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ENDOPHYTES: STRUCTURAL AND FUNCTIONAL DIVERSITY AND BIOTECHNOLOGICAL APPLICATIONS IN CONTROL OF PLANT PATHOGENS

☼ Endophytes are an interesting group of plant-associated bacteria that live inside plants and show neutral or beneficial interaction with their host plants. The structure of bacterial communities in endophytic microenvironments of important crops (different cultivars of potato, lettuce, and sugar beet) and native plants (different bryophyte species) was analyzed by a multiphasic approach at different field sites in Europe. Interestingly, results of the cultivation-independent approaches using Single-Strand Conformation Polymorphism (SSCP) and/or Terminal Restriction Fragments Length Polymorphism (T-RFLP) of 16S rRNA genes amplified by universal as well as group-specific and functional primers revealed a high diversity and specificity of endophytic bacterial communities.

The antagonistic potential of endophytic bacteria, which was determined by screening for *in vitro* antagonism against different pathogens (bacteria, fungi, protists, and nematodes) ranged from 5 to 43 %. An impressive, phylogenetically diverse spectrum of antagonistic strains was found. The indigenous antagonistic potential of endophytic bacteria was influenced by the plant genotype and developmental stage, the internal microenvironment, and the soil type. A screening strategy for biocontrol strains resulted in the selection of promising candidates. These strains were evaluated in greenhouse and field trials regarding their efficiency to control pathogens under *in situ* conditions. One product (RhizoStar[®]) on the basis of *Serratia plymuthica* HRO-C48 to control *Verticillium* wilt on different host plants was developed. For other promising candidates like *Pseudomonas trivialis* 3Re2-7 (B3) and *Serratia plymuthica* 3Re4-18 (B4) a biological control strategy against the soil-borne pathogen *Rhizoctonia solani* will be established.

INTRODUCTION

Endophytic bacteria have been defined by Hallmann et al. (Hallmann et al., 1997) as those bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plant and do not visibly harm the plant. Criteria to identify endophytes were published, which include an additional microscopic evidence to visualize bacteria inside plant tissues (Reinhold-Hurek and Hurek, 1998). However, for ecological studies this criterion is difficult to apply. Endophytic bacteria occur in the endosphere, the internal microenvironment of the shoot and leaves, as well as in the internal microenvironment of the root defined as endorhiza. Recently, it has been demonstrated that bacterial endophytes may have beneficial effects on host plants, such as growth promotion and biological control of pathogens (Sturz et al., 2000; Berg and Hallmann, 2006). It has been suggested that bacteria might interact more closely with the host plant and therefore could be efficient biological control agents in sustainable crop production (Rosenblueth and Martinez-Romero, 2006). However, further characterization of bacterial communities and their antagonistic potential is necessary to fully understand their structure and their function for plant health and growth. Furthermore, strategies to exploit endophytes in biotechnological applications have to be developed.

In this review, our work on the diversity and specificity of endophytic communities was summarized. Strategies to screen antagonistic endophytes as Biological Control Agents are presented as well as studies to use endophytes in biological plant protection.

RESULTS

Endophytic bacteria in plants show a high diversity and specificity

In different studies analyzing field-grown plants (potato, sugar beet and lettuce) or native plants like bryophytes, a high diversity of endophytic bacteria was found. Furthermore, only specific bacterial populations were able to invade and live inside plant ecosystems.

In the majority of studies, potato (*Solanum tuberosum* L.), an important crop plant originated from Peru, was investigated. In the first study analyzing potato-associated endophytes, culturable bacteria, which were obtained after plating on R2A, the endophytic populations averaged in 10^3 and 10^5 CFU g [fresh wt]⁻¹ in the endosphere and endorhiza, and therefore were lower than in ectophytic microenvironments with 10^5 and 10^7 CFU g [fresh wt]⁻¹ for the phyllosphere and rhizosphere, respectively (field trial *cv.* Cilena in Rostock, Germany; Krechel et al., 2002). The occurrence and diversity of potato-associated bacteria was additionally monitored by a cultivation-independent approach using T-RFLP analysis of 16S rDNA, which suggested the existence of microenvironment-specific communities (Krechel et al., 2004).

In another study, the composition and properties of bacteria colonizing the endosphere of field-grown potato (*cv.* Bionta) in Neustift, Lower Austria was analyzed by a multiphasic approach (Sessitsch et al., 2004). Using T-RFLP analysis of 16S rDNA, the patterns obtained revealed a high heterogeneity of community composition and confirmed the existence of plant-specific communities. However, endophytic populations correlated to a certain extent with the plant growth performance. Endophytes were also isolated from good and poor growing plants and were identified by partial sequencing of the 16S rRNA genes. A broad phylogenetic spectrum was found among isolates and differently growing plants hosted different bacterial populations. Endophytic 16S rRNA genes showed high homology to known sequences belonging to the α -, β -, and γ -*Proteobacteria*, to *Firmicutes* und *Actinobacteria* as well as to the *Cytophaga/Flavobacterium/Bacteroidetes* phylum. Sixteen different genera were found; however, only four species/genera could be detected in both plant groups: *Clavibacter michiganensis*, *Frigoribacterium* sp., *Pantoea ananatis* and *Sphingomonas* sp. In better growing plants *Actinobacteria* and γ -*Proteobacteria* were highly prominent, whereas from other plants mainly *Firmicutes* were isolated. α -*Proteobacteria* were equally abundant in strong and weak plants.

