Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode *Meloidogyne incognita* (Kofoid & White) Chitwood

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Abstract: To study the effect of microenvironments on potato-associated bacteria, the abundance and diversity of bacteria isolated from the rhizosphere, phyllosphere, endorhiza, and endosphere of field grown potato was analyzed. Culturable bacteria were obtained after plating on R2A medium. The endophytic populations averaged 10^3 and 10^5 CFU/g (fresh wt.) for the endosphere and endorhiza, respectively, which were lower than those for the ectophytic microenvironments, with 10^5 and 10^7 CFU/g (fresh wt.) for the phyllosphere and rhizosphere, respectively. The composition and richness of bacterial species was microenvironment-dependent. The occurrence and diversity of potato-associated bacteria was additionally monitored by a cultivation-independent approach using terminal restriction fragment length polymorphism analysis of 16S rDNA. The patterns obtained revealed a high heterogeneity of community composition and suggested the existence of microenvironment-specific communities. In an approach to measure the antagonistic potential of potato-associated bacteria, a total of 440 bacteria was screened by dual testing for in vitro antagonism towards the soilborne pathogens *Verticillium dahliae* and *Rhizoctonia solani*. The proportion of isolates with antagonistic activity was highest for the rhizosphere (10%), followed by the endorhiza (9%), phyllosphere (6%), and endosphere (5%). All 33 fungal antagonists were characterized by testing their in vitro antagonistic mechanisms, including their glucanolytic, chitinolytic, pectinolytic, cellulolytic, and proteolytic activity, and by their BOX-PCR fingerprints. In addition, they were screened for their biocontrol activity against *Meloidogyne incognita.* Overall, nine isolates belonging to *Pseudomonas* and *Streptomyces* species were found to control both fungal pathogens and *M. incognita* and were therefore considered as promising biological control agents.

Key words: biocontrol, antagonistic potential, plant-associated bacteria.

Résumé : Afin d'étudier l'impact des microenvironnements sur les bactéries associées à la pomme de terre, nous avons analysé l'abondance et la diversité de bactéries isolées de la rhizosphère, de la phyllosphère, des endorhizes et de l'endosphère de pommes de terre cultivées sur le terrain. Les bactéries cultivables ont été obtenues à la suite d'un étalement sur géloses R2A. Les populations endophytiques étaient en moyenne de 10^3 et 10^5 CFU/g (poids frais) pour l'endosphère et les endorhizes et étaient donc inférieures aux microenvironnements ectophytiques contenant en moyenne 10^5 et 10^7 CFU/g (poids frais) respectivement pour la phyllosphère et la rhizosphère. La composition et la richesse des espèces de bactéries dépendaient du microenvironnement. La fréquence et la diversité des bactéries associées aux pommes de terre ont été en outre mesurées par une approche indépendante de la culture et basée sur l'analyse par polymorphisme de la longueur des fragments de restriction terminaux de l'ADNr 16S. Les profils obtenus ont révélé une hétérogénéité importante de la composition des communautés et ont signalé l'existence de communautés spécifiques aux microenvironnements. Dans une approche visant à mesurer le potentiel antagoniste de bactéries associées à la pomme de terre, nous avons criblé un total de 440 bactéries par double analyse de l'antagonisme in vitro envers les pathogènes du sol *Verticillium dahliae* et *Rhizoctonia solani*. La proportion des isolats ayant une activité antagoniste était la plus élevée dans la rhizosphère (10%) suivie des endorhizes (9%), de la phyllosphère (6%) et de l'endosphère (5%). Les 33 antagonistes fongiques ont été caractérisés en analysant leurs mécanismes d'antagonisme in vitro, soit leur activité glucanoytique, chitinolytique, pectinolytique, cellulolytique et proteolytique, de même que par leur empreinte de

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BOX-PCR. De plus, ces bactéries ont été criblées pour leur activité de biocontrôle contre *Meloidogyne incognita*. Somme toute, neuf isolats appartenant aux espèces *Pseudomonas* et *Streptomyces* se sont avérés être efficaces dans le contrôle des deux champignons pathogènes et de *M. incognita* et constituent par conséquent à nos yeux des agents de lutte biologique prometteurs.

Mots clés : contrôle biologique, potentiel antagoniste, bactéries associée aux plantes.

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Introduction

The study of plant-associated bacteria and their antagonistic potential is important not only for understanding their ecological role and interaction with plants, but also for any biotechnological applications, e.g. biological control of plant pathogens. *Verticillium dahliae* Kleb., *Rhizoctonia solani* Kühn, and root-knot nematodes of the genus *Meloidogyne* are important diseases causing dramatic yield losses in many crops, including potato (Barker et al. 1998; Tjamos et al. 2000). Disease severity can even be enhanced by co-infection of *Verticillum* spp. with root-knot nematodes resulting in synergistic yield losses (Overman and Jones 1970). In the coming years, the phasing out of methyl bromide as a control measure for Verticillium wilt and plant parasitic nematodes will have a great impact on the accumulation of these pathogens in the soil (Tjamos et al. 2000). Therefore, efficacious control methods for soilborne pathogens are urgently needed for commercial potato production.

An environmentally friendly alternative to protect roots against fungal and nematode pathogens is bacteria-mediated biological control (Weller 1988; Hasky-Günther et al. 1998; Bloemberg and Lugtenberg 2001). Numerous studies have demonstrated the ability of several rhizobacteria to suppress diseases caused by fungal plant pathogens and plant-parasitic nematodes (Becker et al. 1988; Weller 1988; Emmert and Handelsman 1999). One of the difficulties in developing rhizobacteria as an alternative control measure is that many biological control agents are found to be too variable in their performance. According to Raaijmakers and Weller (2001), variable expression of genes involved in disease suppression and poor root colonization are the major factors contributing to this inconsistency. Mechanisms of bacterial antagonism towards plant pathogens include the competition for nutrients and space, the production of antibiotics and toxins, or the production of host cell-wall-degrading enzymes (Fravel 1988; Chet et al. 1990). Successful biological control not only requires a better understanding of the complex regulation of disease suppression by antagonists in response to environmental factors but also a better picture of the dynamics and composition of plant-associated bacterial communities and what triggers plant colonization. Thus, little is known about microenvironment specificity of antagonistic plant-associated bacteria and their biocontrol potential, especially for internal plant colonizers. Endophytic bacteria have been defined by Hallmann et al. (1997) as those bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plant and, additionally, do not visibly harm the plant. Recently, it has been demonstrated that bacterial endophytes may have beneficial effects on host plants, such as growth promotion and biological control of pathogens (Frommel et al. 1991; Chen et al. 1994; Sturz et al. 1997; Downing and Thomson 2000; Ashikari et al. 2001). It has been suggested that bacteria might interact more closely with the host plant than first expected and, therefore, could be efficient biological control agents in sustainable crop production (Sturz et al. 2000). Most information about the occurrence of endophytic bacteria and their community structure has been obtained using cultivation-dependent approaches. Therefore, the use of cultivation-independent methods or a combination of methods might provide new information on community structure. Garbeva et al. (2001) showed differences in the potato-associated bacterial community from stem peel and roots using denaturing-gradient gel electrophoresis of 16S rDNA-based PCR, and following cloning and sequencing, unknown bacteria were detected. Therefore, further characterization of these communities might provide new information about their potential role for plant health and growth.

To examine the impact of the microenvironment on the abundance and diversity of bacterial communities, especially those from the important functional group of antagonists, isolates from the rhizosphere, phyllosphere, endorhiza, and endosphere were analyzed by a multiphasic approach. A cultivation-independent approach, terminal restriction fragment length polymorphism (T-RFLP), of 16S rDNA was combined with bacterial cultivation on R2A medium (Difco, Detroit, Mich.). For the latter, the bacterial isolates obtained were screened for antagonism against *V. dahliae* and *R. solani*. Isolates with antagonistic activity obtained a comprehensive phenotypic and genotypic characterization to provide new data on microenvironment-dependent diversity. Additionally, the antagonists were studied for their biocontrol potential against *Meloidogyne incognita* to identify biological control agents active against both types of pathogens.

Materials and methods

Isolation of bacteria and determination of colony-forming units (CFU)

On 19 July 2000, six samples of potato (*Solanum tuberosum*) cv. Cilena composed of three plants each were taken from a field in Böhlendorf near Rostock, Germany. Roots with adhering soil and leaves were collected in sterile bags and then transported to the laboratory. To extract the plant-associated microorganisms, 5 g each of roots and leaves were transferred into a sterile Erlenmeyer flask containing 45 mL of a sterile 0.85% NaCl solution. The samples were shaken for 10 min. Roots and leaves were then removed for isolation of the endorhiza and endosphere colonizers, and the remaining suspension was filtered through Stomacher

Table 1. Plate counts (CFU/g (fresh wt.)) and percentages of antagonistic bacteria against the plant pathogenic fungi *Verticillium dahliae* and *Rhizoctonia solani*.

