The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site

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Abstract

Rhizobacteria with antagonistic activity towards plant pathogens play an essential role in root growth and plant health and are influenced by plant species in their abundance and composition. To determine the extent of the effect of the plant species and of the site on the abundance and composition of bacteria with antagonistic activity towards *Verticillium dahliae*, bacteria isolated from the rhizosphere of two *Verticillium* host plants, oilseed rape and strawberry, and from bulk soil were analysed at three different locations in Germany over two growing seasons. A total of 6732 bacterial isolates screened for *in vitro* antagonism towards *Verticillium* resulted in 560 active isolates, among which *Pseudomonas* (77%) and *Serratia* (6%) were the most dominant genera. The rhizosphere effect on the antagonistic bacterial community was shown by an enhanced proportion of antagonistic isolates, by enrichment of specific amplified ribosomal DNA restriction analysis types, species and genotypes, and by a reduced diversity in the rhizosphere in comparison to bulk soil. Such an effect was influenced by the plant species and by the site of its cultivation. Altogether, 16S rRNA gene sequencing of 66 isolates resulted in the identification of 22 different species. Antagonists of the genus *Serratia* were preferentially isolated from oilseed rape rhizosphere, with the exception of one site. For isolates of *Pseudomonas* and *Serratia*, plant-specific and site-specific genotypes were found.

Introduction

The rhizosphere was defined by Hiltner in 1904 as the portion of soil influenced by the root, where processes mediated by microorganisms which are of central importance for plant nutrition and health take place. The rhizosphere effect describes the phenomenon that, in comparison to bulk soil, the biomass and activity of microorganisms is enhanced as a result of exudation of compounds by the root (Lynch, 1990; Sørensen, 1997).

Extremely complex interactions occur in the rhizosphere (Whipps, 2001). The interactions between soil-borne pathogens and their antagonistic counterparts are of fundamental importance for plant nutrition and health. Our model pathogen is one of the most important soil-borne fungi, *Verticillium dahliae* Kleb., which causes Verticillium wilt, which is responsible for high yield losses in a broad host range, including many important crops such as strawberry and oilseed rape (Tjamos *et al.*, 2000). With the impending phase-out of the fumigant methyl bromide worldwide, there is no possibility to suppress this pathogen (Martin, 2003). Antagonists are naturally occurring organisms with traits enabling them to interfere with pathogen growth, survival, infection or plant attack (Chernin & Chet, 2002). Mechanisms responsible for antagonistic activity include: (1) inhibition of the pathogen by antibiotics, toxins and biosurfactants (antibiosis); (2) competition for colonization sites, nutrients and minerals; (3) parasitism that may involve production of extracellular cell-wall-degrading enzymes such as chitinase and β-1,3 glucanase; and (4) mycophagy (Fravel, 1988; Bloemberg & Lugtenberg, 2001; Whipps, 2001; De Souza *et al.*, 2003; De Boer *et al.*, 2005). Antagonistic bacteria play an important role in the suppression of soil-borne plant diseases, and can be used as biological

control agents (Whipps, 1997; Emmert & Handelsman, 1999; Weller *et al.*, 2002). Therefore, knowledge of the autochthonous antagonistic potential and of the factors by which it is influenced is the key for a successful and reproducible biological control.

Several studies have identified different biotic and abiotic factors influencing the structural and functional diversity of bacterial communities (Siciliano, 2001; Heuer *et al.*, 2002; Kowalchuk *et al.*, 2002; Reiter *et al.*, 2002; Graner *et al.*, 2003; Garbeva *et al.*, 2004; Sessitsch *et al.*, 2004). Studies in which the effect of soil or plant type was analysed were recently reviewed by Garbeva *et al.* (2004). For example, the occurrence of *Pseudomonas* genotypes in the rhizosphere of flax and tomato was clearly plant-species-dependent (Lemanceau *et al.*, 1995). In a previous 3-year field study, the effects of plant species on the rhizosphere-associated bacterial communities and on bacterial antagonists of *Verticillium* host plants were analysed at one location in Germany. The proportion and composition of bacterial antagonists of potato, oilseed rape and strawberry was shown to be influenced by the plant species and growth stage (Berg *et al.*, 2002). The strawberry rhizosphere was characterized by a high proportion and a low diversity of *Verticillium* antagonists. In contrast, a lower proportion and a high diversity of bacterial antagonists were obtained from the rhizosphere of oilseed rape and potato. Plant specificity of the rhizosphere-associated bacterial communities was also shown by analysing 16S rRNA gene fragments amplified from community DNA and separated by denaturing gradient gel electrophoresis (DGGE) (Smalla *et al.*, 2001). Thus, although it is known that plant species influence the structure of the antagonistic bacterial community, the extent to which this plant specificity can be observed at different sites is still unclear.

The objective of this work was to analyse the rhizosphere effect and the diversity of rhizosphere-associated antagonistic bacteria of two different *Verticillium* host plants, strawberry and oilseed rape, in comparison to bulk soil at three different sites, and to determine the extent to which the plant species and the site influence the proportion and composition of antagonistic bacteria in the rhizosphere. Bacteria were isolated from R2A or enrichment in high-molecular-weight substrates from the rhizosphere and bulk soil over a 2-year period (2002 and 2003) at three different growth stages of both host plants (young, flowering and senescent plants) at three locations in Germany (Berlin, Braunschweig and Rostock). A total of 6732 bacterial isolates were screened for antagonistic activity towards *V. dahliae*. Antagonists were genotypically characterized by amplified ribosomal DNA restriction analysis (ARDRA) and BOX patterns, and representatives of each ARDRA type, location and microenvironment were identified by partial 16S rRNA gene sequencing.

Materials and methods

Experimental design

Two different crop plants, oilseed rape (*Brassica napus* L. (family: *Brassicaceae*)) cv. Licosmos and strawberry (*Fragaria* × *ananassa* (Duchense) Decaisne & Naudin (family: *Rosaceae*)) cv. Elsanta were grown in a randomized block design with four replicates per crop plant during two vegetation periods (2002, 2003). The field trials were located at Berlin (52°31′N, 13°24′E), Braunschweig (52°16′N, 10°31′E) and Rostock (54°05′N, 12°07′E). Soil parameters for all locations were analysed by the Institute for Agricultural Analysis and Research (LUFA, Rostock, Germany). In Berlin, the soil texture was sand with pH = 6.4, 2.3% organic matter and 5% clay, and with the following contents of nutrients (mg $100\,g^{-1}$ soil): P_2O_5 51, K_2O 12, Mg 7. In Braunschweig, the soil texture was weakly loamy sand with pH = 5.6, 1.6% organic matter and 6% clay, and with the following contents of nutrients (mg $100\,g^{-1}$ soil): P_2O_5 35, K_2O 19, Mg 7. In Rostock, the soil texture was weakly loamy sand with pH = 6.0, 2.6% organic matter and 10% clay, and with the following contents of nutrients (mg $100\,g^{-1}$ soil): P_2O_5 41, K_2O 12, Mg 5.

