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Endophytic Colonization of Potato (*Solanum tuberosum* L.) by a Novel Competent Bacterial Endophyte, *Pseudomonas putida* Strain P9, and Its Effect on Associated Bacterial Communities▽

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***Pseudomonas putida* strain P9 is a novel competent endophyte from potato. P9 causes cultivar-dependent suppression of *Phytophthora infestans*. Colonization of the rhizoplane and endosphere of potato plants by P9 and its rifampin-resistant derivative P9R was studied. The purposes of this work were to follow the fate of P9 inside growing potato plants and to establish its effect on associated microbial communities. The effects of P9 and P9R inoculation were studied in two separate experiments. The roots of transplants of three different cultivars of potato were dipped in suspensions of P9 or P9R cells, and the plants were planted in soil. The fate of both strains was followed by examining colony growth and by performing PCR-denaturing gradient gel electrophoresis (PCR-DGGE). Colonies of both strains were recovered from rhizoplane and endosphere samples of all three cultivars at two growth stages. A conspicuous band, representing P9 and P9R, was found in all *Pseudomonas* PCR-DGGE fingerprints for treated plants. The numbers of P9R CFU and the P9R-specific band intensities for the different replicate samples were positively correlated, as determined by linear regression analysis. The effects of plant growth stage, genotype, and the presence of P9R on associated microbial communities were examined by multivariate and unweighted-pair group method with arithmetic mean cluster analyses of PCR-DGGE fingerprints. The presence of strain P9R had an effect on bacterial groups identified as *Pseudomonas azotoformans*, *Pseudomonas veronii*, and *Pseudomonas syringae*. In conclusion, strain P9 is an avid colonizer of potato plants, competing with microbial populations indigenous to the potato phytosphere. Bacterization with a biocontrol agent has an important and previously unexplored effect on plant-associated communities.**

The colonization of plant tissue is of paramount importance for successful application of plant-growth-promoting bacteria. For instance, efficient root colonization was shown to be important for the control of *Fusarium oxysporum* in tomato by a phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* strain (6). The migratory response of bacterial inoculants to compounds released by plant roots is often the first step required for establishment of the bacteria in the rhizosphere and rhizoplane (29, 48, 50). Following the initial colonization by introduced bacteria, these organisms may spread further to the aerial parts of the plant (35). The degree of root colonization by bacterial inoculants depends on, in addition to the mode of application (35), factors intrinsic to the organism used, like the presence of flagella (11) and/or the presence of particular outer membrane lipopolysaccharides (13). Such features may differ from strain to strain. Once attached to plant roots, the inoculant bacteria may evoke "protective" responses in the plants, enabling them to resist phytopathogen attack (4).

Endophytic colonization, characterized by colonization of internal plant tissues concomitant with growth and systemic spread, can be an important factor for plant growth (23, 24). For instance, cells of *Rhizobium etli* G12 (used as a biocontrol agent) marked with a green fluorescent protein were visible in root hairs, around epidermal cells, and within the vascular tissue of *Arabidopsis thaliana* plants (22), and these plants exhibited maximum control of the nematode *Meleidogyne incognita*. Moreover, cells of the green fluorescent protein-labeled plant-growth-promoting bacterial strain *Burkholderia phytofirmans* PsJN were present in xylem vessels and different plant organs, including inflorescences, of grape plants (7). Endophytic colonization was also observed for the nitrogen-fixing bacteria *Acetobacter diazotrophicus* in sugarcane (12) and *Serratia marcescens* in rice (20). Hence, different types of bacteria apparently have the capacity to colonize the internal compartments of plants and eventually interact with their hosts, thus occupying niches inside plants where they may evoke responses that are important for plant health maintenance and nutrient acquisition.

Introduced bacteria can communicate with each other using a range of signaling systems, including quorum sensing, and most likely also with (related) bacteria indigenous to plants and even with their host plant (41). We hypothesize that introduced strains also impact plant-associated indigenous microbial communities by cross talk with members of these communities, by competition for nutrients or space, or by production of antibiotics. Shifts in endophytic bacterial communi-

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ties are therefore expected to occur after bacterial inoculation. This is a phenomenon that has been observed after inoculation of Madagascar periwinkle (*Catharanthus roseus*) and Cleveland tobacco (*Nicotiana clevelandii*) plants with *Methylobacterium mesophilicum* (1).

In situ microscopic detection of endophytes allows determination of the preferred colonization site. However, it is hard to detect cells (e.g., cells marked with *gfp*) at levels below certain threshold levels in plants, especially when they are grown under nonsterile conditions. For this reason it is not possible to precisely determine in situ interactions between inoculants and indigenous bacterial populations. Molecular fingerprinting techniques, like PCR-denaturing gradient gel electrophoresis (DGGE), are suitable for studying microbial communities in the rhizospheres (14, 26, 44, 51), rhizoplanes (48), and endospheres (18, 38, 42, 51) of different plant species. The use of bacterial group-specific primer systems has been proposed for studies of different taxonomic groups in the plant endosphere (52). The impact of factors like crop history, plant growth stage, and cultivar (genotype) on plant-associated microbial populations can be established by using multivariate statistical analyses (17, 40, 51). The combination of molecular fingerprinting techniques and multivariate analyses enabled us to show that the plant growth stage and cultivar contributed strongly and significantly to the composition of plant-associated microbial communities (37, 51).

The aim of this study was to establish the fate and impact of strain P9 during endophytic colonization of potato plants. The polyphasic approach used allowed us to investigate the presence of strain P9 at culture-dependent and -independent levels in plants.

MATERIALS AND METHODS

Isolation and characterization of P9. Strain P9 was isolated from potato (*Solanum tuberosum* L.) cv. Aziza from an experimental field plot near Heelsum, The Netherlands. A surface-sterilized and subsequently peeled part of a stem (peeled by removing the epidermis) from a single potato plant was homogenized by hammering it in a sterile plastic bag filled with 3 ml of tap water. The resulting homogenate was dilution plated onto R2A (BD, Franklin Lakes, NJ), and the plates were incubated (27°C, 5 days). Colonies from the highest dilutions were picked on the basis of differences in morphology and streaked until pure cultures were obtained. The 13 resulting isolates were tested to determine their capacity to suppress *Phytophthora infestans* lesion growth using a nondetached potato leaf assay (54). Briefly, in vitro transplants of *S. tuberosum* cv. Robijn (R plants) and *S. tuberosum* cv. Eersteling (E plants) (HZPC Research, Metslawier, The Netherlands) were incubated for 1 h with their roots either in pure-culture suspensions (10^8 cells per ml) of the different isolates (bacterial treatments) or in sterile tap water (control treatment). The resulting bacterially treated and control plants (four replicates per cultivar per treatment) were planted in commercial potting soil (Potgrond 4; Hortimea, Elst, The Netherlands) and allowed to grow until they flowered (i.e., growth stage 6 [21]) in a greenhouse using a daily cycle consisting of 16 h of light (with supplemental illumination provided by Philips HPIT lamps; 80 to 90 W per m^2) at 20°C and 8 h of darkness at 16°C.

