RESEARCH ARTICLE

Effect of bacterial antagonists on lettuce: active biocontrol of *Rhizoctonia solani* and negligible, short-term effects on nontarget microorganisms

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Abstract

The aim of this study was to assess the biocontrol efficacy against *Rhizoctonia solani* of three bacterial antagonists introduced into naturally *Rhizoctonia*-infested lettuce fields and to analyse their impact on the indigenous plant-associated bacteria and fungi. Lettuce seedlings were inoculated with bacterial suspensions of two endophytic strains, *Serratia plymuthica* 3Re4-18 and *Pseudomonas trivialis* 3Re2-7, and with the rhizobacterium *Pseudomonas fluorescens* L13-6-12 7 days before and 5 days after planting in the field. Similar statistically significant biocontrol effects were observed for all applied bacterial antagonists compared with the uninoculated control. Single-strand conformation polymorphism analysis of 16S rRNA gene or ITS1 fragments revealed a highly diverse rhizosphere and a less diverse endophytic microbial community for lettuce. Representatives of several bacterial (*Alpha-*, *Beta-* and *Gammaproteobacteria*, *Firmicutes*, *Bacteriodetes*), fungal (*Ascomycetes*, *Basidiomycetes*) and protist (*Oomycetes*) groups were present inside or on lettuce plants. Surprisingly, given that lettuce is a vegetable that is eaten raw, species of genera such as *Flavobacterium*, *Burkholderia*, *Staphylococcus*, *Cladosporium* and *Aspergillus*, which contain potentially human pathogenic strains, were identified. Analysis of the indigenous bacterial and endophytic fungal populations revealed only negligible, short-term effects resulting from the bacterial treatments, and that they were more influenced by field site, plant growth stage and microenvironment.

Introduction

The soil-borne phytopathogenic fungus *Rhizoctonia solani* Kühn [teleomorphe: *Thanatephorus cucumeris* (A.B. Frank) Donk; basidiomycetes] is responsible for high-yield losses in a number of economically important crops worldwide (Wolf & Verreet, 1999). Based on hyphal anastomosis reactions, *Rhizoctonia* isolates are divided into 13 anastomosis groups (AGs), each having a distinct degree of host specificity (Carling *et al.*, 2002). In addition to causing late sugar-beet rot and damping-off diseases on various vegetables, *Rhizoctonia* causes bottom rot on lettuce, an economically important disease resulting in yield losses of up to 70% (Davis *et al.*, 1997). *Rhizoctonia solani* isolates of groups AG1-IB (Germany), AG2-1 (UK, the Netherlands) and AG4 (the

Netherlands, UK, USA) have isolated from diseased lettuce plants (Maplestone *et al.*, 1991; Grosch *et al.*, 2004). However, strategies to control *Rhizoctonia* diseases are limited because *Rhizoctonia*-resistant cultivars are currently unavailable (Li *et al.*, 1995). Furthermore, control of the pathogen is difficult because of its ecological behaviour, its extremely broad host range, and the high survival rate of sclerotia under various environmental conditions. There are broad-spectrum as well as *Rhizoctonia*-specific fungicides available for some crops, but many chemical pesticides will be phased out over the next few years, leaving no efficient solutions for future plant protection. Moreover, there is an increasing concern for environmental protection and a demand for healthy food without chemical residues, especially for vegetables eaten raw.

The use of naturally occurring antagonists to biologically control plant pathogens might offer alternative environmentally friendly plant protection strategies (Whipps, 2001). In previous studies, we successfully isolated bacteria with antagonistic activity towards *Rhizoctonia* from the endosphere and rhizosphere of potato plants (Berg *et al.*, 2005b) that showed *ad planta* biocontrol activity against *R. solani* on lettuce (Grosch *et al.*, 2005). From these studies, the three most promising bacterial isolates, *Serratia plymuthica* 3Re4-18, *Pseudomonas trivialis* 3Re2-7 and *Pseudomonas fluorescens* L13-6-12, were selected in order to assess their biocontrol activity further under commercial production conditions.

Although originating from plant-associated microenvironments, beneficial bacteria, if applied to plant roots in sufficient numbers, may perturb indigenous microbial populations and the important ecological functions associated therewith (Blouin-Bankhead *et al.*, 2004; Winding *et al.*, 2004). Therefore, unwanted, unspecific actions of the introduced beneficial microorganisms against nontarget organisms have to be assessed. To this end, knowledge concerning the microbial ecology of the target habitats is necessary for reasonable risk assessment studies relating to the release of beneficial microorganisms. As only a small proportion of the microorganisms can be analysed by common cultivation techniques, several DNA-based, cultivation-independent methods had been developed to overcome the limitations of cultivation techniques (reviewed in Smalla, 2004). The use of such molecular methods is urgently needed to include the greatest possible number of total microorganisms in risk assessment studies regarding nontarget effects of introduced beneficial bacteria. To date, only a few cultivation-independent studies have focused on the effects of genetically modified microorganisms (GMOs) such as *Pseudomonas* (Glandorf *et al.*, 2001; Viebahn *et al.*, 2003) and *Sinorhizobium* (Schwieger & Tebbe, 2000) on nontarget microorganisms. Little is known, however, about these effects for biological control agents (BCAs) based on naturally occurring strains as well as for commercialized BCA products (Winding *et al.*, 2004).

Our study aimed (1) to assess the biological control properties of three bacterial strains antagonistic to *R. solani* under conditions similar to those used in commercial lettuce production, and (2) to analyse their interaction with the indigenous microbial communities of field-grown lettuce using cultivation-independent PCR-single-strand conformation polymorphism (PCR-SSCP) analysis combined with gaining new information about autochthonous lettuce-associated microbial communities. So far, studies on lettuce-associated microorganisms have been performed mainly on processed lettuce and using common microbiological cultivation practices (Maloney *et al.*, 1997; Fiddaman *et al.*, 2000). We report here for the first time on the indigenous endophytic and rhizosphere plant-associated microbial community of unprocessed field-grown lettuce.