Isolation of bacterial strains and determination of CFU

Plant roots with adhering soil taken from five or more plants per plot were sampled into sterile Stomacher bags and treated as one sample. Prior to cell extraction, 5 g of each pooled sample were transferred into a new Stomacher bag (Interscience, St Nom, France). Samples were extracted in a Stomacher laboratory blender (BagMixer, Interscience). Twenty-five millilitres of de-mineralized water were added to a 5 g rhizosphere sample. After 1 min of treatment (BagMixer), the supernatant was decanted into a 50 mL tube. The Stomacher treatment was repeated for 1 min after adding 20 mL of demineralized water to the sample, and the resulting supernatant was also decanted into the 50 mL tube. An aliquot was taken from the combined supernatants, serially diluted and plated onto R2A, a nutrient-poor medium suitable for the growth of diverse plant-associated bacteria (Difco, Detroit, MI). Plates were incubated for 5 days at 20 °C, and CFU were counted to calculate the means of colonies ($\log_{10}$ CFU) based on fresh weight of root or soil. In total, 96 isolates per treatment, location, sampling time and year were randomly selected. Only in 2002, bacteria were enriched in microtiter plates with high-molecular-weight substrates (xylan: Merck, Darmstadt, Germany; casein: Gibco, Paisley, UK; and chitin: Sigma, Deisenhofen, Germany) to obtain bacteria with hydrolytic activities. The plates were filled with: $1.5\,g\,L^{-1}$ peptone of casein (Gibco),

$0.5\,g\,L^{-1}$ peptone of soya (Gibco), $0.5\,g\,L^{-1}$ NaCl (pH 7.3), $0.5\,g\,L^{-1}$ azurine-dyed, cross-linked (AZCL) substrates, chitin and casein. The combined supernatants of six replicates per treatment were inoculated in serial dilutions. The contents of the wells of the last completely grown row were combined after 5 days of incubation, and plated after serial dilution on R2A. Fifteen to 20 colonies were randomly selected per high-molecular-weight substrate, purified and stored in broth containing 15% glycerol at $-70\,^{\circ}C$. Isolated bacteria were encoded by a combination of numbers and letters indicating: (1) location (B = Berlin, BS = Braunschweig, R = Rostock); (2) microenvironment (B = bulk soil, E = rhizosphere of strawberry, R = rhizosphere of oilseed rape); (3) sampling time (1 = young plants 2002, 2 = flowering plants 2002, 3 = early senescent plants 2002, 4 = young plants 2003, 5 = flowering plants 2003, 6 = early senescent plants 2003); (4) number of the plot; and (5) consecutive number of the isolate per plant.

Screening of antagonistic bacteria

Bacterial isolates were screened for their activity towards *Verticillium dahliae* Kleb. 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 (Chemex, München, Germany), 5 g NaCl (Merck), 20 g agar (Difco), distilled water (to 1 L), pH 6.8. Zones of inhibition were measured after 5 days of incubation at 20 °C according to Berg *et al.* (2002). All strains were tested in three independent replicates, with *Verticillium dahliae* V25 isolated from *Brassica napus* L. and *Verticillium dahliae* V35 isolated from *Fragaria* × *ananassa* [Duchense] Decaisne & Naudin (Messner *et al.*, 1996).

Identification of antagonistic bacteria

Bacterial DNA was extracted following the protocol of Anderson & McKay (1983), modified for genomic DNA. Small-subunit rRNA gene fragments were amplified with a TGradient thermocycler (Biometra, Göttingen, Germany). The 50 µL reaction mixture contained at least 10 µL PCR SuperMIX Taq & Go (Qbiogene, Carlsbad, CA), 1 µL of primer EubI-forward (5′-GAG TTT GAT CCT GGC TCA G-3′), 1 µL EubII-reverse (5′-AGA AAG GAG GTG ATC CAG CC-3′) and 1 µL template. The PCR products were purified with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), as recommended by the manufacturer. The amplicons were sequenced using the DTCS CEQ™ Quick Start Kit (Beckman Coulter, Fullerton, CA). The fragments were sequenced with Beckman Coulter System using the forward primer EubI. The sequences were edited and aligned with CEQ™ 2000 XL Analysis Systems and, for phylogenetic analysis and identification of related sequences, a BLAST algorithm according to Altschul *et al.* (1997).

ARDRA was used to group isolates at the genus level. Aliquots of PCR products containing 200 ng of amplified DNA were digested with 5 units of the endonuclease *Hha*I (Gibco) for 3 h at 37 °C. The resulting DNA fragments were analysed by gel electrophoresis in 2.5% agarose gels.

BOX-PCR fingerprints

BOX-PCR was performed as described by Rademaker & De Bruijn (1997) using the BOXA1R primer 5′-CTA CGG CAA GGC GAC GCT GAC G-3′. PCR amplification was performed with a TGradient thermocycler (Biometra). A 12-µL aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels as described in Berg *et al.* (2002). The reproducibility of the results was verified in at least two independent experiments. The genotypic diversity of the bacterial communities was measured by the Shannon information theory function (Shannon & Weaver, 1949). Instead of species, the isolates with a similarity of more than 80% were put together and characterized as one genotype group. According to the formula, the coefficient between the number of genotype groups, instead of the originally used number of species, and the number of isolates indicates the diversity in a sample. The diversity index $\langle H' \rangle$ is expressed on an unlimited scale, where high numbers represent a high diversity.

