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Assessing the risk of biological control agents on the indigenous microbial communities: *Serratia plymuthica* HRO-C48 and *Streptomyces* sp. HRO-71 as model bacteria

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Abstract. The phytopathogenic fungus *Verticillium dahliae* Kleb. causes high yield losses in strawberry production. As effective chemical control of this fungus is no longer available, biological control based on natural antagonists might provide new control strategies. The aim of this study was to assess the impact of the two biological control agents *S. plymuthica* HRO-C48 and *Streptomyces* sp. HRO-71 on the rhizosphere community of the *Verticillium* host plant strawberry in field trials at two different sites in Germany. Therefore, we determined the abundances of culturable bacteria and investigated the community structure of the total rhizosphere microbiota by PCR-single strand conformation polymorphism analysis of the 16S rRNA and fungal ITS1 region. The abundances of culturable rhizobacteria on R2A medium as well as the proportion of *in vitro Verticillium* antagonists did not differ significantly. Additionally, no treatment specific differences were obtained in the composition of species of the non-target antagonistic bacteria in the rhizospheres. The culture-independent analysis revealed only transient differences between the bacterial communities not due to the treatments rather than to the plant growth stage. Fungal and bacterial community fingerprints showed the development of a microbiota, specific for a field site. However, no sustainable impact of the bacterial treatments on the indigenous microbial communities was found using culture-dependent and -independent methods.

Key words: biocontrol, rhizosphere, risk assessment, *Serratia*, SSCP, *Streptomyces*

Introduction

The soilborne fungus *Verticillium dahliae* Kleb., the agent of Verticillium wilt, causes worldwide a dramatic yield loss in many crops, e.g.

strawberry, potato and oilseed rape (Tjamos, 2000). As negative effects on the climate phased out the fungicide methyl bromide, efficacious control methods are urgently needed (Martin, 2003). To control soilborne diseases in an environmentally friendly way biological control using antagonistic microorganisms became more and more attentive. Much research has been done on antifungal bacteria naturally occurring in the rhizosphere (reviewed in Whipps, 2001; Weller et al., 2002). Recently we reported the successful selection of rhizobacteria antagonistic to *Verticillium dahliae in vitro* as well as *ad planta* (Berg et al., 2000; Berg et al., 2001). The Gram-negative strain *Serratia plymuthica* HRO-C48 proved to be an effective antagonistic bacterium because of its plant growth promoting and chitinolytic activity (Berg et al., 1999; Frankowski et al., 2001; Kurze et al., 2001; Müller et al., 2004) and was successfully developed as a product called Rhizostar® (produced by E-nema GmbH Raisdorf, Germany). The Gram-positive strain *Streptomyces* sp. HRO-71 was described as an efficient biological control organism toward several plant pathogenic fungi by the production of siderophores, antibiotics, indole-3-acetic acid (IAA), and chitinases (Berg and Lüth, 1999; Berg et al., 2001).

Although the two antagonistic strains originate from the rhizosphere itself, their application in large densities could probably have negative effects on the indigenous rhizosphere microbiota. Consequently, the impact of applied microorganisms on the non-target root-associated microorganisms should be assessed. Up to now only a minority of 0.1 to 3.0% of the total microbiota can be analyzed using culture-dependent methods (Ward et al., 1990; Smalla, 2004). During the last decade, several molecular techniques, allowing the whole microbial communities to be investigated, have been developed, e.g. molecular fingerprint techniques on the basis of 16S/18S rRNA and related genes like denaturing gradient gel electrophoresis (Muyzer and Smalla, 1998), terminal restriction fragment length polymorphism analysis (Liu et al., 1997) and single strand conformation polymorphism analysis (Schwieger and Tebbe, 1998).

The objective of our study was to analyze the impact of biological control agents (BCAs) on the indigenous microbiota by comparing bacterial as well as fungal communities of treated and non-treated plants using cultivation-dependent and cultivation-independent methods. To find out, if the bacterial treatment has effects on the indigenous antagonistic potential, the group of natural occurring *Verticillium* antagonists was examined more in detail using cultivation techniques. We investigated the microbial rhizosphere communities of

field-grown strawberry (cv. Honeye and Elsanta) in field trials at two different sites in Germany (Rostock and Herten) by applying two bacterial antagonists. Samples were taken from young, flowering and senescent plants. The following treatments were carried out: (i) the wildtype strain of *Serratia plymuthica* HRO-C48 as single treatment, (ii) a rifampicin resistant mutant of *Serratia plymuthica* HRO-C48 as single treatment, (iii) the wildtype strain of *Streptomyces* sp. HRO-71 as single treatment, and (iv) the rifampicin resistant mutant of HRO-C48 and HRO-71 as combined treatment.

Material and methods

Biological control organisms

The strain *Serratia plymuthica* HRO-C48 (DSMZ 12502; Rhizostar®) was isolated from the rhizosphere of oilseed rape (Kalbe et al., 1996). The Gram-positive strain *Streptomyces* sp. HRO-71 (DSMZ 12424; Rhizovit®) originated from the rhizosphere of strawberry (Berg et al., 2000). For the preparation of the inoculums, the strains HRO-C48 and HRO-71 were grown in nutrient broth (Oxoid, Hamshire, U.K.) and DSM medium 65, containing 4 g glucose (Roth, Karlsruhe, Germany), 4 g yeast extract (Sifin, Berlin, Germany), 10 g malt extract (Merck, Darmstadt, Germany), 2 g $CaCO_3$ (Roth) per liter (pH 7.2), respectively. After one day of pre-incubation in 100 ml medium, the BCAs were inoculated in a 10 l Biostat B fermenter (B. Braun Biotech International, Melsungen, Germany) and grown at 30 °C and 150 rpm for two (HRO-C48) and four (HRO-71) days in the same medium.

