Soil Biol. Biochem. Vol. 19, No. 4, pp. 443–449, 1987
Printed in Great Britain. All rights reserved

0038-0717/87 $3.00 + 0.00
Copyright © 1987 Pergamon Journals Ltd

BIOASSAY FOR STUDYING THE ROLE OF SIDEROPHORES IN POTATO GROWTH STIMULATION BY *PSEUDOMONAS* SPP IN SHORT POTATO ROTATIONS

PETER A. H. M. BAKKER, ALBERT W. BAKKER, JOEY D. MARUGG,[1]
PETER J. WEISBEEK[1] and BOB SCHIPPERS

Willie Commelin Scholten Phytopathological Laboratory, Department of Plant Pathology, State University Utrecht and University of Amsterdam, Javalaan 20, 3742 CP Baarn and [1]Department of Molecular Cell Biology, State University Utrecht, Padualaan 8, Trans 3, 3584 CH Utrecht, The Netherlands

(*Accepted* 1 *December* 1986)

Summary—A bioassay is described for studying the mechanisms of growth stimulation by fluorescent pseudomonads using rooted potato stem cuttings. Root development of potato stem cuttings was inhibited in short potato-rotation soil compared to development in long potato-rotation soil. Treatment with *Pseudomonas fluorescens* isolate WCS374 or *Pseudomonas putida* isolate WCS358 increased root development in short potato-rotation soil, whereas siderophore-negative Tn5 transposon mutants of isolate WCS358 had no effect. Both siderophore-producing and siderophore-negative Tn5 mutants of isolate WCS358 could be recovered in similar numbers from the root systems. These results were obtained in a bioassay of 8 days duration.

It is postulated that failure of siderophore-negative Tn5 transposon mutants of isolate WCS358 to induce growth stimulation demonstrates that siderophore production is a prerequisite for growth stimulation in short potato-rotation soil.

INTRODUCTION

In The Netherlands, potato yield losses in short potato-rotations have been demonstrated in rotational experiments, which began in 1963 at the Experimental Farm "De Schreef" (Hoekstra, 1981) and in 1973 at the Research Station for Arable Farming and Field Production of Vegetables (PAGV) near Lelystad (Kupers, 1979; Lamers, 1981). The yield decreases seem to be due to as yet unknown harmful rhizosphere microorganisms other than regular soil-borne potato pathogens (Schippers *et al.*, 1985; Scholte *et al.*, 1985).

Seed tuber treatment with selected isolates of *Pseudomonas fluorescens* or *P. putida* increased tuber yield in pot experiments using field soil continuously cropped to potato (Geels and Schippers, 1983b, c) and in field experiments in field plots cropped to potato once every 3 yr (Geels and Schippers, 1983c; Schippers *et al.*, 1985). It was suggested that competition for Fe^{3+} ions plays an important role in plant growth stimulation after bacterization with fluorescent pseudomonads (Kloepper *et al.*, 1980; Geels and Schippers, 1983b). Siderophores produced by pseudomonads are supposed to sequester iron in the rhizosphere, making it unavailable to other rhizosphere microorganisms, some of which are harmful to plant growth (Kloepper *et al.*, 1980).

We describe a bioassay, using rooted potato stem cuttings, that was developed to study the mechanisms of potato growth stimulation by fluorescent pseudomonads. With this bioassay the role of siderophores in potato growth stimulation by *P. putida* isolate WCS358 was studied using siderophore-negative mutants of this isolate obtained through Tn5 transposon mutagenesis (Marugg *et al.*, 1985).

MATERIALS AND METHODS

Cultivation of rooted potato stem cuttings

Potato (*Solanum tuberosum* L.) plants, cv. Bintje, were grown in a greenhouse (±20°C, 16 h light period) for 3–4 weeks. Then tops of plants were removed and shoots, originating from axillary buds, were allowed to grow for about 2 weeks. Shoots of about 10 cm length were cut off and placed in wet vermiculite (Agravermiculite, grade 2). After 1–2 weeks roots developed and the stem cuttings were ready for use.