Then plants were infected with *P. infestans* isolate IPO82001 (mating type A2), which was chosen because of its aggressive infection of potato plants (16). Leaves at positions 9, 10, and 11 (54) of each plant were treated with 10 μl of a suspension containing 10^4 *P. infestans* IPO82001 spores per ml. For each leaf, the size of the *P. infestans* lesion was measured 3, 4, and 5 days after inoculation. The increase in the lesion size over time was used as a measure of *P. infestans* conduciveness. Strain P9 was chosen as the prime candidate for further ecological studies of potato because treatment with it resulted in the smallest increase in the size of *P. infestans* lesions in R plants. The analysis of the effect of P9 on *P. infestans* lesion growth in treated and control E and R plants was repeated in three separate experiments. The antagonistic activity of strain P9 against different phytopathogens was further tested by dual plate testing with *P. infestans* IPO82001, *Rhizoctonia solani* AG 3, and *Ralstonia solanacearum* biovar 2 (51). Also, the presence of antibiotic production loci in strain P9 was examined by PCR using primers that target phenazine, phloroglucinol, pyrrolnitrin, and pyoluteorin biosynthetic operons (10).

The identity of P9 was determined by PCR amplification of a major part of the 16S rRNA gene using primers 27F and 1492R (39) and PCR amplification of the *gacA* gene using primers gacA-1F (8) and gacA2 (10), followed by DNA sequencing of the PCR amplicons obtained by Greenomics (Plant Research International, The Netherlands). Phylogenetic distances were calculated for the 16S rRNA gene sequences of strain P9, the 20 type strains showing the highest levels of similarity with the 16S rRNA gene of P9, and the type strains of 120 different selected *Pseudomonas* species, all obtained from the Silva database (34), release 95 (accessed 14 September 2008), using the ARB software package (30). Reconstruction of phylogenetic relationships was based on neighbor joining with the Jukes-Cantor correction by using 1,000 bootstraps.

A spontaneous rifampin (Rp)-resistant derivative of P9, designated P9R, was selected by growing cells on R2A amended with 50 μg per ml Rp (Sigma-Aldrich, St. Louis, MO). PCR amplicons, generated with primers 27F and 1492R, and BOX-PCR and repetitive extragenic palindromic PCR fingerprints (36) of P9 and P9R were constructed for comparisons. The fitness of P9R was tested in a competition assay with wild-type P9 by mixing equal numbers of cells (10^6 cells per ml) of the two strains in 0.1× tryptic soy broth (0.1× TSB) (Becton-Dickinson, Maryland) followed by two subsequent growth and transfer steps with 1:1,000 dilutions in fresh flasks with 0.1× TSB. After the last transfer-growth step, late-exponential-phase cells were serially diluted in 0.1× TSB and plated onto R2A and R2A containing Rp. The number of wild-type P9 CFU was calculated by subtracting the number of P9R CFU obtained on R2A containing Rp from the total number of CFU recovered on R2A. P9 and P9R were stored in 20% glycerol at −70°C.

Introduction of P9 and P9R into potato plants and plant growth. Two separate experiments were performed, using strain P9 in experiment 1 and P9R in experiment 2, to study the establishment of these two strains on the roots (rhizoplane) and inside the stem base (endosphere) of potato. Potato transplants were treated with freshly grown P9 and P9R cells by dipping their roots. For this purpose, in vitro-grown transplants of three potato cultivars, cultivars Eersteling (E plants), Robijn (R plants), and Karnico (K plants) (kindly provided by J. van der Haar, HZPC, Metslawier, The Netherlands), were incubated for 1 h with their roots in washed cell suspensions at a density of 1.8×10^8 P9 CFU per ml (P9 treatment) or 2.5×10^8 P9R CFU per ml (P9R treatment) or in sterile tap water (control treatment). Then the P9- and P9R-treated and control plants were separately planted in 3-liter pots containing potting soil (four pots per treatment per time). The plants were allowed to grow until they flowered (growth stage 6 [21]) in the greenhouse under the same light-darkness regimen. The plants were watered twice a week during the first 3 weeks and daily after 3 weeks, and the soil moisture was kept constant by weighing the pots before and after addition of tap water. Nutrients (NPK) were administered weekly to the plants at the time of watering to avoid nutrient deficiencies.

Sampling of potato plants, sample processing, and plating. Plants that appeared to be healthy and were in the juvenile stage (growth stage 1) and the flowering stage (growth stage 6), according to the potato development system described by Hack et al. (21), were harvested. Four replicate plants per cultivar per treatment were removed from the pots, and the soil was carefully shaken from the roots. Then the roots were washed under running tap water, and the adhering water was removed by blotting the roots on sterilized tissue paper. For each plant, parts of roots were cut off and mixed, after which approximately 1 g was transferred to a sterile plastic bag containing 3 ml tap water. The roots were homogenized by hammering, and the homogenates were considered "rhizoplane"' samples.

For each plant, part of the stem between 1 and 5 cm above the soil level (stem base) was removed and washed under running tap water. The washed parts of the stems were then treated by subsequent submersion in a 1.5% chlorine solution (1 min), in 96% ethanol (1 min), and finally in sterile tap water (1 min). The effectiveness of surface sterilization was checked by placing treated parts of stems on R2A and removing them after 5 min. The resulting surface-sterilized parts of stems (approximately 2 g) were transferred into sterile plastic bags containing 3 ml tap water and homogenized by the hammering procedure. The resulting homogenates were considered "endosphere" samples.

For plating, rhizoplane and endosphere samples were serially 10-fold diluted in a sterile 0.85% NaCl solution, after which dilutions were plated onto the following media: King's B agar (KBA) containing 20 g proteose peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot H_2O$, 10 g glycerol, 15 g agar, and 1 liter water and, after autoclaving, supplemented with the antibiotics (all obtained from Sigma-Aldrich) ampicillin (100 mg), chloramphenicol (13 mg), vancomycin (10 mg), and Delvo-

cid (active ingredient, natamycin; DSM Food Specialties, Delft, The Netherlands) (0.5 g) (experiment 1); R2A containing Rp and Delvocid (experiment 2); and R2A containing Delvocid (both experiments). The plates were incubated for 5 days at 27°C.

Only colonies showing morphological resemblance to P9 colonies were counted, and between one and five colonies selected from plates of all samples in both experiments were analyzed by BOX-PCR. The colonies producing fingerprints identical to that of strain P9 were considered P9 positive.