Plants and field trials

We investigated the microbial rhizosphere communities of the *Verticillium* host plant field-grown strawberry (*Fragaria×ananassa* Duch.). Frigo plants of strawberry (cv. Honeye and Elsanta) were planted in cooperation with two strawberry farms integrated in commercial strawberry production on fields near Rostock (trial 1) and Herten (trial 2) (both Germany) in may 2001 and 2002. In trial 1 single treatments of the wildtype strain of *Serratia plymuthica* HRO-C48 (C48-I) and *Streptomyces* sp. HRO-71 (71-I) as well as a water control (ctrl-I) were applied. A rifampicin resistant mutant of HRO-C48 (C48-II), a combination of HRO-C48 and HRO-71 (71 + C48-II) and

a water control (ctrl-II) were applied in trial 2. The plants were arranged in rows in a distance of 30 cm with five replicates per treatment and about 50 plants per replicate. The plant roots were dipped into the bacterial suspensions and water (control) for 15 to 20 min prior planting. The rhizosphere samples were taken, each with five replicates per treatment, from young plants, flowering plants and senescent plants, three times a year in about 5 week intervals. A sample consists of 5 g roots with adhering soil which were combined from three plants each.

Abundances of bacteria and Rifampicin resistant mutants

The samples were pre-treated for 60 s, in a bag mixer using Stomacher bags (Interscience, St. Nom, France.), after the addition of 2× 25 ml of sterile *A. dest.* and then diluted in sterile PBS (130 mM NaCl, 10 mM sodiumphosphate buffer). Determination of colony forming units (CFU) was performed by plating on R2A agar (Difco, Detroit, USA) containing 100 ppm nystatin (Fluka, Neu-Ulm, Germany). Rifampicin resistant mutants of HRO-C48 were reisolated on nutrient agar containing 100 ppm rifampicin (Fluka). Agar plates were incubated at 20 °C for 4 days prior to enumeration of Colony Forming Units (CFU). The Wilcoxon matched pairs signed rank test ($\alpha = 0.05$) was applied for statistical analyses.

Screening for antifungal in vitro *antagonism*

To analyze the antagonistic potential of the rhizosphere bacterial community, 150 bacterial isolates per treatment, originating from the CFU determination experiment, were selected and screened for antifungal *in vitro* antagonism towards *Verticillium dahliae* Kleb. By selecting the strains we tried to consider phenotypically different colonies and took care of dominating colony morphologies. Screening was carried out using a dual culture assay (Berg, 1996) in 24-well-microtiter plates on Waksman agar containing 5 g proteose-peptone (Merck), 10 g glucose (Merck), 3 g meat extract (Oxoid), 5 g NaCl (Merck), and 20 g agar (Difco) per liter of distilled water. Waksman agar was used because it is equally suitable for the cultivation of fungi and bacteria. The *Verticillium dahliae* strain (culture collection of the Department of Microbiology, University of Rostock) was routinely grown at 20 °C and 130 rpm in Czapeck Dox medium (Difco). Per well, 20 μl of the *Verticillium* suspension and a bacterial isolate were co-incubated for four days at 20 °C on microtiter plates. The

formation of an inhibition zone of fungal growth around the tested isolate indicated an antagonistic activity of the isolate. Bacterial strains that generated inhibition zones were tested a second time on petri dishes to exactly asses the antagonistic activity according to Berg (1996). Statistically significant differences were determined by the Wilcoxon matched pairs signed rank test ($\alpha = 0.05$).

Characterization of the antagonistic strains by BOX-PCR fingerprints

All detected *Verticillium* antagonists were characterized by the molecular means of BOX-PCR fingerprints (Martin et al., 1992). Bacterial DNA was prepared following the protocol of Andersen and McKay (1983) modified for genomic DNA. The BOX-PCR using the BOX A1R primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) was done according to Rademaker and De Bruijn (1997) and finally the BOX-PCR fingerprints were clustered using GelCompar® software (version 4.1, Applied Math, Kortrijk, Belgium). The cluster analyses were used to calculate the diversity of antagonistic isolates applying the Shannon's diversity index $H' = -\sum[(n_i/N)^* \ln(n_i/N)]$ with n_i = number of individuals for each species and N = total number of individuals in the survey (Shannon and Weaver, 1949) at 80% similarity of fingerprints.

Identification of antagonistic strains by fatty acid methyl ester profiles and sequencing of 16S rRNA gene

Bacterial antagonists showing a high genotypic similarity of their BOX pattern (more than 80%) were grouped together. Representatives of each group were selected for identification (i) by their fatty acid methyl ester (FAME) profiles (Sasser, 1990), or (ii) by partly sequencing of 16S rRNA gene followed by an alignment with reference sequences using the BLAST algorithm (Altschul et al., 1997). The FAME profiles were characterized by the MIDI system (Microbial Identification System, Inc., Newark, USA). For the sequencing of 16S rRNA gene fragments the universal eubacterial primer 27F (Lane, 1991) and the DTCS CEQ^{TM}Quick Start Kit (Beckman Coulter) were used, according the manufacturer's manual. The sequences were analyzed by the CEQ^{TM} 2000 XL DNA analysis system in a Beckman Coulter sequencing machine. The Diversity of identified antagonistic isolates of different treatments was analyzed by computing the Simpson's index of diversity (Simpson, 1949) $D = 1 - \sum[n_i^*(n_i - 1)]/[N^*(N - 1)]$ with

S = number of species, n_i = number of individuals and N = total number of individuals in the survey. The Simpson's diversity index D accounts for richness and proportion of each species, focusing in particular on the abundance of the dominant species.

Analysis of the community structure by single strand conformation polymorphism (SSCP)-analysis