Microenvironment	CFU ± SD	Isolates tested	Isolates (%) active against		
			V.d.	*R.s.*	both
Rhizosphere	$5.2 \times 10^7 \pm 1.4 \times 10^7$	110	9	9	11
Phyllosphere	$3.2 \times 10^5 \pm 9.9 \times 10^4$	110	2	7	7
Endorhiza	$1.9 \times 10^5 \pm 1.1 \times 10^5$	110	3	9	10
Endosphere	$6.4 \times 10^3 \pm 6.4 \times 10^3$	110	1	5	5

Note: *V.d., Verticillium dahliae*; *R.s., Rhizoctonia solani*.

bags (Interscience, U.K.) prior to the isolation of the rhizosphere and phyllosphere colonizers. For the isolation of endophytic colonizers, roots and leaves were surface-sterilized in 1.5% NaOCl for 3 min, followed by homogenization with mortar and pestle. Before homogenization, the plant material was imprinted on nutrient agar to serve as a sterility check. If bacterial growth occurred within 24 h, the sample was discarded.

All samples were serially diluted with sterile 0.85% NaCl and plated onto R2A medium. Plates were incubated for 5 days at 20°C. CFU were counted and expressed as $\log_{10}$ CFU per gram fresh weight. For each microenvironment, 110 colonies with visually different colony morphology were transferred on nutrient agar, purified, and stored at –70°C in nutrient broth containing 15% glycerol until further use.

DNA extraction and T-RFLP analysis of bacterial communities

Bacterial pellets were obtained by differential centrifugation and stored at –70°C. Total DNA was extracted from these pellets using Ultra Clean Soil DNA Kit (Mo Bio Laboratories Inc, Solana Beach, Calif.). 16S rDNA fragments from community DNA were amplified with primer 8–27f fluorescently labeled with 6-FAM and with primer 926r (Lui et al. 1997). Each 50-μL reaction mixture contained 1× PCR buffer, 1.75 μM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 25 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Applied Biosystems, Foster City, Calif.). DNA amplification was performed by using the following program: 3 min denaturation at 95°C, 25 cycles of 30 s at 94°C, 40 s at 50°C, and 90 s at 72°C, and a final extention for 8 min at 72°C. The PCR products were purified with a Qiaquick PCR purification kit (Qiagen Inc., Valencia, Calif.). Approximately 100 ng of amplified 16S rDNA was digested with *Hha*I (New England Biolabs Inc., Beverly, Mass.) and precipitated with ethanol. Precipitated DNA was washed with 70% ethanol, vacuum-dried, and resuspended in 20 μL water. The sample was mixed with 0.1 μL of GeneScan 1000 Rox size standard (Applied Biosystems), denaturated at 95°C for 2 min, immediately placed on ice, and evaluated following electrophoresis in POP6 polymer with an ABI 310 DNA sequencer (Applied Biosystems). Terminal fragment sizes between 40 and 950 bp were determined using the GeneScan Analytical Software v.3.1.2 (Applied Biosystems).

Identification of plant-associated bacteria and determination of diversity indices

All isolates were identified based on their cellular fatty acids following fatty acid methyl ester gas chromatography (FAME-GC), as described by Sasser (1990), and characterized by the MIDI system (Microbial Identification System, Inc., Newark, Del.). Antagonistic isolates were additionally identified by 16S rDNA sequencing aligned with the reference 16S rRNA gene sequence using the BLAST algorithm according to Altschul et al. (1997). The general species diversity of the bacterial communities was measured by the Shannon information theory function (Shannon and Weaver 1949). Species richness, expressed as the number of species (*S*) as a function (ratio) of the total number of individuals (*N*), was determined by the index (*d*) proposed by Menhinick (1964).

Screening of bacteria for antagonism towards *V. dahliae* and *R. solani*

All bacterial isolates were screened for their activity towards *V. dahliae* Kleb. and *R. solani* Kühn by a dual culture in vitro assay on Waksman agar containing 5 g proteose–peptone (Merck, Darmstadt, Germany), 10 g glucose (Merck), 3 g meat extract (Oxoid, Hampshire, England), 5 g NaCl (Merck), and 20 g agar (Difco), filled up to 1 L with distilled water and adjusted to pH 6.8. After 5 days of incubation at 20°C, zones of inhibition were measured according to Berg (1996). The two pathogenic fungi, originating from the culture collection of the Department of Microbiology, Rostock University, were routinely grown on Czapek Dox medium (Difco).

Screening of bacteria for antagonism towards *M. incognita*

Bacterial isolates, which showed antagonistic activity towards *V. dahliae* and (or) *R. solani*, were further tested for their antagonism towards *M. incognita*. Potato tubers cv. Hansa that are susceptible to plant-parasitic nematodes were pregerminated in the dark for 2 weeks. The tubers were incubated in the bacterial suspension (OD_{560} = 1.0; approx. $\log_{10}$ CFU/mL) for 30 min and planted in 400 mL plastic pots filled with a heat-treated field soil–sand mixture (1:1, v/v). An additional 3 mL of the bacterial suspension was pipetted onto the soil to ensure bacterial establishment. Three days later, the potatoes were inoculated with 3000 eggs of *M. incognita*. Nematode inoculum was produced by extracting eggs from galled tomato roots using the NaOCl technique described by Hussey and Barker (1973). Eggs were inoculated by pipetting 3 mL tap water containing the requisite amount of inoculum into the root zone around the potato tuber. Six weeks after nematode inoculation, the potato plants were evaluated for shoot and root fresh weight, number of galls, and egg masses. Nonbacterized potatoes

Table 2. List of bacterial species isolated from microenvironments of potato.

	Occurrence				
Bacterial species*	Rhizosphere	Phyllospere	Endorhiza	Endosphere	Σ
Arthrobacter atrocyaneus			1		1
Arthrobacter aurescens			1		1
Arthrobacter crystallopodietis				1	1
Arthrobacter globiformis	2				2
Arthrobacter ilicis	3				3
Arthrobacter pascens		1	1		2
Arthrobacter protoformiae		1			1
Aureobacterium barkeri	2				2
Aureobacterium esteroaromaticum	2	9	1		12
Aureobacterium liquefaciens	3		2		5
Aureobacterium testaceum	1				1
Bacillus amyloliquefaciens		1			1
Bacillus citinusporus	1	1	1		3
Bacillus coagolans	1				1
Bacillus atrophaeus		1		1	2
Bacillus laterosporus			1		1
Bacillus megaterium	4	1	3	1	9
Bacillus mycoides		5	5		10
Bacillus pumilus		2	4	1	7
Bacillus psychrophilus	1				1
Bacillus sphaericus		1			1
Brevibacterium acetylicum			1		1
Brevibacterium linens		1			1
Chryseobacterium balustinum	2		2		4
Chryseobacterium indologenes			1		1
Chryseobacterium menigosepticum			1		1
Clavibacter michiganense	10	4	5	1	20
Comamonas acidovorans	1		2		3
Curtobacterium flaccumflaciens	4	5		3	12
Curtobacterium citreum	2				2
Curtobacterium pusillum	1				1
Cytophaga johnsonae	2		1		3
Dactylosporangium fulvum	1				1
Enterobacter intermedius			2		2
Flavobacterium resinovorum	1		1		2
Flavobacterium aquatile			1		1
Hydrogenophaga pseudoflora		1		1	2
Hydrogenophaga pseudoflava		2			2
Kocuria kristinae	1	2	1		4
Micrcococcus agilis	1	3		1	5
Micrococcus halobius	1		1		2
Micrococcus luteus	1	4	11	1	17
Micrococcus lylae		1			1
Micrococcus varians		2		1	3
Micromonospora carbomonaceca	1				1
Paenibacillus macerans	6	4		3	13
Pantoea agglomerans		3	2	31	36
Pseudomonas aeruginosa				1	1
Pseudomonas chichorii				4	4
Pseudomonas chlororaphis	1		10	14	25
Pseudomonas fluorescens		2	4	4	10
Pseudomonas marginalis				2	2
Pseudomonas putida	1	2	7	6	16
Pseudomonas rubisubalbicans	1				1
Pseudomonas syringae		3	2	11	16
Pseudomonas viridiflava		1	4	4	9

Table 2. (*concluded*).

Bacterial species*	Occurrence				Σ
	Rhizosphere	Phyllospere	Endorhiza	Endosphere	
Rhodoccocus erythropolis	1				1
Shingobacterium heparinum	1		1		2
Sphingobacterium spiritivorum	2				2
Staphylococcus haemolyticus			1		1
Stenotrophomonas maltophilia			11		11
Streptomyces halstedii	4	2	5		11
Streptomyces violaceusniger	3	1	2		6
Streptomyces cyaneus			2		2
Streptoverticillium reticulum	2				2
Variovorax paradoxus	2				2
Xantomonas campestris	1				1
Σ number of species	35	28	35	20	
Σ number of isolates	73	66	101	92	
Richness	4.1	3.4	3.5	2.1	
Diversity	3.2	3.1	3.2	1.9	

*Identification by fatty acid methyl ester (FAME) analysis.

with and without *M. incognita* served as control. Each treatment was replicated five times and the experiment was conducted twice.

Bacterial characterization based on production of hydrolytic enzymes and secondary metabolites

Production of cell-wall-degrading enzymes and secondary metabolites are common mechanisms bacteria use to inhibit fungal growth and (or) nematode reproduction. To better characterize the antagonistic bacterial isolates, their potential to produce hydrolytic enzymes and secondary metabolites was studied. Chitinase activity (β-1,4-glucosamine polymer degradation) and pectinase activity were tested in minimal medium, according to Chernin et al. (1995). Clearing zones were detected 5 days after incubation at 20°C. β-glucanase and cellulase activity was tested using chromogenic azurine-dyed cross-linked and Remazol Brilliant Blue R substrates (Megazyme, Bray, Ireland), respectively. Formation of blue haloes was recorded until 5 days after incubation. Protease activity indicated by casein degradation was determined from clearing zones in skim milk agar (50 mL sterilized skimmed milk mixed at 55°C with 50 mL 1/5 tryptic soy ager and 4% agar) after 5 days of incubation at 20°C.

