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DISPERSAL OF WILD-TYPE AND GENETICALLY-MODIFIED *PSEUDOMONAS* spp FROM TREATED SEEDS OR SOIL TO AERIAL PARTS OF RADISH PLANTS

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Summary—Dispersal of wild-type and genetically-modified strains of plant growth-promoting fluorescent *Pseudomonas* spp to aerial parts of radish plants was investigated using different inoculation strategies. *P. putida* strain WCS358rr colonized the surface of cotyledons of radish plants when seeds were coated with this strain or when non-coated seeds were sown in soil treated with WCS358rr. The cotyledons were not colonized by WCS358rr when suspensions of this strain were inoculated onto roots of radish seedlings that had already grown for 4 d in soil. Strain WCS358rr not only colonized the surface, but also the interior of cotyledons. For all types of inoculation strategies tested, strain WCS358rr colonized the root surface but did not penetrate the interior of the root. These results indicate that WCS358rr was not transported up, or downwards through the vascular system. When coated on seed, dispersal to cotyledons of radish was also demonstrated for *P. fluorescens* WCS374 and its genetically-modified derivative WCS374pMR. Plasmid pMR was maintained in WCS374pMR throughout the experiment. No differences were observed between modified strain WCS374pMR and wild-type strain WCS374 with regard to dispersal to and colonization of cotyledons and roots of radish. Strains WCS358rr, WCS374 and WCS374pMR survived on cotyledons for a period of 21–31 d. Dispersal of the applied bacterial strains to the first true leaves of radish could not be demonstrated. The results indicate that cotyledons of radish become contaminated with strains of *Pseudomonas* spp during germination of treated seeds or during seedling emergence from treated soil. The implications of dispersal of wild-type and genetically-modified pseudomonads to aerial parts of the plant are discussed.

INTRODUCTION

Root-colonizing fluorescent *Pseudomonas* spp have received much attention for their possible environmental applications, including their use in the culture of crops to control a wide variety of plant-pathogenic bacteria and fungi (Weller, 1988; Schippers, 1992). Under field conditions, however, the degree of disease control by introduced strains of fluorescent *Pseudomonas* spp varies greatly from test to test (Weller, 1988). Genetic engineering offers the potential to improve the biocontrol activity of particular *Pseudomonas* strains by the introduction or amplification of specific genes. So far, the effectiveness of most genetically-engineered strains still has to be evaluated under field conditions. When releasing such modified strains into the environment many other important issues also must be considered, including the potential ecological consequences. In this context, considerable effort has been directed towards monitoring survival of modified strains in soil and rhizosphere environments and towards transfer of recombinant DNA from the modified strains to the indigenous microflora (Alexander, 1986; Trevors *et al.*, 1987; Fry and Day, 1990; Parke, 1991). However, the role of dispersal of genetically-modified and also wild-type strains of *Pseudomonas* spp to environments outside the area of release is still poorly understood.

Introduced micro-organisms are only dispersed if an effective route for transmission exists. Kluepfel and Tonkyn (1990) demonstrated that the rhizobacterium *Pseudomonas aureofaciens* strain RN migrates from soil by the invasion of the plant vascular system and direct transport via insects. The rhizosphere-inhabiting *Azospirillum* spp were able to contaminate non-inoculated soybean plants up to 6 m from the inoculation source, possibly due to airborne transmission (Bashan, 1991). Under controlled conditions of high relative humidity, bacteria can reach leaves of plants from seed and soil surfaces (Leben, 1965; Andrews, 1992; Kluepfel, 1993). In this context, the data of Robertson and Alexander (1994) strongly suggest that rain and wind-blown soil are important means for transmitting *Azorhizobium caulinodans*, a bacterium that nodulates the stem of *Sesbania rostrata*. The success of subsequent establishment of

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dispersed micro-organisms depends on their ability to survive adverse conditions encountered in new environments (Andow, 1986). Bacteria originating from soil can survive and grow on leaves, but under the fluctuating environmental conditions on leaves their populations tend to decline more rapidly than those of bacteria isolated from the phyllosphere (O'Brien and Lindow, 1989).