The high specificity of endophytic bacterial communities of potato (field trial *cv.* Cilena, Bonn, Germany) was also found in a study comparing four microenvironments: namely rhizosphere, phyllosphere, endorhiza and endosphere (Berg et al., 2005 b). Analysis of bacterial communities by T-RFLP of 16S rDNA clearly revealed the existence of discrete microenvironment-specific patterns. In addition,

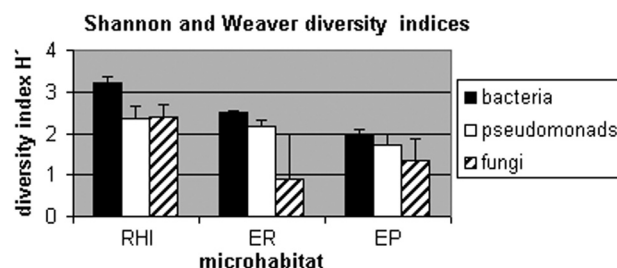


Fig. 1. Shannon Weaver diversity indices of lettuce-associated microhabitats, calculated from the SSCP-derived fingerprints of the total bacterial, fungal and *Pseudomonas* communities.

RHI = rhizosphere, ER = endorhiza, EP = endophyllosphere

the plant growth stage showed a strong influence on the T-RFLP pattern.

A similar picture was found in sugar beet (*Beta vulgaris* L.), an herbaceous dicotyledonous plant and important crop belonging to the Chenopodiaceae family (Zachow et al., 2007). The aim of this study was to analyze the composition of microorganisms isolated from the rhizosphere, phyllosphere, endorhiza, endosphere and the sugar beet body of field-grown sugar beet plants (*cv.* Philippa, Dorena and Laetitia) at three different plant development stages from six locations in Europe. The analysis of microbial communities by Single Strand Conformation Polymorphism (SSCP) of 16S/18S rDNA clearly showed the existence of discrete microenvironment- and site-specific patterns. An influence of the cultivar was also seen.

The plant-associated bacterial communities of unprocessed field-grown lettuce *cv.* Nadine were analyzed 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 (Scherwinski et al., 2008). As expected, within the SSCP profiles, a much higher genetic diversity was found within the rhizosphere communities compared to 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 or 1.9 ± 0.1 for the rhizosphere, endorhiza or endophyllosphere samples; whereas, diversity indices of $\langle H' \rangle = 2.4 \pm 0.3$, 2.2 ± 0.2 or 1.7 ± 0.3 were determined from the band patterns of the *Pseudomonas* populations, respectively (Fig. 1). The complexity of the rhizosphere *Pseudomonas* 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 approx. 70 % between fingerprints from different samplings and approx. 60 % between rhizosphere and endorhiza samples. SSCP profiles from endophyllosphere communities formed a unique group at 50 % similarity, apart from all