Materials and methods

Bacterial strains

The bacterial strains *Pseudomonas fluorescens* L13-6-12, *Pseudomonas trivialis* 3Re2-7 and *Serratia plymuthica* 3Re4-18 were obtained from the Culture Collection of Antagonistic Microorganisms (SCAM) at Graz University of Technology and stored at $-70\,^\circ$C in nutrient broth (Oxoid Limited, Basingstoke, UK) containing 15% glycerol. The three strains originate from the rhizosphere (L13-6-12) (Lottmann *et al.*, 1999) and the endorhiza (3Re4-18, 3Re2-7) (Berg *et al.*, 2005b) of potato plants and show antifungal activity *in vitro* as well as *ad planta* (Faltin *et al.*, 2004; Grosch *et al.*, 2005).

Field experiments

The effect of the three BCAs *P. fluorescens* L13-6-12, *P. trivialis* 2Re3-7 and *S. plymuthica* 3Re4-18 on bottom rot in lettuce was tested in two experimental fields (100 m × 24 m), both of which are naturally infested with *R. solani* AG1-IB (Grosch *et al.*, 2004). These fields are located at the Institute of Vegetable and Ornamental Crops in Golzow (lat. 52°N/loamy clay) and in Grossbeeren (lat. 52°N/sandy loam). The experimental fields were divided into 12 beds each containing 10 plots of 17.28 m² with 11.1 plants m⁻². Treatments were given randomly with seven replications, and the neighbouring plot of each treatment was left untreated to exclude any treatment influences. Fertilization was based on soil chemical properties, analysed before planting. Standard fertilizer was used 1 day before planting (N 100 kg ha⁻¹ and K 120 kg ha⁻¹). Weeds were removed by hand during the first 3 weeks. Lettuce seedlings *cv*. 'Nadine' were grown to the three to four leaf stage in plant containers holding 92 seedlings in a greenhouse at 20/15 °C (16/8 h day night⁻¹) and then transplanted by hand into the field (2004 July 10, Golzow; 2004 July 24, Grossbeeren). Overhead sprinkler irrigation was applied immediately after planting and then as required to supplement rainfall and maintain good crop growth. For preparing the bacterial solutions for the plant treatments, BCAs were grown in nutrient broth (Sifin, Berlin, Germany) for 12 h at 29 °C. BCA solutions were first applied 7 days before planting at the two to three leaf stage. The seedlings ($n = 150$) were drenched with 1.0 L of bacterial suspension (10⁷ CFU mL⁻¹). The second application was applied with a back sprayer (BASF, Gloria tank-back-sprayer), 5 days after planting, with 1.0 L of bacterial suspension (10⁹ CFU mL⁻¹) per m² of soil surface. Approximately 5 (Golzow) and 6 (Grossbeeren) weeks after planting, the lettuce plants were harvested

by hand and the dry weight (DW) of 35 plants was measured. In addition, disease severity (DS) of 15 plants per plot was assessed on a scale ranging from 1 to 7 (Kofoet *et al.*, 2001). The effectiveness of the individual BCAs was compared with the effect of a systemic fungicide (BAS 516 00 F), which was also tested with a single and double application. The first application was carried out 3 days after planting, and a second application was performed 10 days after planting with a sprayer ($1 \, L \, m^{-2}$, $0.15 \, g \, m^{-2}$).

Statistical analysis

The STATISTICA program (StatSoft Inc., Tulsa, OK) was used for statistical analysis. The DW of lettuce plants was compared with the control after ANOVA using Tukey's procedure (HSD) with $P = 0.05$. The DS of lettuce plants with bottom-rot symptoms in the field experiments was analysed using the Kruskal–Wallis nonparametric test with $P < 0.05$.

Sampling and extraction of microorganisms

Samples were taken 2 days after the first treatment from *c.* 3-week-old plants, and 5 (Golzow) or 6 (Grossbeeren) weeks after transplantation in the field with three replicates per treatment. Material from three plants was combined to one sample, each consisting of 5 g of roots with adhering soil for rhizosphere analysis, 10 g of leaves for the analysis of the endophyllosphere, and 5 g of roots for the analysis of the endorhiza. Serial centrifugation steps were applied to extract the microorganisms from the rhizosphere samples as described elsewhere (Scherwinski *et al.*, 2006). For the analysis of the endophytic microorganism communities, the roots or leaves were washed and then surface-sterilized by incubation in 20% hydrogen peroxide for 10 min followed by four washing steps using sterile water. The sterility of roots and leaves was checked on nutrient agar (Sifin, Berlin, Germany). To extract the endophytic microorganisms, 2 mL of $1 \times$ phosphate buffered saline (PBS) was added to the sterilized plant material before grinding using mortar and pestle. After centrifugation of the resulting suspension at $10.000 \, \boldsymbol{g}$ for 20 min, the microorganism pellets were stored at $-70 \, °C$.

DNA extraction and amplification

Total DNA extraction was carried out according to Martin-Laurent *et al.* (2001): the procedure involved mechanical cell disruption in an extraction solution containing 100 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% (w/v) polyvinylpyrrolidone and 2% (w/v) sodium dodecyl sulfate, and use of the FastPrep® Instrument (Qbiogene, BIO101® Systems, Carlsbad). The genetic diversity within the rhizosphere, endophyllosphere and endorhiza communities from all BCA-treated samples, except those from the