We have investigated the dispersal of wild-type and genetically-modified strains of plant growth-promoting fluorescent *Pseudomonas* spp to aerial plant parts of radish. We developed a method for the recovery of introduced *Pseudomonas* spp from the surface and the interior of radish leaves. Dispersal to and subsequent colonization of leaves of radish plants by introduced strain *P. putida* WCS358rr was studied for three types of inoculation strategies: seed-, soil-or root-bacterization. Finally, dispersal of genetically-modified *Pseudomonas* spp to aerial plant parts was examined using derivative *P. fluorescens* WCS374pMR as a model strain. Dispersal to and subsequent colonization of leaves of radish plants by derivative WCS374pMR was compared to that of its wild-type strain.

MATERIALS AND METHODS

Bacterial strains and growth media

Wild-type strains *P. putida* WCS358 and *P. fluorescens* WCS374 are plant growth-promoting and disease-suppressing rhizobacteria isolated from the roots of potato (Geels and Schippers, 1983; Bakker *et al.*, 1986; Schippers, 1992). Both strain WCS374 and strain WCS358 are potential biocontrol agents of fusarium wilt of radish in commercial greenhouses (Leeman *et al.*, 1995). Strain WCS358rr is a spontaneous rifampicin-resistant derivative of WCS358 (Glandorf *et al.*, 1992). The rifampicin-resistance of strain WCS358rr is very stable as was demonstrated in field studies conducted by Glandorf *et al.* (1992). WCS374pMR is a derivative of strain WCS374 modified with plasmid pMR which encodes resistance to tetracycline and harbors the siderophore receptor PupA (Pseudobactin uptake protein A) of strain WCS358 (Marugg *et al.*, 1989; Bitter *et al.*, 1991). For the preparation of inoculum, bacterial strains were cultured on King's medium B agar (KB) (King *et al.*, 1954) for 48 h at 27°C, harvested in 0.1 M $MgSO_4$ and washed twice by centrifugation (10 min, 5000*g*).

Recovery of bacterial strains from the surface and interior of leaves and roots

To determine the efficiency of recovery of introduced bacterial strains from leaves of radish (*Raphanus sativus* cv. Saxa Nova), a suspension of strain WCS358rr was inoculated onto the cotyledons of 4-d-old radish seedlings to a final density of 10^6 cfu per cotyledon. After 30 min at 20°C, cotyledons were excised from the plants and shaken vigorously for 30 s in glass test tubes (22 mm dia, Sigma) containing 4.5 ml of 0.1 M $MgSO_4$ or 4.5 ml of 0.1 M $MgSO_4$–0.05% Tween 80, respectively. Tween 80 was added to the solution to reduce the surface tension of leaves (Bunster *et al.*, 1989). The efficiency of recovery of WCS358rr was determined by dilution plating suspensions on KB-agar supplemented with rifampicin (150 $\mu g\ ml^{-1}$) (KBRif). The efficiency of surface sterilization was tested by treatment of inoculated cotyledons for 15 s with 10% H_2O_2 or 20% H_2O_2, respectively. Subsequently, cotyledons were rinsed twice in sterile water and pressed on KBRif-agar or pour plated in KBRif-agar (40°C). Growth of strain WCS358rr was determined after 48 h at 27°C.

In colonization studies with strains WCS358rr, WCS374 and WCS374pMR, their population densities in the leaf-interior were determined by homogenizing surface-sterilized leaves with a mortar and pestle. The homogenate was suspended in 2.0 ml of sterile 0.1 M $MgSO_4$ and after appropriate dilutions, population densities of the introduced bacterial strains were determined. To study colonization of the root surface by the introduced bacterial strains, segments (0.5 g) of radish roots were sampled at random from a depth of 0–8 cm from the stem base. These segments were suspended in 5 ml sterile 0.1 M $MgSO_4$ and shaken vigorously for 30 s in glass test tubes containing 2.5 g of glass beads (0.17 mm dia). Colonization of the root interior was determined by surface sterilization of the roots with 10% H_2O_2 for 15 s (Van Peer *et al.*, 1990). After rinsing twice with sterile distilled water, roots were homogenized with a mortar and pestle. The homogenate was suspended in 2.0 ml of sterile 0.1 M $MgSO_4$ and after appropriate dilutions, population densities of the introduced bacterial strains were determined.