Microorganisms of the rhizosphere communities were extracted by serial centrifugation steps. The microorganism suspension, obtained after the Bagmixer treatment of the samples (5 g), was applied in a first centrifugation step at 450× *g* for 5 min to remove larger particles. The supernatant from this step was kept and centrifuged a second time at 10,000× *g* for 20 min to obtain the microorganism pellet. Afterwards the pellet was washed in 10 ml NaCl solution (0.85%) and stored at −70 °C. The DNA was isolated as described by Martin-Laurent et al. (2001). For mechanical lysis samples were homogenized in a FastPrep® Instrument (Qbiogene, BIO101® Systems, Carlsbad, USA) for 30 s at speed 5.0. DNA was purified by the Geneclean Turbo Kit (Qbiogene, BIO101® Systems, Carlsbad, USA) containing the special binding buffer guanidine thiocyanate for humic acid reduction. The procedure of single strand conformation polymorphism analysis (SSCP) was done according to Lieber et al. (2002). Table 1 shows the used primers and the PCR conditions. Bacterial communities were analyzed using the universal eubacterial primer pair Unibac-II-515f/Unibac-II-927rP (Lieber et al., 2002) in a reaction mixture of 52.2 μl PCR Mastermix (Tag&Go, Qbiogene, BIO101®), 0.5 μM each primer, 2.5 mM $MgCl_2$ and about 20 ng template. To obtain genetic fingerprints of fungal as well as *Pseudomonas* communities a nested PCR was applied. In a first PCR the fungal-specific primer pair ITS1f/ITS4rP (White et al., 1990) or the *Pseudomonas* specific primer pair F311Ps/1459rPs (Milling et al., 2004) were used in a 20 μl reaction mixture containing 11 μl PCR Mastermix (Tag&Go, Qbiogene, BIO101®), 0.5 μM each primer, 2.5 mM $MgCl_2$and 6 μl PCR-product from the first PCR as template. The PCR products were purified by the GeneClean Turbo Kit (Qbiogene, BIO101®), before an exonuclease digestion and DNA single strand folding according to Lieber et al. (2002). The polyacrylamide gel electrophoresis was performed on a TGGE apparatus (Biometra, Göttingen, Germany) at 26 °C and 400 V for about 19.5 h (fungi) or 26.5 h (eubacteria, pseudomonads) using 8% (wt/vol) and 8.5% (wt/vol) acrylamide gels for bacterial or fungal communities, respectively. Afterwards the Gels were

Table 1. Universal and specific primers and PCR conditions used for single strand conformation polymorphism analysis of the rhizosphere communities

Specifity	Primer	Primer sequence* (5′–3′)	Annealing temp. (°C)	PCR cycles	Reference
Eubacteria	Unibac-II-515f	GTG CCA GCA GCC GC	53	36	Lieber et al., 2002
	Unibac-II-927rP**	CCC GTC AAT TYM TTT GAG TT			
Pseudomonads	F311Ps	CTG GTC TGA GAG GAT GAT CAG T	63	25	Milling et al., 2004
	1459rPs	AAT CAC TCC GTG GTA ACC GT			
Fungi (ITS 1)	ITS1f	TCC GTA GGT GAA CCT GCG G	58	37	White et al., 1990
	ITS2rP**	GCT GCG TTC TTC ATC GAT GC			
Fungi (ITS2)	ITS1f	TCC GTA GGT GAA CCT GCG G	54	37	White et al., 1990
	ITS4rP**	TCC TCC GCT TAT TGA TAT GC			

*IUPAC-code: Y = T/C; M = A/C
**5′phosphoryled primer

silver-stained according to the procedure of Bassam et al. (1991). Conspicuous bands were eluted from the gel by suspending the gel slice in 70 μl elution buffer, containing 0.5 M ammonium acetate (Sigma), 10 mM magnesium acetate (Sigma), 1 mM EDTA (AppliChem) and 0.1% SDS (AppliChem), for at least 4 h at 37 °C and then identified by cloning according to Minkwitz and Berg (2000) and sequencing as described above. DNA sequences from SSCP bands were compared with available sequences from database using BLASTN program (Altschul et al., 1997) and the ARB software package (Department of Microbiology, Technical University of Munich, Munich, Germany) (Ludwig et al., 2004).

Computer-analysis of SSCP gels

The silver-stained SSCP gels were scanned using a transmitted light scanner (Casba^{TM}4 scanner, Spiral Biotech Inc., USA) to obtain digitized gel images that could be processed using GelCompar® software (Applied Math, Kortrijk, Belgium). After gel normalization, which allowed band pattern of different gels to be compared, and background subtraction similarity matrices were calculated using the band-based Dice similarity coefficient (Dice, 1945). Finally, dendrograms, representing the similarity of the microbial communities, were constructed with the hierarchic cluster method of unweighted pair group method using average linkages (UPGMA).

Nucleotide sequence accession numbers

Sequence accession numbers for sequences submitted to the EMBL nucleotide sequence database are AM000003-AM000021 for identified bacteria and AM084400-AM084413 for identified SSCP bands.

Results

Culture dependent analysis

Abundances of rhizosphere bacteria and establishment of biocontrol agents

The plate counts on R2A agar of the strawberry rhizosphere samples ranged from $\log_{10}$ 7.4 to 8.2 CFU g^{-1} root fresh weight [rfw] for the 71-I and 71 + C48-II treatment, $\log_{10}$ 7.1 to 7.7 CFU g^{-1} rfw for the C48-I and C48-II treatment, and $\log_{10}$ 7.4 to 8.0 CFU g^{-1} rfw for the control

plants. Samples from treated plants and the control did not differ significantly ($\alpha = 0.05$). The establishment of the rifampicin resistant mutant of strain HRO-C48 was exemplarily calculated in field trial 2. The strain was reisolated in numbers of $\log_{10}$ 6.1 to 4.5 CFU g^{-1} rfw with a seasonal decrease during the vegetation period (Figure 1).

Proportion of bacterial isolates antagonistic towards Verticillium *dahliae*

The proportion of bacterial antagonists in all investigated strawberry roots varied strongly but did not differ significantly between the treatments and the control ($\alpha = 0.05$). Altogether 2162 isolates were tested, resulting in 316 *Verticillium* antagonists. The proportion of bacterial antagonists was significantly higher in trial 1 compared to trial 2. The highest proportions of antagonists were $40.8\% \pm 21.6$ (SD) for the C48-I treatment, $23.7\% \pm 11.2$ for the 71-I treatment and $23.0\% \pm 12.2$ for the water control. Whereas in trial 2 the highest proportions of *Verticillium* antagonists amounts to $14.2\% \pm 1.6$ for the C48-II treatment, $16.9\% \pm 6.7$ for the 71 + C48-II treatment and $8.5\% \pm 3.9$ in the water control.