The ability of bacterial isolates to produce indole-3-acetic acid was determined using the microplate method developed by Sawar and Kremer (1995). The production of fluorescent siderophores was tested by growing the bacteria on King's medium B (King et al. 1954) for 2 days at 25°C and by checking for fluorescence after excitation at 366 nm.

The antifungal antibiotic 2,4-diacetylphloroglucinol (Phl) is responsible for the antifungal activity of many *Pseudomonas* strains. To detect the *phlD* gene, a PCR approach was used. Amplification with the gene-specific primers Phl2 (Raaijmakers et al. 1997) was performed in the following mix: 1 μL target DNA, 10 pmol of each primer, and 17 μL PCR SuperMix High Fidelity (Gibco). The PCR was performed under the following conditions: 3 min at 95°C, followed by 29 cycles consisting of 1 min at 94°C, 45 s at 48°C, and 45 s at 72°C. PCR was finished by a primer extension step at 72°C for 5 min. PCR-amplified DNA was detected by gel electrophoresis using a 1.5% agarose gel. The gels were stained with ethidium bromide for 30 min, and the PCR products were visualized with a UV transilluminator. The reproducibility of the results was verified in two independent experiments.

Bacterial plant growth promotion

A newly developed microplate assay was used to measure the potential of the antagonistic bacteria to promote plant growth (Berg et al. 2001). In previous experiments, this test provided reliable data for plant growth promotion by bacteria of different plants, including potato. Seeds of strawberry (*Frageria ×ananassa)* cv. Rügen Selecta (Erfurter Samenzucht, Erfurt, Germany) were pregerminated in moist chambers at 20°C for 6 days. Three standard 24-well microplates (Roth, Karlsruhe, Germany) were filled with 1 mL water agar containing 2% agar (Difco) and adjusted to pH 6.8. One pregerminated seed was transferred into each well, to which 10 μL of a bacterial suspension was added. The bacterial suspension was prepared by growing the bacteria in nutrient broth for 18 h, centrifuging at 5000 × *g* for 10 min, dissolving the bacterial pellet in water, and adjusting the suspension to 10^5 CFU/mL. The control received 10 μL of distilled water. Five weeks after incubation at 22 h light : 16 h dark photoperiod in a growth chamber (Percival Scientific, Boone, Iowa,), the following plant growth parameters were evaluated: total number of first leaves, total number of radial roots, stem length, and root length development of roothairs. The isolates were tested in six replicates at each concentration, and the experiment was repeated three times. Data were analyzed for significance using the Mann–Whitney *U* test ($P \leq 0.05$) the software provided by Statistical Product and Service Solutions for Windows, Rel. 9. 0. 1. (SPSS Inc., Chicago, Ill.).

BOX-PCR genomic fingerprints

In a final step, the antagonistic bacterial isolates were characterized by molecular means. Bacterial DNA was prepared

Table 3. Identification of antagonists.

Strain	FAME	SI	Sequencing 16S rDNA	AN*	SI	Phylogenetic assignment
RR5	*Curtobacterium flaccumfaciens*	0.539	*Rhizomonas suberifaciens*	D13737	94.48	α-*Protobacteria*
RR7	*Streptomyces violaceusniger*	0.277	*Streptomyces deastatochromogenes*	D63867	98.48	*Firmicutes*
RR8	*Chryseobacterium balustinum*	0.775	*Streptococcus mitis*	AY005045	98	*Firmicutes*
RR10	*Streptomyces halstedii*	0.696	*Streptomyces griseus*	AB030572	98	*Firmicutes*
RR12	no match					
RR20	*Streptomyces halstedii*	0.586	*Streptomyces turgidiscabies*	AB026221	99	*Firmicutes*
RR33	*Paenibacillus macerans*	0.435	*Kitasatosporia cystargenia*	U93318	98	*Firmicutes*
RR68	*Streptomyces halstedii*	0.101	*Streptomyces galilaeus*	AB045878	98	*Firmicutes*
RR76	*Streptomyces violaceusniger*	0.449	*Streptomyces griseus*	AB030572	99	*Firmicutes*
RR77	*Curtobacterium pusillum*	0.491	*Ralstonia paucula*	AF085226	98	β-*Proteobacteria*
RR110	*Streptomyces halstedii*	0.801	*Streptomyces turgidiscabis*	AB026221	98	*Firmicutes*
RP27	*Pseudomonas viridiflava*	0.917	*Pseudomonas syringae*	AB001444	98	γ-*Proteobacteria*
RP35	*Bacillus mycoides*	0.814	*Bacillus cereus*		98	*Firmicutes*
RP37	*Pseudomonas putida*	0.878	*Pseudomonas veronii*	AB056120	99	γ-*Proteobacteria*
RP41	*Curtobacterium flaccumfaciens*	0.585	*Sphingomonas adhaesiva*	D13722	95.89	α-*Protobacteria*
RP58	*Streptomyces violaceusniger*	0.203	*Streptomyces lavendulae*	D85109	98	*Firmicutes*
RP67	*Pseudomonas syringae*	0.921	*Pseudomonas graminis*	Y11150	98	γ-*Proteobacteria*
RP87	*Streptomyces halstedii*	0.722	*Streptomyces setonii*	D63872	98.28	*Firmicutes*
RRE1	*Pseudomonas putida*	0.453	*Pseudomonas veronii*	AB056120	99	γ-*Proteobacteria*
RRE7	*Pseudomonas chlororaphis*	0.835				γ-*Proteobacteria*
RRE8	*Pseudomonas chlororaphis*	0.87				γ-*Proteobacteria*
RRE9	*Pseudomonas chlororaphis*	0.846				γ-*Proteobacteria*
RRE41	*Pseudomonas putida*	0.866				γ-*Proteobacteria*
RRE42	*Pseudomonas fluorescens*	0.9	*Pseudomonas jessenii*	AF068259	98	γ-*Proteobacteria*
RRE87	*Pseudomonas chlororaphis*	0.849	*Pseudomonas veronii*	AB056120	100	γ-*Proteobacteria*
RRE96	no match		*Amycolatopsis mediterranei*	AMP16SRR	98	*Firmicutes*
RRE101	*Chryseobacterium balustinum*	0.771	*Stenotrophomonas maltophilia*	AJ293463	100	γ-*Proteobacteria*
RRE109	*Pseudomonas chlororaphis*	0.559	*Pseudomonas putida*	AF094737	98	γ-*Proteobacteria*
RPE30	*Pseudomonas marginalis*	0.95	*Pseudomonas veronii*	AB056120	99	γ-*Proteobacteria*
RPE47	*Pseudomonas marginalis*	0.903	*Pseudomonas synxantha*	D84025	99	γ-*Proteobacteria*
RPE55	*Pseudomonas aeruginosa*	0.742	*Pseudomonas putida*	AF094744	98	γ-*Proteobacteria*
RPE57	*Pseudomonas chlororaphis*	0.853	*Pseudomonas rhodesiae*	AF064459	99	γ-*Proteobacteria*
RPE88	*Pseudomonas putida*	0.839	*Pseudomonas fluorescens*	AF094725	98	γ-*Proteobacteria*

Note: FAME, fatty acid methyl ester (analysis). SI, % similarity to the closest relative.
*Representative strain (GenBank assession number).

using Quiagen Genomic tips (Quiagen, Hilden, Germany). BOX PCR was done as described by Rademaker and De Bruijn (1997) using the BOXA1R primer 5′-CTACGG-CAAGGCGACGCTGACG-3′. PCR amplification was performed with a Peltier Thermal Cycler PTC-200 (Biozym Diagnostic, Hessisch Oldendorf, Germany) using an initial denaturation step at 95°C for 6 min, and subsequent 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 65°C for 8 min, followed by a final extension at 65°C for 16 min. A 10-µL aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5× Tris–borate–EDTA (TBE) buffer (0.0445 M Tris base, 0.0445 M boric acid, and 0.001 M EDTA) for 4 h, stained with ethidium bromide, and photographed under UV transillumination. The reproducibility of the results was verified in three independent experiments. Computer-assisted evaluation of BOX-PCR-generated fingerprints was made using the GelCompar program Version 4.1 (Applied Math, Kortrijk, Belgium). The cluster analysis was performed with the unweighted pair-group average (UPGMA) algorithm.

Results

Molecular fingerprinting of potato-associated bacterial communities

For cultivation-independent analysis, total community DNA was extracted from the microbial pellet recovered from different microenvironments. 16S rDNA fragments amplified by PCR were analyzed by T-RFLP. All T-RFLP patterns displayed a complex community composition (Fig. 1). The highest diversity was found in the rhizosphere. Furthermore, the T-RFLP patterns of all microenvironmants differed clearly, whereas the endosphere and the endorhiza communities were more similar to each other than to the other microenvironments.

Table 4. Antifungal activities and antifungal mechanisms of antagonistic isolates.

