Plant and Soil **147**: 135–142, 1992.

PLSO 9539

Stability of rifampicin resistance as a marker for root colonization studies of *Pseudomonas putida* in the field

D.C.M. GLANDORF[1], I. BRAND[2], P.A.H.M. BAKKER[1] and B. SCHIPPERS[1]
[1]*Department of Plant Ecology and Evolutionary Biology, Section of Plant Pathology, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands and* [2]*Department of Plant Molecular Botany, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands*

Received 23 March 1992. Revised August 1992

Key words: PGPR, plant growth promotion, potato, *Pseudomonas*, marker stability, rhizobacteria, rifampicin resistance, root colonization, soil

Abstract

The stability of rifampicin resistance in plant growth-promoting *Pseudomonas putida* strain WCS358 was studied in potato rhizosphere in the field. Three out of seven rifampicin-resistant mutants of strain WCS358 were selected in this study. Their specific growth rate, competitive growth in liquid medium and colonization of potato roots in non-sterile soil, was comparable to that of their parental strain. These rifampicin-resistant mutants were used to treat potato seed tubers, which were thereafter sown in the field. To test the stability of the rifampicin resistance in the field, about 1200 fluorescent *Pseudomonas* isolates obtained from underground plant parts at 82, 95, 109 and 130 days after seeding, were tested for rifampicin resistance and for agglutination with an antiserum specific for strain WCS358. All fluorescent *Pseudomonas* isolates that showed a positive agglutination reaction with the antiserum, were also rifampicin-resistant. Twelve agglutination-positive isolates, selected at random, were all identified as strain WCS358 from patterns of lipopolysaccharides after sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Thus, rifampicin resistance seems to be a stable marker in the mutants of strain WCS358 tested, also under field conditions.

It is concluded that rifampicin resistance can be used as a reliable marker for ecological studies on rhizosphere pseudomonads.

Introduction

Efficient root colonization by introduced bacteria is essential for biocontrol of root pathogens (Bull et al., 1991) and for plant growth promotion (Bakker et al., 1986; Schippers et al., 1987). Rifampicin resistance is the most commonly used marker to study population dynamics and survival of plant growth-promoting and disease-suppressing *Pseudomonas* spp. after their introduction in the rhizosphere (Bahme and Schroth, 1987; Bull et al., 1991; Burr et al., 1978; Geels and Schippers, 1983; Kloepper et al., 1980; Loper et al., 1985; Suslow and Schroth, 1982; Weller, 1984). However, it cannot be excluded that rifampicin resistance can change characteristics in the *Pseudomonas* strains used, or is unstable under field conditions.

Among *Rhizobium* spp., spontaneous rifampicin resistance (Rifr) was associated with diminished symbiotic effectiveness and nodulating competitiveness (Lewis et al., 1987; Pankhurst, 1977). Certain Rifr mutants of *P. fluorescens* were less able to compete with the parental strain in sterile soil (Compeau et al., 1988). No detailed studies have been reported concerning

the stability of rifampicin resistance in fluorescent pseudomonads under field conditions.

The present study investigates the reliability of resistance to rifampicin as a marker to monitor the population dynamics of plant growth-promoting *Pseudomonas putida* strain WCS358 in the rhizosphere of field-grown potato. Rifampicin-resistant (Rifr) mutants of strain WCS358 and the wild-type strain were compared with respect to their specific growth rates and competitive growth in vitro, composition of lipopolysaccharides (LPS), and to their root colonization in non-sterile soil. Stability of rifampicin resistance in *P. putida* WCS358 was determined at regular intervals up to four months after introduction of the Rifr mutants in the rhizosphere of field-grown potato by reisolating *Pseudomonas* isolates from the rhizosphere and assaying these isolates on both rifampicin resistance and immunological reactions specific for strain WCS358.