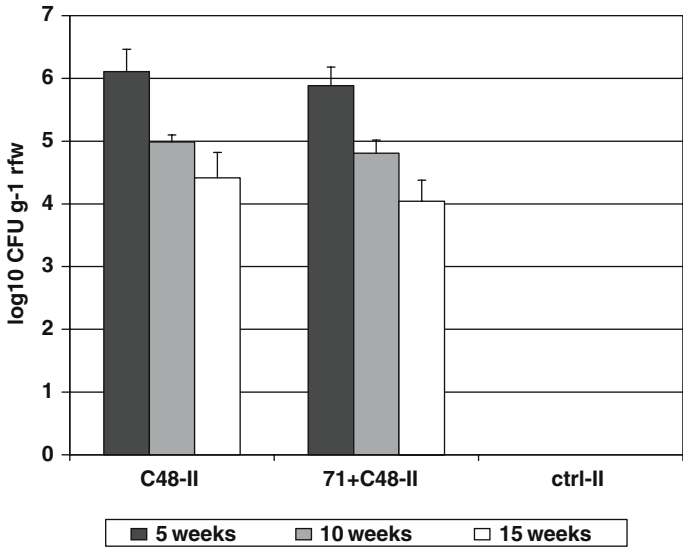

Figure 1. Abundance of re-isolated rifampicin resistant mutants of the strain HRO-C48 determined on nutrient agar containing Rifampicin (100 ppm) after three days incubation at 20 °C.

Spectrum of naturally occurring Verticillium *antagonists*

A total of 184 selected antagonistic strains, which represents all of the genotypic groups, that could be found using BOX-PCR fingerprinting, were identified by FAME and partial 16S rDNA sequencing to find out, if there is an influence on the spectrum of antagonistic species. All in all 34 bacterial species could be identified (Table 2) belonging to γ-proteobacteria ($n = 12$), high G + C group ($n = 12$) and low G + C group ($n = 10$). While *Pseudomonas putida* was an abundant species during the whole vegetation period in all rhizosphere samples in both trials, 14 species occurred only once. Altogether five *Pseudomonas* species and seven *Streptomyces* species could be identified. Strains of the genera *Paenibacillus, Xanthomonas* and *Streptoverticillium* were exclusively isolated in trial 1 and mainly isolated from the rhizosphere after 15 weeks, whereas strains belonging to the genera *Bacillus* and *Streptomyces* were found predominantly in trial 2.

Based on these data, the Simpson's index of diversity was calculated for each treatment (Table 2). In trial 1 we found no differences in the diversity of antagonistic bacteria caused by the treatments, whereas in trial 2 the diversity of *Verticillium* antagonists tended to decrease in the C48-II treatment and in the 71 + C48-II treatment after fifteen weeks.

Genotypic characterization of antagonistic bacteria

The 316 selected *Verticillium* antagonists (10–30 isolates per treatment) were characterized on the genotypic level using BOX-PCR. GelCompar® was used for the comparison of BOX patterns. The cluster analysis of BOX-fingerprints of the antagonistic isolates showed a high genotypic diversity of the antagonistic strains. The BOX pattern of test strains, analyzed by independent BOX-PCR reactions, showed a similarity of more than 80%. Therefore we assume that all strains with a higher similarity represent one genotype.

To analyze whether or not there is an impact on the non-target bacterial population, the Shannon's diversity index for the bacterial communities of each treatment was determined at about 80% similarity of the BOX pattern (Table 3). The cluster groups including the BOX pattern of strain HRO-C48 were not considered. In trial 1 and trial 2 the number of clusters increased during the vegetation period. This indicates an increased genotypic diversity of *Verticillium* antagonists in general. In both trials antagonistic bacterial strains with BOX pattern similar to that of HRO-C48 were frequently isolated in the

Table 2. Diversity of identified antagonistic isolates from different treatments of both trials

Species	Rostock 2001									Herten 2002								
	5 weeks			10 weeks			15 weeks			5 weeks			10 weeks			15 weeks		
	C48-I	71-I	ctrl-I	C48-I	71-I	ctrl-I	C48-I	71-I	ctrl-I	C48-II	71 + C48 -II	ctrl-II	C48-II	71 + C48 -II	ctrl.II	C48-II	71 + C48 -II	ctrl-II
low G + C																		
Bacillus cereus																	1	
Bacillus lentimorbus											3			1		1		
Bacillus simplex																1		
Bacillus subtilis											1			2		1		
Paenibacillus macerans								1	1									
Paenibacillus papuli				1														
Paenibacillus peoriae								1										
Paenibacillus polymyxa					3		8	5	2									
Staphylococcus hominis								2	1							1		
Staphylococcus warneri	1			1														
high G + C																		
Kitasatosporia kifunense											1			1				
Kytococcus sedentarius				1														
Microbacterium barkeri									1									
Micromonospora carbonacea							1		1									
Streptomyces lavendulae												1						
Streptomyces phaeochromogenes				1				1								2	3	1

Table 2. Continued

Species	Rostock 2001									Herten 2002								
	5 weeks			10 weeks			15 weeks			5 weeks			10 weeks			15 weeks		
	C48-I	71-I	ctrl-I	C48-I	71-I	ctrl-I	C48-I	71-I	ctrl-I	C48-II	71 + C48 -II	ctrl-II	C48-II	71 + C48 -II	ctrl.II	C48-II	71 + C48 -II	ctrl-II
Streptomyces scabies										1					1			
Streptomyces somaliensis														1				
Streptomyces subrutilus															1			
Streptomyces tauricus												1						
Streptomyces tumescens													1					
Streptoverticillium reticulum			2				2	1	1									
γ-proteobacteria																		
Lysobacter sp.	1						1											
Pseudomonas chlororaphis		1								1								
Pseudomonas fluorescens		1				2					2							1
Pseudomonas putida			3	2	6				1			1	2	2				
Pseudomonas syringae		1																
Pseudomonas veronii														1				
Serratia grimesii											1	1						
Serratia odorifera										5	4	1	1					
Serratia plymuthica															3			1
Serratia proteamaculans										1	1							