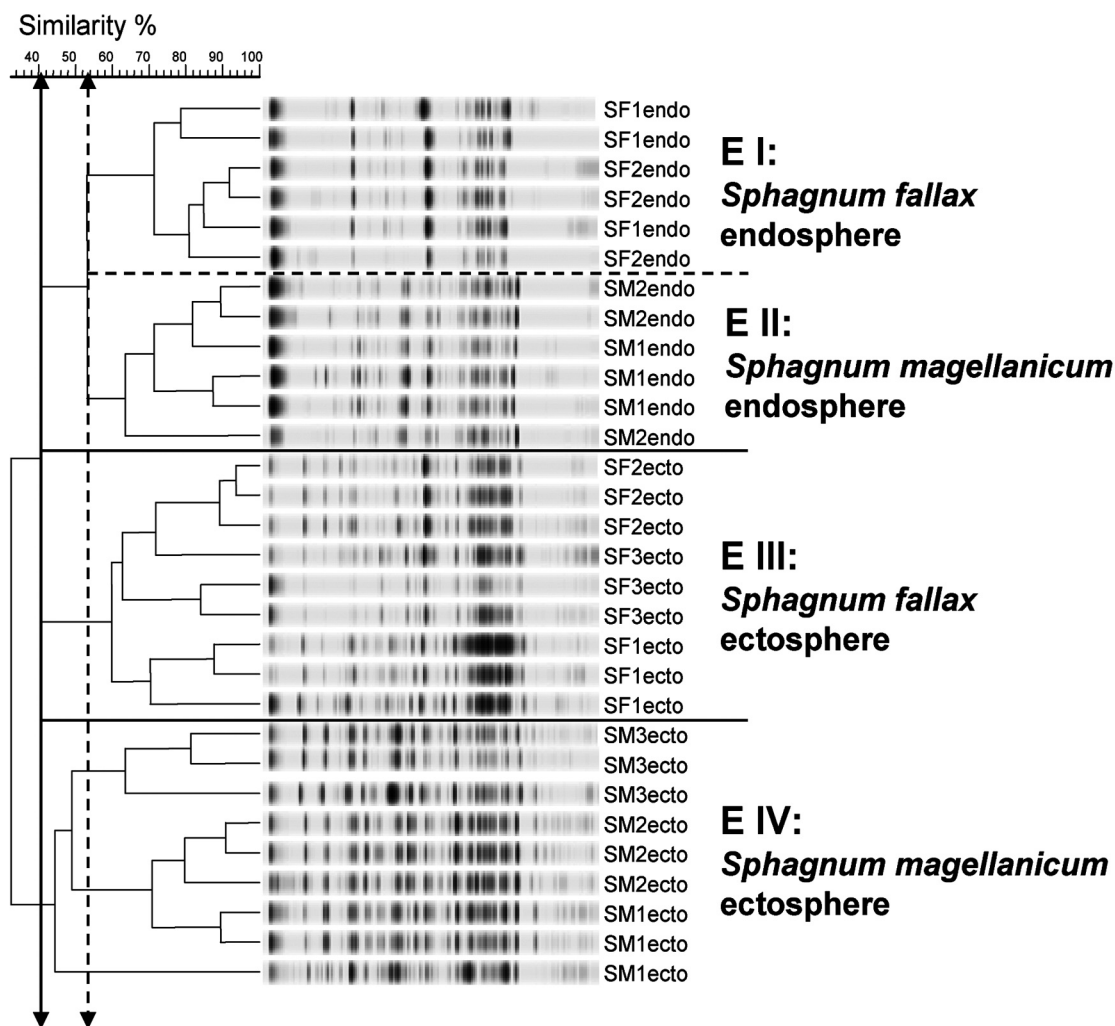


Fig. 2. Dendrogram based on amplified 16S rDNA fragments of the ectophytic bacterial communities associated with *Sphagnum magellanicum* (SM1ecto-SM3ecto), of ectophytic bacterial communities associated with *S. fallax* (SF1ecto-SF3ecto), of the endophytic bacterial communities of *S. magellanicum* (SM1endo-SM3endo), and of the endophytic bacterial communities *S. fallax* from the different sites in the bog ecosystem in Ribnitzer Großes Moor, Germany (SF1endo-SF3endo).

The fragments were obtained in three independent replicates by using eubacterial primers and separated by SSCP. The patterns obtained were grouped by UPGMA. Double-headed vertical arrows indicate the similarity for the groupings

root-associated community patterns. Representatives of several bacterial (α -, β - and γ -proteobacteria, firmicutes, bacteroidetes), fungal (ascomycetes, basidiomycetes) and protist (oomycetes) groups were present inside or on lettuce plants. Surprisingly, as lettuce belongs to raw-eaten vegetables, species of the genera *Flavobacterium*, *Burkholderia*, *Staphylococcus*, *Cladosporium* and *Aspergillus*, which contain potentially human pathogenic strains, were identified (Berg et al., 2005a).

Bryophytes represent ecological niches, which harbor a hitherto largely uncharacterized microbial diversity. The bryophyte genus *Sphagnum* consisting of approx. 300 different species is worldwide distributed and the dominant

component of the peat bog vegetation. It is phylogenetically old, and *Sphagnum* bogs belong to the oldest vegetation forms with more or less constant conditions for more than 1000 years. Not surprisingly, *Sphagnum* bogs are unique habitats for a lot of plants and animals, even though they form an extreme habitat for microorganisms characterized by high acidity (pH 3.5 to 5.0), low temperature, and extremely low concentration of mineral nutrients. The objective of this work was to analyze the bacteria associated with two different *Sphagnum* species, *S. magellanicum* and *S. fallax* (class *Sphagnopsida*, family *Sphagnaceae*), which were isolated from six bog ecosystems at different sites in Europe (Opelt et al., 2007). The SSCP gels performed with

eubacterial primers showed the abundance of diverse 16S rDNA fragments of bacteria. The patterns suggested that the microbial diversity of ecto- as well as endophytic bacterial communities at *Sphagnum* is immense (Fig. 2). Each bryophyte species was found to display its particular community profile for the ectophytic as well as endophytic bacterial communities. Using statistical analysis of the community profiles, the ectophytic community patterns of *S. fallax* formed one cluster (EIII) and the patterns of *S. magellanicum* formed another cluster (EIV) at fingerprint similarity of 40 %, suggesting that specific bacterial communities are associated with each of the *Sphagnum* species. The high plant specificity was confirmed by SSCP patterns of the endophytical communities. The endophytic communities of *S. fallax* formed one cluster (EI) and the endophytes of *S. magellanicum* belonged to another cluster (EII) at a similarity of 54 %. For both *Sphagnum* species a higher diversity index H' for the ectophytic than for the endophytic bacterial communities was calculated (Opelt et al., 2007). The endophytic communities showed a high degree of species specificity. In addition, using *Burkholderia*-specific primers we found a high diversity of *Burkholderia* isolates in the endophytic and ectophytic habitats of *Sphagnum*. Furthermore, a high diversity of nitrogen-fixing bacteria was detected by using *nifH*-specific primers, especially inside *Sphagnum* mosses. The latter results suggest an important function of endophytic bacteria for the *Sphagnum* bog ecosystem.

Altogether, using cultivation dependent and independent methods in all studies diverse endophytic bacterial populations were found, especially by applying DNA-dependent methods. In our investigations we found an influence of plant species, plant developmental stage, and field site on the composition of the endophytic bacteria.