Methods

Pseudomonas putida strain WCS358 and selection of rifampicin-resistant mutants

Wild-type *P. putida* WCS358 has been studied extensively in relation to its plant growth-promoting properties and root colonization (Bakker et al., 1986; Geels et al., 1986; Geels and Schippers, 1983; Hofstad et al., 1985; Marugg et al., 1985; de Weger et al., 1986). Rifampicin-resistant mutants of *P. putida* WCS358 were obtained by transferring colonies of this strain to King's medium B (King et al., 1954) agar plates containing increasing concentrations (50, 100, 150, 200, 250 μg mL^{-1}) of rifampicin (Serva). The stability of these Rifr mutants, including a rifampicin-resistant mutant of WCS358 (358-G) obtained by Geels and Schippers (1983), was tested by subculturing the Rifr mutants on King's B (KB) agar 15 times for 48 h at 27°C and comparing the number of colony forming units (cfu) after dilution plating of suspensions on KB and KB with rifampicin after each subculturing. The Rifr mutants were stored at 4°C on KB supplemented with rifampicin (250 μg mL^{-1}).

Specific growth rates

Specific growth rates of *P. putida* WCS358 and the Rifr mutants were determined in liquid King's B and minimal medium (Evans et al., 1970) supplemented with 0.2% glucose (MM) at 21°C by measuring the optical density of the cells at 660 nm after a 20-fold dilution in fresh medium of an overnight culture of the cells in appropriate media. Three replicates per treatment were used.

WCS358 antiserum

Immunoglobuline was prepared against *P. putida* WCS358 in rabbit at the Research Institute for Plant Protection at Wageningen, the Netherlands as described by Vruggink and Maas-Geesteranus (1975). Antiserum was harvested two weeks after the last injection.

Specificity of the antiserum

High specificity of an antiserum obtained for *P. fluorescens* strain WCS374 was described by Bakker (1989). Specificity of the antiserum obtained for strain *P. putida* WCS358 was tested by growing twenty-five potato stem-cuttings in soil cropped to potato once every three years derived from the Experimental Farm 'De Schreef', located near Lelystad, the Netherlands as described by de Weger et al. (1987). After 7 days, rhizosphere suspensions were prepared by shaking root segments (0.3 g) of each stem-cutting vigorously for 30 sec in glass tubes containing 2 mL 0.1% (w/v) proteose peptone (Difco) with 2 g glass beads (0.11 mm diameter). Dilutions of suspensions were plated on modified KB medium (Geels et al., 1986) and after 48 h incubation at 27°C, 300 colonies were selected at random from the KB plates. Agglutination of these 300 isolates with the antiserum (1 : 400 dilution in PBS −0.9% NaCl buffered with 10 m*M* sodium phosphate-pH 7.2) was tested on 10-well multitest slides (Flow Laboratories, UK.).

Assay for competitiveness

Growth of Rifr mutants in competition with the parental strain was studied after inoculation of 25 mL of KB or minimal medium with 0.1 mL (10^9 cfu mL^{-1}) of parental strain WCS358 and of the Rifr mutants. Immediately after inoculation of the media and at 6, 10 and 24 h of incubation at 21°C, samples were taken and serial dilutions were plated on KB agar plates containing 0 or 150 μg mL^{-1} rifampicin to determine the population densities of the parental strain and the Rifr mutants. At 24 h, 0.1 mL of the mixed cultures was added to 25 mL of fresh medium and this procedure was repeated three times. Three replicates per treatment were used. Results were analyzed by analysis of variance. The T-method (Sokal and Rohlf, 1981) was used to calculate minimum significant difference (MSD).

Isolation and analysis of LPS

Isolation and characterization of LPS of the parental strain and of Rifr mutants of WCS358 was determined as described by de Weger et al. (1986). Cell envelope samples were obtained by differential centrifugation of the cells after ultrasonic treatment. Samples containing 10 to 20 μg protein were treated with proteinase K, after which 10-fold dilutions were applied per lane. Gels were stained with silver reagent (Tsai and Frasch, 1982).