	Antagonistic activity*			Cellular production†									Plant growth promoting effect on ‡			
				Hydrolytic enzyme					Metabolite							
Strain	*V.d.*	R.*s.*	*M.i.*	Cellulases	Chitinases	Glucanases	Pectinases	Proteases	King's B	Siderophore	DAPG	Auxin	root length	stem length	no. of roots	root hairs
RR5	+	–	–	+	+++	+++	–	++	–	–	n.d.	–	–	–	–	–
RR7	+++	++	–	++	–	++	–	–	–	+	n.d.	–	–	–	–	–
RR8	+	–	+	–	–	+	–	+++	–	+	n.d.	–	+	+	–	–
RR10	–	+	–	+	+++	+	–	++	–	+	n.d.	–	–	–	–	–
RR12	+	+	+	+	+	++	–	+	–	+	n.d.	–	+++	+++	–	++
RR20	+	+	+	+	+	+++	–	+	–	+	n.d.	–	–	–	–	–
RR33	+	+	–	+	–	++	–	–	–	–	n.d.	–	++	++	+++	+
RR68	+++	++	–	++	–	+++	–	–	–	+	n.d.	–	++	+	–	–
RR76	+++	++	+	++	–	+++	–	–	–	+	n.d.	–	–	–	–	–
RR77	–	+	–	–	–	+++	–	++	–	+	n.d.	–	–	–	–	–
RR110	+	+++	–	+	–	++	–	+	–	+	n.d.	–	–	–	–	–
RP27	–	++	–	–	–	–	–	++	+	+	+	+	–	–	–	–
RP35	–	++	–	–	+	–	–	++	+	+	n.d.	–	–	–	–	–
RP37	+	+++	–	–	–	–	–	++	+	+	+	–	–	–	–	–
RP41	–	+	–	+	–	+++	–	++	+	+	n.d.	–	–	–	–	–
RP58	–	+	+	–	–	–	–	+	+	+	n.d.	–	–	–	–	–
RP67	–	+	+	–	–	+	–	+	+	+	+	+	++	+	–	–
RP87	+++	+++	–	–	–	–	–	–	–	–	n.d.	–	–	++	–	–
RRE1	–	+	–	–	–	–	–	+++	+	+	–	–	–	–	–	–
RRE7	–	+	–	–	–	–	–	+++	+	+	+	+	–	–	–	–
RRE8	–	++	–	+++	–	–	–	+++	+	+	+	+	–	–	–	–
RRE9	–	+	–	–	–	–	–	+++	+	+	+	+	–	–	–	–
RRE41	–	+	–	–	–	–	–	–	+	++	+	–	–	–	–	–
RRE42	–	+	–	–	–	–	–	+++	+	+	+	–	–	–	–	–
RRE87	+	+	+	–	–	–	–	–	+	++	+	–	–	–	–	–
RRE96	+	–	–	+	+	+	–	+	+	+	n.d.	–	–	–	–	–
RRE101	+	+	–	–	–	–	–	++	–	+	n.d.	+	–	–	–	–
RRE109	–	+++	–	–	–	–	–	++	+	+	+	–	–	–	–	–
RPE30	–	+	–	–	–	–	–	++	+	+	+	–	–	–	–	–
RPE47	–	+	–	–	–	–	–	+++	+	+	+	–	–	–	–	–
RPE55	++	+	–	–	–	–	–	–	+	+	+	–	–	–	–	–
RPE57	–	+	–	–	–	–	–	+++	+	+	+	+	–	–	–	–
RPE88	–	+	–	–	–	–	–	+++	+	+	+	–	–	–	–	–

Note: *V.d.*, *Verticillium dahliae*; *R.s.*, *Rhizoctonia solani*; *M.i.*, *Meloidogyne incognita*; n.d., not done; DAPG, detection of the 2,4-diacetylphloroglucinol gene by PCR.

*Radius of zone of inhibition in dual culture assay: +, 0–5 mm; ++, 5–10 mm; +++, >10 mm; –, absent.

†Radius of zone of hydrolysis in plate assay: +, 0–5 mm; ++, 5–10 mm; +++, >10 mm ; –, absent.

‡Effect on strawberry seedlings. Root length: +, 7–8 mm; ++, 8–10 mm; +++, >10 mm. Stem length: +, 12.5–15 mm; ++, 15–17 mm; +++, >17 mm. No. of roots: +, 7.7–8; ++, 8–9; +++, >9. Root hairs: +, 1.4–1.6; ++, 1.6–1.8; +++, >1.8; –, absent.

Table 5. Biocontrol potential of antagonistic isolates against *Meloidogyne incognita* infestation of potato.

	Reduction of *M. incognita* (%)		Plant growth promotion (%)	
Isolates	Galls	Egg masses	Shoot weight	Root weight
RR8	78	80	124	11
RR12	72	–	–	–
RR20	50	45	150	123
RR76	56	–	126	93
RP58	85	100	111	136
RP67	71	–	116	115
RRE7	66	–	178	140
RRE8	84	40	121	114
RRE87	71	80	100	95

Fig. 1. Terminal restriction fragment length polymorphism (T-RFLP) profiles of four bacterial communities isolated from the rhizosphere, phyllosphere, endorhiza, and endosphere of potato plants. Terminal fragments were generated from an *Hha*I digestion of 16S rDNA amplified from total bacterial community DNA using a fluorescently tagged 8-27f primer and an unlabeled 926r primer.

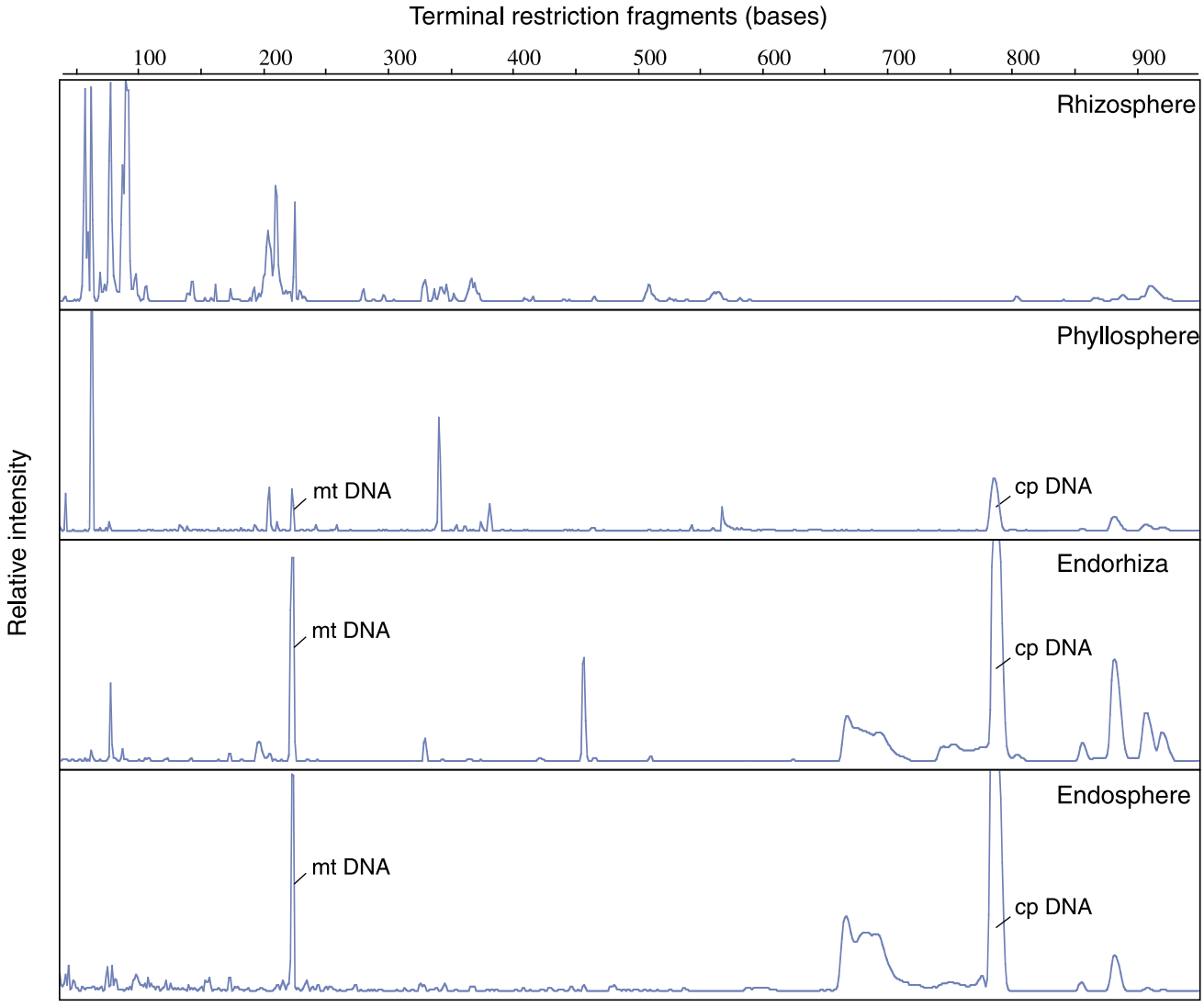


Table 6. Concluding assessment of antagonistic isolates.