Isolates and mutants of Pseudomonas *spp*

P. putida isolate WCS358 and *P. fluorescens* biotype A isolate WCS374 were used as wild-type isolates. The *in vitro* and *in vivo* antagonistic activities of these isolates have been well characterized (Geels and Schippers, 1983a, b). The siderophore biosynthesis (Marugg *et al.*, 1985) and structure (van der Hofstad *et al.*, 1986) of isolate WCS358 have been studied in detail. Outer membrane proteins of isolates WCS 358 and WCS 374 have been studied by de Weger *et al.* (1986).

Tn5-mediated mutagenesis of isolate WCS358 was carried out as described by Simon *et al.* (1983), with slight modifications (Marugg *et al.*, 1985). Tn5 transposon mutants showing no fluorescence, no growth on 0.8 mM bipyridyl and no *in vitro* growth inhibition of *Escherichia coli* S17-1 on a Fe^{3+} deficient agar medium (Marugg *et al.*, 1985), were considered to be siderophore-negative. Two of these mutants, JM217 and JM218, were used in this study. Tn5 mutant WCS358 Tn5:B, which does not differ from the wild-type isolate in siderophore production and

specific growth rate in batch cultures in King's medium B and minimal medium (unpublished results), was used as a control.

The presence of a unique Tn5 transposon insertion in the chromosomal DNA of the mutants of isolate WCS358 was demonstrated by fractionating *Eco*R1-digested chromosomal DNA on a 0.8% agarose gel. The DNA fragments were transferred to nitrocellulose paper, and this blot was hybridized with [α-^{32}P]-labeled pACK1, a pACYC184 derivative containing the kanamycin resistance gene of Tn5 (Marugg *et al.*, 1985). Hybridization with the probe was demonstrated in an autoradiogram.

Since the Tn5 transposon mutants carry resistance markers against kanamycin and streptomycin, they can be distinguished from the wild type isolate by isolation on a medium containing these antibiotics.

Bacterial suspensions were prepared by diluting 2-day-old cultures, grown on King's medium B agar plates (KB) (King *et al.*, 1954) at 27°C, in sterile tapwater to approximately 10^9 cells ml^{-1}.

Bioassay

In the initial experiments, roots of potato stem cuttings were dipped into a suspension of isolate WCS374 or into sterile tapwater. The treated stem cuttings were planted in transparent root observation boxes (van Vuurde and Schippers, 1980) containing a sieved soil (2 mm mesh). In each experiment only two plants were used per treatment, because of the large amount of work involved with this method. Two soils were used; a continuous potato soil (described as "short potato-rotation soil") and a soil cropped to potato once every 6 yr (long potato-rotation soil) since 1973. Both soils were obtained from rotation experimental fields of the PAGV. They are heavy, sandy clays, pH (KCl) 7.4, rich in lime (8%) and with 2.9% organic matter. Before use, 5 kg portions of these soils were amended with 3 ml (1 M) $Ca(NO_3)_2$, 1.6 ml (3 M) NH_4NO_3, 12 ml (1 M) KNO_3, 13.3 ml (0.5 M) KH_2PO_4, 2 ml (1 M) $MgSO_4$ and 1 ml of a solution containing (0.24 M) H_3BO_3, (60 mM) $MnSO_4 \cdot H_2O$, (9 mM) $ZnSO_4 \cdot 7H_2O$, (2 mM) $CuSO_4 \cdot 5H_2O$ and (2 mM) $Na_2MoO_4 \cdot 4H_2O$, to avoid nutrient limitation for plant growth. The rooted stem cuttings were allowed to grow for 2 weeks in a controlled environment chamber with a 16 h light period (irradiance 60,000 mW m^{-2}) and 16°C and a relative humidity of 73% followed by an 8 h dark period at 12°C and a relative humidity of 86%. During this period development of the roots was traced visually through the transparent wall of the observation box. After this period root dry weight was determined.