DNA extraction from rhizoplane and endosphere samples and PCR-denaturing gradient gel electrophoresis (DGGE) analyses. Total genomic DNA was extracted from late-exponential-phase cultures of P9 and P9R cells using the procedure of Ausubel et al. (3). Total rhizoplane and endosphere DNA was extracted using a DNeasy plant mini kit (Qiagen Benelux B.V., Venlo, The Netherlands) according to the manufacturer's protocol, and the resulting DNA pellets were dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). For bacterial PCR, a nested system was used. This system included primers F799 (5) and R1492 (39) in the first PCR and primers F968 and R1401 (27) with a GC clamp (32) in primer F968 in the second PCR. For *Pseudomonas*-specific PCR, a seminested setup was used, which included primers PsF and PsR in the first PCR and primers F968-GC and PsR in the second PCR as described by Garbeva et al. (19). PCR amplification was performed as described by van Overbeek and van Elsas (51).

DGGE was performed with PCR products from analyses of rhizoplane and endosphere DNA samples as described previously (17, 47). Gels were loaded with 50 ng of P9 and P9R and 200 ng of rhizoplane and endosphere amplicons per lane. After electrophoresis of the gels and staining with SYBR Gold (Molecular Probes, Leiden, The Netherlands), the gels were photographed under UV light, after which digitized fingerprints were analyzed and compared using GelcomparII software (Applied Maths, Belgium).

A conspicuous band that occurred in rhizoplane and endosphere fingerprints and comigrated with P9 and P9R bands was sliced out from a total of six fingerprints of samples obtained in both experiments. Additionally, bands at five other positions were sliced out from a total of 11 fingerprints of samples obtained in experiment 2. DNA was PCR amplified with relevant primers, and the resulting amplicons were cloned into the vector pGEM-T (Promega, Leiden, The Netherlands). Randomly selected clones (one clone from each of the 17 libraries) were PCR amplified with relevant primers and run in DGGE gels to reconfirm their positions in the gel before sequencing at the facilities of Greenomics (Wageningen, The Netherlands). The sequences obtained were examined to determine similarities with the 16S rRNA genes of either strain P9 or other bacteria. The DNA sequences were first checked for the presence of chimeras using the Check Chimera tool, and nonchimeric sequences were assessed to determine similarities to database sequences available at the NCBI website (http://www.ncbi.nlm.nih.gov) by BLAST searching.

Bands in the individual lanes were normalized by using a PCR-DGGE marker (51) loaded at four different positions in the gel. The number, mobility, and intensity of bands in each fingerprint were used as input variables for calculation of the Shannon diversity of the dominant populations and multivariate analyses.

Statistical and cluster analyses. The following factors were examined by using analysis of variance (Genstat, 10th ed.; Rothamsted Experimental Station, Harpenden, United Kingdom): (i) the effect of inoculation of E and R plants on *P. infestans* lesion growth, (ii) the effect of plant growth stage, cultivar, and treatment on the numbers of total and Rp-resistant CFU in rhizoplane and endosphere samples at both growth stages, and (iii) the effect of plant growth stage, cultivar, and treatment on bacterial and *Pseudomonas* Shannon diversity values calculated using PCR-DGGE fingerprints of rhizoplane and endosphere samples with Canoco software (Biometris, Wageningen, The Netherlands). PCR-DGGE fingerprints were also analyzed by multivariate analyses by considering plant growth stage, cultivar, and treatment "environmental" variables and the positions and intensities of individual bands in PCR-DGGE fingerprints "species" variables (46). For this purpose, separate modules of Canoco software were used for detrended correspondence analysis (DCA) (to calculate gradient lengths between "environmental" and "species" variables) and canonical correspondence analysis (CCA) (to establish eventual correlations between "environmental" and "species" variables). The significance of correlations between "environmental" and "species" variables was determined by inclusion of a Monte Carlo permutation test with 499 unrestricted permutations. Differences were considered significant at a P value of ≤ 0.05. Cluster analysis was performed for *Pseudomonas* PCR-DGGE fingerprints of rhizoplane and endosphere samples from both growth stages using the unweighted pair group method with arithmetic mean (UPGMA) algorithm in GelcompareII.

Nucleotide sequence accession number. The DNA sequence of the partial 16S rRNA gene of strain P9 (1,491 bp) has been deposited in the EMBL database (http://www.ebi.ac.uk) under accession number FM211694.

RESULTS

Characterization of strain P9 and of its Rp-resistant derivative P9R. The rates of *P. infestans* lesion growth in 13 different bacterially treated and control E plants were between 90 and 150 mm^2 per day, and the rates in bacterially treated and control R plants were between 30 and 100 mm^2 per day. The average lesion growth rate was about twofold higher for the control E plants (98 mm^2 per day) than for the R plants (52 mm^2 per day), which indicates that the resistance to *P. infestans* was higher in plants R plants than in E plants. No significant differences in lesion growth were found between control plants and plants treated with each of the 12 isolates tested for the R plants or between control plants and plants treated with each of the 13 isolates for the E plants. However, a significant difference was found between P9-treated R plants and all other bacterium-treated and control R plants. Specifically, on average, the *P. infestans* lesion growth in the P9-treated R plants was 45% (31 to 59%) less than that in control plants in three separate experiments, whereas no suppressive effect of P9 on *P. infestans* lesion growth was found in E plants in any experiment.

Testing of strain P9 with the phytopathogens *P. infestans*, *R. solani* AG3, and *R. solanacearum* biovar 2 on plates showed that there was a 1-mm zone of inhibition with *P. infestans* and no inhibition with the other two pathogens. PCR tests done with P9 genomic DNA revealed a positive signal for the locus for production of pyoluteorin, but no evidence was found for the presence of phenazine, phloroglucinol, and pyrrolnitrin biosynthetic loci.

On the basis of its almost complete 16S rRNA gene sequence (1,491 bp), strain P9 showed 99.5% similarity with *Pseudomonas putida* strain GB-1 (database accession number CP000926) and 99.3% similarity with two uncultured bacterium clones (database accession numbers DQ158114 and DQ158119). Based on a 526-bp stretch of the *gacA* gene, strain P9 showed the highest level of identity (87%) with *Pseudomonas entomophila* strain L48 (database accession number CT573326) and 86% identity with *P. putida* strain F1 (database accession number CP000712). The 16S rRNA genes of P9 and two *P. putida* type strains clustered separately from the 16S rRNA genes of all other *Pseudomonas* type strains (Fig. 1). In all likelihood, strain P9 belongs to *P. putida*.

The derivative of P9, strain P9R, had a 16S rRNA sequence identical to that of its wild-type parent strain; also, the BOX-PCR and repetitive extragenic palindromic PCR profiles of the two strains were identical. In a competition experiment with strains P9 and P9R in liquid medium without Rp, neither strain outcompeted the other after an estimated 30 generations of growth, indicating that the two strains were the same in terms of fitness under conditions that supported growth.

