Microbial Ecology

# Sugar Beet-Associated Bacterial and Fungal Communities Show a High Indigenous Antagonistic Potential Against Plant Pathogens

**Christin Zachow[1,2], Ralf Tilcher[3] and Gabriele Berg[1,2]**

(1) University of Rostock, Microbiology, Albert-Einstein-Str. 3, 18051, Rostock, Germany
(2) Institute of Environmental Biotechnology, Graz Technical University, Petersgasse 12/1, 8010, Graz, Austria
(3) KWS SAAT AG, Grimsehlstr. 31, 37555, Einbeck, Germany

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## Abstract

The aim of this study was to analyze microbial communities in/on sugar beet with special focus on antagonists toward plant pathogens. For this purpose, the composition of microorganisms isolated from the rhizosphere, phyllosphere, endorhiza, and endosphere of field-grown sugar beet plants was analyzed by a multiphasic approach at three different plant development stages at six locations in Europe. The analysis of microbial communities by Single Strand Conformation Polymorphism (SSCP) of 16S/18S rRNA clearly revealed the existence of discrete microenvironment- and site-specific patterns. A total of 1952 bacterial and 1344 fungal isolates screened by dual testing for antagonism toward the pathogens *Aphanomyces cochlioides*, *Phoma betae*, *Pythium ultimum*, and *Rhizoctonia solani* resulted in 885 bacterial (=45%) and 437 fungal (=33%) antagonists. In general, the indigenous antagonistic potential was very high and influenced by (a) the location, (b) the plant developmental stage, and (3) the 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 the 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.

## Introduction

The sugar beet (*Beta vulgaris* L.) is a herbaceous dicotyledonous plant belonging to the Chenopodiaceae family. The plant species is indigenous to the Mediterranean area, but beet has been cultivated in temperate areas for a long time mainly as source for sweets [14]. During the last years, the importance of sugar beets as a basis for biotechnological processes and as energy plant has increased. The crop has a biennial life cycle. Only the first year is of importance for sugar production, when the plant produces economically important saccharose concentrations up to 18% [45]. Pathogenic fungi are responsible for economically important yield losses in sugar beet production [14]. For example soil-borne pathogens like *Aphanomyces cochlioides* Dreschler and *Pythium ultimum* Trow cause root and seedling rot. The pathogen *Rhizoctonia solani* Kühn is able to cause different diseases depending on the time of infection and the anastomosis group (AG) of strain. While AG4 causes the early beet rot on *Beta vulgaris*, strains of AG2-2 IIIB cause late sugar beet rot and lead to yield losses at a late stage of plant development. This pathogen is characterized by a high aggressiveness against sugar beets, a high tolerance against low and high temperatures, and a low sensitivity against fungicides [12]. In addition, the seed-borne pathogen *Phoma betae* A. B. Frank causes preemergence damping-off and severe hypocotyl infections of emerged seedlings [1]. The sugar beet pathogens belong to different phylogenetical groups, while *P. betae*

*Correspondence to*: Gabriele Berg; E-mail: gabriele.berg@TUGraz.at

belongs to ascomycota; *R. solani* is the anamorph of *Thanatephorus cucumeris* (A. B. Frank) Donk, which belongs to basidiomycota. *A. cochloides* and *P. ultimum* are oomycetes, which are classified as protists and not as fungi anymore. Although fungicides against the pathogens and resistant cultivars are partly available, there is an interest to biologically control the pathogens and to minimize the impact of pesticides in sugar beet production.

Plants colonized inside and out by large bacterial and fungal communities, which have a beneficial interaction with their host plant [19, 46, 47]. They interact with their host plants via diverse mechanisms, and those with antagonistic properties against plant pathogens can be indirectly responsible for plant health [13, 25, 49]. Bacterial and fungal communities in the rhizosphere show a high degree of plant specificity [7, 21, 27, 41] including those microorganisms with antagonistic properties [5, 6]. Therefore, to analyze the indigenous antagonistic potential of each plant species is important for understanding the natural self-protection of plants. On the basis of this knowledge, it is possible to manage the indigenous antagonistic potential, optimize biological approaches to control pathogens, and to detect new, plant-specific antagonists. There are some reports about the indigenous sugar beet-associated microorganisms that mainly focused on bacteria [9, 15, 31, 36, 37, 43]. All studies indicate a high colonization rate of the sugar beet plant by microorganisms including their seeds [15, 42]. However, little is known about bacteria living in the endophytic microenvironments [33] and fungal antagonists associated with sugar beet plants.

The objective of the study was to analyze the indigenous microbial communities of sugar beets, especially of those with antagonistic activity against plant pathogens, and to determine the influence of different biotic and abiotic parameters. To do this, we have taken samples from the rhizosphere, phyllosphere, endorhiza, and endosphere samples from leaves and internal parts of sugar beets three times over the first year of the biannual vegetation period at six locations in Europe and analyzed by a multiphasic approach including cultivation-dependent and -independent methods. Three different cultivars were included in the experimental design. In addition, we screened for bacterial and fungal isolates that displayed antagonistic activity against four important soil- and seed-borne pathogens of sugar beet plants which cause yield losses in commercial sugar beet production.