To study the role of siderophore production in plant growth stimulation, roots of potato stem cuttings were dipped into suspensions of isolate WCS358 or one of the transposon mutants or into sterile tapwater. Six stem cuttings were used per treatment. Excess suspension was allowed to drip off and the cuttings were planted in plastic Petri dishes (9 cm dia) with a hole in the side. Each Petri dish was filled with 100 g of sieved soil (2 mm mesh). The moisture content of the soil was adjusted to 50% of the field capacity. The soils and growth conditions were similar to those described in the initial experiments. Root dry weight was measured 8 days after planting.

Root colonization by siderophore-producing and siderophore-negative transposon mutants

Roots of potato stem cuttings were treated with water, JM217, JM218 or WCS358 Tn5:B and planted in either long or short potato-rotation soil. Ten stem cuttings were used per treatment. The soils and growth conditions were as before. The number of colony forming units (cfu) of Tn5 transposon mutants on the root systems was determined 9 days after planting. Root pieces were shaken vigorously for 30 s in glass test tubes containing 2.5 g of 3 mm dia glass beads and 2 ml sterile 0.1% proteose peptone in distilled water. The suspensions were dilution plated on KB supplemented with 200 mg kanamycin sulfate (Sigma) and 200 mg streptomycin sulfate (Pharmachemie BV) l^{-1}.

Statistics

Results were analyzed by analysis of variance. Student's *t*-test was used to calculate minimum significant difference (MSD). In case of heterogeneity of variances or non-normal distribution the Kruskal–Wallis test was used (Sokal and Rohlf, 1981).

RESULTS

Differential effects of soil and isolate WCS374 treatments on root development in the root observation boxes could be detected visually after 1 week. After 2 weeks, root dry weight in short potato-rotation soil was reduced compared to that in long potato-rotation soil (Table 1). No other visually detectable differences, e.g. lesions or discolourations, were observed between roots from short and long potato-rotation soil. Isolate WCS374 increased root development in short potato-rotation soil (Fig. 1 and Table 1). In long potato-rotation soil root development was decreased by WCS374 (Fig. 2 and Table 1). These results were reproduced three times.

Treatment of rooted stem cuttings with the wild type isolate WCS358 or the siderophore-producing transposon mutant WCS358 Tn5:B increased root dry weight in the continuous potato soil, whereas siderophore-negative mutants had no effect (Table 2). Bacterial root treatments with WCS358, WCS358 Tn5:B, JM217 or JM218, decreased root dry weight in long potato-rotation soil (Table 2). These results were reproduced twice.

No statistical significant differences (Kruskal–Wallis test $P = 0.05$) were observed in the recovery of siderophore-producing and siderophore-negative mutants from potato stem cutting roots (Table 3). Bacteria recovered from roots treated with JM217 or

Table 1. Effects of WCS374 treatment of potato stem cuttings on root dry weight after 2 weeks growth in root observation boxes in short (1:1) and long (1:6) potato-rotation soil. Mean values of two plants and standard deviation are given

Treatment	Root dry weight (mg)	
	1:1 soil	1:6 soil
Control	215 ± 50	520 ± 95
WCS374	394 ± 96	257 ± 74