Root colonization assay

Root colonization of rifampicin-resistant mutants and their parental strain was determined as described by de Weger et al. (1987). Ten rooted potato stem-cuttings per treatment were bacterized and grown in soil cropped to potato once every 3 years (1 : 3 rotation) at the Experimental Farm 'De Schreef'. Characteristics of this soil are described by Hoekstra (1981). Stem-cuttings were grown for 7 days in a controlled environment chamber with a 16 h light period (irradiance 60.000 mW m^{-2}) at 16°C and 73% RH followed by an 8 h dark period at 12°C and 86% RH. Root samples (0.1 g) were then taken at 0–2 cm, 2–4 cm and 6–10 cm from the stem-bases and vigorously shaken for 30 sec in glass tubes containing 2 mL 0.1% (w/v) proteose peptone and 2 g glass beads (0.11 mm diameter). Appropriate dilutions of these suspensions were plated on modified KB supplemented with 300 μM pseudobactin 358 (KBPs358-medium) (Raaijmakers et al., 1991) and on modified KB supplemented with 150 μg mL^{-1} rifampicin for determining populations of the parental strain and of the rifampicin-resistant mutants of strain WCS358, respectively. KBPs358-medium has been developed as a selective medium to reisolate *P. putida* strain WCS358 (Raaijmakers et al., 1991). Colonies growing on the KBPs358-medium were further identified by selecting approximately 100 isolates and testing them for agglutination with the antiserum against strain WCS358 (1 : 400 dilution in PBS pH 7.2) on 10-well multitest sides (Flow laboratories). Results were analyzed by the Kruskal-Wallis test followed by non-parametric multiple comparisons by the simultaneous test procedure (Sokal and Rohlf, 1981).

Stability of the rifampicin-resistance marker under field conditions

Pregerminated seed tubers (*Solanum tuberosum* c.v. *Bintje*) were treated with a mixture of rifampicin-resistant mutants of strain WCS358 (358-1B, 3B and G) as described by Geels et al. (1986). Seed tubers treated with 1% (w/v) sodium carboxymethylcellulose (CMC) or with the rifampicin-resistant mutants of WCS358 were seeded by hand in different plots in a 1 : 3 rotation of potato at the Experimental Farm 'De Schreef'. Each plot contained 10 treated plants divided over two rows and was guarded by 14 non-treated plants. Each treatment was replicated in four plots. At 82, 95, 109 and 130 days after seeding, for each treatment 10 plants from a single plot were harvested. Samples taken from roots (0–50 cm depth), underground stems, stolons and progeny tubers were shaken vigorously for 30 sec in glass tubes containing 2 g 0.11 mm (diameter) glass beads and 2 mL sterile 0.1% (w/v) proteose peptone in distilled water. Dilutions of suspensions were plated on modified KB plates. After growth for 48 h at 27°C, ap-

proximately 300 isolates were randomly selected from the modified KB plates and tested for agglutination reaction with antiserum directed against strain WCS358 and for growth on modified KB with 150 μg mL^{-1} rifampicin. A random selection of twelve isolates that reacted positively with the antiserum and that were also able to grow on KB containing 150 μg mL^{-1} of rifampicin, was analyzed by SDS-PAGE to determine if these isolates were identical to *P. putida* WCS358.

Results

Growth characteristics of the rifampicin-resistant mutants of strain WCS358

Six different Rifr isolates were derived from *P. putida* WCS358 by stepwise increases in rifampicin concentrations in KB plates. Rifr mutants, selected from the medium containing 250 μg mL^{-1} rifampicin, behaved differently when grown on KB with rifampicin concentrations up to 250 μg mL^{-1}. Four of the six isolates could only withstand concentrations of rifampicin up to 50 μg mL^{-1} and showed a diminished colony size. Two Rifr mutants (358-1B, 358-3B) and a Rifr derivative obtained by Geels and Schippers (1983) (358-G), which were resistant to rifampicin up to 250 μg mL^{-1} and showed normal colony size, were selected for further studies. The three rifampicin-resistant isolates, inoculated separately or as a mixture, exhibited comparable specific growth rates as the parental strain in both liquid KB and minimal medium. The specific growth rates of the wild-type strain, mutants 358-1B, 358-3B and 358-G and a mixture of the Rifr mutants, were respectively, 0.34, 0.32, 0.38, 0.32 and 0.34 h^{-1} in KB and 0.20, 0.19, 0.22, 0.23 and 0.23 h^{-1} in minimal medium.