L13-6-12-treated samples, which were not considered for analysis of endophytes, were analysed by SSCP. Amplification of bacterial 16S rRNA gene fragments was carried out as described previously (Scherwinski *et al.*, 2006) using the eubacterial primers Unibac-II-515f/Unibac-II-927rP (5′-GTG CCA GCA GCC GC-3′) and Unibac-II-927rP (5′-CCC GTC AAT TYM TTT GAG TT-3′; Lieber *et al.*, 2002) in the reaction mixture: 52.2 μL of PCR Mastermix (Tag&Go, Qbiogene, BIO101®), 0.5 μM of each primer, 2.5 mM MgCl$_2$ and about 20 ng of template. *Pseudomonas*-specific primers F311Ps/1459rPs (Milling *et al.*, 2004) as well as the fungal primer pairs ITS1f/ITS4r (White *et al.*, 1990) were applied in a 20-μL reaction mixture containing 11 μL of PCR Mastermix (Tag&Go, Qbiogene, BIO101® Systems, Carlsbad), 0.5 μM of each primer, 2.5 mM MgCl$_2$ and about 20 ng of template. To obtain smaller fragments, allowing a better separation during electrophoresis, a nested PCR was carried out using primers Unibac-II-515f/Unibac-II-927rP (Lieber *et al.*, 2002) for pseudomonads and ITS1f/ITS2r (White *et al.*, 1990) for fungi. The reaction mixture was 52.2 μL of PCR Mastermix (Tag&Go, Qbiogene, BIO101®), 0.5 μM of each primer, 2.5 mM MgCl$_2$ and 1 : 100 diluted product from the first PCR as template.

SSCP analysis

SSCP analysis of amplified bacterial 16S rRNA gene or ITS1 fragments was carried out according to Schwieger & Tebbe (1998) modified by Lieber *et al.* (2002). Exonuclease digestion (10 U λ Exonuclease, New England BioLabs Inc., Beverly) of purified PCR products was applied before the electrophoretic separation of folded DNA single strands using the TGGE MAXI system (Biometra, Göttingen, Germany). Triplicates of each treatment along with pure cultures of the antagonistic strains and a DNA standard (Ferments GmbH, St Leon-Rot) were loaded onto 8% (bacterial communities) or 9% (fungal communities) acrylamide gels. Electrophoretic separation was performed in $1 \times$ Tris-borate-EDTA (TBE) buffer for 26 h (bacteria) or 19 h (fungi). After silver-staining according to Bassam *et al.* (1991), gels were digitalized using a transillumination scanner (Casba™4 scanner, Spiral Biotech. Inc.).

Computer-assisted cluster analysis

In order to compare SSCP fingerprints of the microbial communities, a computer-assisted cluster analysis was carried out using the GELCOMPAR® software (Applied Math, Kortrijk, Belgium). DNA standards were loaded onto each gel to allow a postrun correction of gel-specific differences between gel-runs using the normalization function of the GELCOMPAR® software. After background subtraction and normalization of the digitalized images of the SSCP gels, similarities between the SSCP fingerprints were calculated

using the band-based Dice similarity coefficient (Dice, 1945). Afterwards, the fingerprints were grouped according to their similarity using the hierarchical cluster method unweighted pairwise grouping method using arithmetic means (UPGMA). The Shannon and Weaver diversity index (H') was calculated from the SSCP banding patterns as $H' = -\Sigma[(n_i/N)\,\ln(n_i/N)]$, with n_i the intensity of a band relative to the total intensities of all bands of the analysed lane, and N the total number of bands in the analysed lane. Band intensity values were determined from densitometric curves generated from the GELCOMPAR® software, considering equal sensitivities for all analysed SSCP gel pictures.

Sequencing of SSCP bands

For further characterization of the analysed microbial communities, constantly dominant or variably emerging SSCP bands were excised from the gels. DNA was eluted from the gel slices by overnight incubation in sterile H_2O at 4 °C, and reamplified using the same primer pairs as described above for the SSCP analysis. PCR products were cloned into a pGEM® T-vector (Promega, Madison, WI) according to Minkwitz & Berg (2001). To identify comigrating DNA fragments represented by one band, amplified ribosomal DNA restriction analysis (ARDRA) of four to six clones per excised SSCP band was carried out for bacterial inserts to reveal the number of different DNA fragments possibly combined in one single band. After reamplification of the cloned inserts (412 bp) using the plasmid-specific M13 primer pair (Promega), ARDRA was carried out according to Costa *et al.* (2006) using the restriction enzymes AluI and MspI. As the ITS regions are highly variably in sequence and length, excised and reamplified ITS fragments were separated in high-concentration (3.5%) agarose gels to detect different ribotypes possibly represented by the same SSCP band. Inserts showing different ARDRA patterns or different sequence length were selected for sequencing using the plasmid-specific primer M13 reverse (Promega) and analysed in a Beckman Coulter sequencing machine (Beckman Coulter Inc., Fullerton, CA). The sequences were aligned with available sequences of the NCBI database (Altschul *et al.*, 1997) or the ARB database (Ludwig *et al.*, 2004).

The nucleotide sequences obtained within this study have been submitted to the EMBL database (http://www.ebi.ac.uk/embl/) under the accession numbers AM411171–AM411212.

Results

Biocontrol effects in field trials

The three antagonistic bacteria, *S. plymuthica* 3Re4-18, *P. trivialis* 3Re2-7 and *P. fluorescens* L13-6-12, were analysed for disease-suppressive activity against *R. solani* in two