High individual differences of endophytic bacterial communities and antagonists

To study differences among individual field grown potato plants regarding root-associated bacteria, the whole bacterial community as well as bacteria with antagonistic activity against fungal plant pathogens were analyzed by a polyphasic approach over a two year period. Great differences were found between bacterial communities of analyzed plants. In general, these differences were higher for the endorhiza than for the rhizosphere. Analysis of whole bacterial communities by T-RFLP of 16S rDNA revealed the existence of different communities in individual plants. The cluster analysis of the T-RFLP profiles, that considered both the absence/presence and the relative abundance of T-RFs, clearly demonstrated differences between individual plants (Fig. 3 a-f). Bacterial communities of the endorhiza showed a higher variability between individual plants than those of the rhizosphere. Similarities in rhizosphere communities were 76 % for young plants, 76 % for flowering plants and 53 % for early senescent plants, compared with 60 %, 32 % and 46 % for the endorhiza,

respectively. Only 11 % of the total T-RFs of the endorhiza and 49 % of the T-RFs of the rhizosphere could be found in all plants. A total of 1,330 bacterial isolates were screened by dual testing for *in vitro* antagonism toward the soil-borne pathogenic fungi *Verticillium dahliae* Kleb. and *Rhizoctonia solani* Kühn. The proportion of antagonists among the endorhiza of individual plants varied between 6 % and 33 %, whereas the percentage of antagonistic isolates from the rhizosphere ranged from 10 % to 16 %. The identification of 158 antagonistic strains resulted in 30 different bacterial species which belong to 12 genera. The diversity index of antagonistic bacteria in the endorhiza varied between 0.7 and 1.7 and in the rhizosphere between 0.5 and 1.

The demonstrated differences in bacterial communities of individual plants contribute to a better understanding of the autochthonous antagonistic potential, the prerequisite for a better assessment as well as management of biocontrol agents.

Endophytic bacterial populations possess a high antagonistic potential against plant pathogens

Plant pathogens often act inside plants. Endophytic bacteria occur in the same ecological niche and therefore most likely interact with plant pathogens. In different studies, a high antagonistic potential against different groups of pathogens – bacteria, fungi, algae and nematodes – was detected. In all plants, which were investigated, antagonistic bacteria were found.

In an approach to measure the antagonistic potential of potato-associated bacteria, a total of 440 bacteria was screened by dual testing for *in vitro* antagonism towards the soil-borne pathogens *V. dahliae* and *R. solani* (Krechel et al., 2002). The proportion of isolates with antagonistic activity was highest for the rhizosphere (10 %) followed by the endorhiza (9 %), phyllosphere (6 %) and endosphere (5 %). In addition, they were screened for their biocontrol activity against the plant pathogenic nematode *Meloidogyne incognita*. Overall nine isolates belonging to *Pseudomonas* and *Streptomyces* species were found to control both fungal pathogens and *M. incognita* and were therefore considered as promising biological control agents (Hallmann et al., 2004).

In another approach to investigate the plant growth-promoting potential of potato-associated bacteria, a total of 35 bacteria were screened by dual testing for *in vitro* antagonism towards the fungal pathogens *Verticillium dahliae* and *Sclerotinia sclerotiorum* (ascomycetes), and *Rhizoctonia solani* (basidiomycetes), the algal pathogen *Phytophthora cactorum* as well as against the bacterial pathogens *Erwinia carotovora*, *Streptomyces scabies* and *Xanthomonas campestris* (Sessitsch et al., 2004). The proportion of isolates with antagonistic activity was highest against *Streptomyces* (43 %) followed by those against *Xanthomonas* (29 %). As all plants showed more or less severe disease symptoms of scab disease caused