Statistics

All statistical analyses were performed at $P < 0.05$. Bacterial counts were $\log_{10}$-transformed before they were analysed. All data (CFU, percentage of *Verticillium* antagonists, diversity indices) were analysed for two-factor analysis of variance and significance using the χ^2 test and ANOVA by Statistical Product and Service Solutions for Windows, release 9.0.1. (SPSS Inc., Chicago, IL). BOX-PCR-generated fingerprints were evaluated in a computer-assisted manner by the GELCOMPAR program, version 4.1 (Applied Maths, Kortrijk, Belgium). The cluster analysis was performed with a Pearson correlation matrix and the unweighted pair-group method using arithmetic averages (UPGMA) algorithm. Finally, collector's curves for amplified rDNA restriction analysis (ARDRA) and BOX patterns (the number of patterns detected vs. the number of strains analysed) were constructed to compare the diversity between locations and microenvironments.

Nucleotide sequence accession numbers

Sequence accession numbers for sequences submitted to the EMBL nucleotide sequence database are AJ851191–AJ851214 and AJ852031–AJ852072.

Results

Isolation of bacteria from rhizosphere and bulk soil

CFU counts on R2A were in the range of $\log_{10}$ 7.5 CFU g^{-1} and 8.5 CFU g^{-1} root or soil fresh weight (fw). The CFU numbers determined for rhizosphere samples were rather similar for the different plants (strawberry, $\log_{10}$ 7.63 ± 0.18 CFU g^{-1} root fw; oilseed rape, $\log_{10}$ 8.0 ± 0.15 CFU g^{-1} root fw). In comparison to bulk soil ($\log_{10}$ 7.33 ± 0.19 CFU g^{-1} root fw), the CFU counts were significantly enhanced in both rhizospheres at all sites. The bacterial counts were higher at the end of the vegetation time, especially in the rhizospheres. In bulk soil, no significant seasonal changes were found. A total of 96 isolates per treatment (strawberry, oilseed rape and bulk soil), location (Berlin, Braunschweig and Rostock), sampling time (young, flowering and senescent plants) and year (2002, 2003) was used in the initial screening of bacteria with antagonistic activity towards *V. dahliae* Kleb. In addition, 96 isolates were obtained after enrichment from each substrate (xylan, casein and chitin) per sampling time.

Screening for isolates antagonistic to *Verticillium dahliae*

In total, 6732 bacterial isolates which were directly isolated from R2A (4459) or obtained after enrichment in high molecular weight substrates (2273) were screened for their ability to suppress *V. dahliae* in a dual-culture *in vitro* assay.

From R2A agar, 341 (= 7.7%) isolates were found which suppress *Verticillium* growth by inhibition of mycelium or microsclerotia formation. The proportion of antagonistic isolates with antagonistic activity was rather different for each site and microenvironment. Their proportion varied between 4% and 13% and was highest on average for Braunschweig ($11.1 \pm 0.57\%$; $n = 133$), followed by Berlin ($9.8 \pm 0.41\%$, $n = 103$) and Rostock ($7.8 \pm 0.36\%$; $n = 105$). The proportion of isolates with antifungal activity was significantly lower for bulk soil ($4.2 \pm 0.27\%$) than for both rhizospheres ($9.6 \pm 0.36\%$). A higher proportion of antagonists was found on average in the strawberry rhizosphere ($10.2 \pm 0.51\%$) than in the rhizosphere of oilseed rape ($8.9 \pm 0.52\%$), although this difference was statistically not significant. Additionally, a seasonal shift was observed for the proportion of antagonists in both rhizospheres. The highest proportion of antagonists in the strawberry rhizosphere was observed at the first sampling (Fig. 1), whereas the number of antagonists decreased slightly during flowering and at the end of the growing season. The highest proportion of antagonists in the oilseed rape rhizosphere was found during flowering of plants. The proportion of antagonists in bulk soil was significantly lower compared with both rhizospheres and was nearly constant over the growing season.

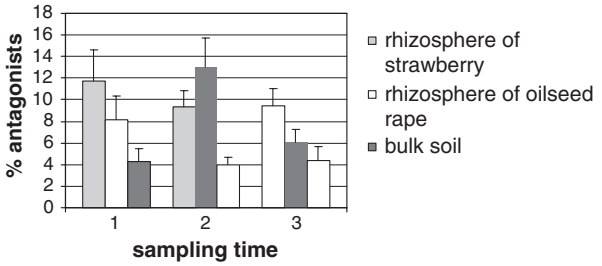

Fig. 1. Proportion of *in vitro Verticillium* antagonists determined in dual-culture assay in the rhizosphere of strawberry and oilseed rape and bulk soil during two vegetation periods (2002, 2003).

After enrichment in high-molecular-weight substrates, the proportion of bacteria with antagonistic activity against *Verticillium* was on average higher (9.7% = 219 isolates) after enrichment in high-molecular-weight substrates. However, the number of antagonists varied among different carbon sources, locations and microenvironments. The carbon source from which the highest proportion of antagonistic bacteria was obtained was casein (14.2%), followed by xylan (10.4%) and chitin (8.0%). In addition, a strong location-dependent effect was found. The highest proportion of antagonistic bacteria was detected in soil from the Berlin site (19.3%), whereas in Rostock and Braunschweig a lower proportion was recovered (8.4% and 4.9%, respectively). In soil samples from Berlin and Rostock, most of the antagonistic bacteria were isolated from casein enrichment, while in Braunschweig xylan was the carbon source from which the highest proportion of antagonists was obtained. In addition, differences between the investigated microenvironments were observed. Two-fold more *Verticillium* antagonists were obtained from casein and xylan enrichment of the rhizospheres in comparison to bulk soil, whereas the number of antagonists enriched in chitin was nearly the same for all habitats. Altogether, the statistical analysis of all data showed a statistically significant influence ($P < 0.05$) of the location and of the investigated microenvironment on the proportion of antagonists.