Stenotrophomonas maltophilia			1															
Xanthomonas axonopodis					1		3	3	7									
Number of species	2		4	3	5	3	5	7	8	4	7	5	6	3	7	1	2	3
Number of isolates	2		4	6	6	11	15	14	15	8	13	5	8	4	9	2	4	3
Simpson's index of diversity	1.0	n.d.	1.0	0.7	0.9	0.7	0.7	0.8	0.8	0.6	0.9	1.0	0.9	0.8	0.9	0	0.5	1.0

Table 3. Shannon Diversity indices calculated by grouping the BOX-PCR-fingerprints of the *Verticillium* antagonists at 80% similarity

Trial/treatment:	Trial 1			Trial 2		
Growth stage:	C48-I	71-I	ctrl-I	C48-II	71 + C48-II	ctrl-II
Young plant (5 weeks)	1.8	1.4	1.6	1.6	1.9	1.7
Flowering plant (10 weeks)	1.5	0.9	1.9	1.6	2.1	1.8
Senescent plant (15 weeks)	2.3	2.6	2.3	1.6	2.5	1.4

according treatments (C48-I, C48-II, 71 + C48-II) after 5 and 10 weeks, whereas in the third sampling (fifteen weeks) no HRO-C48-like pattern was found. Compared to the control, in the 71-I treatment (trial 1) the diversity apparently decreased after 5 and 10 weeks. The same results were obtained after 10 weeks in the C48-I and C48-II treatment. Nevertheless, compared to the control, after 15 weeks we detected an identical or even increased diversity in all treatments of both trials.

Figure 2 exemplarily shows the molecular BOX-PCR fingerprints of all antagonistic *Pseudomonas* strains. Strains of this genus were distributed over all treatments. At 78% similarity of BOX pattern nine clusters were formed. A preferential isolation of a certain genotype associated to a treatment was not found. Two clusters (cluster I and V) included strains of either trial 1 or trial 2 and two clusters a combination of strains from both trials. Cluster V contained genotypically, highly similar *Pseudomonas* isolates, originated from only one sampling time. However, in this cluster we also found no selection of specific genotypes due to the treatments. These results suggest that treatment, sampling time and field site tendentiously did not influence the distribution of *Pseudomonas* strains extensively.

Culture-independent analysis

Bacterial community fingerprints

Using universal eubacterial primers, the SSCP patterns suggest, that the microbial diversity in strawberry rhizosphere is immense (Figure 3). We found between 20–40 bands for the total bacterial community patterns and about 10–25 bands for the *Pseudomonas* specific and fungal community patterns. In general, bacterial SSCP patterns showed a high similarity and only a few single bands occurred occasionally, but this could not be associated to one of the

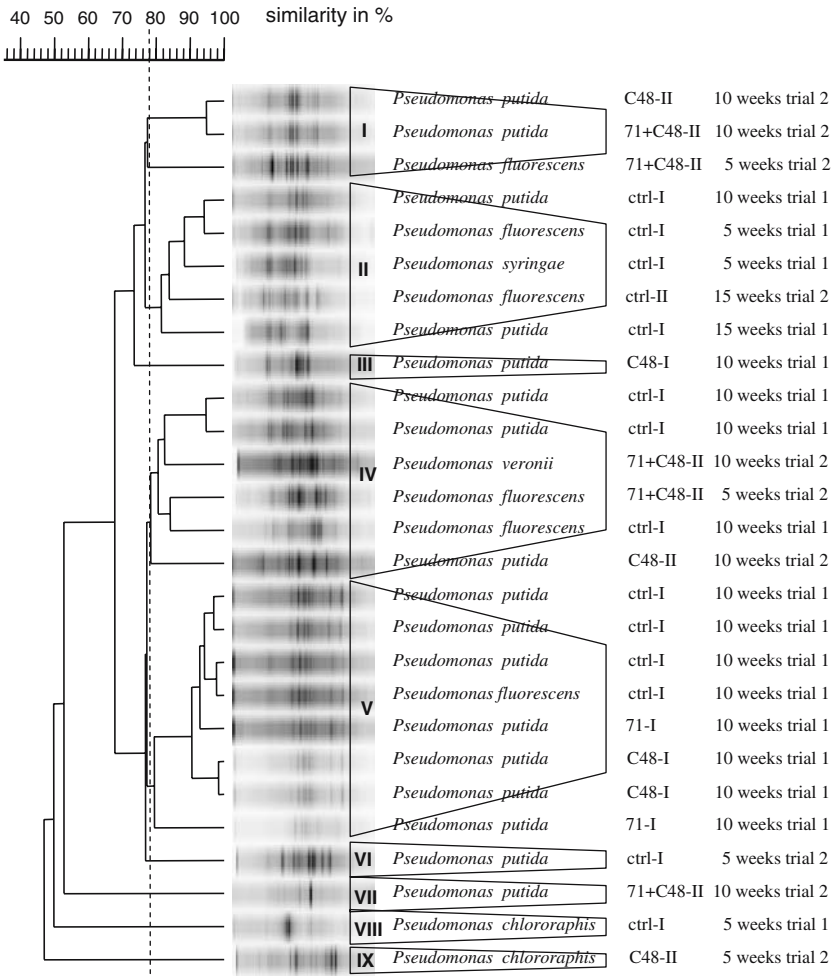

Figure 2. Dendrogram of antagonistic *Pseudomonas* strains obtained by cluster analysis with the UPGMA algorithm of BOX-PCR fingerprints using GelCompar® software.

treatments. Over the vegetation period the community composition changed. Band 1U-a identified as *Streptomyces* sp. (Table 4) became more intensive at second and third sampling while the bands 1U-b, 1U-f and 1U-g reflecting the bacterial genus/species *Lysobacter* sp., *Pseudomonas* sp. and *Sphingomonas subarctica*, could be detected with the same intensity in the rhizosphere samples of all plant growth stages. A strong band (1U-e) corresponding to the band of the reference strain HRO-C48 was detected in the fingerprints of the HRO-C48 treatment (C48-I, C48-II, 71 + C48-II) at the first and sec-