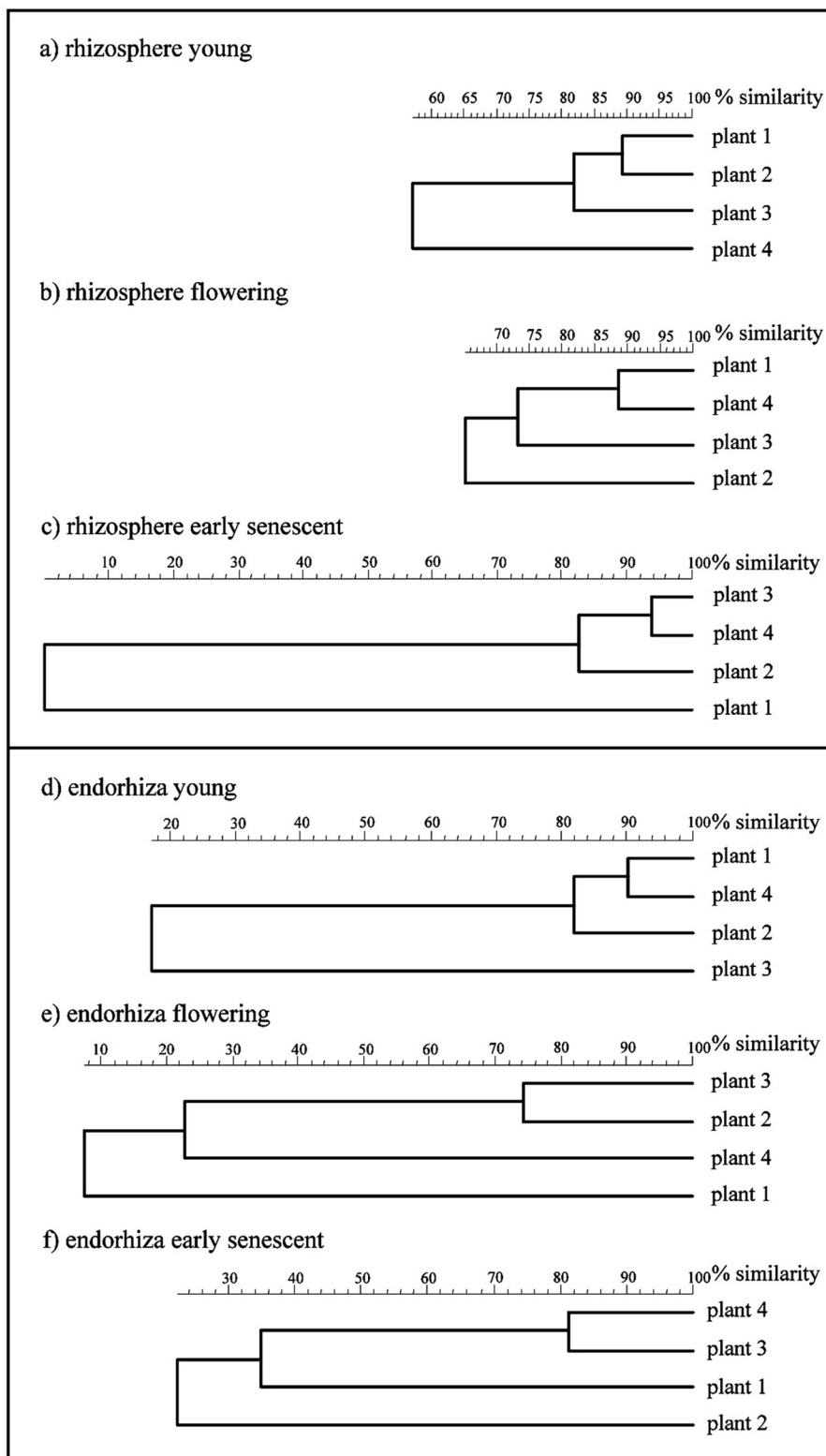


Fig. 3. Dendrograms showing the differences in rhizosphere (a-c) and endorhiza (d-e) between the single potato plants *cv.* Cilena (1-4) at different plant stages (young plants, flowering plants, early senescent plants) from the field trial in Bonn, Germany. The dendrograms are based on 16S rDNA T-RFLP profiles and were generated using a Pearson correlation matrix and clustering by the Ward algorithm.

by *S. scabies*, it was assumed that the presence of the pathogen induced the colonization of antagonists. The biotechnological potential of endophytic isolates assessed by their antagonistic activity and *in vitro* production of enzymes, antibiotics, siderophores and the plant growth hormone indole-1,3-acetic acid was generally high. Overall, seven endophytes were found to antagonize fungal as well as bacterial pathogens and showed a high production of active compounds and were therefore considered as promising biological control agents.

In a large study, also conducted on potato-associated endophytes, the antagonistic potential against soil-borne pathogens was analyzed (Berg et al., 2005 b). A total of 2,648 bacteria were screened by dual testing for antagonism towards *V. dahliae* and *R. solani*. In general, composition and diversity of bacterial antagonists were specific for each microenvironment. The rhizosphere and endorhiza were the main reservoir for antagonistic bacteria, and showed the highest similarity regarding their colonization with antagonists. The most prominent species of both microenvironments was *Pseudomonas putida*. Those isolates when characterized at the genotypic and phenotypic level showed microenvironment-specific BOX-PCR fingerprints. *P. putida* isolates from the rhizosphere and endorhiza gave nearly identical fingerprints thus confirming the high similarity of the bacterial populations for these two microenvironments (Fig. 4). Evaluation of the bacterial isolates for biocontrol potential as based on fungal antagonism and physiological characteristics resulted in the selection of five promising isolates from each microenvironment. The most effective isolate was *Serratia plymuthica* 3Re4-18 isolated from the endorhiza.

In order to analyze the antagonistic potential of sugar-beet associated micro-organisms, a total of 1,952 bacterial and 1,344 fungal isolates screened by dual testing for antagonism towards the pathogens *Aphanomyces cochlioides* and *Pytium ultimum* (oomycetes), *Phoma betae* (ascomycetes) and *Rhizoctonia solani* (basidiomycetes) and 885 bacterial (=45 %) and 437 fungal (=33 %) antagonists were identified. In general, the indigenous antagonistic potential was very high and influenced by i) plant location, ii) plant developmental stage, and iii) microenvironment. Furthermore, we showed for the first time that the antagonistic potential was highly specific for each target pathogen. The majority of antagonistic microorganisms suppressed only one pathogen (bacteria: 664 = 75 %; fungi: 256 = 59 %) whereas a minority showed a broad host range (bacteria: 4 = 0.5 %; fungi: 7 = 1.6 %). The bacterial communities harbored the highest antagonistic potential against *P. ultimum* whereas the fungal communities contained more antagonists against *A. cochlioides* and *R. solani*. In contrast to their high proportion, only a low diversity of antagonists at genotypic and species level was found. Novel antagonistic species, e. g. *Subtercola pratensis* or *Microbacterium testaceum* were found in the internal part of the sugar beet body.