Enumeration of introduced bacterial strains

Population densities of WCS374 and WCS374pMR were determined by immunofluorescence colony-staining (IFC) according to the method described by Van Vuurde and Roozen (1990) and modified by Leeman *et al.* (1991). Aliquots of leaf or root suspensions (0.2 ml) were mixed homogeneously in 12-well tissue culture plates with 1.0 ml of molten (40°C) modified KB agar (KB^+, Geels and Schippers, 1983). After 24 h at 27°C, the agar was dried into a thin film and the colonies of WCS374 or WCS374pMR, that had developed in the media, were stained overnight with antibodies conjugated to fluorescein-isothiocyanate (FITC). Immunoglobulins were prepared in rabbit against whole cells of WCS374 according to the method described by Vruggink and Maas-Geesteranus (1975). Polyclonal antiserum Ab9061 is highly specific for WCS374 (P. A. H. M

Bakker, unpubl. Ph.D. thesis, Utrecht Univ., 1989; Leeman *et al.*, 1991). The optimal dilution of the FITC-conjugated antiserum for enumeration of strain WCS374 was 100 times as was determined in a titration procedure. Stained colonies were enumerated using a Zeiss Axioskop microscope with epifluorescence-illumination.

Population densities of WCS358rr were determined by mixing aliquots (0.2 ml) of leaf-or root-suspension with 1.0 ml molten KB^+-agar supplemented with rifampicin (150 $\mu g\ ml^{-1}$). Colonies of strain WCS358rr were stained for 30 min with ethidium bromide (1 $mg\ ml^{-1}$) and enumerated by epifluorescence-illumination as described above.

Colonization studies with P. putida strain WCS358rr

For soil bacterization, suspensions of strain WCS358rr were inoculated into non-sterile sandy soil collected from a commercial greenhouse (Leeman *et al.*, 1991) to a final density of 10^6 cfu g^{-1}. PVC tubes (14 cm high, 5 cm dia) were filled with the treated soil and two radish seeds were sown in soil at a depth of 1 cm. Radish plants were grown under controlled conditions (70% RH) with a 16 h light period (irradiance 60 W m^{-2}) at 24°C followed by an 8 h dark period at 20°C. For seed bacterization, radish seeds were first surface sterilized with 0.5% NaHClO for 5 min, washed twice with sterile distilled water and coated with a suspension of WCS358rr (10^8 cfu ml^{-1}) in 1% methyl cellulose. Coated seeds were dried for 24 h at room temperature. The final density of WCS358rr was approximately 5×10^5 cfu $seed^{-1}$. Two coated seeds were sown in PVC tubes containing non-sterile sandy soil (Leeman *et al.*, 1991) and grown under controlled conditions as described above. For root bacterization, two radish seeds were sown in PVC tubes containing non-sterile sandy soil and after growth for 4 d under controlled conditions the roots were bacterized by injecting 2 ml of a suspension of WCS358rr (10^7 cfu ml^{-1}) with a syringe 2 cm below the soil surface in the vicinity of the roots. Colonization of the surface and the interior of both roots and leaves by strain WCS358rr was determined after 4, 14, 21 and 26 d of plant growth.

Colonization studies with P. fluorescens WCS374 and WCS374pMR

For wild-type strain WCS374 and its derivative WCS374pMR, dispersal to aerial plant parts was studied only for inoculum coated on seeds. Seeds were coated with 1% methyl cellulose (control), or with a suspension of WCS374 or WCS374pMR (10^{10} cfu ml^{-1}) in 1% methyl cellulose as described above. The final density for both WCS374 and WCS374pMR was 5×10^7 cfu $seed^{-1}$. Colonization of the surface of leaves and of the surface and the interior of roots was determined after 4, 11 and 31 d of plant growth.