Plant growth and recovery of CFU from the rhizoplane and endosphere. Growth stage 1 occurred at 32 days after planting for all plants of the three potato cultivars in both experiments, whereas growth stage 6 occurred later for R and K plants (at 71 days in experiment 1 and at 73 days in experiment 2) than for

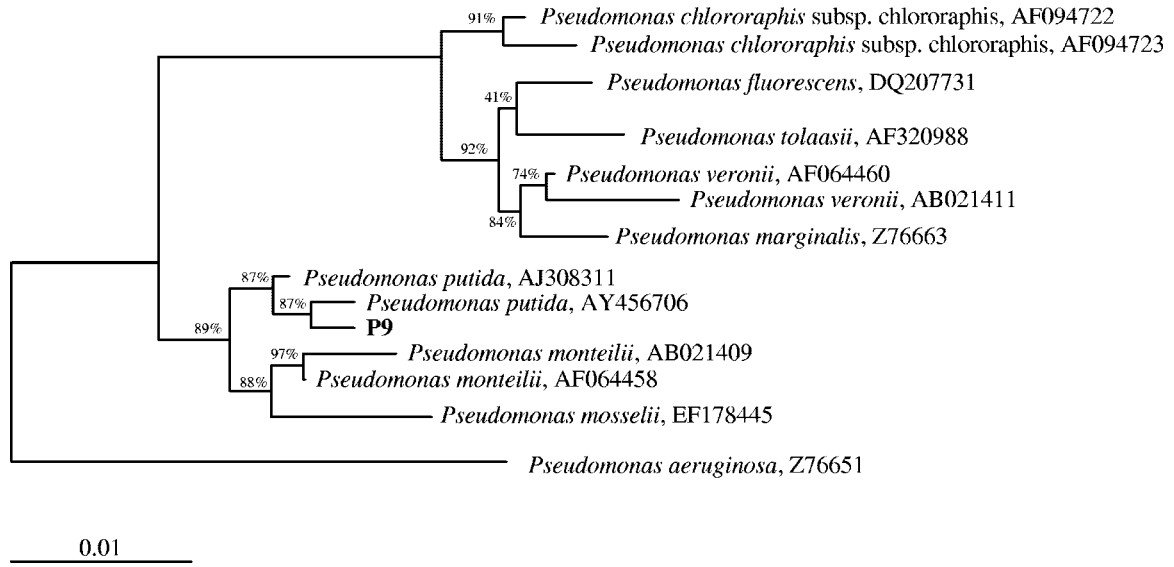

FIG. 1. Phylogenetic tree constructed using partial (1,491 bp) 16S rRNA gene sequences of strain P9 and 13 type strains of different *Pseudomonas* species. The partial 16S rRNA gene of *Pseudomonas aeruginosa* Z76651 was used as an outgroup, and boot strap values are indicated at the nodes.

E plants (at 59 days in experiment 1 and at 61 days in experiment 2). No differences in plant weight and height were observed between nontreated and P9- or P9R-treated plants at the two growth stages in both experiments.

(i) Experiment 1. At growth stage 1, the numbers of rhizoplane bacteria (on R2A) were between log 6.28 and log 6.56 CFU per g of fresh tissue, and at growth stage 6 they were between log 6.33 and log 7.08 CFU per g of fresh tissue. There were no significant differences in the total numbers of bacteria between cultivars or plant treatments at either growth stage. The numbers of rhizoplane fluorescent bacteria (on KBA) were between log 3.64 and log 4.89 CFU per g of fresh tissue at growth stage 1 and between log 3.16 and log 4.59 CFU per g of fresh tissue at growth stage 6. Individual fluorescent colonies with the morphology typical of P9 (i.e., white and slightly translucent with sharp edges) were observed for all undiluted samples of P9-treated plants taken at growth stage 1 and for 8 of 12 10-fold-diluted samples of P9-treated plants taken at growth stage 6. No colonies resembling P9 colonies were observed for undiluted (growth stage 1) and 10-fold-diluted samples (growth stage 6) of nontreated plants. The BOX-PCR fingerprints of 48 selected colonies resembling P9 colonies were all identical to the P9 BOX-PCR fingerprint. The calculated numbers of P9 bacteria in the rhizoplane at growth stage 1 were between log 3.52 and log 4.87 CFU per g of fresh tissue, and at growth stage 6 the levels were below the detection limit for P9-treated K plants (the limit of detection was log 3.2 CFU per g fresh tissue), log 3.40 CFU per g of fresh tissue for P9-treated R plants, and log 4.14 CFU per g of fresh tissue for P9-treated E plants. There were no significant differences in the numbers of P9 CFU between cultivars at both growth stages.

Standard checks for the effectiveness of surface sterilization of potato stems showed that there was no colony formation upon exposure of surface-sterilized stems to R2A, demonstrating that all of the stems analyzed had sterile surfaces. In the endosphere, the numbers of bacteria were between log 2.56 and log 5.15 CFU per g of fresh tissue for growth stage 1 and between log 2.57 and log 4.24 CFU per g of fresh tissue for growth stage 6. The numbers of bacteria were significantly higher in P9-treated K plants (log 5.15 CFU per g of fresh tissue) than in all other plants (between log 2.57 and 4.39 CFU per g of fresh tissue) at growth stage 1, whereas there were no significant differences in the numbers of CFU between cultivars or plant treatments at growth stage 6. The numbers of fluorescent bacteria were between log 2.25 and log 5.09 CFU per g of fresh tissue at growth stage 1 and between log 2.03 and log 4.87 CFU per g of fresh tissue at growth stage 6. Individual colonies resembling P9 colonies were found for 10 of 12 undiluted samples of P9-treated plants taken at growth stage 1 and for 2 of 12 10-fold-diluted samples taken at growth stage 6. No colonies resembling P9 colonies were observed for undiluted samples (growth stage 1) and 10-fold-diluted samples (growth stage 6) of nontreated plants. The BOX-PCR fingerprints of 23 selected colonies were all identical to that of P9. The numbers of P9 bacteria in the endosphere at growth stage 1 were between log 2.06 and log 4.80 CFU per g of fresh tissue, and at growth stage 6 the levels were below the detection limit for P9-treated E and R plants and log 3.20 CFU per g of fresh tissue for P9-treated K plants (two P9 colonies from different replicate samples). There were no significant differences in the numbers of P9 CFU between cultivars or plant treatments for the potato endosphere at both growth stages.