naturally infested lettuce fields. All bacterial and fungicide treatments significantly reduced the DS at both field sites at levels within the range 20–42% (Fig. 1a). Interestingly, the applied BCAs showed, in comparison with the untreated control, the same efficiency as the fungicide treatment. The best disease reduction was achieved by the fungicide double treatment (42%) in the Grossbeeren trial, and by the *P. fluorescens* L13-6-12 treatment (24%) in the Golzow trial. A generally higher DS was observed for all treatments in Golzow. Compared with the experiment in Golzow (20–24% reduced DS), disease was better suppressed by the various treatments at the Grossbeeren trial (35–42%). Correlated with the lower DS in Grossbeeren, the DW was generally higher at this site than at Golzow. However, a significantly increased DW was determined for all treatments at both sites with the exception of the *P. trivialis* 3Re2-7 treatment in Golzow (Fig. 1b). Interestingly, the same treatment (3Re2-7) achieved the best DW increase at Grossbeeren (19%), whereas the *S. plymuthica* 3Re4-18 treatment at Golzow (20%) was responsible for the best DW. Again, for bacterial and fungicide treatments equal effects could be attained, with all bacterial antagonists
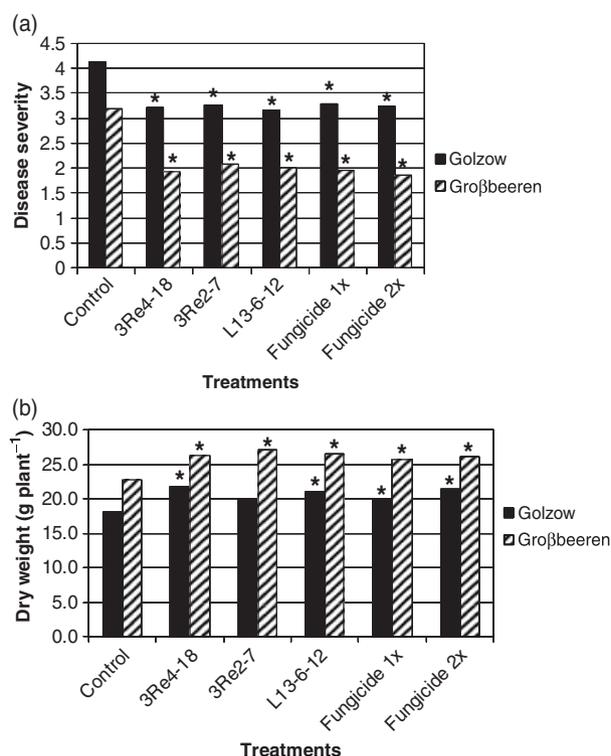

Fig. 1. Disease severity (a) and dry weights (b) of inoculated and uninoculated lettuce plants determined at harvest 5 (Golzow) or 6 (Grossbeeren) weeks after planting in the field. Disease severity (DS) is based on a scale ranging from 1 (low DS) to 7 (high DS) according to Kofoet *et al.* (2001). Asterisks mark significant differences from the uninoculated control treatment ($P = 0.05$).

showing a slightly better impact on the DW at the Grossbee-ren trial compared with the fungicide treatments.

Plant-associated microbial communities of field-grown lettuce

The plant-associated bacterial communities of unprocessed field-grown lettuce were analysed by SSCP using universal bacterial as well as *Pseudomonas*-specific primers. Generally, the community structures were strongly related to the field site, plant growth stage and the microenvironment (Figs 2–5). As expected, within the SSCP profiles, a much higher genetic diversity was found within the rhizosphere communities compared with the communities from the endophytic habitats. Shannon and Weaver diversity indices calculated from the SSCP banding patterns were $\langle H' \rangle = 3.2 \pm 0.2$, 2.5 ± 0.05 and 1.9 ± 0.1 for the rhizosphere, endorhiza and endophyllosphere samples, whereas diversity indices of $\langle H' \rangle = 2.4 \pm 0.3$, 2.2 ± 0.2 and 1.7 ± 0.3 were determined from the band patterns of the corresponding *Pseudomonas* populations. The complexity of the rhizosphere *Pseudomo-nas* communities (represented by number of bands) was found to increase from the first to the second sampling. However, computer-assisted comparison of the fingerprints showed similarities of *c.* 70% between fingerprints from different samplings and of *c.* 60% between rhizosphere and endorhiza samples. SSCP profiles from endophyllosphere communities formed a unique group at 50% similarity, apart from all root-associated community patterns (Fig. 4).

Altogether, 22 bacterial ribotypes could be further char-acterized by sequencing (Table 1). Cloned 16S rRNA gene fragments were assigned to the phylogenetic groups of *Proteobacteria* (*Alpha*, *Beta* and *Gamma* subgroups), *Firmi-cutes* and *Bacteriodetes*. The list contains some genera commonly associated with plants, for example *Pseudomo-nas*, *Rhodanobacter*, and *Burkholderia*. Most of the obtained sequences were phylogenetically affiliated to the group of *Proteobacteria*. Many of the sequenced inserts showed high-est homologies to clone sequences derived from other cultivation-independent analyses of environmental samples (e.g. clones US28_5, US31_2, US41_1, PS27_2). For two inserts (clones US10_5, PS21_1) derived from endosphere
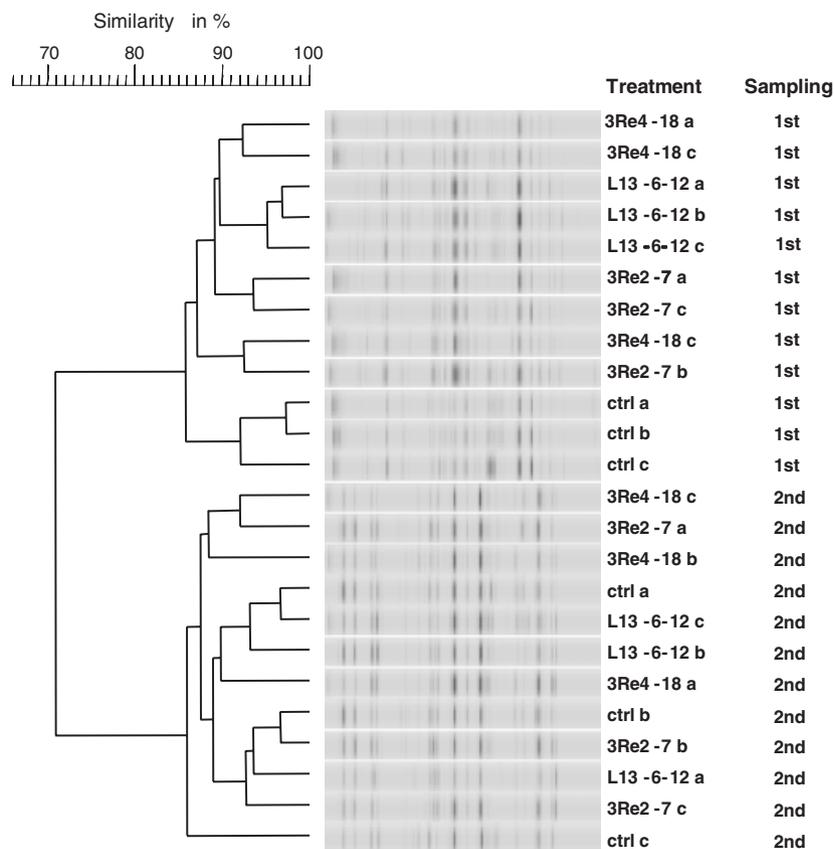