Table 1. Efficiency of recovery of *P. putida* strain WCS358rr from cotyledons of radish*

	Introduced (log cfu $leaf^{-1}$)	Recovered (log cfu $leaf^{-1}$)	Efficiency of recovery (%)
$MgSO_4$	5.9 (0.1)a	5.1 (0.3)a	17 (11)a
$MgSO_4$–Tween 80	5.9 (0.1)a	6.0 (0.1)b	106 (19)b

*WCS358rr was inoculated on cotyledons of 4-d-old radish plants to a final density of approximately 6 log cfu g^{-1}. After 30 min at 20°C, cotyledons were shaken in 0.1 M $MgSO_4$ or 0.1 M $MgSO_4$–0.05% Tween 80. Mean values and SDs (in parentheses) of 3 replicates are presented. For each column different letters indicate a statistically significant difference ($P = 0.05$).

Statistical analysis

Populations of introduced bacteria on roots and leaves approximate a lognormal distribution (Hirano *et al.*, 1982; Loper *et al.*, 1984). Enumerated values, therefore, were submitted to logarithmic transformation prior to analysis. Transformed values were analysed by analysis of variance followed by Fisher's least significant difference test (SAS Institute Inc., Cary, NC). Experiments were performed twice.

RESULTS

Efficiency of recovery and surface sterilization

When cotyledons that had been inoculated with a suspension of WCS358rr were shaken for 30 s in a solution containing 0.1 M $MgSO_4$–0.05% Tween 80, recovery of WCS358rr approximated 100% (Table 1). If Tween 80 was omitted from the solution, the efficiency of recovery was reduced to approximately 17% (Table 1). Surface sterilization of cotyledons with 20% H_2O_2 for 15 s efficiently removed strain WCS358rr, as confirmed by leaf prints on KBRif-agar plates and by pour-plating cotyledons in molten KBRif-agar (Table 2). These procedures for recovery and surface sterilization were used throughout this study.

Dispersal of strain P. putida WCS358rr

In all treatments (Fig. 1), strain WCS358rr colonized the root surface of radish, whereas colonization of the root interior was never observed. WCS358rr was dispersed to the surface of cotyledons of radish plants only when it was introduced into soil prior to sowing or when coated on seed. Surprisingly, strain WCS358rr was not only present on the surface,

Table 2. Surface sterilization of cotyledons after inoculation with WCS358rr (log 5.9 cfu $cotyledon^{-1}$) in 10 or 20% H_2O_2 for 15 s (mean values of 3 replicates are presented)

Surface sterilization (% H_2O_2)*	Recovered (log cfu $leaf^{-1}$)	Growth from leaf print	Growth in pour-plates
0	6.0	+	+
10	1.7	+	+
20	†	–	–

*The efficiency of surface sterilization was checked by: (i) recovery of WCS358rr after shaking cotyledons in 0.1 M $MgSO_4$–0.05% Tween 80; (ii) growth from prints of cotyledons on KBRif-agar plates; or (iii) growth on cotyledons pour-plated in molten KBRif – agar.

†Below detection limit (< log 1.4 cfu $leaf^{-1}$)

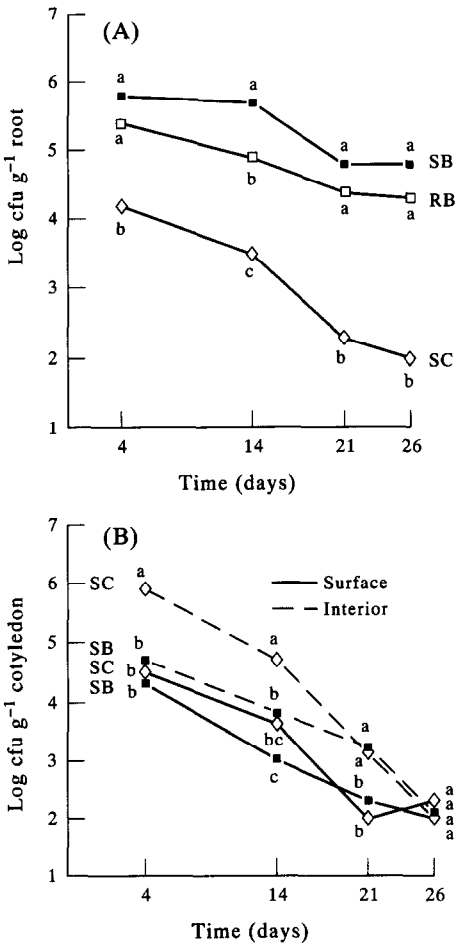

Fig. 1. Colonization of the surface and the interior of radish roots (A) and of cotyledons (B) by *P. putida* WCS358rr after 4, 14, 21 and 26 d of plant growth. The inoculum strategies used were root-bacterization (RB), soil-bacterization (SB) and coating on seeds (SC). For each sampling day, different letters indicate statistically significant differences ($P = 0.05$).