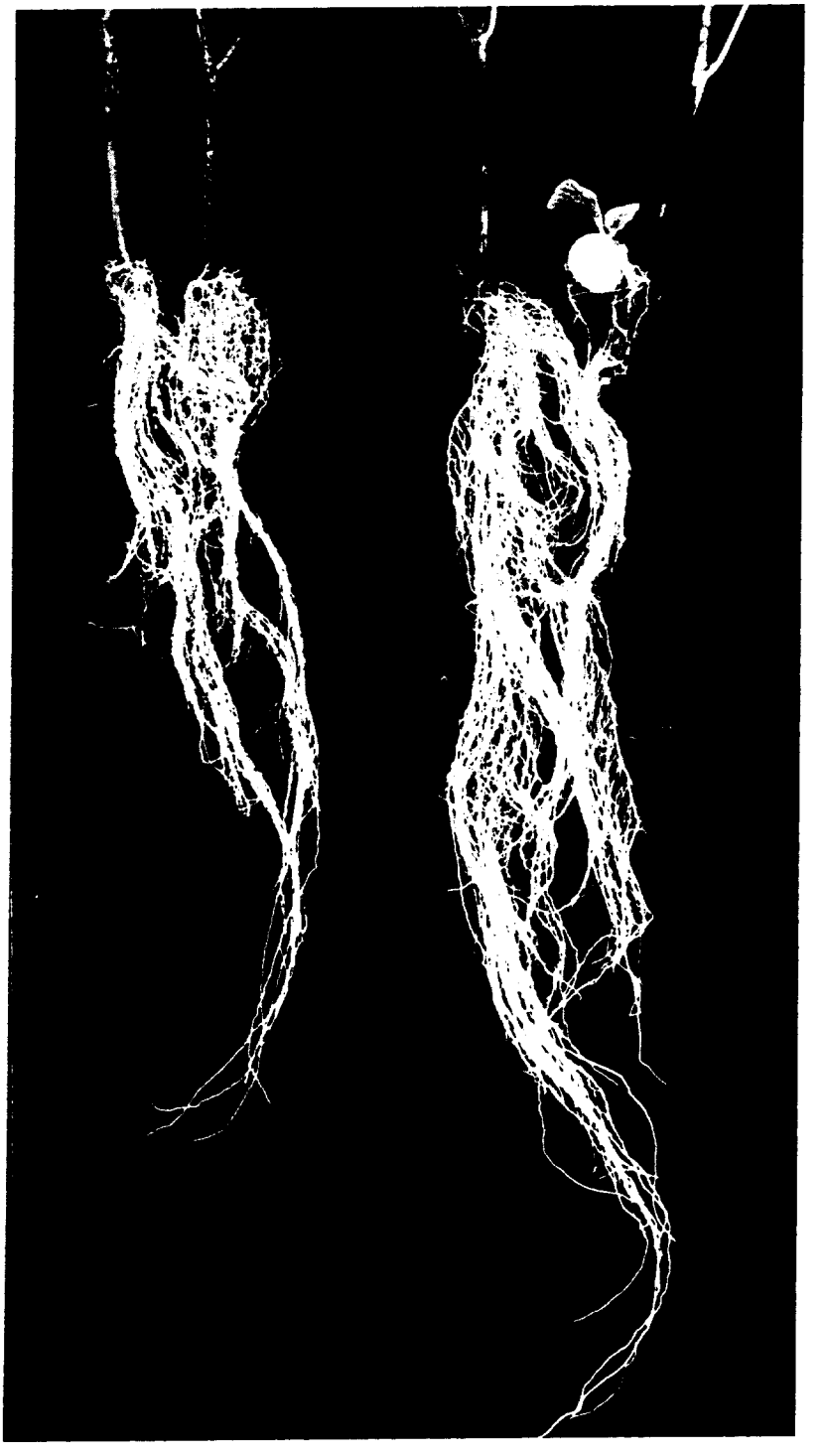

Fig. 1. Root systems of potato stem cuttings after 2 weeks growth in short potato-rotation soil. Left is control, right is treated with WCS374.