Fig. 2. Cluster analysis of the rhizosphere bacterial samples after the various treatments (*Serratia plymuthica* 3Re4-18, *Pseudomonas trivialis* 3Re2-7, *Pseudomonas fluorescens* L13-6-12 7, fungicide) from the first and second samplings (Grossbeeren). Similarities between SSCP fingerprints were calculated using the band-based Dice similarity coefficient and grouped according to their similarity using the hierarchical UPGMA. Letters a–c mark the three independent replicates per treatment.
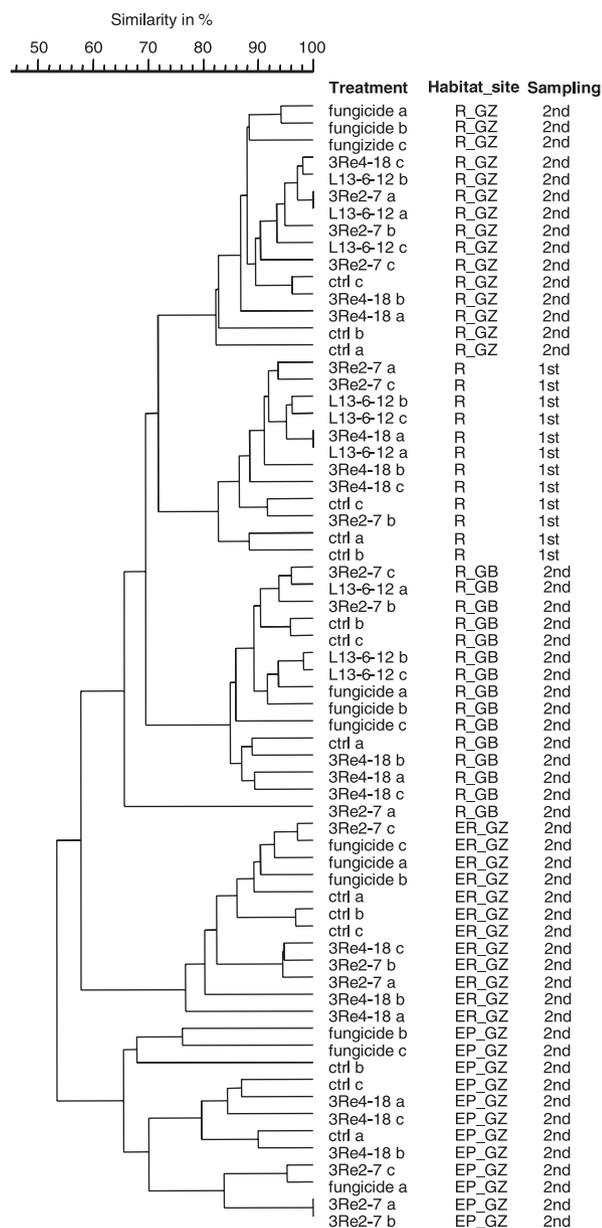
Fig. 3. Cluster analysis of all fingerprints obtained from *Pseudomonas* communities. SSCP profiles were grouped (UPGMA method) according to their similarity, which was calculated using the band-based Dice similarity coefficient. GZ, Golzow; GB, Grossbeeren; R, rhizosphere; ER, endorhiza; EP, endophyllosphere. Letters a–c mark the three independent replicates per treatment.

Fig. 4. Cluster analysis of the eubacterial community fingerprints of endophytic and rhizosphere samples based on the Dice similarity coefficient and the UPGMA. GZ, Golzow; GB, Grossbeeren; R, rhizosphere; ER, endorhiza; EP, endophyllosphere. Letters a–c mark the three independent replicates per treatment.

samples, high sequence homologies were found to 16S rRNA gene fragments from lettuce chloroplasts. One sequenced ribotype was found as a dominant band in the profiles of the rhizosphere samples from the first sampling and of the endophyllosphere samples from the second sampling (bands BR1 and BEP2).

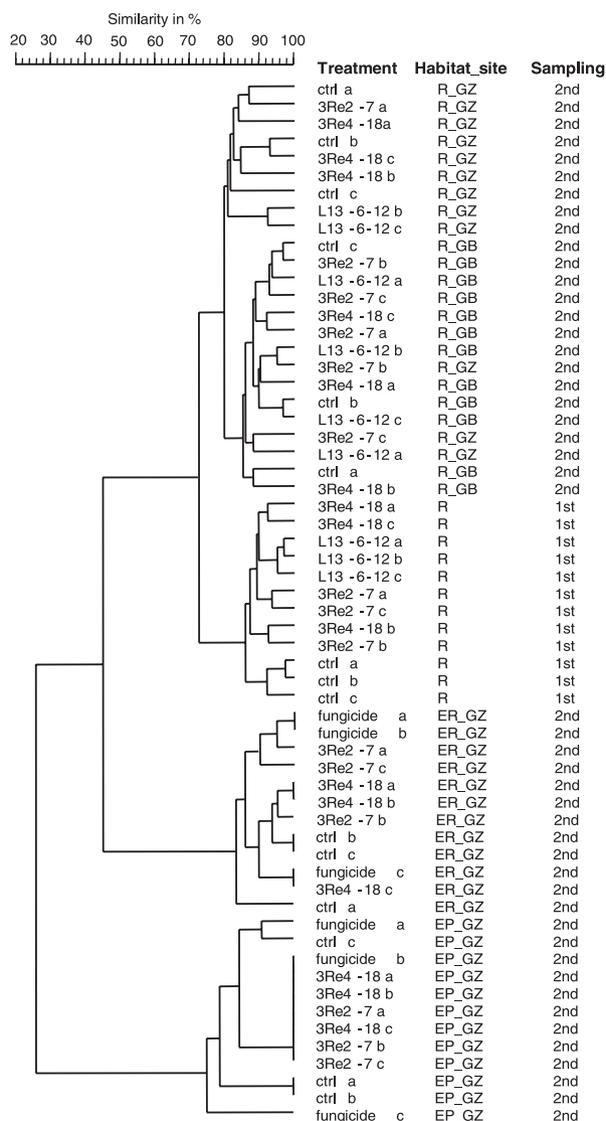As for the bacterial communities, we found more complex fungal communities in the rhizosphere (20–25 SSCP bands) than in the endophytic microhabitats (two to seven SSCP bands). The patterns were also specific for the different microenvironments. For the rhizosphere communities, a Shannon and Weaver diversity index of $\langle H' \rangle = 2.4 \pm 0.3$ was calculated. A much lower diversity was determined for the fungal communities of the endorhiza ($H' = 0.9 \pm 0.9$) and the endophyllosphere ($H' = 1.4 \pm 0.5$). Altogether, 15 bands were extracted from SSCP gels and sequenced successfully. Most sequences showed similarities to ascomycetous fungi such as *Cladosporium herbarum*, *Aspergillus flavus* and *Tetracladium maxilliforme* and to cloned inserts from environmental samples (Table 1). Other