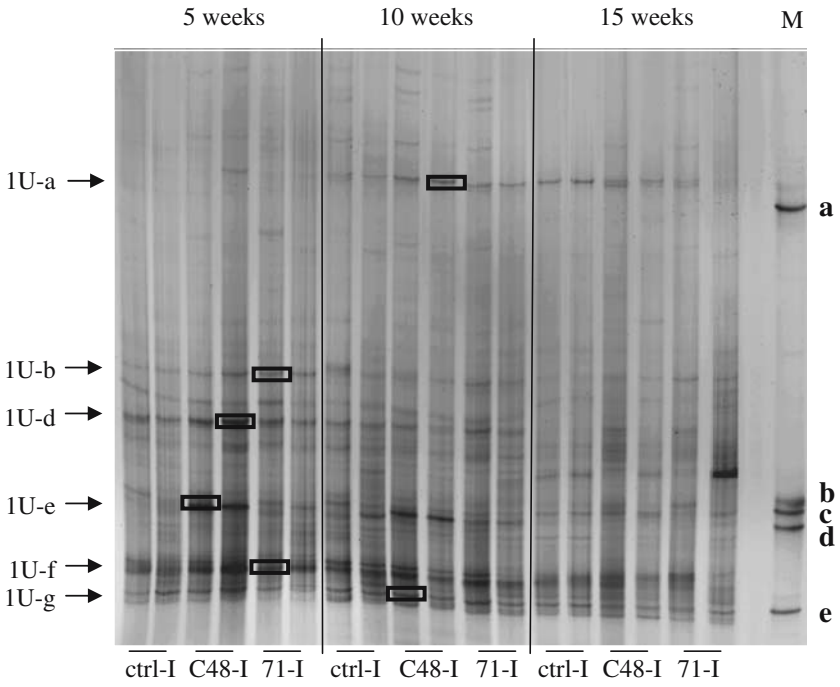

Figure 3. SSCP pattern from trial 1 obtained after HRO-C48 (C48-I), HRO-71 (71-I) and water control (ctrl-I) treatment using universal eubacterial primers at the sampling times (i) after 5 weeks, (ii) after 10 weeks, (iii) after 15 weeks. Lane marked M contains PCR-products from pure bacterial cultures: a = *Streptomyces* sp. HRO-71, b = *Collimonas fungivorans*, c = *Serratia plymuthica*, d = *Bacillus subtilis*, e = *Pseudomonas putida*; Black framed bands were excised for identification by sequencing (Table 4).

ond sampling time (for about ten weeks). Sequencing of those bands revealed a similarity of about 98% (trial 1) and 100% (trial 2) to the applied strain of *Serratia plymuthica* HRO-C48.

Band pattern of *Pseudomonas* communities showed an even higher similarity than the universal bacterial communities. The *Pseudomonas* community fingerprints of the treatments and the control were nearly identical. We recognized hardly any changes in community composition or diversity due to plant growth stages. However, the two trials differ greatly and indicate the development of a field site specific community (Figure 4).

Fungal rhizosphere community fingerprints

Fungal community profiles were obtained by nested-PCR amplification of the ITS1 region. As shown for the bacterial communities the composition

Table 4. Identification of DNA bands extracted from SSCP gels

Band	Origin	Closest database match (NCBI or ARB DataBank)		
		Species	Accession No.	SI (%)
1U-a	R*-eubacterial	*Streptomyces* sp.	Y15495	97.0
1U-b	R-eubacterial	*Lysobacter* sp.	AF472556	99.0
1U-c	R-eubacterial	*Arthrobacter* sp.	AY383043	96.0
1U-d	R-eubacterial	*Flavobacterium pectinovovorum*	D12669	99.0
1U-e	R-eubacterial	*Serratia plymuthica*	AJ233433	98.0
1U-f	R-eubacterial	*Pseudomonas* sp.	AB051695	98.0
1U-g	R-eubacterial	*Sphingomonas subarctica*	X94102	95.0
2U-a	H**-eubacterial	*Acinetobacter johnsonii*	X89775	99.3
2U-d	H-eubacterial	*Serratia plymuthica*	AJ233433	95.0
2U-g	H-eubacterial	*Sphingomonas rosa*	D13945	98.0
1P-c	R-Pseu	*Pseudomonas syringae*	AF326379	99.0
1P-e	R-Pseu	*Xanthomonas* sp.	AB016762	93.0
2P-c	H-Pseu	uncultured bacterium	AY328768	98.0
2P-d	H-Pseu	*Pseudomonas fluorescens*	AY785748	99.0
2I-b	H-ITS	*Geomyces* sp. FF130	AJ608960	98.0
2I-c	H-ITS	*Exidiopsis* sp.	AF291282	97.0
2I-d	H-ITS	Mycorrhizal fungal sp. pkc11	AY394892	94.0
1I-c	R-ITS	*Cladosporium herbarum*	AF177734	99.0

*R = Rostock
**H = Herten

of fungal communities changed depending on the plant growth stage. These changes were more distinct in the samples from trial 1 (Figure 5), whereas community fingerprints from trial 2 (data not shown) were found to be more similar concerning replicates and different treatments. Fungal SSCP fingerprints showed a high degree of heterogeneity. In contrast to the bacterial community fingerprints, the SSCP patterns of fungal communities showed a certain variability within the replicates which even increased during the vegetation period. Therefore it was quite difficult to obtain clear results concerning a possible impact on the diversity of the indigenous fungal communities.

Computer-assisted comparison of SSCP profiles

The GelCompar® software was used to compare the fungal and bacterial SSCP profiles generated from samples of different field sites, samplings and treatments. For all three microbial groups (bacteria, fungi, pseudomonads) investigated, a clear differentiation of patterns