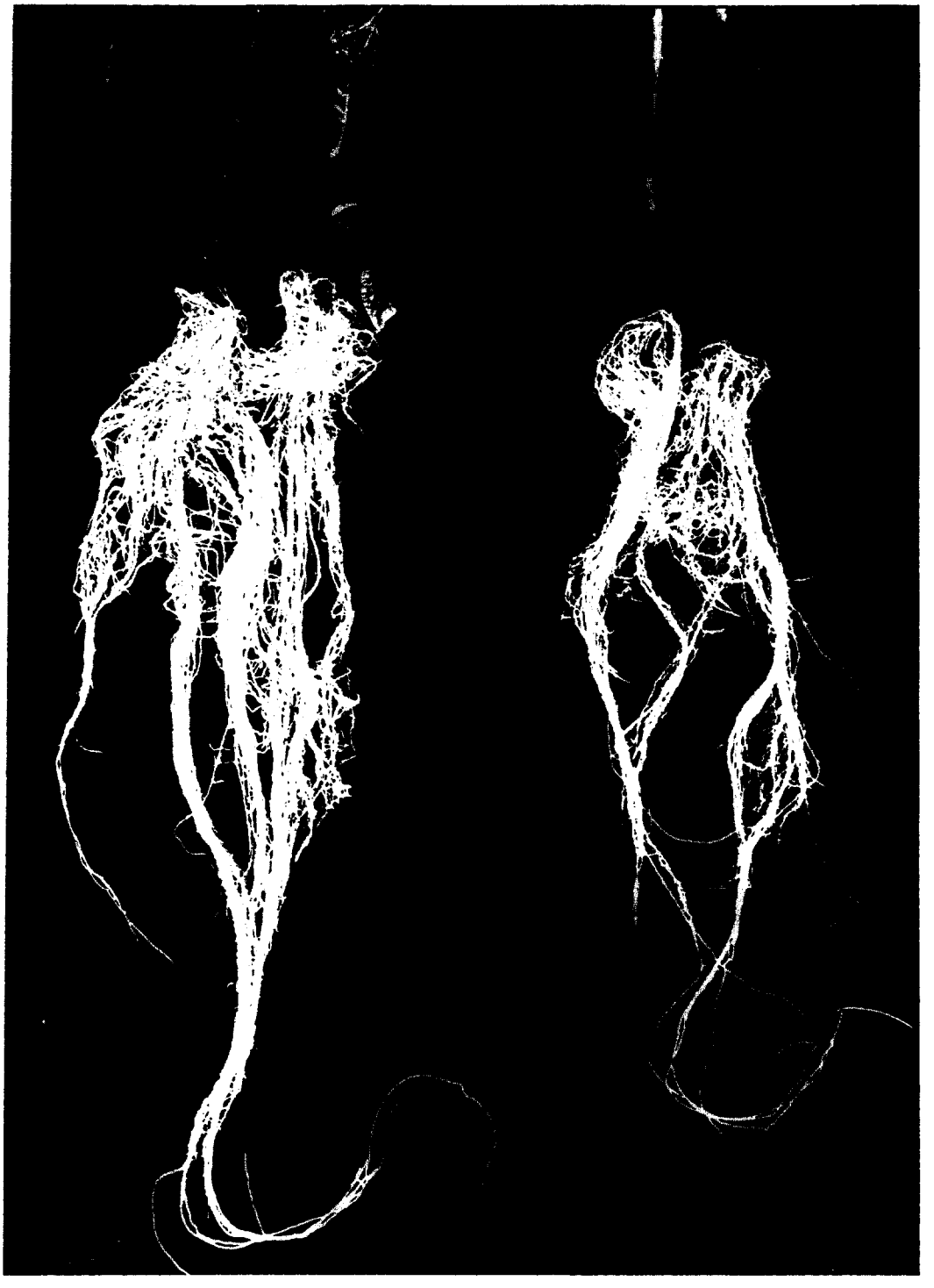

Fig. 2. Root systems of potato stem cuttings after 2 weeks growth in long potato-rotation soil. Left is control, right is treated with WCS374.