(ii) Experiment 2. At growth stage 1, the numbers of rhizoplane bacteria ranged from log 6.68 to log 7.65 CFU per g fresh tissue. The differences between cultivars were significant, but the differences between plant treatments were not significant; the interaction of cultivar and plant treatment also was not significant (Table 1). At growth stage 6, the numbers of rhizoplane bacteria were between log 6.86 and log 7.33 CFU per g root, and there were no significant effects of cultivar or treatment on the number of CFU. No Rp-resistant CFU were

TABLE 1. Numbers of rhizoplane and endosphere bacteria for nontreated and strain P9R-treated potato plants[a]

Growth stage	Plants (treatment)[b]	No. of rhizoplane bacteria (log CFU per g plant)[c]		No. of endosphere bacteria (log CFU per g plant)[c]	
		R2A	R2A with Rp	R2A	R2A with Rp
1	E	7.05 B	BD[d]	5.25 B	BD
	R	6.68 C	BD	6.10 A	BD
	K	7.65 A	BD	4.73 B	BD
	E (P)	7.19 B	4.45	6.04 A	3.48 A, Y
	R (P)	7.01 C	4.46	6.55 A	3.88 A, Y
	K (P)	7.50 A	4.99	5.00 B	2.09 B, Y
6	E	7.12	BD	3.90 B	BD
	R	7.06	BD	5.29 A	BD
	K	7.27	BD	5.13 A	BD
	E (P)	7.06	4.07	4.96 A	2.58 A, Z
	R (P)	7.33	4.38	4.97 A	2.06 A, Z
	K (P)	6.86	4.22	4.96 A	0.77 B, Z

[a] Plants were inoculated by dipping the roots in a preparation containing log 8.4 CFU/ml.
[b] P in parentheses indicates that plants were inoculated with P9R.
[c] R2A with Rp was selective for Rp resistance (Rp concentration, 50 μg/ml). Statistical differences ($P < 0.05$) between plants treated differently (cultivar and treatment) for growth stages are indicated, where A > B > C, as are statistical differences between growth stages for treated plants, where Y > Z.
[d] BD, below the detection limit, which was log 0.5 CFU per g plant.

found in any of the control (nontreated) rhizoplane samples, whereas Rp-resistant CFU were found in all samples from P9R-treated plants. The numbers of Rp-resistant bacteria in the rhizoplane of P9R-treated plants were between log 4.45 and log 4.99 CFU per g plant at growth stage 1 and between log 4.07 and log 4.38 CFU per g plant at growth stage 6. There were no morphological differences between Rp-resistant colonies, and the morphology of all of the colonies resembled the morphology of P9R colonies; also, no differences in BOX-PCR fingerprints were found for 48 colonies picked from potato rhizoplane plates. P9R was the only isolate recovered from all rhizoplane samples under Rp selection conditions.

At growth stage 1, the numbers of endosphere bacteria on R2A were between log 4.73 and log 6.55 CFU per g plant, and the numbers of bacteria in control R plants and P9R-treated E and R plants were significantly higher than the numbers in control E and K plants and in P9R-treated K plants. The numbers of endophytic bacterial CFU in P9R-treated E plants were significantly higher than the numbers in control E plants. The total numbers of endophytic bacteria at growth stage 6 were between log 3.90 and log 5.29 CFU per g plant tissue. Again, the numbers of CFU in P9R-treated E plants were significantly higher than the numbers of CFU in control E plants. The numbers of endophytic Rp-resistant bacteria in P9R-treated plants were between log 2.09 and log 3.88 CFU per g plant at growth stage 1 and between log 0.77 and log 2.58 CFU per g plant at growth stage 6. All Rp-resistant colonies had the morphology expected for P9R. The BOX-PCR fingerprints of 48 selected colonies were identical to that of P9R, indicating that P9R was the only organism from the endosphere that grew under Rp selection conditions. No Rp-resistant colonies were found in endospheres of control plants. In two P9R-treated plants (E and K plants) at growth stage 6, also no colonies were found. The numbers of endophytic P9R CFU were significantly higher in E and R plants than in K plants at both growth stages.

(iii) Comparison of experiments 1 and 2. The colony growth of fluorescent bacteria that were different from P9 hampered recovery of individual P9 colonies from rhizoplane and endosphere samples taken from growth stage 6 plants in experiment 1. This demonstrates that KBA is rather unselective for P9, making this medium less suitable for recovery of P9 in older plants. Therefore, it was decided to repeat the experiment with strain P9R using R2A amended with Rp for colony selection. This medium was selective for P9R, and P9R colonies were recovered from undiluted rhizoplane and endosphere samples of plants taken at growth stage 6 in experiment 2. Although P9 colonies were hardly detectable in endosphere samples of plants at growth stage 6, the presence of two P9 colonies in K plants demonstrates that P9 persists in the endosphere of potato, and this observation was confirmed in experiment 2 by using an isogenic relative of P9, P9R.

Molecular detection of strains P9 and P9R by PCR-DGGE. **(i) Experiment 1.** PCR-DGGE with bacterial and *Pseudomonas*-specific primers was performed with rhizoplane and endosphere DNA extracts to assess the fate of strain P9 during growth in different cultivars. A clear and conspicuous band comigrating with the P9 band was found in the bacterial and *Pseudomonas* rhizoplane and endosphere fingerprints of all P9-treated plants. DNA sequences identical to that of strain P9 were found in clones of all 10 libraries constructed from the bands from different PCR-DGGE fingerprints. This indicated that the band represented P9. The band representing P9 was not found in any fingerprint for nontreated plants. In the bacterial fingerprints for the rhizoplanes of P9-treated plants this band was found for all four replicates of E, K, and R plants at growth stage 1 and for four of the E plant replicates, three of the R plant replicates, and three of K plant replicates at growth stage 6. This band was also found in *Pseudomonas* fingerprints for rhizoplanes of all P9-treated plants. In the bacterial endosphere fingerprints, the band representing P9 was found in four replicates of E and K plants and in three replicates of R plants at growth stage 1 and in three replicates of K plants, two replicates of E plants, and two replicates of R plants at growth stage 6. This band was found in all *Pseudomonas* endosphere fingerprints for the three cultivars at both growth stages. Introduced strain P9 was always observed in *Pseudomonas* fingerprints, but this was not the case for bacterial fingerprints. Hence, the *Pseudomonas*-specific PCR-DGGE method was better suited for detection of strain P9 in the potato rhizoplane and endosphere.