Characterization and identification of antagonistic bacterial isolates

A total of 468 bacterial isolates obtained from R2A plates (273) and enrichment procedures (195) was characterized by 16S rRNA RFLP and could be assigned to 13 ARDRA types (A–M). Eleven different ARDRA types were found for antagonists isolated from R2A and nine different types for those originating from enrichment in high-molecular-weight substrates (Table 1). In addition, the composition of

Table 1. Number and distribution of different amplified rDNA restriction analysis (ARDRA) groups using plating on R2A (P) and enrichment in high-molecular-weight substrates (E)

		Locations*						Microenvironment					
		B		BS		R		Bulk soil		Strawberry		Oilseed rape	
ARDRA group†	Genera‡	P§	E¶	P	E	P	E	P	E	P	E	P	E
A	*Pseudomonas*	60	83	87	33	54	43	17	32	98	73	86	54
B	*Serratia*	0	0	15	2	11	1	1	0	4	3	21	0
C	*Streptomyces* I	3	0	5	0	6	0	10	0	3	0	1	0
D	*Streptomyces* II	3	0	3	0	3	1	2	0	6	1	1	0
E	*Bacillus pumilus*	2	2	1	0	5	0	7	1	1	3	0	1
F	*Paenibacillus*	1	0	2	0	2	0	2	0	3	0	0	0
G	*Flavobacterium*	3	0	0	0	0	0	1	0	2	0	0	0
H	*Pantoaea/Erwinia*	0	3	0	0	2	2	1	1	0	0	1	1
I	*Stenotrophomonas*	0	2	2	1	0	0	0	1	0	0	2	2
J	*Bacillus cereus*	1	12	1	0	0	0	1	6	1	2	0	4
K	*Streptomyces* III	0	0	0	0	1	0	0	0	1	0	0	0
L	*Delftia*	0	0	0	2	0	1	0	1	0	3	0	4
M	*Citrobacter*	0	0	0	0	0	0	0	2	0	0	0	0
Σ ARDRA groups		7	5	8	4	8	5	9	7	9	6	6	6
Σ isolates		73	109	116	38	84	48	42	44	119	85	112	66

*Different locations: B = Berlin, BS = Braunschweig, R = Rostock.

†Letters represent different restriction patterns of the 16S rDNA using *Hha*I.

‡Genera were identified by partial sequencing of the 16S rDNA (for detailed results, see Table 2).

§Isolates obtained after plating from R2A agar.

¶Isolates obtained after enrichment in high-molecular-weight substrates.

ARDRA types was different for both isolation methods. Fewer ARDRA types were found in the rhizosphere of strawberry and oilseed rape than in bulk soil. The lower number of ARDRA types indicated a reduced bacterial diversity in the rhizosphere in comparison to bulk soil.

For identification, the 16S rRNA genes of 66 representative isolates comprising all ARDRA types, locations and microenvironments were partially sequenced, and sequences were compared to entries available in public databases (Tables 2 and 3). The 16S rRNA genes showed high homology to known sequences belonging to the *Alpha-*, *Beta-* and *Gammaproteobacteria*, to low and high G+C gram-positive bacteria as well as to the *Cytophaga/Flavobacterium/Bacteroides* phylum. Twenty-two different species belonging to 15 genera were found. At the Rostock and Berlin sites, a higher number of ARDRA types belonging to the group of *Verticillium* antagonists (12) was found than at the Braunschweig site (8). The number of different ARDRA types obtained from isolates with antagonistic activity was slightly lower in the rhizosphere (strawberry, 10; oilseed rape, 12) than in soil (13). Based on sequence similarities, it was possible to identify putative species for all ARDRA groups and label them as shown in the second column of Table 1.

Whereas the genus *Pseudomonas*, represented by ARDRA type A, was dominantly found by both isolation methods, *Serratia* (ARDRA type B) and *Streptomyces* I (ARDRA type C) antagonists were mainly isolated from R2A, and *Bacillus* (ARDRA type J) and *Delftia* (ARDRA type L) originated mainly from the enrichments. On R2A medium, no *Delftia* and *Citrobacter* were found, whereas the ARDRA types C, F, G and K were missing among the antagonists from the enrichments.

The main ARDRA group A included the majority of isolates (360 of 468 = 77%). All the representatives which were sequenced and aligned belonged to the four different *Pseudomonas* species *P. filiscindens*, *P. fluorescens*, *P. marginalis*, and *P. lini*, or could be identified only on the genus level (*Pseudomonas* spp.). Isolates of this group occurred in soil of each location as well as in each of the investigated rhizospheres. However, a clear enrichment in the rhizospheres of both plants was found in comparison to bulk soil. This observation was made for antagonists obtained from both isolation methods. *Serratia* isolates (ARDRA group B) were found as typical inhabitants of the rhizosphere of oilseed rape at the Rostock and Braunschweig sites and were preferentially isolated from R2A plates. In Berlin, none of the *Verticillium* antagonists belonged to this genus. ARDRA groups C and D belonged to the gram-positive genus *Streptomyces*. Antagonists belonging to ARDRA group C were obtained only from R2A plates, and isolates of both groups were mainly found in bulk soil and the strawberry rhizosphere. *Bacillus* isolates represented by ARDRA types E and J were also mainly found in bulk soil. Whereas *Bacillus* was more frequently detected after enrichment, *Paenibacillus* isolates (ARDRA group F) were only found on R2A plates. *Verticillium* antagonists of ARDRA group G

Table 2. Identification of antagonistic isolates obtained from R2A by partial 16S rRNA gene sequence analysis