	Antagonistic activity*			Cellular production† Hydrolytic enzyme					Metabolite		Plant growth promoting effect on‡				
Strain	*V.d.*	R.s.	*M.i.*	Cellulases	Chitinases	Glucanases	Pectinases	Proteases	Siderophores†	Auxin	root length	stem length	no. of roots	root hairs	Assessment
RR5	1	–	–	1	1	1	–	1	–	–	–	–	–	–	5
RR7	3	2	–	1	–	1	–	–	1	–	–	–	–	–	8
RR8	1	–	6	–	–	1	–	1	1	–	1	1	–	–	12
RR10	–	1	–		1	1	–	1	1	–	–	–	–	–	6
RR12	1	1	6	1	1	1	–	1	1	–	3	3	–	2	21
RR20	1	1	6	1	1	1	–	1	1	–	–	–	–	–	13
RR33	1	1	–	1	–	1	–	–	–	–	2	2	3	1	12
RR68	3	2	–	1	–	1	–	–	1	–	2	1	–	–	11
RR76	3	2	6	1	–	1	–	–	1	–	–	–	–	–	14
RR77	–	1	–	–	–	1	–	1	1	–	–	–	–	–	3
RR110	1	3	–	1	–	1	–	1	1	–	–	–	–	–	8
RP27	–	2	–	–	–	–	–	1	1	1	–	–	–	–	5
RP35	–	2	–	–	1	–	–	1	1	–	–	–	–	–	5
RP37	1	3	–	–	–	–	–	1	1	–	–	–	–	–	6
RP41	–	1	–	1	–	1	–	1	1	–	–	–	–	–	5
RP58	–	1	6	–	–	–	–	1	1	–	–	–	–	–	9
RP67	–	1	6	–	–	1	–	1	1	1	2	1	–	–	14
RP87	3	3	–	–	–	–	–	–	–	–	–	2	–	–	8
RRE1	–	1	–	–	–	–	–	1	1	–	–	–	–	–	3
RRE7	–	1	–	–	–	–	–	1	1	1	–	–	–	–	4
RRE8	–	2	–	1	–	–	–	1	1	1	–	–	–	–	6
RRE9	–	1	–	–	–	–	–	1	1	1	–	–	–	–	4
RRE41	–	1	–	–	–	–	–	–	1	–	–	–	–	–	2
RRE42	–	1	–	–	–	–	–	1	1	–	–	–	–	–	3
RRE87	1	1	6	–	–	–	–	–	1	–	–	–	–	–	9
RRE96	1	–	–	1	1	1	–	1	1	–	–	–	–	–	6
RRE101	1	1	–	–	–	–	–	1	1	1	–	–	–	–	5
RRE109	–	3	–	–	–	–	–	1	1	–	–	–	–	–	5
RPE30	–	1	–	–	–	–	–	1	1	–	–	–	–	–	3
RPE47	–	1	–	–	–	–	–	1	1	–	–	–	–	–	3
RPE55	2	1	–	–	–	–	–	–	1	–	–	–	–	–	4
RPE57	–	1	–	–	–	–	–	1	1	1	–	–	–	–	4
RPE88	–	1	–	–	–	–	–	1	1	–	–	–	–	–	3

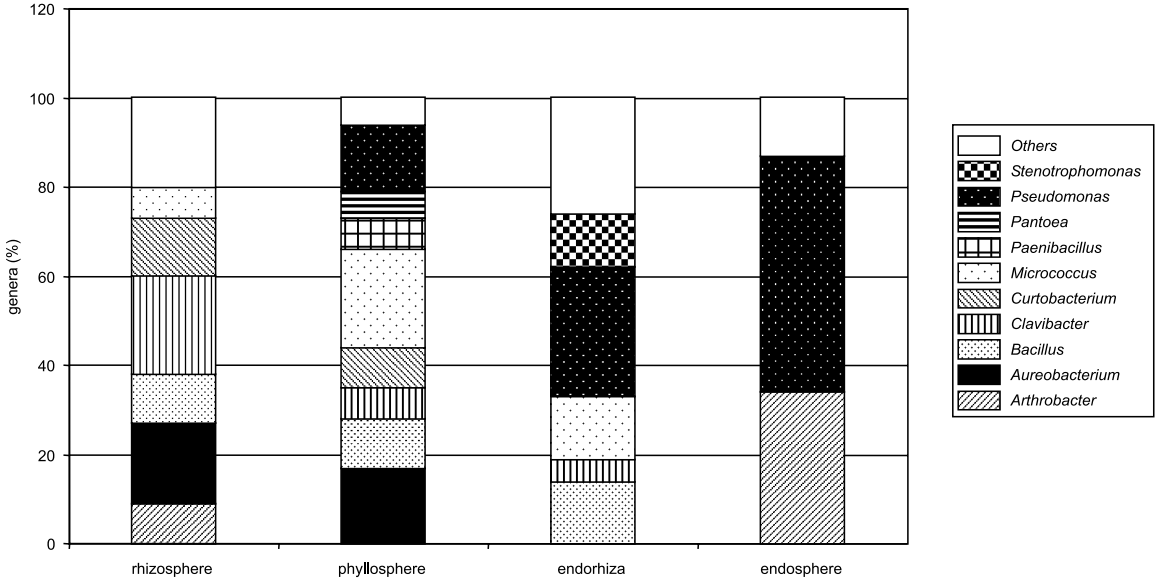
Note: *V.d.*, *Verticillium dahliae*, *R.s.*, *Rhizoctonia solani*, *M.i.*, *Meloidogyne incognita.*

*Radius of zone of inhibition in dual culture assay: 1, 0–5 mm; 2, 5–10 mm; 3, >10 mm; 6, efficient suppression of the pathogen; –, no suppression.

†Plate assay: 1, hydrolysis; –, no hydrolysis.

‡Effect on strawberry seedlings. Root length: 1, 7–8 mm; 2, 8–10 mm; 3, >10mm. Stem length: 1, 12.5–15 mm; 2, 15–17 mm; 3, >17 mm. No. of roots: 1, 7.7–8; 2, 8–9; 3, >9. Root hairs: 1, 1.4–1.6; 2, 1.6–1.8; 3, >1.8; –, absent.

Fig. 2. Distribution of bacterial genera in different microenvironments of potato identified by fatty acid methyl ester (FAME) with SI ≤ 0.5 (SI, % similarity to the closest relative).



Population densities of potato-associated bacteria in different microenvironments

The highest abundance of culturable bacteria was found in the potato rhizosphere with 5.2×10^7 CFU/g (fresh wt.) (Table 1), followed by the phyllosphere and endorhiza with 3.2×10^5 and $1.9\ 10^5 \times$ CFU/g (fresh wt.), respectively. The lowest population density occured in the endosphere with 6.4×10^3 CFU/g (fresh wt.).

Diversity of potato-associated bacteria in different microenvironments

Of the 440 bacterial isolates, 332 were identified at the species level (SI (% similarity to the closest relative) > 0.5) by FAME-GC, composing 66 species (Table 2). The number of isolates with no match varied between 9 for the endorhiza and 44 for the phyllosphere. The highest number of species was isolated from the rhizosphere and endorhiza (35), while only 28 and 20 species were found in the phyllosphere and endosphere, respectively. Richness and diversity indices confirmed the specificity of microenvironments and the high diversity in the rhizsophere. Only four species were obtained from all microenvironments: *Bacillus megaterium*, *Clavibacter michiganense*, *Micrococcus luteus*, and *Pseudomonas putida.* The most frequently isolated isolates were characterized as *Pantoea agglomerans* (36 isolates), *Pseudomonas chlororaphis* (25 isolates), and *C. michiganense* (20 isolates). Regarding the microenvironment, commonly isolated species included *C. michiganense* for the rhizosphere (10 isolates), *Aureobacterium esteroaromaticum* for the phyllosphere (9 isolates), *M. luteus* and *Stenotrophomonas maltophilia* for the endorhiza (11 isolates), and *Pantoea agglomerans* for the endosphere (31 isolates). A high number of species (36 species, 45%) was exclusively found in one microenvironment. The majority of these species (15 species, 24%) only occurred in the rhizosphere. Twenty-four out of sixty-six species were only isolated from individual plants and usually occured once. The distribution of bacterial genera in different microenvironments is shown in Fig. 2. Differences between microenvironments were clearly shown by the composition of genera.

Screening of bacteria for antagonism towards *V. dahliae* and *R. solani*

A total of 440 bacterial isolates were screened for their ability to suppress growth of *V. dahliae* and *R. solani* in an in vitro dual culture assay (Fig. 3). A total of 33 isolates were found to be antagonistic against one or both of these pathogens. Seven of these isolates expressed strong activity, with inhibition zones larger than 10 mm. Although similar numbers of isolates from each microenvironment were tested, the relative number of isolates with antagonistic activity varied among microenvironments (Table 1). The relative number of isolates with antifungal activity against *V. dahliae* was highest for the rhizosphere (8%), followed by the endorhiza (3%), phyllosphere (2%), and endosphere (1%). Antagonism towards *R. solani* was higher in general, although the proportion was similar: highest for the rhizosphere and endorhiza (8%), followed by the phyllosphere (6%) and endosphere (5%).