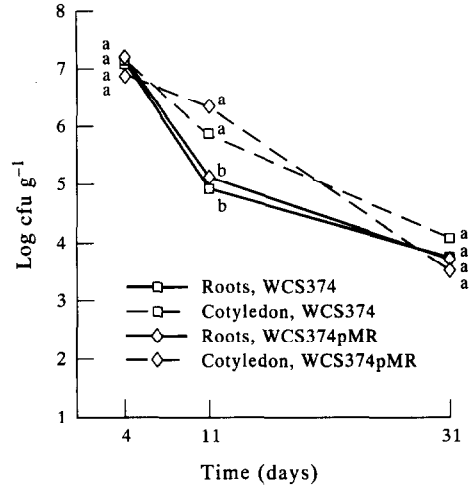

Fig. 2. Colonization of the surface of radish roots and cotyledons by wild-type strain *P. fluorescens* WCS374 and its derivative WCS374pMR. Both strains were coated on seeds to a final density of 5×10^7 cfu seed^{-1}. After 4, 11 and 31 d of plant growth, population densities of WCS374 and WCS374pMR were determined. For each sampling day, different letters indicate statistically significant differences ($P = 0.05$).

but also in the interior of cotyledons of radish. Transport of strain WCS358rr upwards to the interior of cotyledons was not observed when this strain was applied onto the roots of seedlings. When inoculated onto the cotyledons of 4-d-old radish seedlings, strain WCS358rr was not transported downwards to the root interior (data not shown). For both soil-and seed treatments, populations of WCS358rr present on the surface and in the interior of cotyledons decreased in time and reached the detection limit after 26 d of plant growth. Throughout the experiment, strain WCS358rr was only detected on cotyledons and not on the first true leaves of radish. Moreover, strain WCS358rr did not disperse from inoculated treatments to cotyledons or first true leaves of non-inoculated control plants.

Dispersal of P. fluorescens WCS374 and its genetically-modified derivative WCS374pMR

Colonization of cotyledons and roots of radish by derivative WCS374pMR and its wild-type strain WCS374 was examined after coating these strains onto seeds. Colonization of the interior of cotyledons was not determined. Both wild-type strain WCS374 and derivative WCS374pMR colonized the surface of the root and the cotyledons (Fig. 2). Neither of the strains colonized the root interior during the experiment. No differences were observed in colonization of the surface of the roots and cotyledons between derivative WCS374pMR and wild-type strain WCS374. Comparison between the population densities of WCS374pMR determined by IFC in KB$^+$ and in KB$^+$ supplemented with tetracycline, did not reveal any significant differences (Table 3). These results indicate that plasmid pMR was maintained in derivative WCS374pMR throughout the experiment.

DISCUSSION

Dispersal of plant growth-promoting fluorescent *Pseudomonas* spp to aerial plant surfaces has received

Table 3. Maintenance of plasmid pMR in populations of strain WCS374pMR present on the surface of roots and cotyledons of radish*

	Population density (log cfu g^{-1} root)		Population density (log cfu g^{-1} cotyledon)	
Time (d)	KB$^+$	KB$^+$Tet	KB$^+$	KB$^+$Tet
4	7.2a	7.2a	6.8a	6.9a
11	5.0a	5.1a	6.5b	6.3b
31	3.8a	3.7a	3.7a	3.5a

*Plasmid pMR encodes resistance to tetracycline. After 4, 11 and 31 d of plant growth, population densities of WCS374pMR were enumerated by IFC in KB$^+$, and in KB$^+$ supplemented with tetracycline (KB$^+$Tet), respectively. Mean values of 6 replicates are presented. For each sampling day, different letters indicate statistically significant differences ($P = 0.05$).