Table 2. Effects of WCS358 and some Tn5 transposon mutants of this isolate on root dry weight of potato stem cuttings in short (1:1) and long (1:6) potato-rotation soil. Dry weight was measured 8 days after planting. The values given are the mean values of six replicates. S^+; siderophore-producing isolate or mutant, S^-; siderophore-negative mutant

Treatment	Root dry weight (mg) 1:1 soil	1:6 soil
Control	10	70
WCS358 (S^+)	32*	34**
JM217 (S^-)	14	34**
JM218 (S^-)	11	26**
WCS358 Tn5:B (S^+)	26*	38**

* and **, significantly different from control at the 0.05 level by Student's *t*-test. *MSD = 10.6, **MSD = 29.7.

Table 3. Bacterial colonization of potato stem cuttings roots by siderophore producing (S^+) and siderophore-negative (S^-) Tn5 transposon mutants of *P. putida* isolate WCS358 in short and long potato-rotation soil. The numbers of cfu were determined on KB medium supplemented with kanamycin and streptomycin at 9 days after planting. Mean values of 10 replicates are given

Treatment	cfu of Tn5 transposon mutants cm^{-1} root 1:1 soil	1:6 soil
Control	0	0
JM217 (S^-)	4.10^5	4.10^4
JM218 (S^-)	2.10^5	7.10^4
WCS358 TN5:B (S^+)	3.10^4	3.10^5

JM218 were not fluorescent, whereas those from roots treated with WCS358 Tn5:B were fluorescent. In the control treatment no bacteria could be isolated on the KB medium supplemented with kanamycin and streptomycin.

Hybridization of fractionated *Eco*R1-digested chromosomal DNA with a [α-^{32}P]-labeled probe revealed one band for each mutant, as is shown for JM217 in the autoradiogram (Fig. 3b). With wild type DNA no hybridization occurred (Fig. 3a). These results demonstrate the insertion of Tn5 sequences in the chromosome of the mutants. Only one insertion was found for each mutant.

DISCUSSION

Root development of potato stem cuttings appeared to be very sensitive to the unknown harmful microbial factor in short potato-rotation soil. After 8 days a marked difference in root development was detected between long and short potato-rotation soil. Treatments with the wild type isolates WCS374 and WCS358 of *P. fluorescens* and *P. putida* stimulated root development in short potato-rotation soil. These observations agree with those of Geels and Schippers (1983b), who reported increased tuber production after seed tuber treatment with the same WCS isolates in short potato-rotation soil in a pot experiment. The most obvious difference between the bioassay and the pot experiments (Geels and Schippers, 1983b) is the time factor (8 days vs 8 weeks), which makes this bioassay suitable for quick screening and analysis of potato growth inhibition in short potato-rotation soil and of potato growth stimulation by fluorescent pseudomonads. The use of root observation boxes (van Vuurde and Schippers, 1980) appeared to be very useful during screening for these phenomena.

While the siderophore-producing isolates WCS374 and WCS358 increased root development in short potato-rotation soil, siderophore-negative mutants of isolate WCS358 had no effect. Since both siderophore-producing and siderophore-negative mutants colonized the roots similarly, it is concluded that siderophores are a prerequisite for growth stimulation in short potato-rotation soil. Evidence for siderophore production in potato rhizosphere by WCS358 has been presented by Bakker *et al.* (1986b).

The absence of symptoms, such as discolourations or lesions on roots from short potato-rotation soil, indicates a role for harmful microorganisms which affect root functioning without being parasitic (Schippers *et al.*, 1986a). These harmful microorganisms are thought to be HCN-producing pseudomonads (Schippers *et al.*, 1986a, b; A. W. Bakker and B. Schippers, 1987).