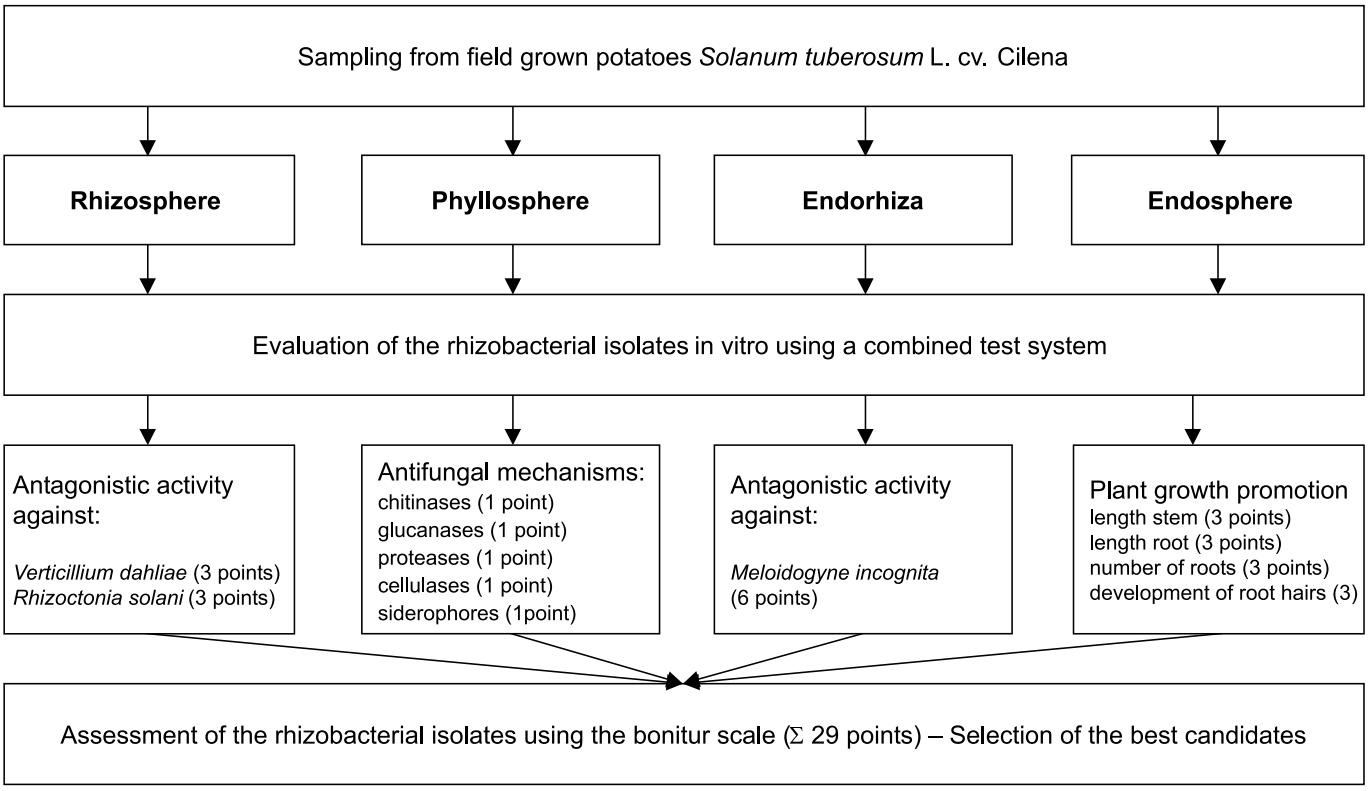
Screening of bacterial antagonism towards *M. incognita*

Nine of the thirty-three antagonistic isolates significantly reduced *M. incognita* infestation on potato (Table 3). Reductions in gall formation ranged from 50 to 85%, compared with the nontreated control. Bacterial treatment also reduced egg mass formation between 40 and 100%. The most efficient biocontrol isolate was *Streptomyces lavendulae* RP58. Several isolates also improved shoot and root fresh weights compared with the *M. incognita*-infested control. Maximum growth promotion achieved by *Pseudomonas jessenii* RRE7 was 178% for shoot fresh weight and 140% for root fresh weight.

Bacterial characterization based on production of hydrolytic enzymes and secondary metabolites

All 33 antagonistic isolates were characterized based on

Fig. 3. Scheme for the screening and assessment strategy.



their production of hydrolytic enzymes and secondary metabolites (Table 4). Protease production was shown for most isolates (76%), and 14 out of 33 isolates (42%) showed β-1,3-glucanase activity. Chitinolytic activity was detected for 9 isolates and cellulolytic activity for 10 isolates. Nine out of twelve isolates with cellulolytic activity originated from the rhizosphere. Siderophores were produced by 91% of the antagonistic isolates. Only *Pseudomonas* isolates produced fluorescent metabolites on King's medium B. Furthermore, PCR was used to detect the presence of the *phlD* gene encoding the antifungal antibiotic 2,4-diacetylphloroglucinol (Phl). The *phlD* gene was well distributed within our *Pseudomonas* isolates. Bacterial production of auxins was measured as an indicator for plant growth promotion. Seven isolates belonging to *Pseudomonas syringae*, *Pseudomonas graminis*, *Pseudomonas veronii*, *Pseudomonas rhodesiae*, and *Stenotrophomonas maltophilia* produced auxin in vitro.

Bacterial plant growth promotion

The effect of the 33 antagonistic isolates to promote plant growth was evaluated in a seedling bioassay. Six isolates caused a significant promotion in plant growth compared with the nontreated control (Fig. 4). The main effect was seen on the length of stem and roots. The highest effect was found for isolate RR12, which enhanced root length by 78%, compared with the nontreated control, which enhanced stem length by 65% and positively affected root hair development.

Grouping according to the BOX fingerprints and identification of antagonistic isolates

To explore the diversity of antagonistic isolates at the molecular level, they were characterized by BOX-PCR fingerprints. BOX-PCR obtained from genomic DNA yielded fingerprints with 12–35 amplification products ranging from 100 to 3000 bp (Fig. 5). Further analysis of the BOX-PCR fingerprints by UPGMA using GelCompare showed a high genotypic diversity and, based on a cut-off level of 85%, resulted in 28 groups, of which 26 represented a single isolate.

Antagonistic isolates with different fingerprint patterns were additionally characterized by 16S rDNA sequencing (Table 5). Based on this technique, all isolates, with exception of RR12, could be identified with a high identity of 95–100%. The majority of identified species belonged to γ-*Proteobacteria* (18 isolates) and the *Firmicutes* group (10 isolates).

Assessing the beneficial capacities of potato-associated bacteria

In an attempt to better select bacterial isolates with high antagonistic potential and to compare the antagonistic potential of bacteria from different microhabitats, points were given for each bacterial trait determined within this study as shown in Fig. 3. Up to three points each were given for antagonistic activity towards *V. dahliae* and *R. solani*, six points for antagonistic activity towards *M. incognita*, and one point each for production of hydrolytic enzymes (chitinases, cellulases, glucanases, pectinases, and proteinases), siderophores, and auxin. A total number of 29 points was possible. For the 33 antagonistic isolates, the number of points varied between 2 (isolate RRE41) and 21 (isolate RR12) (Table 6). On average, the highest antagonistic potential was achieved for bacterial isolates from the rhizosphere (10.3), followed by isolates from the phyllosphere (7.4), the endorhiza (4.7), and the endosphere (3.4). The low rating of

Fig. 4. Effect of antagonistic bacteria on the plant growth (A, length; B, length of roots; C, number of roots; and D, development of root hairs) of strawberry seedlings in a microchamber assay untreated control. Bacteria were added at a concentration of 1×10^5 CFU/mL using seed dipping for 15 min. Each treatment was replicated three times, 10 plants per pot. After 5 weeks, the plants were monitored. Statistically significant differences ($P \leq 0.05$) were analyzed by Mann–Whitney and indicated by asterisks (*).