very little attention compared to their dispersal and survival in soil and in rhizosphere environments. This is surprising because of the interest in the environmental fate of of introduced strains of fluorescent pseudomonads, including of those that are genetically-modified. Studying the reported ability of particular *Pseudomonas* strains to induce plant resistance against pathogens (Van Peer *et al.*, 1991; Wei *et al.*, 1991; Zdor and Anderson, 1992) also requires knowledge about the dispersal of these strains on and in plant tissues. Our study demonstrates that introduced *P. putida* strain WCS358rr, *P. fluorescens* strain WCS374 and genetically-modified WCS374pMR are dispersed to the cotyledons of radish plants (Figs 1 and 2). Dispersal to cotyledons appears to depend on the inoculation strategy used. Populations of WCS358rr could only be detected on radish cotyledons after introduction of this strain into soil prior to sowing or after coating this strain onto seeds [Fig. 1(B)]. Throughout the experiments, the dispersal of these strains to the first true leaves of radish could not be demonstrated. Although colonization of the root interior by strains of *Pseudomonas* spp and by other bacterial genera is regarded as a common phenomenon (Van Peer and Schippers, 1989; Kloepper *et al.*, 1992), strains WCS358rr, WCS374 and WCS374pMR did not penetrate to the root interior. Therefore, it is unlikely that the applied bacterial strains colonized the cotyledons via internal transport from the roots to the shoot. These results strongly indicate that cotyledons of radish become contaminated with WCS358rr, WCS374 or with WCS374pMR either during the germination of coated seeds or during seedling emergence from inoculated soil.

Upward dispersal of bacterial strains to aerial plant parts has also been reported for antagonistic strain *Erwinia herbicola*, but this strain was only detectable for up to 10 d after planting (Kempf and Wolf, 1989). Populations of WCS358rr could still be detected on cotyledons after 21 d of plant growth [Fig. 1(B)]. Wild-type strain *P. fluorescens* WCS374 and derivative WCS374pMR maintained relatively high population densities on the surface of cotyledons for up to 31 days of plant growth (Fig. 2). Because of differences in inoculum density on coated seeds between WCS358rr and WCS374, conclusions regarding, for example, strain-specific tolerance to environmental conditions encountered on cotyledons cannot be drawn from our experiments. It should be emphasized, however, that the experiments described in our study were performed under controlled conditions in the greenhouse. Under field conditions, the phylloplane is a very dynamic environment with cyclic and noncyclic variables, including temperature, relative humidity, dew, wind, rain and radiation (Andrews, 1992). These variables may adversely affect the survival of the dispersed *Pseudomonas* strains. Moreover, natural antagonists or competitors may affect the population density of the applied *Pseudomonas* strains even further.

Bacteria carried on the surface of leaves may subsequently enter the vascular system through natural openings such as stomata, hydathodes and stem lenticils (Huang, 1986). Invasion of leaves has been reported for many strains, including pathogenic strain *Xanthomonas campestris* (Graham *et al.*, 1992) and epiphytic bacteria (Underwood, 1991). Also the uptake of *Rhizobium* in pea leaf protoplasts has been described (Davey and Cocking, 1972). Our results demonstrate that also non-pathogenic *P. putida* strain WCS358rr colonized the interior of radish cotyledons after soil- or seed-inoculation [Fig. 1(B)].

In conclusion it can be stated, that specific strains of fluorescent *Pseudomonas* spp that are regarded as rhizobacteria, can be dispersed to aerial plant parts depending on the type of inoculation strategy used. Moreover, they are able to survive on the surface and in the interior of cotyledons of radish for a considerable period. This should be taken into account in studies on *Pseudomonas*-induced plant resistance against leaf pathogens, which require spatial separation of the *Pseudomonas* strain and the pathogen. Moreover, future applications of genetically-modified *Pseudomonas* spp in crop culture to control plant pathogens deserve special attention with respect to dispersal of the organism and its recombinant DNA. In our study, plasmid pMR was maintained in the genetically-modified strain WCS374pMR during 31 d of plant growth and did not affect colonization and dispersal of derivative WCS374pMR compared to that of its wild-type strain WCS374 (Table 3; Fig. 2). As was stated by Kluepfel (1993), it should be remembered that introduced strains of genetically-modified *Pseudomonas* spp, including their recombinant DNA, may be further dispersed from aerial plant parts by various biotic vectors and abiotic vehicles encountered under field conditions.