Specificity of WCS358 antiserum

Except for strain WCS358 and its Rifr mutants, none of three hundred *Pseudomonas* isolates derived from rhizospheres of potato stem-cuttings grown in non-sterile soil obtained from the Experimental Farm 'De Schreef' reacted with the antiserum raised against strain WCS358.

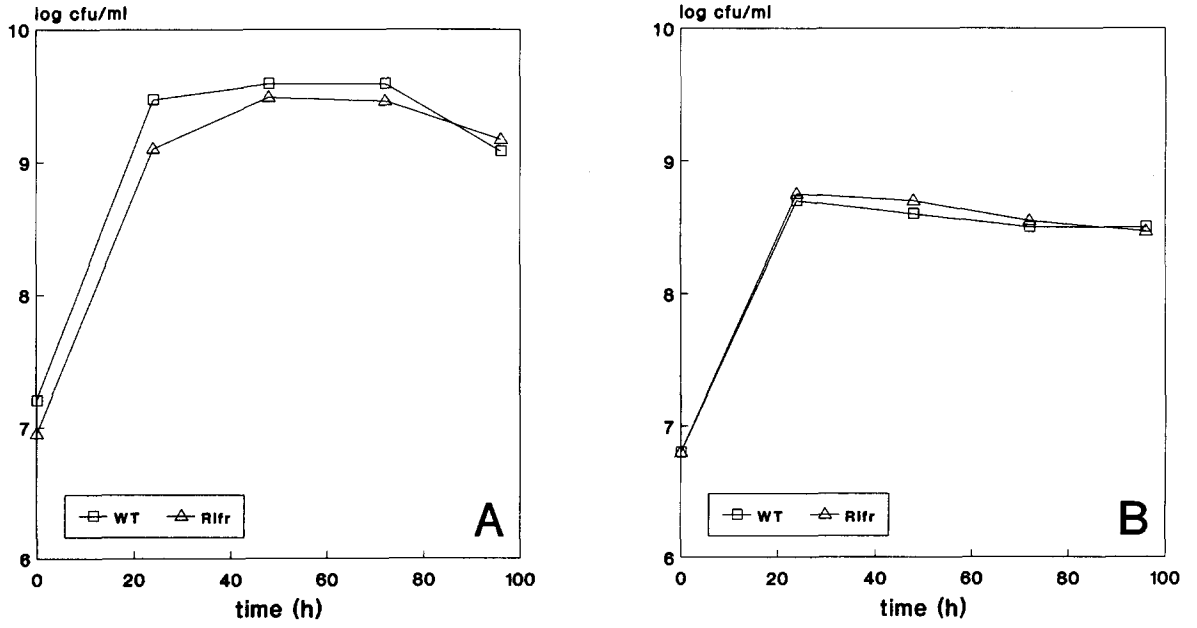

Fig. 1. Competition between wild-type *P. putida* WCS358 (WT) and a mixture of rifampicin-resistant mutants 358-1B, 358-3B and 358-G (Rifr) in KB (**A**) and MM (**B**) during 3 daily transfers in fresh medium. 25 mL of KB or MM was inoculated with 0.1 mL of a mixture of WT and Rifr mutants (1 : 1 ratio). After 24 h of growth, 0.1 mL of the mixed culture was transferred into fresh medium (25 mL) and this was repeated two times.

Assay for competition

The initial proportion of the cfu mL^{-1} of Rifr mutants and the wild-type strain WCS358 were maintained in both KB and minimal medium throughout the experiment (Fig. 1).