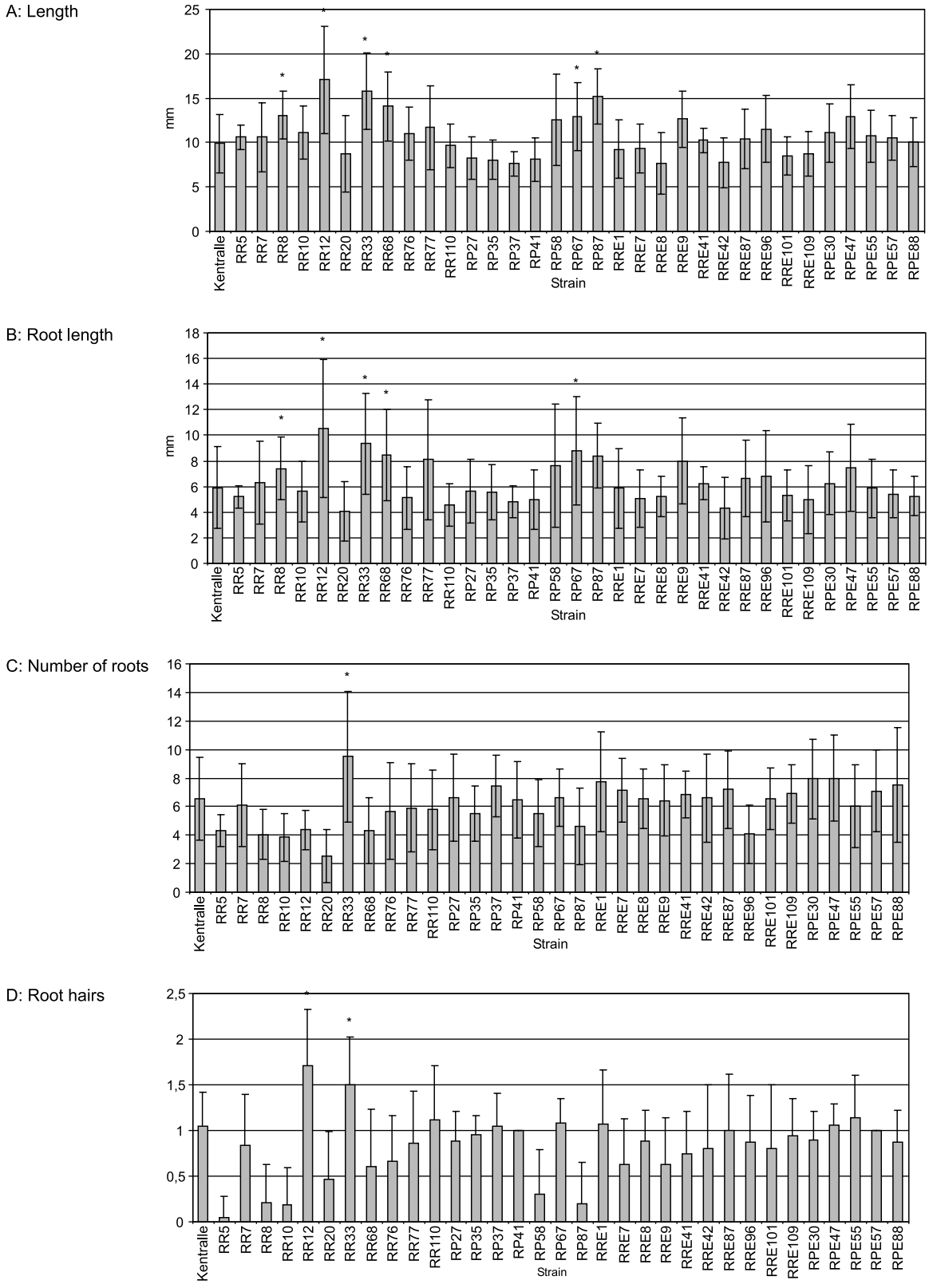
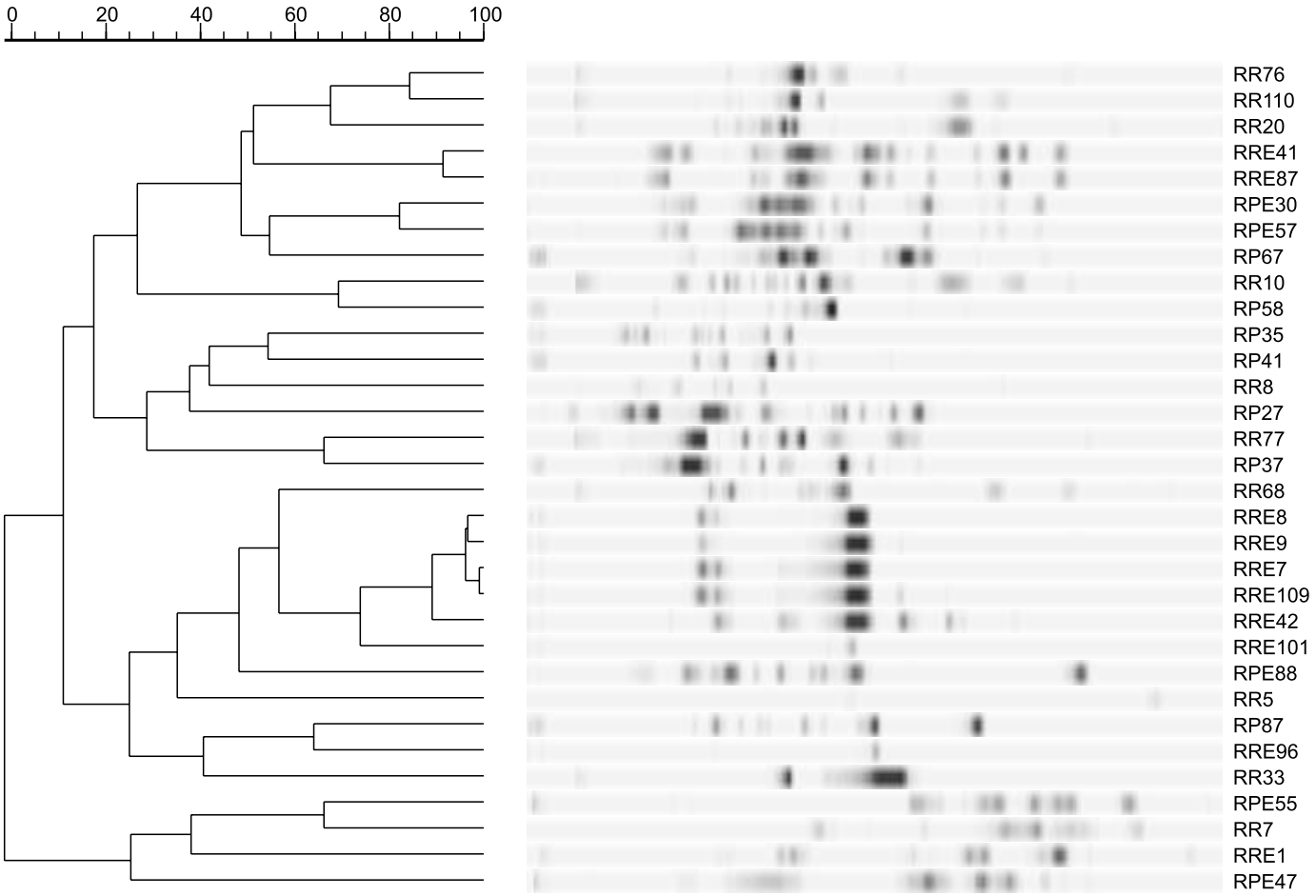


Fig. 5. Dendrogram showing the relationship of antagonistic isolates from different microenvironments of potato based on BOX-PCR fingerprints using cluster analysis determined by the unweighted pair-group average (UPGMA) algorithm.



the bacterial isolates within the latter two microhabitats was mainly caused by low or no antagonistic activity against the plant pathogens and absence of lytic enzymes.

Discussion

The potato-associated bacterial communities of the four microenvironments, rhizosphere, phyllosphere, endorhiza, and endosphere, were analyzed using a multiphasic approach, combining cultivation-dependent (FAME-GC) and cultivation-independent (T-RFLP) techniques. For culturable bacteria, the population densities determined for each microenvironment confirm previous reports (Hallmann et al. 1997). However, when comparing both approaches, similar conclusions had to be made regarding the specificity of bacterial communities in relation to their microenvironment. Based on FAME-GC analysis of culturable bacteria, each microenvironment was predominantly colonized by a different bacterial species. The most frequently isolated bacterium from the rhizosphere was *C. michiganense*, and from the phyllosphere, *A. esteroaromaticum*, which both belong to the *Firmicutes* group. The endorhiza and endosphere were dominated by *S. maltophilia* and *Pantoea agglomerans*, respectively, which belong to the γ-*Proteobacteria* group. *Pantoea agglomerans* is known to occur endophytically in corn and other plant species (Quadt-Hallmann et al. 1997*a*) and is reported as the most frequently isolated endophyte from citrus (Araújo et al. 2001) and pea (Elvira-Recuenco and van Vuurde 2000), whereas *S. maltophilia* is a common endophytic colonizer of potato (Garbevera et al. 2001). Only 4 out of 66 bacterial species occurred in all microenvironments. These four species were identified as *B. megaterium*, *C. michiganense*, *M. luteus*, and *Pseudomonas putida*, which are all known as ubiquitous colonizers of the soil–plant environment.

Microenvironment specificity of the bacterial communities was also expressed by their diversity indices based on FAME-GC identification. The greatest diversity was found in the rhizosphere, while the lowest was in the endosphere. A question often raised is what is the origin of endophytic colonizers? Assuming that seedborne endophytes do not play a major role (Hallmann 2001), bacteria have to colonize the plant from the outside, such as the rhizosphere or phyllosphere. Therefore, a comparison of the external and internal colonizing species might give valuable information concerning their origin. For the endorhiza, 16 out of 35 bacterial species (46%) were found in the rhizosphere and in the phyllosphere. For the endosphere, 15 out of 20 bacterial species (75%) were found in the phyllosphere, but only 8 (40%) were found in the rhizosphere, thus favoring the phyllosphere as the main source for endophytic colonization. Similar results were achieved with a T-RFLP pattern analysis of nonculturable bacteria, which showed a higher

correspondance of the endophytic community with the phyllosphere than with the rhizosphere. However, these results are in contrast with standard literature favoring the rhizosphere as the predominant source of endopyhtic colonization, where bacteria enter the root either actively by dissolving the plant cell-wall compounds (Quadt-Hallmann et al. 1997*b*), or by using natural openings and wounds as avenues of entrance (Hallmann 2001; James and Olivares 1998). The unexpected low similarity of culturable bacteria from the endorhiza compared with the rhizosphere is based on one sampling and, therefore, should not be overinterpreted, considering the continuous changes in community structure over time. For nonculturable bacteria, knowledge regarding their entrance is almost nonexistent, and it can only be speculated if the genetic similarity in bacterial communities between phyllosphere and internal colonizers might be caused by nonculturable bacteria entering the plant from the phyllosphere. However, further research is required to prove or disprove this hypothesis.