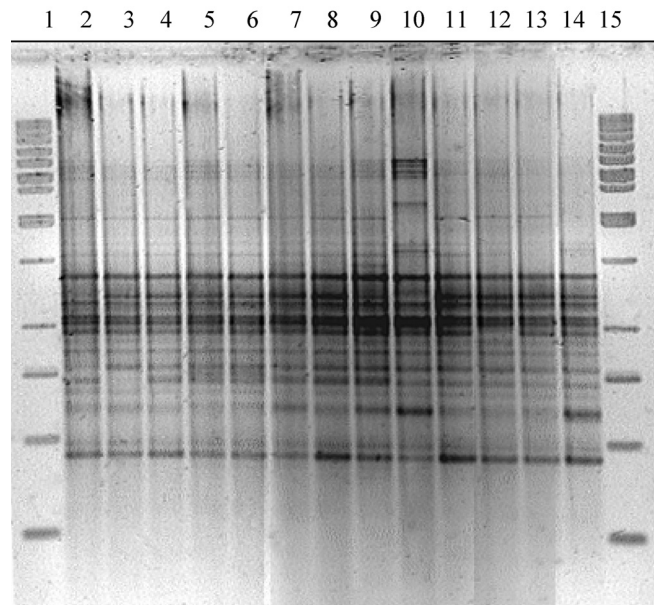


Fig. 4. BOX-PCR fingerprints of *Pseudomonas putida* isolates from the rhizosphere and the endorhiza of potato plants *cv.* Cilena at different plant stages (5 = flowering plants, 6 = early senescent plants) from the field trial in Bonn, Germany.

Lanes containing the following: 1: marker; 2: 5R2-4; 3: 5R2-27; 4: 5R2-21; (all rhizosphere) 5: 5Re4-2; 6: 5Re4-27; 7: 5Re4-19; 8: 5Re2-27; 9: 5Re4-3; 10: 6Re2-8; 11: 5Re1-25; 12: 6Re2-20; 13: 6Re3-9; 14: 6Re4-27, (all endorhiza) 15: marker

The phylogenetically oldest land plants, bryophytes, contain an extremely high proportion of antagonistic bacteria in their associated bacterial communities. We analyzed bacteria associated with three bryophyte species *Tortula ruralis*, *Aulacomnium palustre*, and *Sphagnum rubellum*, which represent typical moss species of three nutrient poor plant communities at the southern Baltic Sea coast in Germany (Opelt and Berg, 2004). The proportion of isolates with antagonistic activity towards the pathogenic model fungus *V. dahliae* was highest for *S. rubellum* (31 %) followed by *A. palustre* (17 %) and *T. ruralis* (5 %). A high percentage (99 %) of moss-associated antagonistic bacteria produced antifungal compounds. For *Sphagnum* mosses, the screening of 1,222 isolates for antagonistic activity resulted in 326 active isolates (Opelt et al., 2007). The bacterial communities harbored a high proportion of antifungal (26 %) but a low proportion of antibacterial isolates (0.4 %). The proportion of isolates with antifungal activity against *V. dahliae* was higher for *S. fallax* (21 %) than for *S. magellanicum* (12 %). Also the proportion of isolates with antifungal activity against *R. solani* was higher for *S. fallax* (19.5 %) than for *S. magellanicum* (13 %). Furthermore, the proportion of isolates with antibacterial activity, tested in dual culture assay against *S. aureus*, was higher for *S. fallax* (2 %). For *S. magellanicum* no isolate with the ability to suppress

growth of *S. aureus* was found. The majority of the antagonistic bacteria (37.5 %) showed an activity against both phytopathogenic fungi while none of the strains with antibacterial activity showed antifungal activity.

In conclusion, all investigated plants harbour a high proportion of microorganisms antagonistic to plant pathogens. At this point it is necessary to mention, that the antagonistic activity was shown *in vitro*. Antagonists (greek = foe, opponent) are microorganisms, which are able to suppress, inhibit or kill other microorganisms. If this antagonistic activity is able to suppress microorganisms on the plant themselves is a second question, which could be only answered by performing greenhouse or field studies.

Biocontrol of plant pathogens using endophytic bacteria

Endophytes with antagonistic activity against plant pathogens are potential Biological Control Agents (BCAs), which can be used in agricultural practice for an environmentally friendly and sustainable plant protection. Therefore, antagonistic strains have to be selected and evaluated under field conditions.

A screening strategy was developed to assess the potential of plant-associated bacteria to control diseases caused by *Rhizoctonia solani* Kühn (Faltin et al., 2004). About 434 already characterized antagonistic bacterial strains isolated from diverse plant species and microenvironments were evaluated for biocontrol and plant growth promotion by a hierarchical combination of assays. Analyzing *in vitro* antagonism towards different *Rhizoctonia* isolates resulted in a selection of 20 potential biocontrol agents. The strains were characterized by their antagonistic mechanisms *in vitro* as well as their production of the plant growth hormone indole-3-acetic acid. The plant growth promoting effect by antagonistic bacteria was determined using a microtiter plate assay on the basis of lettuce seedlings. Lettuce and sugar beet as host plants, were included in the biocontrol experiments in which the antagonistic effect of seventeen bacterial isolates could be confirmed *in vivo*. Sequencing of the 16S rDNA gene and/or FAME-GC was used to identify the antagonistic isolates. Molecular fingerprints of isolates obtained by BOX-PCR were compared to avoid further investigation with genetically very similar strains and to obtain unique molecular fingerprints for quality control and patent licensing. According to our strategy an assessment scheme was developed and four interesting biological control agents namely *Pseudomonas trivialis* B3, *P. fluorescens* B1, *Serratia plymuthica* B4 and *S. ordorifera* B6 were found. While *S. plymuthica* B4 was the best candidate for biological control of *Rhizoctonia* in lettuce, *P. trivialis* B3 was the best candidate to suppress the pathogen in sugar beet. Interestingly, although in screening only a small proportion of endophytic bacteria was included, two strains (B3, B4) were originally isolated from the endorhiza of potato.