(ii) Experiment 2. The strain P9R band comigrated with the P9 band in PCR-DGGE performed with bacterial and *Pseudomonas*-specific primers. The band representing P9R was not found in any fingerprint for nontreated plants. In the bacterial fingerprints for the rhizoplane of P9R-treated plants this band was found in all four replicates of E, K, and R plants at growth stage 1 and in four replicates of E plants, four replicates of R plants, and two replicates of K plants at growth stage 6. This band was also found in all *Pseudomonas* fingerprints for rhizoplanes of P9R-treated plants. In the bacterial endosphere fingerprints, the band representing P9R was found in two replicates of E plants, two replicates of K plants, and four repli-

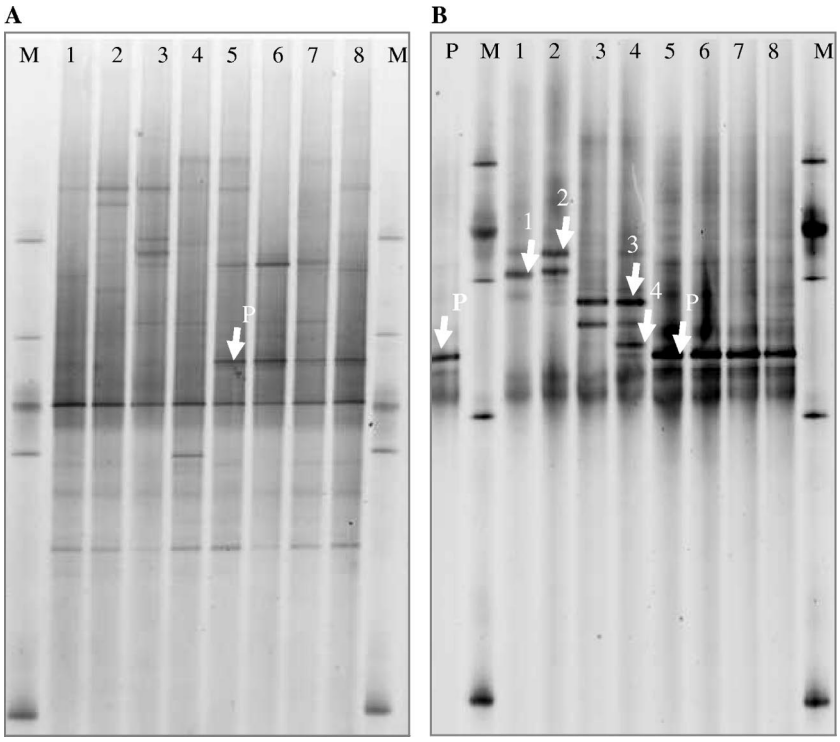

FIG. 2. PCR-DGGE fingerprints obtained with bacterial primers (A) and *Pseudomonas* primers (B) for the endosphere of nontreated (lanes 1 through 4) and P9R-treated (lanes 5 through 8) R plants at growth stage 6. Lanes M contained the bacterial marker, and lane P contained the PCR-amplified products from strain P9R cells. Arrows P indicate the band representing P9R, arrow 1 indicates the *P. azotoformans* band, arrow 2 indicates the *P. proteolytica* band, arrow 3 indicates the *P. syringae* band, and arrow 4 indicates the *P. veronii* band.

cates of R plants at growth stage 1 and in three replicates of E plants, two replicates of K plants, and one replicate of R plants at growth stage 6. This band was found in all *Pseudomonas* endosphere fingerprints of P9R-treated plants (Fig. 2).

(iii) Comparison between experiments 1 and 2. P9 and P9R were always detected in *Pseudomonas* PCR-DGGE fingerprints of treated plants belonging to the three cultivars. This demonstrates the consistency of the endophytic colonization of strain P9 and its isogenic relative, P9R, in two separate experiments. In experiment 1, the poor recovery of P9 colonies from treated plants at growth stage 6 did not allow further quantitative comparisons between the cultivation-dependent and -independent approaches. In experiment 2, it was possible to make such a comparison.

Comparison between numbers of culturable P9R cells and P9R-specific band intensities for PCR-DGGE. There was consistency in the presence of P9R CFU and the P9R-specific band in *Pseudomonas* PCR-DGGE fingerprints in most (46 of 48) samples. However, for two replicate endosphere samples (one from an E plant and one from a K plant, both at growth stage 6), no P9R CFU were recovered, whereas a P9R-specific band was present in the *Pseudomonas*-specific fingerprints. This may indicate that P9R cells were present in a nonculturable form inside the plants.

The quantitative relationship between the number of P9R CFU and P9R-specific PCR-DGGE band intensity was further assessed by using linear regression analysis. Linear correlations were found for all bacterial and *Pseudomonas* rhizoplane and endosphere samples (Table 2). However, the slopes of the regression lines and corresponding constant values differed for different PCR primer systems; the slopes of the lines calculated on the basis of P9R band intensity in bacterial fingerprints were steeper, whereas the corresponding constant values were lower than those for *Pseudomonas* fingerprints. This indicates that more sensitive detection of P9R cells in rhizoplane and endosphere samples is possible with *Pseudomonas* PCR-

TABLE 2. Correlation between numbers of P9R CFU and intensities of P9R-specific bands in bacterial and *Pseudomonas* PCR-DGGE fingerprints[a]

Location	Community	*P* value	% of variance accounted for	Slope (SE)	Constant value (SE)
Rhizoplane	Bacteria	0.003	30.2	10.7 (3.2)	−24.8 (14.4)
Rhizoplane	*Pseudomonas*	0.002	32.7	6.0 (1.7)	−0.58 (5.0)
Endosphere	Bacteria	<0.001	39.0	20.8 (5.2)	−50.2 (23.3)
Endosphere	*Pseudomonas*	0.059	11.4	6.9 (3.5)	40.6 (10.2)

[a] P9R-specific band intensity was used as the response variable.

DGGE than with bacterial PCR-DGGE. Correlations were significant on three of four occasions, whereas for the correlation between the number of P9R CFU and the *Pseudomonas* band intensity for endosphere samples, the *P* value was close to the arbitrary level of significance. The explained variance was less than 40% on all occasions, indicating that other factors also are responsible for variation in P9R band intensities in PCR-DGGE gels.

Effect of growth stage, cultivar, and P9R treatment on microbial communities in the potato rhizoplane and endosphere. The effects of growth stage, treatment, and cultivar on bacterial and *Pseudomonas* communities were further assessed using PCR-DGGE fingerprints obtained in experiment 2. Rhizoplane and endosphere PCR-DGGE fingerprints were analyzed by analysis of variance using Shannon diversity estimates of dominant types, by multivariate analyses using DCA and CCA of "environmental" and "species" variables, and by UPGMA cluster analysis of *Pseudomonas* fingerprints. The P9-specific bands were omitted from all fingerprints to establish the effect of treatment alone on resident microbial communities. Bands in *Pseudomonas* endosphere fingerprints that were present for control plants but not for the corresponding P9R-treated plants were identified by DNA sequencing and BLAST-assisted searches of the NCBI database.