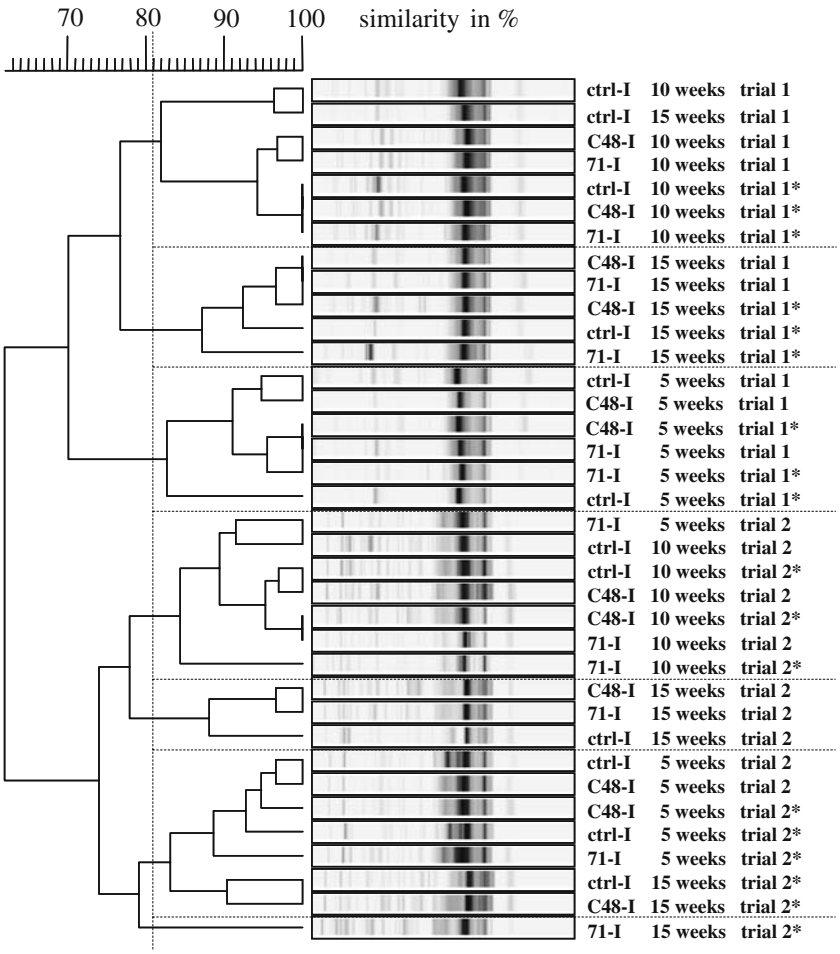

Figure 4. Cluster analysis of SSCP fingerprints of *Pseudomonas* communities from both trials. Dice similarity index was calculated before clustering using UPGMA method. The asterisks (*) mark the replicates.

was found according to the field site. Samples from different trials were grouped into two clusters at fingerprint similarities of 63% for pseudomonads (Figure 3) and approx. 60% for fungal and total bacterial communities, containing exclusively samples from one field site. Samples from different sampling times formed discrete clusters at similarities of 65% and higher for bacterial communities or 70% for fungal communities. Molecular fingerprints of total bacterial and *Pseudomonas* communities from treated and untreated plants showed at 80% to 100% similarity while it was only 70% to 80% for fungal

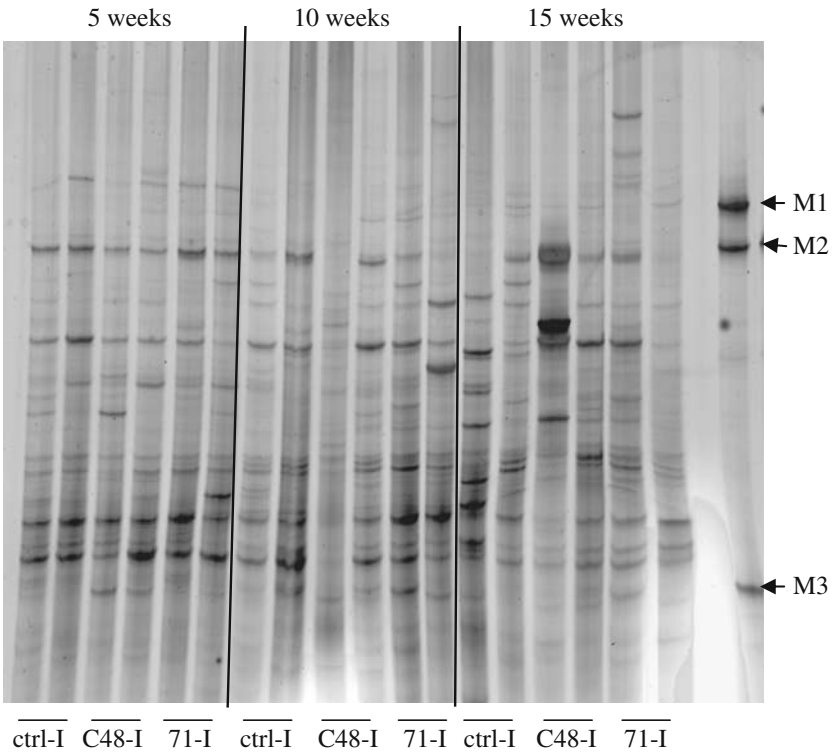

Figure 5. SSCP fingerprint of fungal rhizosphere communities from field trial 1 obtained after amplification of the ITS1 region. Lanes marked M contain PCR-products from pure fungal cultures: M1 = *Trichoderma viride*, M2 = *Trichoderma longibrachiatum*, M3 = *Alternaria alternata*. The different treatments (C48-I, 71-I) and the control (ctrl-I) are marked below.

communities. Apart from the replicates of bacterial community fingerprints, most similar SSCP fingerprints were recognized between samples from different treatments which showed similarities of 78–100%. Despite the high variability within the replicates the same results were obtained for fungal communities. Here, the most similar community fingerprints (69–79% similarity) were detected between different treatments, whereas samples from different vegetation periods showed 70–72% similarity between SSCP band patterns.

Discussion

Biological plant protection is playing an increasing role from an ecological point of view and the sustained dealing with natural

resources. To find a suitable method for risk assessment we compared both, cultivation-dependent and cultivation-independent methods, in this study. Two strains, the Gram negative *Serratia plymuthica* HRO-C48 and the Gram positive *Streptomyces* sp. HRO-71, were applied to strawberry rhizosphere.

A good establishment of the introduced strains is essential for an effective biological control. The rifampicin resistant mutant of the strain HRO-C48 was reisolated from the rhizosphere of strawberry over three months and could also be found among the dominant members in the SSCP fingerprints of the corresponding, HRO-C48-treated communities. This indicated an effective root colonization of the strain HRO-C48 in strawberry rhizosphere, and supports the results of Kurze et al. (2001). The direct establishment of the strain HRO-71 was not determined, because of a lack of a suitable mutant. Further we were not able to find a corresponding band in the HRO-71-treated communities by culture-independent analysis.