No.	ARDRA group*	Isolate number†	Origin location‡	Microenvironment	Closest database match and accession number	Percentage homology
1	A	BSE4-4-1	BS	Rhizosphere strawberry	*Pseudomonas fluorescens* AJ278813.1	99
2	A	BSE4-6-4	BS	Rhizosphere strawberry	*Pseudomonas* sp. AF436229.1	98
3	A	BSR1-3-8	BS	Rhizosphere oilseed rape	*Pseudomonas fluorescens* AJ278813.1	98
4	A	BSR1-3-20	BS	Rhizosphere oilseed rape	*Pseudomonas* sp. AY456704.1	99
5	A	BSR2-1-19	BS	Rhizosphere oilseed rape	*Pseudomonas fluorescens* AJ278813.1	98
6	A	RE3-1-14	R	Rhizosphere strawberry	*Pseudomonas lini* AY035996.1	99
7	A	RR4-5-23	R	Rhizosphere oilseed rape	*Pseudomonas fluorescens* AJ278813.1	98
8	A	RR1-1-1	R	Rhizosphere oilseed rape	*Pseudomonas fluorescens* AJ278813.1	99
9	A	RR2-6-9	R	Rhizosphere oilseed rape	*Pseudomonas fluorescens* AJ278813.1	99
10	A	RR2-6-20	R	Rhizosphere oilseed rape	*Pseudomonas* sp. AF456226.1	98
11	A	RB3-3-12	R	Bulk soil	*Pseudomonas marginalis* AF364098.1	99
12	A	BE4-4-16	B	Rhizosphere strawberry	*Pseudomonas* sp. AY599715.1	99
13	A	BR1-4-15	B	Rhizosphere oilseed rape	*Pseudomonas* sp. AY308052.1	98
14	B	BSR4-4-3	BS	Rhizosphere oilseed rape	*Serratia proteamaculans* AJ233434.1	96
15	B	BSR4-4-11	BS	Rhizosphere oilseed rape	*Serratia proteamaculans* AJ233434.1	98
16	B	BSR1-2-6	BS	Rhizosphere oilseed rape	*Serratia proteamaculans* AJ233434.1	99
17	B	RE2-3-4	R	Rhizosphere strawberry	*Serratia fonticola* AF511435.1	98
18	B	RE3-4-8	R	Rhizosphere strawberry	*Serratia plymuthica* AJ233433.1	99
19	B	RR2-5-21	R	Rhizosphere oilseed rape	*Serratia plymuthica* AJ233433.1	98
20	B	RR1-6-24	R	Rhizosphere oilseed rape	*Serratia plymuthica* AJ233433.1	98
21	B	RR2-5-10	R	Rhizosphere oilseed rape	*Serratia plymuthica* AJ233433.1	99
22	C	RB3-6-13	R	Bulk soil	*Streptomyces* sp. AY465321.1	99
23	D	BSE2-3-6	BS	Rhizosphere strawberry	*Streptomyces* sp. AY623798.1	99
24	D	BSB2-1-17	BS	Bulk soil	*Kitasatosporia kifunense* U93322.1	99
25	E	BSB3-1-9	BS	Bulk soil	*Bacillus pumilus* AY112667.1	99
26	E	RE4-3-5	R	Rhizosphere strawberry	*Bacillus pumilus* AY112667.1	98
27	E	RB3-2-10	R	Bulk soil	*Bacillus pumilus* AY112667.1	99
28	E	BB1-3-5	B	Bulk soil	*Bacillus pumilus* AY112667.1	99
29	F	RE1-4-13	R	Rhizosphere strawberry	*Paenibacillus polymyxa* AY359632.1	99
30	F	RB4-3-10	R	Bulk soil	*Paenibacillus polymyxa* AY302439.1	99
31	F	BB4-1-18	B	Bulk soil	*Paenibacillus polymyxa* AY359632.1	99
32	G	BE3-3-11	B	Rhizosphere strawberry	*Flavobacterium* sp. AF493648.1	98
33	G	BB2-3-14	B	Bulk soil	*Flavobacterium* sp. AF493648.1	98
34	H	RR1-1-6	R	Rhizosphere oilseed rape	*Pantoea agglomerans* AF373197.1	99
35	H	RB2-3-3	R	Bulk soil	*Brevundimonas vesicularis* AY169433.1	99
36	I	BSR4-2-12	BS	Rhizosphere oilseed rape	*Stenotrophomonas maltophilia* AF100734.1	98
37	J	BE4-1-6	B	Rhizosphere strawberry	*Bacillus cereus* AY138270.1	99
38	K	RE2-6-8	R	Rhizosphere strawberry	*Streptomyces tauricus* AB045879.1	99

*The letters represent the different amplified rDNA restriction analysis (ARDRA) patterns (A–K).

†Letters represent the locations and microhabitats: RR = Rostock, oilseed rape; RE = Rostock, strawberry; RB = Rostock, bulk soil; BR = Berlin, oilseed rape; BE = Berlin, strawberry; BB = Berlin, bulk soil; BSR = Braunschweig, oilseed rape; BSE = Braunschweig, strawberry, BSB = Braunschweig, bulk soil; Arabic numerals represent the plots (1–4), the sampling times over 2 years (1–6) and the strain number (1–24).

‡Locations: R = Rostock; B = Berlin; BS = Braunschweig.

(*Flavobacterium*) were only found in bulk soil and in the strawberry rhizosphere at the site in Berlin. The second group of enterics corresponds to ARDRA group H and was found primarily after enrichment. *Stenotrophomonas maltophilia* (group I) was mostly found in the rhizosphere of oilseed rape at two sites. Isolates belonging to *Delftia tsuruhatensis* and *D. acidovorans* (ARDRA group L) were exclusively found after enrichment and preferentially detected in both rhizospheres.

Isolates belonging to *Pseudomonas*, *Serratia* and *Streptomyces* represented 85% of the *Verticillium* antagonists from R2A. Thus, these groups were chosen to be analysed in more detail. The highest number of *Verticillium* antagonists belonging to *Pseudomonas* was found in Braunschweig (Table 4). *Pseudomonas* was more frequently isolated from oilseed rape and strawberry rhizosphere soil than from bulk soils for all sites. At the Rostock and Braunschweig sites, we observed higher numbers of antagonistic *Pseudomonas*

Table 3. Identification of isolates obtained after enrichment in high-molecular-weight substrates by partial 16S rRNA gene sequence analysis