Composition of LPS of Rifr mutants of WCS358

No differences were found in composition of LPS between the wild-type strain and the Rifr mutants when analyzed by SDS-PAGE (Fig. 2).

Root colonization of the Rifr mutants of WCS358

The effect of rifampicin resistance on root-colonization capacity was examined in soil obtained from the experimental fields using a root colonization assay. Numbers of cfu of all strains tested decreased rapidly with increasing depth. No significant differences ($p = 0.05$) were observed between the parental strain and the Rifr mutants (Table 1). All isolates selected from the KBPs358 medium reacted with WCS358 antiserum.

Persistence of rifampicin resistance under field conditions

By using both immunological characteristics and rifampicin resistance as markers for identifying WCS358, the stability of the rifampicin-resistance markers could be monitored throughout the growing season. Fluorescent *Pseudomonas* isolates were isolated from underground parts of potato plants originating from tubers treated

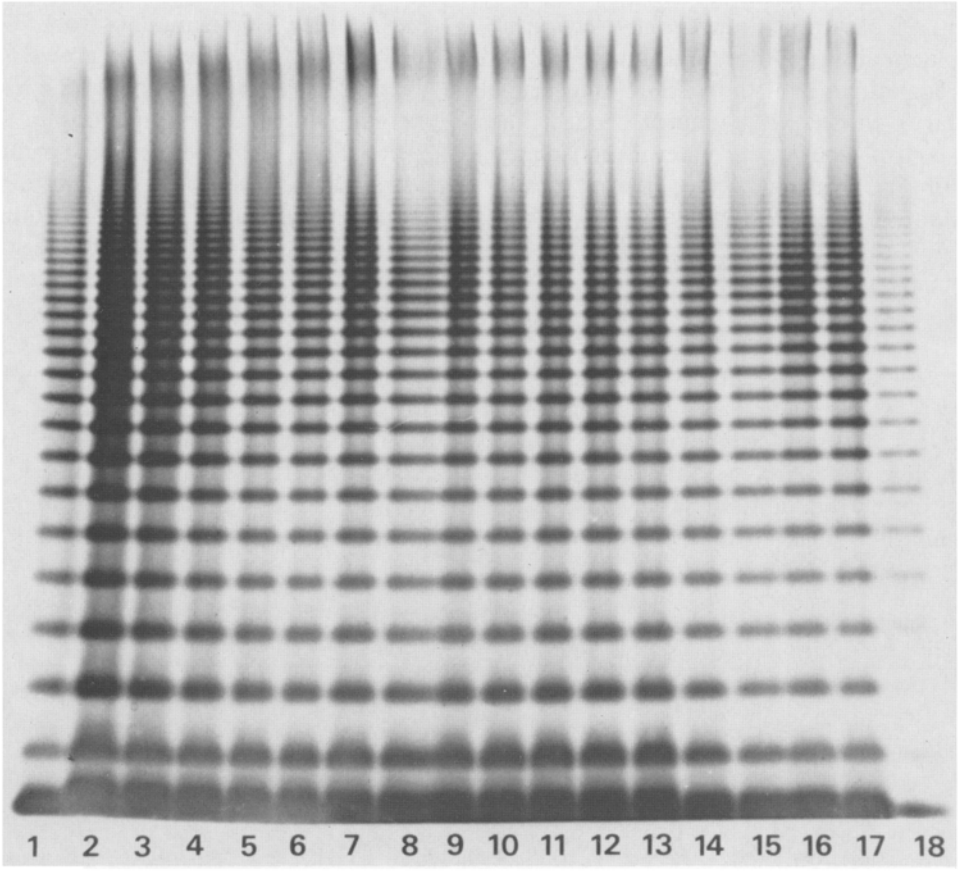

Fig. 2. SDS-PAGE of LPS from wild-type *P. putida* WCS358 (lane 1, 17, 18), Rifr mutants 358-1B (lane 2), 358-3B (lane 3), 358-G (lane 4) and a random selection of twelve Rifr *Pseudomonas* isolates obtained from underground plant parts of bacterized seed tubers 109 and 130 days after seeding, reacting positively with WCS358 antiserum (lane 5–16).