Four cultivation-dependent criteria were investigated: (i) abundances of rhizosphere bacteria, (ii) antagonistic potential against *V. dahliae*, (iii) species composition and (iv) diversity of antagonistic bacteria. The abundances of rhizosphere bacteria and the antagonistic potential were not influenced by the BCA treatments. In both field trials, the bacterial abundances did not differ between the treated and non-treated plants. The proportion of antagonistic bacteria isolated in trial 1 was relatively high compared to the results of Berg et al. (2002, 2005) who found a proportion of *Verticillium* antagonists of 9.5% or 10.2% in the rhizosphere of field-grown strawberries over several vegetation periods. The species composition of the antagonistic strains differed slightly between the treated and non-treated plants. However, these results should be interpreted cautiously because of the limited number of identified isolates in some treatments (Table 2). Nevertheless, the rhizosphere of the young strawberry plant was shown to be colonized mainly by fast growing *Pseudomonas, Serratia* and *Bacillus* strains. A rather high proportion of *Pseudomonas* strains as *Verticillium* antagonists in the strawberry rhizosphere was described by Berg et al. (2002). Pseudomonads are important members of the root-associated microbial community due to their aggressive colonization of the rhizosphere. Their strong plant growth promoting ability could be proved in several studies (Bloemberg and Lugtenberg, 2001; Weller et al., 2002). A high percentage of the antagonistic bacteria could be identified as *Serratia plymuthica* strains by 16S rDNA sequencing and FAME. This species was dominant in this treatment for about

10 weeks confirming the good establishment of the applied strain HRO-C48 in the strawberry rhizosphere. But these differences were not significant due to a high variation in general. The characterization of all antagonistic strains was assessed by the molecular BOX fingerprint. This method, based on repetitive BOX elements in the bacterial genome, is prominent by its simplicity and applicability to a variety of bacterial groups. Slightly differences in the diversity could be found, that arisen only from transient effects (Table 3). Thus, the diversity of the naturally antagonistic isolates of the strawberry rhizosphere was not influenced by the treatments.

An analysis of the community structure by cultivation-independent methods is urgently needed for an impact assessment, because cultivation approaches offer only a limited knowledge of naturally occurring microbial diversity. As a cultivation-independent method the single strand conformation polymorphism (SSCP) analysis was used (Schwieger and Tebbe, 1998). This method avoids the heteroduplex formation during electrophoresis and does not require the construction of compound gradient gels. This is a decisive advantage over denaturing gradient gel electrophoresis (DGGE). It provides the reproducibility and comparability of gels. By this method in both field trials only transient differences in the bacterial communities were observed, but not associated to a certain treatment. Using universal as well as group-specific primers similar patterns in treated and un-treated plants were obtained. Single bands appeared independent of the treatment. The dominance of the strain HRO-C48 in this treatment was confirmed. A strong band corresponding to the HRO-C48 isolate was visible and became weaker during the vegetation period (Figure 4). Similar results were found by the analysis of bacterial rhizosphere communities of transgenic potatoes after the application of an antagonistic *Serratia grimesii* strain (Lottmann et al., 2000). A corresponding band to strain HRO-71 could not be detected in both trials but other members of the *Streptomyces* genus were identified. The application of the strain HRO-71 did not cause any threat to the indigenous *Streptomyces* community of strawberry rhizosphere by competing with other members of this genus. Strains of this genus were frequently detected by cultivation-independent methods in the rhizosphere of strawberry (Smalla et al., 2001) or as endophyt on selective agar plates (Coombs and Franco, 2003). Differences observed between the sampling times and locations were higher than between the treatments. The community structure seemed to be less influenced by the treatment, rather than by seasonal shifts and soil

quality. This could be found for bacterial as well as fungal communities despite of the heterogeneity and replicate variability of the fungal community fingerprints that were also detected by other authors (Girvan et al., 2004; Costa et al., 2005). Changes in the structure of bacterial and fungal communities during the vegetation period are usually observed (Lottmann et al., 2000; Smalla et al., 2001; Smit et al., 2001). A number of biotic and abiotic factors can have a significant effect on the microbial community structure and activity, including soil structure, fertilizer use, crop species and climate as reviewed by Garbeva et al. (2004).

The data presented in this study indicate that the introduction of antifungal strains into the strawberry rhizosphere causes only minor and transient effects on the composition of the rhizosphere microbiota. We compared cultivation-dependent and cultivation-independent criteria to characterize the community structure. We did not observe quantitative effects on the abundances of culturable bacteria and on the proportion of indigenous *Verticillium* antagonists. Although the proportion of antagonists in the control samples was often lower, especially at the beginning of the vegetation period, a significant difference between the treatments and the control could not be determined. The main effect we found was the frequent occurrence of strain HRO-C48 for about 10 weeks in the corresponding treatments. A good establishment with less sustainable effects on the indigenous microbiota is the aim of biological plant protection. Cultivation, testing for antagonistic activity, characterization and identification are time consuming and large-scale processes. Furthermore, it is necessary to extend the investigations on the whole rhizosphere community. Molecular methods like denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) and terminal restriction fragment length polymorphism (T-RFLP) are common and sufficient methods to characterize complex microbial communities (Liu et al., 1997; Muyzer and Smalla 1998; Schwieger and Tebbe, 1998).

In this study, the changes observed were of little relevance and were not caused primarily by the treatment. Similar results were obtained in other culture dependent (Walsh et al., 2001) and culture-independent (Van Dillewijn et al., 2002; Viebahn et al., 2003) studies after the introduction of genetically modified bacteria on various plants. In these studies, seasonal shifts of community composition, but only minor, transient shifts due to the beneficial microorganisms, were detected. Results from previous studies, along with the results

from our culture-dependent and –independent experiments indicate that there is no long-term impact on the indigenous microbial communities due to a root treatment using bacterial antagonists. Therefore, we did not observe any definable non-target risk for indigenous microorganisms arising from an application of wildtype bacterial biocontrol organisms.

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