To develop a biocontrol strategy, three potato-associated ecto- and endophytically living bacterial strains, *P. fluorescens* B1, *P. fluorescens* B2 and *S. plymuthica* B4 were evaluated against *R. solani* in potato and in lettuce (Grosch et al., 2005). The disease suppression effect of the three BCAs was tested in a growth chamber and in the field. In growth chamber experiments, all three BCAs limited completely or significantly the dry weight (DW) losses on lettuce and the disease severity (DS) caused by *R. solani* on potato sprouts. The strain B1 showed with 52 % on average the highest suppression effect on potato. Under field conditions the DS decreased significantly on both crops when bacterized, and biomass losses of lettuce were reduced. The best disease suppression effect on potato was achieved by B1 with 37 %, followed by B2 (33 %) and B4 (31 %), whereas the marketable tuber yield was increased up to 17 % in comparison to the pathogen control at higher disease pressure. Furthermore, in all experiments B1 proved to be the most effective BCA against *R. solani*. Therefore, this BCA is a promising candidate for further development to a commercial product against *Rhizoctonia* diseases. To our knowledge, this is the first report on the high potential of endophytes as BCA against *R. solani* under field conditions.

RhizoStar®: a commercial product on the basis of the endophytic bacterium *Serratia plymuthica* HRO-C48

One of the most important soil-borne pathogens is *V. dahliae* Kleb., causing Verticillium wilt responsible for high yield losses in a wide variety of host plants including important crops such as strawberry, oilseed rape and olive. With the impending phase-out of the fumigant methyl bromide worldwide, there is almost no possibility left to control the pathogen, and therefore alternative management strategies are required. One of these alternatives is the use of beneficial or antagonistic microorganisms which can suppress soil-borne pathogens in the rhizosphere. *S. plymuthica* strain HRO-C48, originally isolated from the rhizosphere of oilseed rape, was selected as biocontrol agent by a hierarchical screening strategy according to plant growth promoting and antagonistic properties. Results were evaluated in a variety of greenhouse and field trials (Berg et al., 1999, Kurze et al., 2001). An important step was to formulate the BCA, and to develop a specific application method for each crop (Müller and Berg, 2008). Furthermore, the mode of action was intensively studied. Interestingly, the interaction with the plant as well as with the fungus was regulated in a quorum-sensing dependent manner (Lui et al., 2007; Müller et al., 2008). The product development was carried out in cooperation with the strawberry farm in Rövershagen, E-nema GmbH (Raisdorf), and Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Hohenlieth, all in Germany).

Biocontrol using endophytes need risk assessment studies

Although originating from plant-associated microenvironments, beneficial bacteria, if applied to plant roots in adequate numbers, may perturb indigenous microbial populations and the important ecological functions associated therewith (Winding et al., 2004). Therefore, unwanted and unspecific actions of the introduced beneficial microbes against non-target organisms have to be assessed. To this end, sufficient knowledge about the microbial ecology of the target habitats is necessary for reasonable risk assessment studies concerning 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 have been developed to overcome the limitations of cultivation (Smalla, 2004). Using such molecular methods is urgently needed to include the greatest possible number of total microorganisms in risk assessment studies regarding non-target effects of introduced beneficial bacteria. We performed two risk assessment studies: one for *Serratia plymuthica* HRO-C48 and another one, in which two endophytic bacteria antagonistic against *Rhizoctonia solani* were integrated.

The BCA *Serratia plymuthica* HRO-C48 was proved to efficiently control *V. dahliae* on strawberry (Kurze et al., 2001). The aim of this study was to assess the impact of the biological control agents *S. plymuthica* HRO-C48 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 microflora by PCR-single-strand-conformation polymorphism analysis of the 16S rDNA 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 microflora, specific for a field site. However, the impact of the treatment on the non-target microflora was lower than that of the soil type, field site and growth stage of the plant (Scherwinski et al., 2006).