Siderophore-negative mutants of isolate WCS358 colonized roots in numbers comparable to those of the siderophore-producing mutant. One might expect that the loss of the ability to produce siderophores influences root colonization negatively. This does not seem to be the case and can be explained by our observations that siderophore negative mutants are

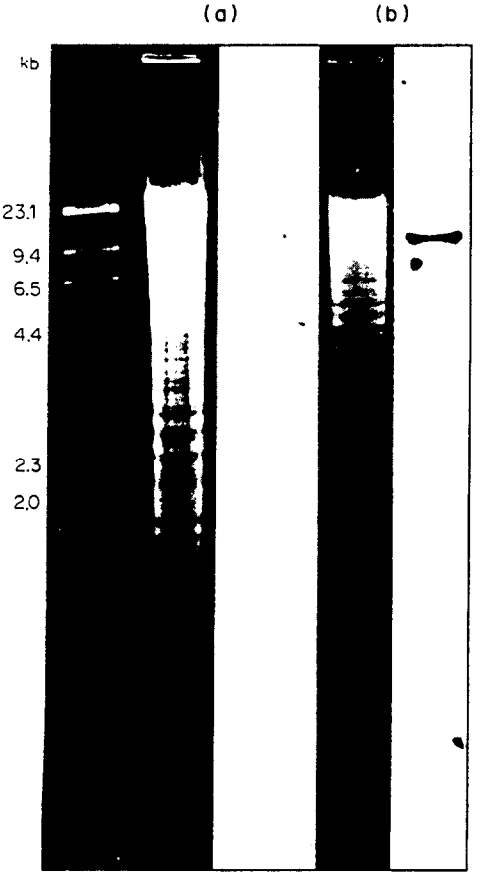

Fig. 3. *Eco*R1 digests of chromosomal DNA of *P. putida* isolate WCS358 and Tn5 mutant JM217 with the corresponding autoradiograms of the hybridization with the Tn5 radiolabeled probe. (A) WCS358 wild-type; (B) JM217. The marker lane is HindIII-digested λ DNA, the sizes are given in kilobases.

able to use siderophores produced by many other fluorescent pseudomonads isolated from potato rhizosphere and also those produced by their wild-type (unpublished results).

Bacterial treatments of roots reduced root development in long potato-rotation soil. In this soil no difference in reduction of root development was observed between the wild-type isolate and siderophore-negative mutants. Apparently, siderophore production has no direct effect on root development. Geels and Schippers (1983b) did not find a negative effect of isolate WCS358 in long potato-rotation soil in a pot experiment of 2 months duration using seed tubers, and did not find such a negative effect in field experiments in long potato-rotations (Geels and Schippers, 1983c; Bakker *et al.*, 1986a). Absence of the seed tuber as a nutrient source for root development apparently makes the bioassay very sensitive to microbial factors. Moreover, the applied pseudomonads colonize the root system in higher numbers in this bioassay compared to root colonization under field conditions (Bakker *et al.*, 1986a). These factors may explain reduced root growth in long potato-rotation soil after bacterization. In short potato-rotation soil suppression of the harmful microbial factor after bacterization results in increased root development despite the direct negative effect of the applied bacteria.

The use of Tn5 transposon mediated mutants seems promising in studying the mechanisms of microbial interactions in the rhizosphere and of plant growth stimulation by fluorescent pseudomonads. One insertion of Tn5 sequences was found for each mutant. This implies that only one gene is not expressed in each mutant, which makes these mutants suitable for studying the function of this single gene in the ecology of the microorganism. The combined resistance to kanamycin and streptomycin facilitated selective isolation of Tn5 mutants, since on a medium containing the two antibiotics no bacteria could be isolated from roots treated with water. The insertion of Tn5 sequences in the chromosomal DNA of WCS358 Tn5:B did not influence the fitness nor the growth stimulating ability of this mutant. Therefore, Tn5 induced kanamycin and streptomycin resistance proves to be a suitable technique in studies of colonization and survival of microorganisms.

Acknowledgements—We thank Anna Williamson for correcting the English and Dr Joop van den Heuvel for critical reading of the manuscript. This work was supported by the Netherlands Technology Foundation (STW).

REFERENCES

Bakker P. A. H. M., Lamers J. G., Bakker A. W., Marugg J. D., Weisbeek P. J. and Schippers B. (1986a) 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.