REFERENCES

Alexander M. (1986) Survival and growth of bacteria. *Environmental Management* **10**, 464–469.

Andow D. A. (1986) Dispersal of microorganisms with emphasis on bacteria. *Environmental Management* **10**, 470–487.

Andrews J. H. (1992) Biological control in the phyllosphere. *Annual Review of Phytopathology* **30**, 603–635.

Bakker P. A. H. M., Lamers J. G., Bakker A. W., Marugg J. D., Weisbeek P. J. and Schippers B. (1986) The role of siderophores in potato tuber yield increase by *Pseudomonas putida* in a short rotation of potato. *Netherlands Journal of Plant Pathology* **92**, 249–256.

Bashan Y. (1991) Airborne transmission of the rhizosphere bacterium *Azospirillum*. *Microbial Ecology* **22**, 257–269.

Bitter W., Marugg J. D., De Weger L. A., Tommassen J. and Weisbeek P. J. (1991) The ferric-pseudobactin receptor PupA of *Pseudomonas putida* WCS358: homology to TonB-dependent *Escherichia coli* receptors and specificity of the protein. *Molecular Microbiology* **5**, 647–655.

Bunster L., Fokkema N. J. and Schippers B. (1989) Effect of surface-active *Pseudomonas* spp on leaf wettability. *Applied and Environmental Microbiology* **55**, 1340–1345.

Davey M. R. and Cocking E. C. (1972) Uptake of bacteria by isolated higher plant protoplasts. *Nature* **239,** 455–456.

Fry J. C. and Day M. J. (1990) *Bacterial Genetics in Natural Environments*. Chapman & Hall, London.

Geels F. P. and Schippers B. (1983) Selection of antagonistic fluorescent *Pseudomonas* spp and their root colonization and persistence following treatment of seed potatoes. *Journal of Phytopathology* **180,** 193–206.

Glandorf D. C. M., Brand I., Bakker P. A. H. M. and Schippers B. (1992) Stability of rifampicin as a marker for root colonization studies of *Pseudomonas putida* in the field. *Plant and Soil* **147,** 135–142.

Graham J. H., Gottwald T. R., Riley T. D. and Achor D. (1992) Penetration through leaf stomata and growth of *Xanthomonas campestris* in citrus cultivars varying in susceptibility to bacterial diseases. *Phytopathology* **82,** 1319–1325.

Hirano S. S., Nordheim E. V., Arny D. C. and Upper C. D. (1982) Lognormal distribution of epiphytic bacterial populations on leaf surfaces. *Applied and Environmental Microbiology* **44,** 695–700.

Huang J. S. (1986) Ultrastructure of bacterial penetration in plants. *Annual Review of Phytopathology* **24,** 141–157.

Kempf H-J. and Wolf G. (1989) *Erwinia herbicola* as a biocontrol agent of *Fusarium culmorum* and *Puccinia recondita* f. sp. *tritici* on wheat. *Phytopathology* **79,** 619–627.

King E. O., Ward M. K. and Raney D. E. (1954) Two simple media for demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44,** 301–307.

Kloepper J. W., Schippers B. and Bakker P. A. H. M. (1992) Proposed elimination of the term endorhizosphere. *Phytopathology* **82,** 726–728.

Kluepfel D. A. and Tonkyn D. W. (1990) Release of soil-borne genetically modified bacteria: biosafety implications from contained experiments. In *Biological Monitoring of Genetically Engineered Plants and Microbes* (D. R. MacKenzie and S. C. Henry, Eds). *Proceedings of the Kiawah Island Conference, Maryland,* pp. 55–67.

Kluepfel D. A. (1993) The behavior and tracking of bacteria in the rhizosphere. *Annual Review of Phytopathology* **31,** 441–472.