fragments could be affiliated to the phylogenetic groups of *Basidiomycetes* or *Oomycetes*. A strong band found in the endophyllosphere fingerprints of all Grossbeeren samples was identified as *Bremia lactucae*, which causes downy mildew on lettuce.

Impact of the treatments on the microbial communities

To detect possible effects arising from bacterial inoculations, the molecular fingerprints of the microbial communities of inoculated and uninoculated lettuce plants were compared using the GELCOMPAR® software. Generally, the community fingerprints derived from samples of differently treated plants had a high similarity. For example, results of the computer-assisted cluster analysis of eubacterial (Figs 2 and 4) and *Pseudomonas* community (Fig. 3) fingerprints are shown. The cophenetic correlation coefficients calculated by the GELCOMPAR® software for the cluster trees generated in this study ranged from 0.85 to 0.98. Note that the closer this value is to 1.0, the more accurately the cluster solution reflects the data.

Within the calculated cluster trees, fingerprints from the rhizosphere samples were most similar to each other (80% and higher) compared with those derived from the endorhiza (70%) or the endophyllosphere (55%) samples (Fig. 4). At a cluster cut-off value of 70%, the profiles of all analysed eubacterial communities formed three distinct groups, each containing exclusively fingerprints from one microhabitat (Fig. 4). For the *Pseudomonas*-specific fingerprints, the same grouping of profiles was found at a cut-off value of *c.* 65% (Fig. 3). Within the rhizobacterial profiles from the first sampling, banding patterns specific, but separate, for the control and the L13-6-12 treatments were found, grouped apart from profiles of the other two bacterial treatments (Figs 2 and 4). However, as shown in both figures, this was only a transient effect, and had disappeared by the second sampling time-point. Within the fingerprints of the second sampling in Golzow, derived from the total endorhiza communities, and in the *Pseudomonas*-specific rhizosphere profiles, all three replicates of the fungicide treatment formed one exclusive cluster. Generally, patterns of samples from different treatments were found to be more similar to each other than the patterns of the three replicates of each specific treatment, suggesting no significant differences arising from the bacterial inoculants. Next to the examples described above, no cluster group was found containing exclusively SSCP fingerprints of one particular treatment.

No effects of the introduced bacteria on the endophytic fungal communities were found. The SSCP fingerprints, consisting of only very few ribotypes, showed in the majority of cases 100% similarity for samples from different treatments (Fig. 5). First results indicate that treatment-
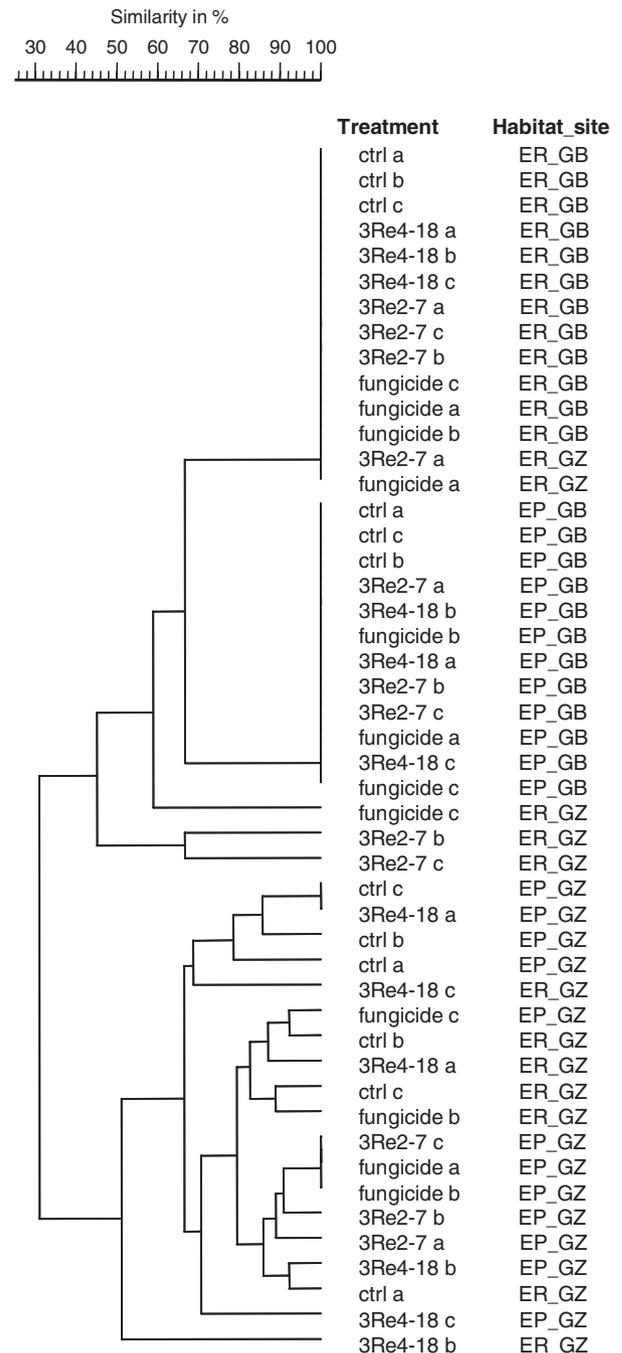

