for the bacteria that were considered loosely adsorbed (△ and ▲), reversibly adherent (▽ and ▼), and firmly anchored (□ and ■) to the rootlet surface. The length of inoculated rootlets (◇) is shown along with the length of the controls (◆). Each value is the mean based on two independent experiments performed with triplicate sets of 10 seedlings. Distinguishing characteristics of bacterial strains are shown in Table 1.

with any combination of strains when one or both of the components produced HCN.

Conclusions. It has been reported widely (4, 12, 15, 27, 31, 34) that the relative importance of biotic and abiotic determinants in microbial and plant-microbe interactions in the rhizosphere can be determined more precisely in gnotobiotic systems than in the field, where heterogeneous, mostly ill-defined factors come into play. The findings of the present work, which were obtained under gnotobiotic conditions, combined with previous reports (1, 2, 18, 20, 32, 35), emphasize that proper assessment of the influence of bacterial properties on the outcome of root colonization requires careful consideration of the test plant and of the indicator effects that must be taken into account. This view is supported by several lines of evidence with respect to production of siderophores and HCN. First, significantly larger populations on cucumber and spinach rootlets were invariably generated by bacterial strains that produced siderophores than by strains that did not. With moderate siderophore producers, however, this effect resulted exclusively from an increase in the fraction of total bacteria that were loosely adsorbed to the rootlet, whereas all fractions of strains that produced high levels of siderophores proliferated more irrespective of the tenacity of their association with the surface of the rootlet. Second, bacterial interactions resulting from the coexistence of different strains on the same rootlet

were or were not reflected in the total population size of each strain on the rootlet, depending on whether the strains differed in production of siderophores or of HCN. Third, inoculation with siderophore producers resulted in greater elongation of rootlets on cucumber seedlings but not on spinach seedlings. And fourth, neither the health nor the elongation of cucumber rootlets was apparently disturbed by HCN producers that induced necroses on the rootlets of spinach. Verification of these findings in long-term experiments with exposure to field soil variables is under way.