Table 1. Potato root colonization of wild-type *P. putida* WCS358 and its rifampicin-resistant mutants

Strain	Log cfu (g^{-1} root) at a depth (cm) of		
	0–2	2–4	6–10
WCS358	6.2a*	3.9a	2.4a
358-1B	6.6a	3.4a	1.9a
358-3B	6.5a	4.2a	2.1a
358-G	6.3a	3.7a	1.1a
Mixture of 358-1B, -3B and -G	6.5a	3.9a	1.1a

* Values in each column with the same letter are not significantly different at $p = 0.05$, based on non-parametric multiple comparisons by STP (Sokal and Rohlf, 1981).

with a mixture of strains 358-1B, 358-3B and 358-G. At 82, 95 and 130 days after seeding, all *Pseudomonas* isolates with a positive response to the antiserum against strain WCS358 also grew on a medium containing 150 μg mL^{-1} rifampicin (Table 2). LPS patterns of a random selection of twelve isolates showing both rifampicin resistance and agglutination with WCS358 antiserum were similar to those of *P. putida* WCS358 (Fig. 2). In the control treatment, none of the isolates reacted with WCS358 antiserum or were rifampicin-resistant.

Discussion

Antibiotic resistance has attained widespread use as a marker for population studies of bacteria. When markers are used for studying survival and population dynamics, however, the fundamental premise is that marked and unmarked organisms behave similarly. Selection of chromosomal-mediated antibiotic resistance often results in associated genetic changes which influence ecologically important traits (Andrews, 1986). Rifampicin resistance has been reported to diminish symbiotic effectiveness (Pankhurst, 1977) and nodulating competitiveness (Lewis et al., 1987) of *Rhizobium* spp. Davies and Whitbread (1989) reported an increase in the number of non-fluorescent colonies when Rifr mutants of strains of fluorescent *Pseudomonas* were cultured in liquid KB medium in the presence of iron. Diminished growth rates in both minimal and enriched media and alterations in membrane protein profile induced by rifampicin resistance were noted for *P. fluorescens* strain Pf1-8 and were associated with a lesser ability to compete with the parental strain in sterile soil (Compeau et al., 1988). This was not observed for rifampicin resistant mutants of *P. putida* strain Pp1-1 in in vitro studies (Compeau et al., 1988). In the present study, Rifr mutants from *P. putida* strain WCS358 exhibited specific growth rates similar to that of the parental strain. No differences were noted in competitiveness of mutants 358-1B, 358-3B and 358-G when compared to the parental strain during 4 successive periods of 24 hours of growth in low-iron medium (KB) and minimal medium (Fig. 1). In addition, no significant differences with the parental strain were noted in the root-colonizing capacities of these strains in non-sterile soil (Table 1). Therefore, it seems unlikely that the fitness of these mutants in potato rhizosphere is significantly different from that of the parental strain.

Another fundamental premise in the use of a marker is the maintainance of its stability for at least the duration of the experiment. Problems in

Table 2. Percentage of fluorescent pseudomonads, derived from underground plant parts grown from bacterized potato seed tubers, identified as WCS358 using immunological procedures and rifampicin resistance[a]

Days after seeding	No. of isolates tested	Isolates reacting with 358 antiserum (%)	Rifampicin-resistant isolates (%)
82	304	25.0	25.0
95	304	17.8	17.8
109	335	33.7	33.7
130	297	29.6	29.6

[a] Rifr mutants of *P. putida* WCS358 were applied to pregerminated seed tubers which were sown in the field. After 82, 95, 109 and 130 days of growth, fluorescent *Pseudomonas* isolates were derived from underground plant parts. Isolates were tested on agglutination with antiserum against WCS358 and on resistance to rifampicin.