Fig. 5. Cluster analysis of endophytic fungal community fingerprints using the hierarchical UPGMA after calculation of the band-based Dice similarity coefficient. GZ, Golzow; GB, Grossbeeren; R, rhizosphere; ER, endorhiza; EP, endophyllosphere. Letters a–c mark the three independent replicates per treatment.

specific rhizosphere communities had developed 6 weeks after planting, as the SSCP fingerprints of the different treatments formed discrete groups after cluster analysis. Interestingly, the additional bands observed in the

Table 1. Sequencing results of cloned 16S rRNA gene or ITS1 fragments derived from selected SSCP bands

Band	Clone	Closest NCBI databank match/accession no.	Identity (%)	Tentative phylogenetic group
BR1	US12_4	Uncultured bacterium clone GL182.3/AY038612	99	*Bacteriodetes*
BR2	US20_2	*Rhodanobacter spathiphylli*/AM087226	98	*Gammaproteobacteria*
BR3	US25_2	*Asticcacaulis benevestidus*/AM087199	97	*Alphaproteobacteria*
BR4	*US27_2*	*Serratia plymuthica DSM 4540*/AJ233433	99	*Gammaproteobacteria*
BR5	US28_5	Uncultured bacterium clone AKAU4071/DQ128543	98	*Alphaproteobacteria*
BR6	US29_6	Gammaproteobacterium Hca1/AY531215	97	*Gammaproteobacteria*
BR7	US30_1	Uncultured bacterium clone Gexpansis25/AF467430 *Staphylococcus* sp.	99	*Firmicutes*
BR8	US31_2	Uncultured bacterium clone 1H31/AY546500	97	*Betaproteobacteria*
	US31_3	*Burkholderia* sp. oral clone AK168/AY005032	99	*Betaproteobacteria*
BR9	PS13_4	*Pseudomonas* sp. isolate R-20805/AM114534	99	*Gammaproteobacteria*
BR10	US32_4	*Flavobacterium columnare*/AY747592	98	*Bacteriodetes*
BR11	PS17_6	Uncultured bacterium clone GOBB-B10-3-2/AF494502 *Pseudomonas* sp.	99	*Gammaproteobacteria*
BEP1	US8_1	Uncultured sheep mite bacterium Llangefui15/AF290479	98	*Gammaproteobacteria*
	US8_4	*Riemerella* sp. Q27/AB192406	99	*Bacteriodetes*
BEP2	US9_2	uncultured bacterium clone GL182.3/AY038612	99	*Bacteriodetes*
	US10_5	*Lactuca sativa* chloroplast/AP007232	98	*Asteraceae*
BEP3	PS21_1	*Lactuca sativa* chloroplast/AP007232	99	*Asteraceae*
	PS21_4	Uncultured bacterium clone EUB40/AY693819 *Ralstonia* sp.	99	*Betaproteobacteria*
BEP4	PS26_6	*Pseudomonas fluorescens* FP9/DQ201400	99	*Gammaproteobacteria*
BEP5	US46_1	*Massilia* sp. Tibet-IIU22/DQ177478	99	*Betaproteobacteria*
BER1	US13_6	*Delftia tsuruhatensis*/AY738262	99	*Betaproteobacteria*
BER2	US14_1	*Acetobacteraceae* bacterium PB 188/AB220147	96	*Alphaproteobacteria*
BER3	US23_2	Uncultured bacterium clone cwr253/AY799982	98	*Proteobacteria*
	US23_4	*Lactuca sativa* chloroplast/AP007232	97	*Asteraceae*
BER4	US41_1	Uncultured bacterium clone 22-0.5D4-3/EF016399 *Burkholderia*	96	*Betaproteobacteria*
BER5	PS27_2	Uncultured bacterium clone/DQ256336	99	*Gammaproteobacteria*
BER6	PS28_3	*Variovorax* sp./DQ205307	99	*Betaproteobacteria*
FR1	IS13_1	*Lactuca sativa*/L13957	98	*Asteraceae*
	IS13_2	*Alternaria tenuissima* (Kunze) Wiltshire/AM159636	100	*Ascomycota*
FR2	IS14_3	*Tetracladium maxilliforme* (Rostr.) Ingold NS170D/DQ068996	99	*Ascomycota*
FR3	IS16_1	*Aspergillus flavus* Link/DQ198161	99	*Ascomycota*
FR4	IS18_1	Uncultured fungus clone DQ182425 *Netriaceae*	98	*Ascomycota*
FR5	IS20_1	*Plectosphaerella cucumerina* (Lindf.) W. Gams/AF176952	98	*Ascomycota*
FR6	IS21_3	*Cladosporium oxysporum* Berk. & M.A. Curtis/DQ912837	99	*Ascomycota*
FR7	IS22_1	Uncultured fungus clone AJ875390	100	*Basidiomycota*
FR8	IS24_2	*Sporobolomyces* sp. TY-247/AY313076	98	*Basidiomycota*
FEP1	IS2_4	*Lactuca sativa*/L13957	97	*Asteraceae*
	IS2_5	*Trichoderma* sp. Ir163C/AY154940	96	*Ascomycota*
FEP2	IS5_2	*Davidiella tassiana* K17 (De Not.) Crous & U. Braun/AM159622	98	*Ascomycota*
FEP3	IS6_1	*Cladosporium herbarum* (Pers.) Link 1c/AM084413	98	*Ascomycota*
FEP4	IS11_1	*Bremia lactucae* Regel/AF241770	99	*Oomycetes*
FER1	IS3_2	*Lactuca sativa*/AY504691	98	*Asteraceae*

Bands derived from BR, bacteria rhizosphere samples; BEP, bacteria endophyllosphere samples; BER, bacteria endorhiza samples; FR, fungi rhizosphere samples; FEP, fungi endophyllosphere samples; FER, fungi endorhiza samples.

fingerprints of inoculated samples suggest differently structured as well as more diverse communities compared with the samples from uninoculated plants.