No.	ARDRA group*	Isolate number†	Origin		Closest database match and accession number	% Homology
			Location‡	Microenvironment		
1	A	BSE1-3-38	BS	Rhizosphere strawberry	*Pseudomonas fluorescens* AJ278813.1	98
2	A	BSR1-1-34	BS	Rhizosphere oilseed rape	*Pseudomonas fluorescens* AY447046.1	99
3	A	RE2-2-43	R	Rhizosphere strawberry	*Pseudomonas fluorescens* AJ278813.1	98
4	A	RE1-3-22	R	Rhizosphere strawberry	*Pseudomonas fluorescens* AJ278813.1	99
5	A	RR3-3-33	R	Rhizosphere oilseed rape	*Pseudomonas* sp. AY456704.1	99
6	A	BE2-2-22	B	Rhizosphere strawberry	*Pseudomonas fluorescens* AJ278813.1	98
7	A	BR2-1-23	B	Rhizosphere oilseed rape	*Pseudomonas filiscindens* AY259924.1	98
8	A	BB2-1-31	B	Bulk soil	*Pseudomonas* sp. AF456214.1	99
9	A	BB4-2-39	B	Bulk soil	*Pseudomonas filiscindens* AY259924.1	98
10	B	BSE2-3-25	BS	Rhizosphere strawberry	*Serratia proteamaculans* AY040208.1	97
11	B	RE2-1-21	R	Rhizosphere strawberry	*Serratia fonticola* AF286869.1	98
12	D	RE1-1-27	R	Rhizosphere strawberry	*Streptomyces* sp. AY465336.1	99
13	E	BR1-3-28	B	Rhizosphere oilseed rape	*Bacillus pumilus* AY373359.1	97
14	H	BE2-1-27	B	Rhizosphere strawberry	*Erwinia rhapontici* Z96087.1	98
15	H	BE4-3-33	B	Rhizosphere strawberry	*Pantoea agglomerans* AB004691.1	98
16	I	BR1-1-30	B	Rhizosphere oilseed rape	*Variovorax paradoxus* D30793.1	98
17	I	BR3-1-28	B	Rhizosphere oilseed rape	*Stenotrophomonas maltophilia* AF100734.1	97
18	J	BE2-3-27	B	Rhizosphere strawberry	*Bacillus cereus* AY138270.1	99
19	J	BR4-2-23	B	Rhizosphere oilseed rape	*Bacillus cereus* AY138271.1	99
20	J	BR4-3-23	B	Rhizosphere oilseed rape	*Bacillus cereus* AY138273.1	100
21	J	BB1-3-25	B	Bulk soil	*Bacillus cereus* AY138270.1	99
22	J	BB3-3-21	B	Bulk soil	*Bacillus thuringiensis* AF501348.1	99
23	L	BSR3-3-39	BS	Rhizosphere oilseed rape	*Delftia tsuruhatensis* AJ606337.1	98
24	L	BSR3-3-29	BS	Rhizosphere oilseed rape	*Delftia tsuruhatensis* AY684787	98
25	L	RB4-2-40	R	Bulk soil	*Delftia acidovorans* AY367028.1	100
26	L	BE4-3-41	B	Rhizosphere strawberry	*Delftia acidovorans* AY367028.1	99
27	L	BR4-2-39	B	Rhizosphere oilseed rape	*Delftia acidovorans* AB074256	98
28	M	BB2-3-32	B	Bulk soil	*Citrobacter freundii* AJ233408.1	98

*The letters represent the different amplified rDNA restriction analysis (ARDRA) patterns (A–M).
†Letters represent the locations and microhabitats: RR = Rostock, oilseed rape; RE = Rostock, strawberry; RB = Rostock, bulk soil; BR = Berlin, oilseed rape; BE = Berlin, strawberry; BB = Berlin, bulk soil; BSR = Braunschweig, oilseed rape; BSE = Braunschweig, strawberry, BSB = Braunschweig, bulk soil; Arabic numerals represent the plots (1–4), the sampling times over two years (1–6) and the strain number (1–48).
‡Locations: R = Rostock; B = Berlin; BS = Braunschweig.

Table 4. Number of isolates belonging to dominant genera isolated from R2A from different habitats and sites

Genus	Number of isolates	Berlin			Braunschweig			Rostock		
		Soil	Strawberry	Rape	Soil	Strawberry	Rape	Soil	Strawberry	Rape
Pseudomonas	201	7	16	30	1	51	38	9	27	22
Serratia	26	0	0	0	0	2	13	1	2	8
Streptomyces	14	1	2	0	4	0	1	5	1	0

strains in the strawberry rhizospheres, whereas at the Berlin site a higher number was isolated from oilseed rape. *Verticillium* antagonists belonging to the second largest genus *Serratia* were most abundant in Braunschweig and Rostock and were not isolated from the Berlin site. At both locations where *Serratia* could be isolated, they were primarily retrieved from the rhizosphere of oilseed rape. In contrast, *Verticillium* antagonists belonging to *Streptomyces* (ARDRA group C) were most frequently isolated from bulk soil.

Genotypic diversity of antagonistic bacterial isolates

All *Verticillium* antagonists (468) were characterized on the genotypic level using BOX-PCR. GelCompar was used for the comparison of BOX patterns. Only the BOX patterns of antagonists from R2A plates (273) were used for calculation of diversity indices. Fingerprints of the dominant genus *Pseudomonas* were selected to examine their diversity. In