REFERENCES

1. **Åström, B.** 1991. Role of bacterial cyanide production in differential reaction of plant cultivars to deleterious rhizosphere pseudomonads. Plant Soil **133:** 93–100.
2. **Åström, B., A. Gustafsson, and B. Gerhardson.** 1993. Characteristics of a plant deleterious rhizosphere pseudomonad and its inhibitory metabolite(s). J. Appl. Bacteriol. **74:**20–28.
3. **Ayers, S. H., P. Rupp, and W. T. Johnson.** 1919. A study of the alkali-forming bacteria in milk. U.S. Department of Agriculture Bulletin 782. U.S. Department of Agriculture, Washington, D.C.
4. **Barazani, O., and J. Friedman.** 1999. Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? J. Chem. Ecol. **25:**2397–2406.
5. **Burd, G. I., D. G. Dixon, and B. R. Glick.** 2000. Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Can. J. Microbiol. **46:**237–245.
6. **Castric, K. F., and P. A. Castric.** 1983. Method for rapid detection of cyanogenic bacteria. Appl. Environ. Microbiol. **45:**701–702.
7. **Cocozza, C., and G. L. Ercolani.** 1997. Produzione di siderofori e caratteristiche associate in pseudomonadi fluorescenti rizosferiche e non rizosferiche. Ann. Microbiol. Enzimol. **47:**17–28.
8. **Davison, J.** 1988. Plant beneficial bacteria. Bio/Technology **6:**282–286.
9. **de Weger, L. A., A. J. van der Bij, L. C. Dekkers, M. Simons, C. A. Wijffelman, and B. J. J. Lugtenberg.** 1995. Colonization of the rhizosphere of crop plants by plant-beneficial pseudomonads. FEMS Microbiol. Ecol. **17:**221–228.
10. **de Weger, L. A., R. van Boxtel, B. van der Burg, R. A. Gruters, F. P. Geels, B. Schippers, and B. Lugtenberg.** 1986. Siderophores and outer membrane proteins of antagonistic, plant growth-stimulating, root-colonizing *Pseudomonas* spp. J. Bacteriol. **165:**585–594.
11. **Dowling, D. N., B. Boesten, P. R. Gill, Jr., and F. O'Gara.** 1994. Developing concepts in biological control: a molecular ecology approach, p. 57–65. *In* F. O'Gara, D. N. Dowling, and B. Boesten (ed.), Molecular ecology of rhizosphere microorganisms. VCH, Weinheim, Germany.
12. **Frommel, M. I., J. Nowak, and G. Lazarovits.** 1991. Growth enhancement and developmental modifications of *in vitro* grown potato (*Solanum tuberosum* ssp. *tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. Plant Physiol. **96:**928–936.
13. **Glick, B. R.** 1995. The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. **41:**109–117.
14. **Guerinot, M. L.** 1994. Microbial iron transport. Annu. Rev. Microbiol. **48:** 743–772.
15. **Han, D. Y., D. L. Coplin, W. D. Bauer, and H. A. J. Hoitink.** 2000. A rapid bioassay for screening rhizosphere microorganisms for their ability to induce systemic resistance. Phytopathology **90:**327–332.
16. **Hemming, B. C.** 1986. Microbial-iron interactions in the plant rhizosphere. An overview. J. Plant Nutr. **9:**505–521.
17. **Höfte, M.** 1993. Classes of microbial siderophores, p. 3–26. *In* L. L. Barton and B. C. Hemming (ed.), Iron chelation in plants and soil microorganisms. Academic Press, San Diego, Calif.
18. **Horwath, W. R., L. F. Elliott, and J. M. Lynch.** 1998. Influence of soil quality on the function of inhibitory rhizobacteria. Lett. Appl. Microbiol. **26:**87–92.
19. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocianin and fluorescin. J. Lab. Clin. Med. **44:**301–307.
20. **Kloepper, J. W., and C. J. Beauchamp.** 1992. A review of issues related to measuring colonization of plant roots by bacteria. Can. J. Microbiol. **38:** 1219–1232.
21. **Kloepper, J. W., R. Liftshitz, and R. M. Zablotowicz.** 1989. Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. **7:**39–44.
22. **Knowles, C. J.** 1976. Microorganisms and cyanide. Bacteriol. Rev. **40:**652–680.
23. **Lugtenberg, B. J. J., L. A. de Weger, and J. W. Bennett.** 1991. Microbial stimulation of plant growth and protection from disease. Curr. Opin. Biotechnol. **2:**457–464.
24. **Lynch, J. M.** 1990. Introduction: some consequences of microbial rhizosphere competence for plant and soil, p. 1–10. *In* J. M. Lynch (ed.), The rhizosphere. John Wiley & Sons, Chichester, United Kingdom.
25. **Meynell, G. G., and E. Meynell.** 1970. Theory and practice in experimental bacteriology, 2nd ed. Cambridge University Press, Cambridge, United Kingdom.
26. **Neilands, J. B., and S. A. Leong.** 1986. Siderophores in relation to plant growth and disease. Annu. Rev. Plant Physiol. **37:**187–208.
27. **Pillay, V. K., and J. Nowak.** 1997. Inoculum density, temperature, and genotype effects on in vitro growth promotion and epiphytic and endophytic colonization of tomato (*Lycopersicon esculentum* L.) seedlings inoculated with a pseudomonad bacterium. Can. J. Microbiol. **43:**354–361.
28. **Schippers, B., A. W. Bakker, and P. A. H. M. Bakker.** 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. **25:**339–358.
29. **Schippers, B., A. W. Bakker, P. A. H. M. Bakker, and R. Van Peer.** 1991. Beneficial and deleterious effects of HCN-producing pseudomonads on rhizosphere interactions, p. 211–220. *In* D. L. Keister and P. B. Cregan (ed.), The rhizosphere and plant growth. Kluwer Academic Publishers, Dordrecht, The Netherlands.
30. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
31. **Simons, M., A. J. van der Bij, I. Brand, L. A. de Weger, C. A. Wijffelman, and B. J. J. Lugtenberg.** 1996. Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. Mol. Plant-Microbe Interact. **9:**600–607.
32. **Sivasithamparam, K., and C. A. Parker.** 1979. Rhizosphere micro-organisms of seminal and nodal roots of wheat grown in pots. Soil Biol. Biochem. **11:**155–160.
33. **Stead, D. E.** 1992. Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. Int. J. Syst. Bacteriol. **42:**281–295.
34. **Timmusk, S., and E. G. H. Wagner.** 1999. The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses. Mol. Plant-Microbe Interact. **12:**951–959.
35. **Vande Broek, A., and J. Vanderleyden.** 1995. The role of bacterial motility, chemotaxis, and attachment in bacteria-plant interactions. Mol. Plant-Microbe Interact. **8:**800–810.
36. **Weller, D. M., and L. S. Thomashow.** 1994. Current challenges in introducing beneficial microorganisms into the rhizosphere, p. 1–18. *In* F. O'Gara, D. N. Dowling, and B. Boesten (ed.), Molecular ecology of rhizosphere microorganisms. VCH, Weinheim, Germany.
37. **Zar, J. H.** 1996. Biostatistical analysis, 3rd ed. Prentice-Hall, London, United Kingdom.