Bakker P. A. H. M., Weisbeek P. J. and Schippers B. (1986b) The role of siderophores in plant growth stimulation by fluorescent *Pseudomonas* spp. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent* **51**, 1357–1362.

Bakker A. W. and Schippers B. (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp-mediated plant-growth stimulation. *Soil Biology & Biochemistry* **19**, 451–457.

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

Geels F. P. and Schippers B. (1983b) Reduction of yield depressions in high frequency potato cropping soil after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp. *Phytopathologische Zeitschrift* **108**, 207–214.

Geels F. P. and Schippers B. (1983c) Reduction of yield depressions in high frequency potato cropping by fluorescent *Pseudomonas* spp. *Les Colloques de l'INRA* **18**, 231–238.

Hoekstra O. (1981) 15 jaar "De Schreef". Resultaten van 15 jaar vruchtwisselingsonderzoek op het bouwplannenproefveld "De Schreef". *Publikatie PAGV* **11**, 1–93. (Lelystad/Alkmaar, Nederland.)

Hofstad G. A. J. M. van der, Marugg J. D., Verjans G. M. G. M. and Weisbeek P. J. (1986) Characterization and structure analysis of the siderophore produced by the PGPR *Pseudomonas putida* strain WCS358. In *Iron, Siderophores and Plant Disease* (T. R. Swinburne, Ed.). 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. *Journal of Laboratory and Clinical Medicine* **44**, 301–307.

Kloepper J. W., Leong J., Teintze M. and Schroth M. N. (1980) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature, London* **286**, 885–886.

Kupers L. J. P. (1979) Agronomic aspects of soil-borne pathogens. In *Soil-Borne Plant Pathogens* (B. Schippers and W. Gams, Eds), pp. 358 370. Academic Press, London.

Lamers J. G. (1981) Continuteelt en nauwe rotaties van aardappelen en suikerbieten. *Publikatie PAGV* **12**, 1–65.

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. *Journal of Bacteriology* **164**, 563–570.

Schippers B., Geels F. P., Hoekstra O., Lamers J. G., Maenhout C. A. A. A. and Scholte K. (1985) Yield depressions in narrow rotations caused by unknown microbial factors and their suppression by selected pseudomonads. In *Ecology and Management of Soil-Borne Plant Pathogens* (C. A. Parker, A. D. Rovira, K. J. Moore, P. T. W. Wong and J. F. Kollmorgen, Eds), pp. 127–130. APS, St Paul, Minnesota.

Schippers B., Bakker A. W., Bakker P. A. H. M., Weisbeek P. J. and Lugtenberg B. (1986a) Plant growth-inhibiting and stimulating rhizosphere micro-organisms. In *Microbial Communities in Soil* (V. Jensen, A. Kjøller and L. H. Sørensen, Eds), pp. 35–48. FEMS Elsevier, London.

Schippers B., Geels F. P., Bakker P. A. H. M., Bakker A. W., Weisbeek P. J. and Lugtenberg B. (1986b) Methods of studying growth stimulating pseudomonads: problems and progress. In *Iron, Siderophores and Plant Disease* (T. R. Swinburne, Ed.), pp. 149–154. Plenum Press, New York.

Scholte K., Veenbaas-Rijks J. W. and Labruyère R. E. (1985) Potato growing in short rotations and the effect of *Streptomyces* spp, *Colletotrichum coccodes*, *Fusarium tabacinum* and *Verticillium dahliae* on plant growth and tuber yield. *Potato Research* **28**, 331–348.

Simon R. U., Priefer U. and Pühler A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**, 784–791.

Sokal R. R. and Rohlf F. J. (1981) *Biometry*. Freeman, San Francisco.

Vuurde J. W. L. van and Schippers B. (1980) Bacterial colonization of seminal wheat roots. *Soil Biology & Biochemistry* **12**, 559–565.

Weger L. A. de, Boxtel R. van, Burg B. van der, 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. *Journal of Bacteriology* **165**, 585–594.