this respect may be anticipated with mutants that are not able to grow on a medium containing the same concentration of rifampicin as the medium from which they were selected. In our study 4 of the 7 Rifr mutants of *P. putida* strain WCS358 showed this diminished resistance to rifampicin in vitro. A similar phenomenon was observed in in vitro studies for *P. putida* Pp1-2 by Compeau et al. (1988). Usually, persistence of antibiotic resistance is screened in vitro, but it is not known to what extent edaphic factors may lead to loss or modification of the marker. Geels and Schippers (1983) suggested that the rapid decrease of the initial high numbers of rifampicin- and nalidixic acid-resistant strains of *Pseudomonas* spp. obtained from the roots of potato plants grown in non-sterile soil under greenhouse conditions might be due to loss of resistance to these antibiotics. Only a few detailed studies concerning the stability of antibiotic resistance in the field have been published, however, they do not involve rifampicin resistance. Brockwell et al. (1977) monitored streptomycin-resistant strains of *R. trifolii* after seed inoculation of clover using serological methods as a second determinant. During 41 months in the field, there was at least a 95% correlation between these two methods. Similar results were obtained by Renwick and Jones (1985), who reported a significant correlation between a fluorescent ELISA procedure and a combined streptomycin and spectinomycin resistance as methods to enumerate *R. trifolii* in root nodules and in soil. The present study notes a 100% correlation between immunological procedures and rifampicin resistance: all *Pseudomonas* isolates which showed a positive response towards the antiserum specific for strain WCS358 were also able to grow on a medium containing rifampicin. Furthermore, a selection of isolates positively responding towards both selection procedures could all be identified as *P. putida* WCS358 by means of SDS-PAGE. These results strongly indicate that no loss of the rifampicin-resistance marker occurred in strain WCS358 during 4 months in the field. It is concluded that rifampicin resistance can be used as a reliable marker for ecological studies on rhizosphere pseudomonads. However, its reliability should be carefully checked in advance for each strain.

Acknowledgements

Thanks are expressed to Ellen Oe for participation in the experiments and to Dr John Leong for critical reading of the manuscript.

These investigations were partly supported by the Foundation for Biological Research (BION), which is subsidized by the Netherlands Organization for Scientific Research (NWO), and partly by the Netherlands Integrated Soil Research Programme (PCBB).

References

Andrews J H 1986 How to track a microbe. *In* Microbiology of the Phyllosphere. Eds. N J Fokkema and J van den Heuvel. pp 14–34. Cambridge University Press, Cambridge, UK.

Bahme J B and Schroth M N 1987 Spatial-temporal colonization patterns of a rhizobacterium on underground organs of potato. Phytopathology 77, 1093–1100.

Bakker P A H M 1989 Siderophore-mediated Plant Growth Promotion and Colonization of Roots by Strains of *Pseudomonas* spp. Ph.D. thesis. Utrecht University, The Netherlands. 100 p.

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. Neth. J. Pl. Pathol. 92, 249–256.

Brockwell J, Schwinghamer E A and Gault R R 1977 Ecological studies of root-nodule bacteria introduced into field environments. V. A critical examination of the stability of antigenic and streptomycin-resistance markers for identification of strains of *Rhizobium trifolii*. Soil Biol. Biochem. 9, 19–24.

Bull C T, Weller D M and Thomashow L S 1991 Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2–79. Phytopathology 81, 954–959.

Burr T J, Schroth M N and Suslow T V 1978 Increased potato yields by treatment of seed pieces with specific strains of *Pseudomonas fluorescens* and *P. putida*. Phytopathology 68, 1377–1383.

Compeau G, Jadoun Al-Achi B, 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.

Davies K G and Whitbread R 1989 In vitro studies of siderophore production by wild type and rifampicin resistant strains of fluorescent Pseudomonads. Plant and Soil 116, 123–125.

Evans C G T, Herbert D and Tempest D W 1970 The continuous cultivation of microorganisms. 2. Construction of a chemostat. *In* Methods in Microbiology, Vol. 2. Eds. J

R Norris and D W Ribbons. pp 277–327. Academic Press, New York.