Leben C. (1965) Influence on humidity on the migration of bacteria on cucumber seedlings. *Canadian Journal of Microbiology* **11,** 671–676.

Leeman M., Raaijmakers J. M., Bakker P. A. H. M. and Schippers B. (1991) Immunofluorescence colony-staining for monitoring pseudomonads introduced into soil. In *Biotic Interactions and Soil-borne Diseases* (A. B. R. Beemster, G. J. Bollen, M. Gerlagh, M. A. Ruissen, B. Schippers and A. Tempel, Eds), pp. 374–380. Elsevier, Amsterdam.

Leeman M., Van Pelt J. A., Den Ouden F. M., Heinsbroek M., Bakker P. A. H. M. and Schippers B. (1995) Biocontrol of Fusarium wilt of radish in commercial greenhouse trials by seed treatment with *Pseudomonas fluorescens* WCS374. *Phytopathology*. In press.

Loper J. E., Suslow T. V. and Schroth M. N. (1984) Lognormal distribution of bacterial populations in the rhizosphere. *Phytopathology* **74,** 1454–1460.

Marugg J. D., De Weger L. A., Nielander H. B., Oorthuizen M., Recourt K., Lugtenberg B., Van der Hofstad G. A. J. M. and Weisbeek P. J. (1989) Cloning and characterization of a gene encoding an outer membrane protein required for siderophore uptake in *Pseudomonas putida* WCS358. *Journal of Bacteriology* **171,** 2819–2826.

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.

Parke J. L. (1991) Root colonization by indigenous and introduced microorganisms. In *The Rhizosphere and Plant Growth* (D. Keister and P. B. Cregan, Eds), pp. 33–42. Kluwer, Dordrecht.

Robertson B. K. and Alexander M. (1994) Mode of dispersal of the stem-nodulating bacterium, *Azorhizobium*. *Soil Biology & Biochemistry* **26,** 1535–1540.

Schippers B. (1992) Prospects for management of natural suppressiveness to control soilborne pathogens. In *Biological Control of Plant Diseases. Progress and Challenges for the Future* (E. C. Tjamos, G. C. Papavizas and R. J. Cook, Eds), pp. 21–34. Plenum Press, New York.

Trevors J. T., Barkay T. and Bourquin A. W. (1987) Gene transfer among bacteria in soil and aquatic environments: a review. *Canadian Journal of Microbiology* **33,** 191–198.

Underwood G. J. C. (1991) Colonization and invasion of leaves of aquatic macrophyte *Ceratophyllum demersum* L. by epiphytic bacteria. *Microbial Ecology* **21,** 267–275.

Van Peer R. and Schippers B. (1989) Plant growth responses to bacterization with selected *Pseudomonas* spp strains and rhizosphere microbial developments in hydroponic cultures. *Canadian Journal of Microbiology* **35,** 456–643.

Van Peer R., Punte W. L. M., De Weger L. A. and Schippers B. (1990) Characterization of root surface and endorhizosphere pseudomonads in relation to their colonization of roots. *Applied and Environmental Microbiology* **56,** 2462–2470.

Van Peer R., Niemann G. J. and Schippers B. (1991) Induced resistance and phytoalexin accu mulation in biological control of fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. *Phytopathology* **81,** 728–734.

Van Vuurde J. W. L. and Roozen N. J. M. (1990) Comparison of immunofluorescence colony-staining in media, selective isolation on pectate medium, ELISA and immunofluorescence cell staining for detection of *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi* in cattle manure slurry. *Netherlands Journal of Plant Pathology* **96,** 75–89.

Vruggink H., Maas-Geesteranus H. P. (1975) Serological recognition of *Erwinia carotovora* var. *atroseptica*, the causal agent of potato blackleg. *Potato Research* **18,** 546–555.

Wei G., Kloepper J. W. and Tuzun S. (1991) Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. *Phytopathology* **81,** 1508–1512.

Weller D. M. (1988) Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* **26,** 379–407.

Zdor R. E. and Anderson A. J. (1992) Influence of root colonizing bacteria on the defense responses of bean. *Plant and Soil* **140,** 99–107.