Discussion

The disease-suppressive effects of three naturally occurring bacterial antagonists towards *Rhizoctonia solani* on bottom rot in lettuce at two field sites in Germany were demon-strated. Furthermore, the plant-associated microbial communities of field-grown lettuce were analysed using a cultivation-independent approach in order to investigate possible nontarget effects on indigenous microorganisms.

Previously, Winding *et al.* (2004) made the criticism that many risk assessment studies for BCAs lack significant results on beneficial effects as well as disease pressure in experimental design. However, this could be because of insufficient colonization or suboptimal expression

of antagonistic traits by the inoculated strains, possibly resulting in an underestimation or masking of side-effects. On the other hand, we found strong biocontrol effects, which we take to endorse the significance of our risk assessment results. In our experiments, the endophytic (3Re4-18, 3Re2-7) as well as the rhizobacterial (L13-6-12) biocontrol strains showed promising biocontrol effects under field conditions. Interestingly, these effects were similar to those obtained by the fungicide treatments, emphasizing the potential of biological control using antagonistic microorganisms. However, further field studies examining the biocontrol efficacy for commercially acceptable levels as well as under changing environmental conditions are needed in order to verify the consistent efficacy of our biocontrol strains.

Negligible, short-term effects on bacterial communities after the introduction of endophytic and rhizospheric BCAs in *Rhizoctonia*-infested lettuce fields were found. This is in agreement with results obtained for genetically modified rhizobacteria (Viebahn *et al.*, 2003; Blouin-Bankhead *et al.*, 2004). However, our data did not allow a clear-cut conclusion on whether the fungal rhizosphere communities are negatively affected, and therefore additional analysis for this microhabitat is needed. Complications may have arisen from the heterogenic distribution of fungi in soils, resulting in highly variable fungal DNA contents. However, the low-abundance endophytic fungal populations were shown not to be influenced by the biocontrol strains.

Primarily, analysis of the lettuce-associated bacterial communities revealed field site-specific and microenvironment-specific community compositions as well as seasonal changes. Seasonal shifts within the microbial rhizosphere communities of various crop plants have been reported in several studies (Smalla *et al.*, 2001; Costa *et al.*, 2006). Maloney *et al.* (1997) found that changes within culturable rhizobacterial populations of lettuce were correlated with the plant growth stage as well as with carbon availability and nitrogen concentrations. In addition, various populations of potato-associated bacteria were reported to be highly specific for microhabitats (Berg *et al.*, 2005b). Comparison of the number of SSCP bands of total bacterial profiles and *Pseudomonas*-specific profiles suggests that pseudomonads represent a large proportion of the total bacterial communities of field-grown lettuce. The relevance and potential of this genus regarding biocontrol have been demonstrated in numerous studies on various crop plants (see the review by Haas & Défago, 2005; Costa *et al.*, 2006). Fiddaman *et al.* (2000) isolated *Pseudomonas* spp. from the rhizosphere and phyllosphere of lettuce and found *in vivo* activity for some of the strains against *R. solani* using a leaf disc bioassay. In contrast, *Pseudomonas marginalis* has been connected with lettuce deterioration (Nguyen-The & Prunier, 1989), and

fluorescent *Pseudomonas* spp. have been implicated in soft rot on butterhead lettuce (Cottyn *et al.*, 2005).

As lettuce is a vegetable that is eaten raw, it is important to investigate and assess the pathogenic potential of plant-associated bacteria (Berg *et al.*, 2005a). Some research has been carried out on lettuce-associated microorganisms with special focus on human health issues. In studies analysing the phyllosphere microbial communities of fresh and partially processed lettuce, several human pathogens including *Shigella* spp., *Salmonella* spp. and *Listeria monocytogenes* were isolated, and thereby partially implicated in disease outbreaks (Davis *et al.*, 1988; Loncarevic *et al.*, 2005). However, some sequenced SSCP ribotypes from this study were affiliated to opportunistic human pathogenic or phytopathogenic bacterial and fungal species, for example *Burkholderia* spp., *Flavobacterium* spp., *Staphylococcus* spp., *Aspergillus* spp., *Cladosporium* spp. and *Sporobolomyces* spp. One band derived from the SSCP profile of the bacterial communities of the endophyllosphere was phylogenetically affiliated to the genus *Riemerella*. So far, only two species have been described within this genus, and these are both assigned to risk group 2 as they are associated with disease in several animal species (Vancanneyt *et al.*, 1999). However, the identification of pathogenic species based on the 16S rRNA gene and not on pathogenicity factors is not evidence that these strains can cause diseases (Berg *et al.*, 2005a).

The three analysed bacterial antagonists were shown to be a promising alternative to chemical fungicides in suppressing bottom rot on field-grown lettuce. SSCP analysis of these strains revealed that their impact on indigenous nontarget bacterial populations was only minor and also transitory. Therefore, the introduction of the three analysed antagonists into the lettuce rhizosphere appears to have no negative consequences for nontarget bacteria and endophytic fungi. The finding of no nontarget effects on indigenous microorganisms in our first field experiments is encouraging for continued product development based on our three effective bacterial antagonists. However, further experiments need to be performed using different application techniques and concentrations as a prelude to optimizing the disease-suppressive activity against *R. solani* under field conditions. Developing specific quantitative real-time PCR assays or applying mutants with selective markers will enable detailed information on the establishment and survival of our introduced BCAs.

Acknowledgements

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