(i) Estimates of the diversity of dominant types. The estimates of the bacterial and *Pseudomonas* diversity of dominant types calculated using replicate rhizoplane and endosphere fingerprints varied between 1.49 and 2.02 for the bacterial rhizoplane fingerprints, between 0.85 and 1.41 for the *Pseudomonas* rhizoplane fingerprints, between 0.86 and 1.81 for the bacterial endosphere fingerprints, and between 0.20 and 1.14 for the *Pseudomonas* endosphere fingerprints. There was an overall significant effect of growth stage on the bacterial and *Pseudomonas* fingerprints for the rhizoplane and on the *Pseudomonas* fingerprints for the endosphere in both experiments, and there was a significant effect of cultivar only on the bacterial fingerprints for the endosphere (not shown). No significant effect of treatment on the bacterial and *Pseudomonas* fingerprints for the rhizoplane and endosphere was found.

(ii) Multivariate analysis. For multivariate analysis, DCA was performed with all fingerprints from experiment 2. The calculated gradient lengths were between 4.58 and 5.25, which justifies the use of CCA. CCA done with the bacterial and *Pseudomonas* fingerprints for rhizoplanes and endospheres of all plants revealed clear effects of plant growth stage, cultivar, and treatment. These effects were significant for all of the samples except the bacterial rhizoplane and endosphere samples, where the effects of cultivar (rhizoplane) and treatment (endosphere) were just above the arbitrary level of significance ($P = 0.062$ for both). The variance explained by these variables differed by community; for the bacterial rhizoplane and endosphere fingerprints, most of the variation was explained by plant growth stage (63 and 73%, respectively), whereas for *Pseudomonas* rhizoplane fingerprints the most important variable was the cultivar selected (39%) and for the *Pseudomonas* endosphere fingerprints the most important variable was plant treatment (51%). The greatest effect of P9R treatment was found for the *Pseudomonas* community of the endosphere.

(iii) Cluster analysis. The effect of treatment on the *Pseudomonas* fingerprints for the rhizoplane and endosphere of all plants in experiment 2 was further assessed by UPGMA cluster analysis. Comparisons of *Pseudomonas* rhizoplane and endosphere fingerprints for nontreated and P9R-treated plants revealed consistent separate clustering based on plant treatment. In two cases (E plant endosphere at growth stage 1 and K plant endosphere at growth stage 6), three of four replicate fingerprints for P9R-treated plants clustered together, whereas in all other cases all four replicate fingerprints clustered together (Fig. 3). This indicates that there was a clear effect of treatment on the composition of *Pseudomonas* communities in the rhizoplane and endosphere, irrespective of the plant growth stage and cultivar selected. Inoculation with strain P9R thus has a clear effect on the composition of *Pseudomonas* communities in the rhizoplane and endosphere of growing potato plants.

(iv) Band identification. Five clear and distinguishable bands present in *Pseudomonas* endosphere fingerprints for nontreated E, K, and/or R plants were absent in the fingerprints for all P9R-treated plants at both growth stages (Fig. 2). Three conspicuous bands, identified as *Pseudomonas azotoformans*, *Pseudomonas syringae*, and *Pseudomonas proteolytica*, were found for nontreated plants of all three cultivars at growth stage 1 and for two or three cultivars at growth stage 6 (Table 3). The absence of these bands for all P9R-treated plants indicates that colonization by these species must be affected by P9R. Other species whose colonization was affected by P9R were found in plants of only one cultivar, and these species were identified as *Pseudomonas azotoformans*/*Pseudomonas fluorescens* and *Pseudomonas veronii*.

DISCUSSION

Strain P9 showed a close resemblance to two uncultured *Pseudomonas* species from soil that were involved in phenol degradation (9). This close resemblance between isolates with such different functions may seem remarkable, but it emphasizes the possible common occurrence of the P9 type of *P. putida* species in different ecological niches. The presence of plant-associated *Pseudomonas* types in soil is common, and whether these types can be distinguished from types that are strictly bound to soil is still being debated (15, 33, 53).

P9 exhibits antagonism against *P. infestans*, and it has been shown that a locus homologous to the pyoluteorin production locus is present in strain P9. It has been demonstrated that pyoluteorin production by *P. fluorescens* strain Pf-5 suppresses growth of *Pythium ultimum* but not the growth of *R. solani* (28). Like strain Pf-5, our strain P9 was able to suppress the growth of an oomycete species, *P. infestans* but not the growth of *R. solani* AG 3, and this makes it likely that P9 possesses an active pyoluteorin production locus. Putative pyoluteorin production by P9 may play a role in the resistance to *P. infestans* in R plants. This possibility should be examined further in studies of strain P9 in potato plants.

Rhizoplane and endosphere colonization of potato plants by P9 and its isogenic relative P9R was demonstrated by using a polyphasic approach (48), including a culture-dependent and -independent detection technique. KBA appeared to be rather unselective for P9, especially when plants were in the flowering stage, which was not the case for R2A amended with Rp, which appeared to be highly selective for P9R. A quantitative relationship between numbers of P9R CFU and P9R-specific band