Geels F P, Lamers J G, Hoekstra O and Schippers B 1986 Potato plant response to seed tuber bacterization in the field in various rotations. Neth. J. Pl. Pathol. 92, 257–272.

Geels F P and Schippers B 1983 Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization and persistence following treatment of seed potatoes. Phytopathol. Z. 108, 193–206.

Hoekstra O 1981 15 jaar 'De Schreef'. Resultaten van 15 jaar vruchtwisselingsonderzoek op het bouwplannenproefveld 'De Schreef'. Publicatie PAGV 11, 1–93.

Hofstad G A J M van der, Marugg J D, Verjans G M C M and Weisbeek P J 1985 Characterization and structure analysis of the siderophore produced by the PGPR *Pseudomonas putida* strain WCS358. *In* Iron, Siderophores and Plant Disease. Ed. T R Swinburn. pp 71–75. Plenum Press, New York.

King E O, Ward M K and Raney D E 1954 Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44, 301–307.

Kloepper J W, Schroth M N and Miller T D 1980 Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. Phytopathology 70, 1078–1082.

Lewis D M, Bromfield E S P and Barran L R 1987 Effect of rifampicin resistance on nodulating competitiveness of *Rhizobium meloti*. Can. J. Microbiol. 33, 343–345.

Loper J E, Haack C and Schroth M N 1985 Population dynamics of soil pseudomonads in the rhizosphere of potato (*Solanum tuberosum* L.). Appl. Environ. Microbiol 49, 416–422.

Marugg J D, Spanje M van, Hoekstra W P M, Schippers B and Weisbeek P J 1985 Isolation and analysis of genes involved in siderophore biosynthesis in plant-growth-stimulating *Pseudomonas putida* WCS358. J. Bacteriol. 164, 563–570.

Pankhurst C E 1977 Symbiotic effectiveness of antibiotic-resistant mutants of fast- and slow-growing strains of *Rhizobium* nodulating lotus species. Can. J. Microbiol. 23, 1026–1033.

Raaijmakers J M, Punte W L M, Bakker P A H M and Schippers B, 1991 A selective medium for reisolation of wild-type *Pseudomonas putida* WCS358 using its siderophore pseudobactin 358. *In* Biotic Interactions and Soil-borne Diseases. Eds. A B R Beemster, G J Bollen, M Gerlagh, M A Ruissen, B Schippers and A Tempel. pp 370–373. Elsevier, Amsterdam, The Netherlands.

Renwick A and Jones D 1985 A comparison of the fluorescent ELISA and antibiotic resistance identification techniques for use in ecological experiments with *Rhizobium trifolii*. J. Appl. Bacteriol. 58, 199–206.

Schippers B, Bakker A W and Bakker P A H M 1987 Interactions of deleterious and beneficial microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. 25, 339–358.

Sokal R R and Rohlf F J 1981 Biometry. W H Freeman, San Francisco.

Suslow T V and Schroth M N 1982 Rhizobacteria of sugar beets: Effects of seed application and root colonization on yield. Phytopathology 72, 199–206.

Tsai C M and Frasch C E 1982 A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115–119.

Vruggink H and Maas-Geesteranus H P 1975 Serological recognition of *Erwinia carotovora* var. *atroseptica*, the causal organism of potato blackleg. Potato Res. 18, 546–555.

de Weger L A, van Boxtel R, van der Burg B, Gruters R A, Geels F P, Schippers B and Lugtenberg B 1986 Siderophores and outer membrane proteins of antagonistic, plant growth-stimulating, root colonizing *Pseudomonas* spp. J. Bacteriol. 165, 585–594.

de Weger L A, van der Vlugt C I M, Wijfjes A H M, Bakker P A H M, Schippers B and Lugtenberg B 1987. Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. J. Bacteriol. 169, 2769–2773.

Weller D M 1984 Distribution of a take-all suppressive strain of *Pseudomonas fluorescens* on seminal roots of winter wheat. Appl. Environ. Microbiol. 48, 897–899.