The aim of the second study was to assess the biocontrol efficacy of three bacterial antagonists against *R. solani* introduced into different naturally *Rhizoctonia*-infested lettuce fields and to analyze their impact on the indigenous plant-associated bacteria and fungi (Scherwinski et al., 2008). Lettuce seedlings were inoculated with bacterial suspensions of two endophytic strains, *Serratia plymuthica* 3Re4-18 and *Pseudomonas trivialis* 3Re2-7,

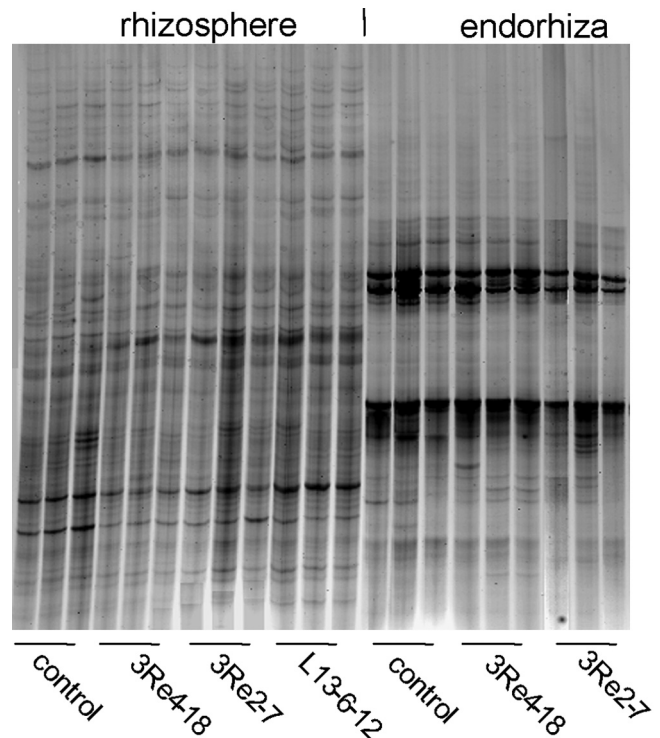


Fig. 5. SSCP profiles of bacterial communities from the rhizosphere (1st sampling) and endorhiza (2nd sampling, field trial in Golzow, Germany) of inoculated (BCAs: *Pseudomonas trivialis* 3Re2-7 (B3) and *Serratia plymuthica* 3Re4-18 (B4)) and non-inoculated lettuce plants *cv.* Nadine. Three independent replicates per treatment were loaded onto the gel

and with the rhizobacterium *Pseudomonas fluorescens* L13-6-12 seven days before and five days after planting in the field. Similar, statistically significant biocontrol effects were observed for all applied bacterial antagonists compared to the non-inoculated control. Single-strand conformation polymorphism (SSCP) analysis of 16S rDNA or ITS1 fragments revealed a highly diverse rhizosphere and a less diverse endophytic microflora of lettuce (Fig. 5). Analysis of the indigenous bacterial and endophytic fungal populations revealed only negligible, short-term effects due to the bacterial treatments, and that they were more influenced by field site, plant growth stage and microenvironment.

CONCLUSIONS

All investigated plants were colonized by a broad spectrum of endophytic bacteria including diverse bacteria antagonistic towards fungal plant pathogens. This enormous potential awaits further exploitation for its use in biotechnological applications, such as environmentally friendly plant control strategies. This requires not only a better understanding of the underlying mechanisms and

their regulation in response to environmental factors, but also a more comprehensive picture of the mechanisms that trigger endophytic colonization as well as of the population dynamics of antagonistic bacterial endophytes within the plant. Continuing research in this area will hopefully lead to new and innovative concepts for biological control of fungal pathogens.

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Эндофиты: структурно-функциональное разнообразие и применение в биотехнологии для контроля патогенов

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✿ РЕЗЮМЕ: Эндофиты представляют собой интересную группу ассоциированных с растениями бактерий, живущих внутри растения, взаимодействие которых с растениями может являться нейтральным или полезным. Структура бактериальных сообществ в эндофитном микроокружении важных сельскохозяйственных культур (различных сортов картофеля, салата и сахарной свеклы) и дико-

растущих растений (различные виды мхов) была проанализирована с помощью многопрофильного подхода на различных полях в Европе. Интересно, что анализ с использованием конформационного полиморфизма однонитевой ДНК (Single-Strand Conformation Polymorphism, SSCP) и/или полиморфизма длин терминальных рестриционных фрагментов (T-RFLP) генов 16S рРНК, амплифицированных при помощи универсальных, а также группспецифических и функциональных праймеров, показал высокое разнообразие и специфичность эндофитных бактериальных обществ. Антагонистический потенциал эндофитных бактерий, определенный при исследовании *in vitro* антагонизма по отношению к различным патогенам (бактериям, грибам, простейшим и нематодам) варьировал, от 5 до 43 %. Интересно, что был выявлен филогенетически разнообразный спектр антагонистических штаммов. На антагонистический потенциал эндофитных бактерий влиял генотип растения и стадия развития, внутреннее микроокружение и тип почвы. При поиске биорегулирующих штаммов были отобраны перспективные штаммы-кандидаты. У этих штаммов оценивали эффективность контроля патогенов в условиях *in situ* в тепличных и полевых экспериментах. На основе HRO-C48 *Serratia plymuthica* был разработан препарат (RhizoStar®), контролирующий вертициллиозный вилт у различных растений-хозяинов. Для других перспективных штаммов-кандидатов, таких как 3Re2-7 (B3) *Pseudomonas trivialis* и 3Re4-18 (B4) *Serratia plymuthica*, в дальнейшем будет определен механизм биологического контроля передающегося через почву патогена *Rhizoctonia solani*.

✿ КЛЮЧЕВЫЕ СЛОВА: картофель, салат, сахарная свекла, мхи, SSCP, T-RFLP, антагонисты, агенты биологического контроля, *Rhizoctonia solani*, *Verticillium dahliae*