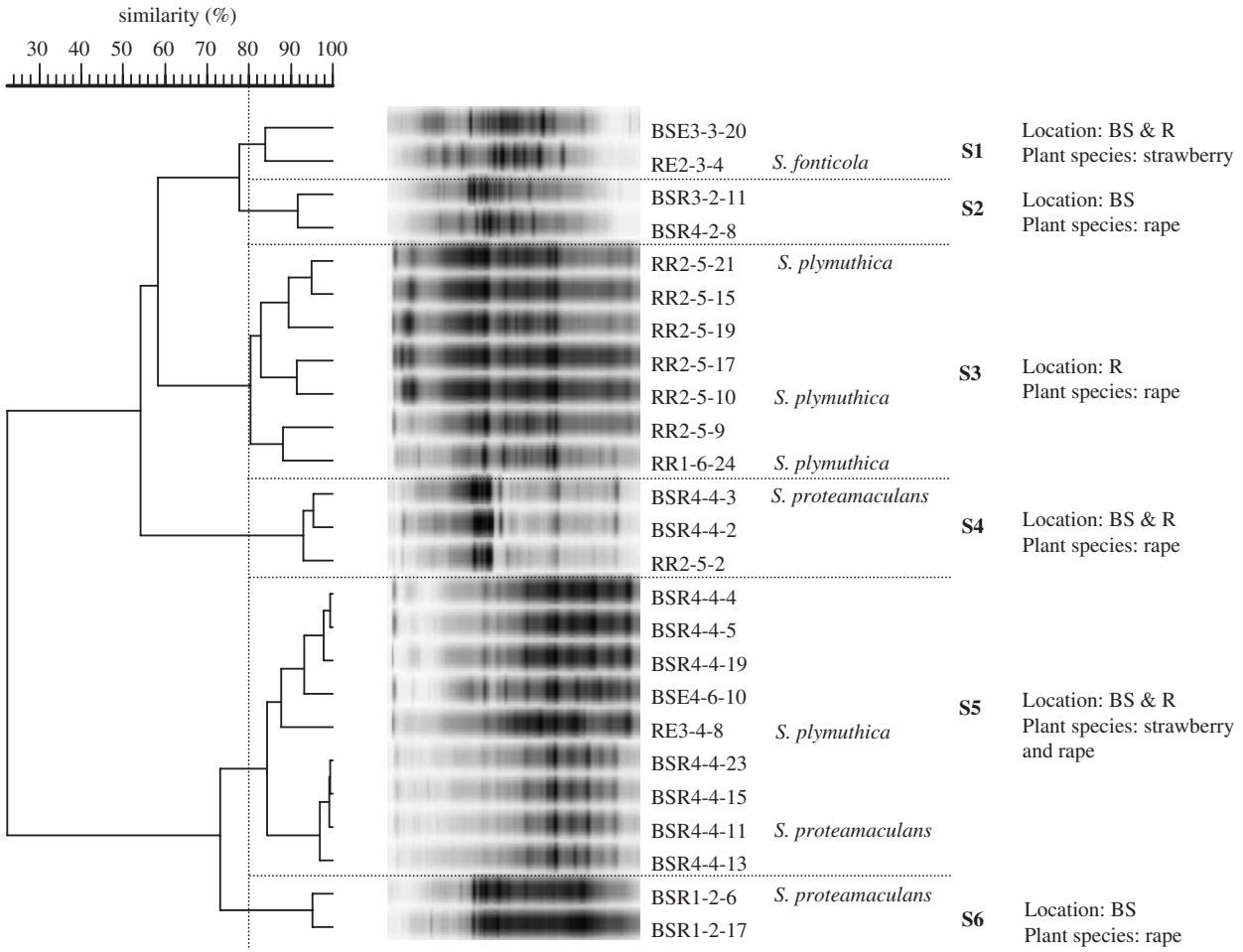

Fig. 2. Dendrogram showing the relationship of *Serratia* isolates obtained from the rhizosphere of oilseed rape and strawberry from the sites in Braunschweig (BS) and Rostock (R) in both years based on BOX-PCR fingerprints using cluster analysis by the unweighted pair group method and arithmetic averages.

comparison to other genera, BOX patterns of 185 *Pseudomonas* isolates from all microenvironments and locations indicated a high intrageneric diversity (data not shown). At a similarity level of 80%, the strains could be divided into 13 different groups and 17 single isolates. Genotype groups contained 2–59 isolates. Whereas four of the 13 groups included only isolates of the same plant species, nine heterogeneous groups with isolates from both plant species and bulk soil were obtained. Four groups comprised isolates from one location. The genotypic diversity of isolates could be also shown for the genus *Serratia*. Altogether, 25 isolates of this genus were isolated from different microenvironments but were not found in samples from Berlin. A comparison of all BOX patterns generated from the *Serratia* antagonists (Fig. 2) resulted, at a similarity level of 80%, in six different clusters or genotypic groups (S1–6). The genotypic groups contained 2–9 isolates. Regarding the distribution of the isolates from different locations, three groups were formed by isolates from one location only: S2, S6 (Braunschweig) and S3 (Rostock). In all other groups, isolates from both locations were present. Furthermore, five groups contained isolates from one rhizosphere independent of the location: S1 (rhizosphere of strawberry) and S2, S3, S4 and S6 (rhizosphere of oilseed rape), with one group containing isolates from both rhizospheres (S5). This last group could be divided into two subgroups at a similarity level of 84%, with one of the subgroups containing only antagonists isolated from the oilseed rape rhizosphere in Braunschweig. Surprisingly, all antagonistic isolates obtained from the Braunschweig site were identified as *Serratia proteamaculans*, whereas the majority of isolates from the Rostock site belonged to the closest relative, namely *S. plymuthica*. The comparison of all BOX patterns obtained from 14 *Streptomyces* antagonists resulted, at a similarity level of 80%, in five different clusters from which four contain isolates from the same location

and three from the same microenvironment (data not shown).

Based on all BOX patterns and their statistical relationship, diversity indices according to Shannon & Weaver (1949) were calculated. In general, the genotypic diversity was higher in bulk soil ($\langle H' \rangle = 2.99$) than in both rhizospheres ($\langle H' \rangle = 2.51$). Comparing both rhizospheres, the diversity of bacterial antagonists for oilseed rape ($\langle H' \rangle = 2.66$) was slightly higher than for strawberry ($\langle H' \rangle = 2.37$). Additionally, the diversity of antagonists was influenced by the location. The indices were on average higher in Rostock, followed by Berlin and then Braunschweig. Collector's curves or ARDRA and BOX abundance curves (the number of patterns detected vs. the number of strains analysed) were constructed to compare the diversity of different microenvironments (data not shown). Full coverage of a sample would be expected to give a plateau-shaped curve. For example, this type of curves was recovered for ARDRA types in the rhizospheres, whereas those obtained for bulk soil showed no plateau. Altogether, the collector's curve derived from pooled data for each location indicated that the recovery of the diversity was greater in Rostock than in Berlin and Braunschweig and in bulk soil than in both rhizospheres.

Discussion

Little is known of how biotic factors influence antagonistic communities in the rhizosphere. Understanding the factors influencing the abundance and activity of bacteria with antagonistic potential is of practical importance because it might enable us in the near future to manage the indigenous microbial communities towards optimal plant growth and health. This knowledge could be the key for a successful biological control by bacterial antagonists.

The proportion of bacteria antagonistic towards *Verticillium dahliae*

In our study, we analysed the functional group of bacteria with antagonistic activity towards *Verticillium dahliae* Kleb. of three different microenvironments and at three different locations. In a study performed under field conditions in Braunschweig for 2 years (1999–2000), the proportion of bacteria isolated from R2A which showed antagonistic activity was highest for strawberry rhizosphere (9.5%), followed by oilseed rape (6.3%), potato (3.7%) and bulk soil (3.3%) (Berg *et al.*, 2002). The same trend was observed in the present study, although the proportion of antagonists on average was higher than in the previous study. Different abiotic conditions can be the reason for this higher percentage, as well as missing crop rotation during the whole experiment. In addition, a higher number of antagonists was found after enrichment in high-molecular-weight substrates such as casein, xylan and chitin. In particular, casein was a carbon source which enhanced the number of antagonists during the isolation procedure, reflecting the frequently observed result that most antagonistic bacteria possess proteolytic activity (Berg *et al.*, 2002). R2A is a nutrient-poor medium suitable to detect a high diversity of plant-associated bacteria. Fast-growing bacteria mainly belonging to the *Gammaproteobacteria* were enhanced in enrichment cultures (Smalla *et al.*, 1998). The number of antagonistic bacteria was enhanced in the rhizosphere in comparison to bulk soil at all three sites. In contrast, such differences were not found for fungi antagonistic towards *Verticillium dahliae* during the same field trials, although their proportion was higher in general (Berg *et al.*, 2005).