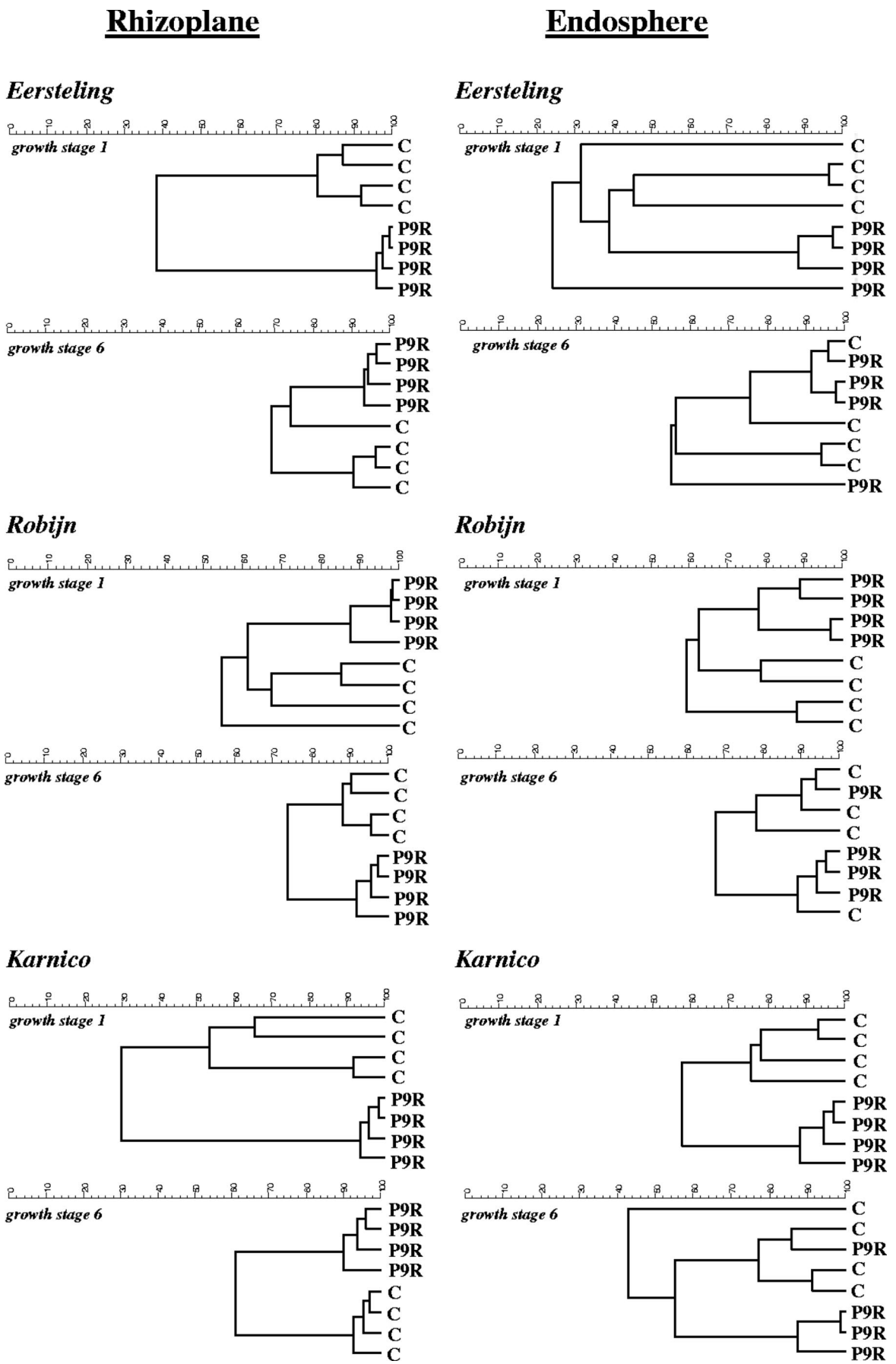

FIG. 3. Dendrograms obtained by using UPGMA for rhizoplane and endosphere PCR-DGGE fingerprints obtained with *Pseudomonas* primers.

TABLE 3. Identities of *Pseudomonas* species whose growth is suppressed by strain P9R in the potato endosphere

Closest match (% similarity)		No. of bands[a]					
		Growth stage 1			Growth stage 6		
Type strain	Non-type strain	E plants	K plants	R plants	E plants	K plants	R plants
P. azotoformans (100)		2	4	1	1	3	1
P. plecoglossicida (99.7)	*P. syringae* (100)	2	2	2		1	2
P. proteolytica (99.7)		1	1	2		2	2
P. azotoformans (96.8)	*P. fluorescens* (96.8)		2			1	
P. veronii (100)				2			1

[a] Numbers of bands (of four) in PCR-DGGE fingerprints that were specifically found for the *Pseudomonas* endosphere of nontreated plants and were not present for the P9R-treated plants.

intensities was found in all but two replicate samples, emphasizing the coherence of the two methods for detection of P9R cells in the potato rhizoplane and endosphere. This demonstrates that phenotypic selection of P9R by recovery of CFU on agar medium with Rp was an appropriate method to follow the fate of the introduced strain in the rhizoplane and endosphere of growing potato plants. This is in contrast to previous reports that claimed that endophytic strains carrying Rp resistance would be "masked" upon recovery from the endospheres of cucumber and cotton plants (31). In our study, discrepancies were found for only two plants at growth stage 6, where no P9R CFU were found but a conspicuous P9R-specific band was present in the *Pseudomonas* fingerprints for the same samples. This may have indicated the presence of nonculturable P9R cells in these samples. The possible appearance of nonculturable P9R cells inside potato stems at flowering coincided with a decrease in the number of P9R CFU. The appearance of nonculturable endophytic cells has been demonstrated previously for an avirulent form of *R. solanacearum* biovar 2 strain 1609 inside tomato plants (49) and may indicate that endophytes encounter stress even in their favored habitat (namely, inside plants).

Clear effects of plant growth stage and cultivar on potato-associated bacterial and *Pseudomonas* communities and P9R populations were observed. Plant growth stage had the greatest effect on indigenous and P9R populations in both the rhizoplanes and endospheres of potato plants, which confirms observations made previously, in which growth stage was the strongest factor affecting indigenous plant-associated communities in field-grown potato plants (51). Plant genotype was another strong factor governing the plant-associated communities and resident P9R populations, corroborating previous data (43, 45, 51). Effects on the composition of plant-associated communities caused by dominating factors like plant growth stage and genotype might override most factors, including P9R inoculation. Nevertheless, clear effects attributable to plant inoculation with P9R on rhizoplane and endosphere communities were observed.

The greatest impact of P9R was its impact on potato-associated *Pseudomonas* populations. This indicates that P9R affected its closest relatives in the potato endosphere most. From the PCR-DGGE bands absent in the potato endosphere when P9R was present it became clear that five species were clearly suppressed by P9R, three of which are common residents of potato plants, as deduced from their presence in all three cultivars. Two of these species, *P. azotoformans* and *P. syringae*, may contribute to plant health either in a positive way (*P. azotoformans*) or in a negative way (*P. syringae*). *P. azotoformans* is a species known to fix nitrogen (2, 55), and it is tempting to speculate that this species may be involved in potato plant growth promotion. Representatives of the *P. syringae* species complex live as commensals or even as pathogens in many different plant species, and in one species it was shown to cause bacterial speck in a taxonomically close relative of potato, tomato (25). Suppression of the growth of both species by P9 in the potato endosphere must be considered an important side effect of the application of biocontrol agents; the growth of other plant-associated bacteria with proposed plant-growth-promoting or pathogenic activities can be suppressed as well. In this study it is demonstrated that disease-suppressing effects caused by application of a potential biocontrol agent result in a shift in plant-associated communities, leading to presumed divergent effects on plant growth and health.

The impact of bacterial inoculation on plant-associated communities is a phenomenon that is not commonly studied. On only one other occasion has the impact of bacterial inoculation on plant-associated community composition been found, when Madagascar periwinkle (*C. roseus*) and Cleveland tobacco (*N. clevelandii*) plants were treated with cells of *M. mesophilicum* strain SR1.6/6 (1). Inoculum-induced shifts in plant-associated microbial communities may even be a valuable side effect of application of biological control agents, as such application might result in replacement of commensal or even deleterious populations with a mutualistic population.

In conclusion, this study demonstrates the importance of the ecology of (proposed) biological control strains for obtaining insight into the performance of these strains under conditions realistic in the field. The effect of an introduced competent endophyte on the natural endophytic community structure in potato plants was shown. We surmise that notable effects on plant growth and resistance caused by inoculation of biological control strains often are attributable not only to the strain itself but also to inoculation-driven shifts in plant-associated communities.

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