Differences among microenvironments were also seen in the relative occurrence of antagonistic isolates. Based on the dual culture assay of fungal pathogens, the highest percentage of antagonistic bacteria was found in the rhizosphere (10%), which was twofold higher than in the endosphere (5%). The spectrum of antagonistic species varied among the microenvironments. Antagonistic isolates from the rhizosphere mainly belonged to *Streptomyces*, while those from the endorhiza and endosphere were pseudomonads. Antagonistic isolates from the phyllosphere were represented by both *Streptomyces* and pseudomonads. Only a few identified species belonged to known antagonistic species, such as *Streptomyces griseus*, *Bacillus cereus*, *Stenotrophomonas maltophilia*, *Pseudomonas fluorescens* and *Pseudomonas putida*, whereas the majority of antagonistic isolates belonged to species which were not previously reported as antagonists, i.e., *Amycolatopsis mediterranei*, *Rhizomonas suberifaciens*, *Ralstonia paucula*, *Pseudomonas graminis* and *Streptomyces* spec.div. Taxonomically, the antagonistic species could be grouped as γ-*Proteobacteria* and *Firmicutes*, which agrees with previous work by Berg et al. (2000). Differences of bacterial antagonists in relation to their microenvironment were also seen based on their genotypic and phenotypic makeup. For example, glucanase and cellulase production was almost exclusively found in rhizosphere bacteria. Overall, comparison of cultivation-dependent and -independent techniques significantly contributed to a better understanding of plant–bacteria interactions.

A second approach of this study followed the characterization and identification of multitarget bacterial antagonists. While the group of plant-associated bacteria include neutral microorganisms with no obvious effect on the host plant, as well as antagonists and mutualistic symbionts, a rapid identification of the latter would be very supportive to enhance screening of efficient strains that promote plant growth and plant health promotion. The identification of strains with a broad control spectrum of different pathogens would be highly appreciated for biocontrol purposes. This target was approached by a multistep screening for biocontrol activity against fungal and nematode pathogens and plant growth promotion, combined with their genotypic and phenotypic characterization. All these data flew into an assessment scheme to identify the best antagonists. Based on this assessment scheme, nine isolates were considered good biocontrol candidates with broad control capacities. These isolates included species of *Streptococcus*, *Streptomyces*, and *Pseudomonas* and an unidentified isolate.

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References

Ashikari, T.B., Joseph, C.M., Yang, G., Phillips, D.A., and Nelson, L.M. 2001. Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling diseases of rice. Can. J. Microbiol. **47**: 916–924.

Araújo, W.L., Maccheroni, W., Aguilar-Vildoso, C.I., Barroso, P.A.V., Saridakis, H.O., and Azevedo, J.L. 2001. Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can. J. Microbiol. **47**: 229–236.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**: 3389–3402.

Barker, K.R., Pederson, G.A., and Windham, G.L. 1998. Plant and nematode interactions. American Society of Agronomy, Madison, Wis., U.S.A.

Becker, J.O., Zavaleta-Meija, E., Colbert, S.F., Schroth, M.N., Weinhold, A.R., Hancock, J.G., and van Gundy, S.D. 1988. Effects of rhizobacteria on root knot nematodes and gall formation. Phytopathology, **78**: 1466–1469.

Berg, G. 1996. Rhizobacteria of oil seed rape antagonistic to *Verticillium dahliae*. J. Plant Dis. Prot. **103**: 20–30.

Berg, G., Kurze, S., Buchner, A., Wellington, E.M., and Smalla, K. 2000. Successful strategy for the selection of new strawberry-associated rhizobacteria antagonistic to Verticillium wilt. Can. J. Microbiol. **46**: 1128–1137.

Berg, G., Fritze, A., Roskot, N., and Smalla, K. 2001. Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. J. Appl. Microbiol. **91**: 963–971.

Bloemberg, G.V., and Lugtenberg, B.J.J. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. **4**: 343–350.

Chen, C., Bauske, E.M., Musson, G., and Kloepper, J.W. 1994. Biological control potential and population dynamics of endophytic bacteria in a cotton/*Fusarium* wilt system. *In* Improving plant productivity with rhizosphere bacteria. *Edited by* M.H. Ryder, R.M. Stephens, and C.D. Bower. Graphic Services, Adelaid, Australia. pp. 191–193.

Chernin, L., Ismailov, Z., Haran, S., and Chet, I. 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl. Environ. Microbiol. **61**: 1720–1726.

Chet, I., Ordentlich, A., Shapira, R., and Oppenheim, A. 1990. Mechanisms of biocontrol of soil-borne plant pathogens by rhizobacteria. Plant Soil, **129**: 85–92.

Downing, K.J., and Thomson, J.A. 2000. Introduction of the *Serratia marcescens chiA* gene into an endophytic *Pseudomonas fluorescens* for the biocontrol of phytopathogenic fungi. Can. J. Microbiol. **46**: 363–369.

Elvira-Recuenco, M., and van Vuurde, J.W.L. 2000. Natural incidence of endophytic bacteria in pea cultivars under field conditions. Can. J. Microbiol. **46**: 1036–1041.

Emmert, E.A.B., and Handelsman, J. 1999. Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiol. Lett. **171**: 1–9.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of plant diseases. Annu. Rev. Phytopathol. **26**: 75–91.

Frommel, M.I., Nowak, J., and Lazarovits, J. 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.

Garbeva, P., van Overbeck, L.S., van Vuurde, J.W.L., and van Elsas, J.D. 2001. Analysis of endophytic bacterial communities of potato by plating and Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA based PCR fragements. Microb. Ecol. **41**: 369–383.

Hallmann, J. 2001. Plant interactions with endophytic bacteria. *In* Biotic interactions in plant–pathogen associations. *Edited by* M.J. Jeger and M.J. Spence. CAB International, Wallingford, U.K. pp. 87–119.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F., and Kloepper, J.W. 1997. Endophytic bacteria in agricultural crops. Can. J. Microbiol. **43**: 895–914.

Hasky-Günther, K., Hoffmann-Hergarten, S., and Sikora, R.A. 1998. Resistance against the potato cyst nematode *Globodera pallida* systemically induced by the rhizobacteria *Agrobacterium radiobacter* (G12) and *Bacillus sphaericus* (B43). Fundam. Appl. Nematol. **21**: 511–517.

Hussey, R.S., and Barker, K.R. 1973. A comparison of methods of collecting inocula for *Meloidogyne* spp. includung a new technique. Plant Dis. Rep. **57**: 1025–1028.

James, E.K., and Olivares, F.L. 1998. Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. Crit. Rev. Plant Sci. **17**: 88.

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.

Lui, W., Marsh, T.L., Cheng, H., and Forney, L.J. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. **63**: 4516–4522.

Menhinick, E.F. 1964. A comparison of some species–individuals diversity indices applied to samples of field insects. Ecology, **45**: 859–861.

Overman, A.J., and Jones, J.P. 1970. Effect of stunt and root-knot nematodes on Verticillium wilt of tomato. Phytopathology, **60**: 1306.

Quadt-Hallmann, A., Hallmann, J., and Kloepper, J.W. 1997*a*. Bacterial endophytes in cotton: location and interaction with other plant-associated bacteria. Can. J. Microbiol. **43**: 254–259.

Quadt-Hallmann, A., Benhamou, N., and Kloepper, J.W. 1997*b*. Bacterial endophytes in cotton: mechanisms of entering the plant. Can. J. Microbiol. **43**: 577–582.

Raaijmakers, J.M., and Weller, D.M. 2001. Exploiting genotypic diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.: characterization of superior root-colonizing *P. fluorescens* strain Q8r1–96. Appl. Environ. Microbiol. **67**: 2545–2554.

Raaijmakers, J.M., Weller, D.M., and Thomashow, L.S. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl. Environ. Microbiol. **63**: 881–887.

Rademaker, J.L.W., and De Bruijn, F.J. 1997. Characterization and classification of microbes by REP-PCR genomic fingerprinting and computer-assisted pattern analysis. *In* DNA markers: protocols, applications and overviews. Edited by G. Caetano-Anollés and P.M. Gresshoff. John Wiley & Sons, Inc., N.Y.

Sasser, M. 1990. Identification of bacteria through fatty acid analysis. *In* Methods in phytobacteriology. *Edited by* Z. Klement, K. Rudolph, and D.C. Sands. Akademiai Kiado, Budapest. pp.199–201.

Sawar, M., and Kremer, R.J. 1995. Determination of bacterially derived auxins using a microplate method. Lett. Appl. Microbiol. **20**: 282–285.

Shannon, C.E., and Weaver, W. 1949. The mathematical theory of communication. University of Illinois Press, Urbana, Ill.

Sturz, A.V., Christie, B.R., and Matheson, B.G. 1997. Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. Can. J. Microbiol. **44**: 162–167.

Sturz, A.V., Christie, B.R., and Nowak, J. 2000. Bacterial endophytes: potential role in developing sustainable system of crop production. Crit. Rev. Plant Sci. **19**: 1–30.

Tjamos, E.C., Rowe, R.C., Heale, J.B., and Fravel, D.R. 2000. Advances in *Verticillium* research and disease management. APS Press, St. Paul, Minn.

Weller, D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26**: 379–407.