Plant specificity and influence of the site on *Verticillium* antagonists

The analysis of bacteria with antagonistic activity on the genotypic level was suitable to detect clear differences owing to the soil type and plant species. Firstly, an effect of the soil type and location on the composition and relative abundance of bacteria was found. A high number of antagonistic bacteria and of ARDRA groups was observed for the Berlin site. Additionally, a very high genotypic diversity was found for *Pseudomonas* as well as for all isolates at the Berlin site. *Verticillium* antagonists belonged to *Pseudomonas* and *Bacillus*, whereas *Serratia* strains were not isolated from microenvironments in Berlin. Antagonists which were isolated only from the site in Berlin were *Bacillus cereus, B. thuringiensis, Erwinia rhapontici, Pseudomonas filiscindens* and *Variovorax paradoxus*. The same soil was characterized by a high number of fungal morphotypes without a dominance of one species and a low genotypic diversity (Berg *et al.*, 2005). For bacterial antagonists from the Braunschweig site, a high number of bacterial (this study) as well as fungal (Berg *et al.*, 2005) antagonists and the lowest diversity in comparison with the other sites were observed. Frequently isolated antagonists belonged to *Pseudomonas* and *Serratia*, whereas the most abundant fungal antagonists in Braunschweig belonged to the genus *Trichoderma*, with the key species *Trichoderma viride* (Berg *et al.*, 2005). The lowest number and the highest diversity of antagonists were observed at the Rostock site. *Verticillium* antagonists were mainly represented by *Pseudomonas* and enterics, among which *Pantoea agglomerans*, *Serratia plymuthica* and *Serratia fonticola* were identified. Fungal *Verticillium* antagonists from the Rostock site were dominated by *Penicillium*, which was found in a high diversity of species and genotypes (Berg *et al.*, 2005). Independent from the site, specificity was found for each of the investigated microenvironments. The strawberry rhizosphere was characterized by a high propor-

tion of *Pseudomonas*, which represented on average 77% of the bacterial antagonists. However, although at two sites (Rostock, Braunschweig) a higher number of *Pseudomonas* antagonists was retrieved from the rhizosphere of strawberry compared with oilseed rape, the opposite was observed for plants grown at the Berlin site. Thus, for Braunschweig the previously reported frequent isolation of *Pseudomonas putida* B from the rhizosphere of strawberries could be confirmed, indicating the effect of the plant (Berg *et al*., 2002). A lower number but a higher diversity of bacterial antagonists was found in the rhizosphere of oilseed rape in comparison to the strawberry rhizosphere at all sites. In addition to *Pseudomonas* and *Serratia*, other enterics and *Stenotrophomonas* isolates were more frequently found. The preferential occurrence of *Serratia* and other related genera with a high genotypic diversity in the oilseed rape rhizosphere was described before (Berg, 2000; Berg *et al*., 2002). The effect of the site became obvious in Berlin, where no *Serratia* could be isolated, in contrast to Braunschweig and Rostock, where *Serratia* was preferentially isolated from the rhizosphere of oilseed rape. Interestingly, whereas from the Rostock site mainly *S. plymuthica* were identified, only *S. proteamaculans* isolates were found at the Braunschweig site. In addition to different root morphology, another reason for plant specificity of root-associated microorganisms is the presence of root exudates such as amino acids, sugars and organic acids, which are an important nutritional source for microbes. The composition of root exudates has been shown to vary depending on the plant species and on the stage of plant development (Neumann & Römhild, 2001). In addition to antagonistic fungi and bacteria, mycorrhiza fungi are an important component of beneficial microorganisms in the rhizosphere. Strawberry roots are colonized by mycorrhiza fungi, and many reports have demonstrated the positive effects of mycorrhiza introduction on the growth and health of strawberries (Taylor & Harrier, 2001). In contrast, no mycorrhiza is known for oilseed rape or other *Brassicaceae* species. This could be another reason for the plant-specific composition of bacterial antagonists in the rhizosphere of the studied plants. Some *Pseudomonas* species and other antagonistic bacteria which occurred preferentially on strawberry roots have been described as mycorrhiza-helper bacteria by Mamatha *et al*. (2002).

Results shown in the present report confirmed the findings of a previous study conducted by Berg *et al*. (2002), in which genera such as *Pseudomonas*, *Serratia*, *Bacillus* and *Streptomyces* were considered the main groups of *Verticillium* bacterial antagonists in the rhizosphere of strawberry and oilseed rape. In contrast, on bryophytes, and especially on *Sphagnum*, a completely different composition of *Verticillium* antagonists occurs, which is dominated by members of the genus *Burkholderia* (Opelt & Berg, 2004).

The influence of plant species and soil type on microbial communities was also assessed for the same samples by DGGE of 16S and 18S rRNA gene fragments amplified from community DNA to obtain a more complete picture of the factors influencing microbial communities in the rhizosphere (Costa *et al*., 2005). Plant specificity was revealed by bacterial DGGE profiles, especially for the actinobacterial communities. *Streptomyces* sp. and *Rhizobium* sp. were the dominant ribotypes in the strawberry rhizospheres, whereas *Arthrobacter* corresponded to dominant ribotypes of the oilseed rape rhizosphere. Correspondingly, *Streptomyces* strains with antagonistic activity were mainly found in the strawberry rhizosphere.

Impact on biocontrol

During this study, basic information about bulk and rhizosphere soil bacteria with antagonistic activity towards *Verticillium* and factors influencing their abundance and diversity was gathered. This knowledge is important for the optimization of biocontrol applications. Successful and consistent biological control requires a better understanding of the dynamics and composition of antagonistic rhizosphere communities. We could show that in all investigated soils an impressive autochthonous antagonistic potential occurred. Concluding our data, both plant species are able to select their antagonists in a plant-dependent manner. The rhizosphere effect on the bacterial community antagonistic towards *Verticillium* was shown (1) by an enhanced proportion of antagonistic isolates, (2) by an enrichment of specific genotypes, especially gram-negative ones, and of specific genotypes of *Pseudomonas* or *Serratia*, and (3) by a reduced diversity on species as well as genotype level. The rhizosphere effect on antagonists was clearly influenced by the plant species and soil type, which was shown for all the above-mentioned parameters (1–3). It is important to emphasize that these data were obtained for microorganisms antagonistic towards *V. dahliae*. Although a long list of *Verticillium* antagonists showed activity against other pathogens in previous assays (Berg *et al*., 2002), results obtained for the rhizosphere effect on *Verticillium* antagonists should be verified for other pathosystems.

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