## Methods

*Experimental Design.* Sugar beet plants (*Beta vulgaris* L.) cv. Philippa, Dorena, and Laetitia were grown in a randomized block design with four replicates per cultivar. In 2004, sugar beet plants were sampled at three sampling times during the first vegetation period as described by Meier *et al.* [35], i.e., corresponding to the growth stages of the plants: (a) young plants, (b) 90% of leaves cover the ground (only 10% of soil is exposed to sunlight), and (c) harvestable size of sugar beet plants. Sugar beet seeds of the different cultivars (gray pill) were covered with fungicides (TMTD; active agent thiram and Tachigaren: active agent hymexazol), insecticides (Chlothianidin, Imidacloprid, Thiamethoxan, and the final color layer) were provided by KWS SAAT AG (structure of the commercial sugar beet seed see http://www.kws.de). The fields for the trials were located in important sugar beet growing areas in Europe: Germany: Klein Wanzleben (KWL), Hilprechtshausen (HILP), and Seligenstadt (SEL), in the Netherlands: Schoondijke (SDK), and in France: Attily (AT) and La Veuve (LV). The soil parameters (soil texture/soil type) of the locations were as follows: Attily and Hilprechtshausen: silty loam/parabrown earth; Klein Wanzleben: coarse silt/black earth of loess; La Veuve: clay/chalk; Schoondijke: loamy sand/marine sediments; and Seligenstadt: silty loam/parabrown earth of loess.

*SSCP Analysis.* Roots with adhering soil and leaves taken from six plants were collected in sterile Stomacher bags, then transported to the laboratory, and than treated as one sample. For the isolation of plant-associated microorganisms, 5 g each of roots and leaves were pooled and transferred into a new Stomacher bag. Samples were extracted in a Stomacher laboratory blender (BagMixer, Interscience, St. Nom, France) after addition of 50 ml 0.85% NaCl solution samples were shaken for 3 min; and then, the supernatants were decanted into 50 ml tubes. The supernatants decanted into 50 ml tubes were used for cultivation and for cultivation-independent investigation procedures. For the latter, after centrifugation at low speed (5 min, $500\times g$), the supernatant was collected and transferred in a new Falcon tube. The resulting supernatants were centrifuged at high speed ($10{,}000\times g$) for 20 min to collect the microbial pellet. The resulting microbial pellet was stored at -70°C. DNA of bacterial and fungal cell consortia was extracted using the FastDNA Spin Kit for Soil (BIO 101, Carlsbad, USA) according to the manufacturer's protocol. Fingerprinting of the sugar beet communities by SSCP was carried out as described by Schwieger and Tebbe [40]. Briefly, 16S rRNA gene sequences were amplified by using the primers Unibac-II-515f (5′-GTG CCA GCA GCC GC-3′) and Unibac-II-927rP (5′-CCC GTC AAT TYM TTT GAG TT-3′) [32]. These primers amplified 16S rRNA genes from nucleotide 515 to nucleotide 927 (*Escherichia coli* numbering), including the variable regions from v4 to v5. The polymerase chain reaction (PCR) was performed by using a total volume of 60 µl containing 12 µl of Taq&Go (Qbiogen), 6 µl of the purified DNA, 2 µl of 25 mM $MgCl_2$, 2.4 µl of each primer,

and 25.2 µl of water (95°C, 5 min; 35 cycle of 95°C, 20 s; 53°C, 30 s; 72°C, 1 min; and elongation at 72°C, 10 min). In the amplification of 18S rRNA gene sequences, the first reaction (1 µl DNA, 1.2 µl, 25 mM $MgCl_2$, 4 µl Taq&Go, 12.2 µl water, 0.8 µl per primer) contains primer ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) [48] (94°C, 5 min; 36 cycle of 94°C, 30 s; 54°C, 35 s; 72°C, 40 s; and elongation at 72°C, 10 min). Six microliters of this reaction product was applied in the second reaction with primer ITS1 and ITS2 (5′ GCT GCG TTC TTC ATC GAT GC 3′) [48]. Furthermore, 12 µl Taq&Go, 2 µl of 25 mM $MgCl_2$, 25.2 µl of water, and 2.4 µl per primer were added (94°C, 5 min; 36 cycle of 94°C, 30 s; 58°C, 35 s; 72°C, 40 s; and elongation at 72°C, 10 min). On the polyacrylamide gel, three replicates of each sample were applied. Silver staining was used for the routine detection of DNA bands in SSCP gels.

*Isolation of Bacteria and Determination of Colony Forming Units.* For each sample, the solutions (50 ml, prepared according to the same procedure as for SSCP described in the chapter above) were serially diluted and plated onto synthetic nutrient agar (SNA) or R2A (Difco, Detroit, USA). SNA is used especially for the cultivation of fungi and contains 1 g $KH_2PO_4$, 1 g $KNO_3$, 0.5 g $MgSO_4 \times 7\ H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 1 l *Aqua distilled*, 0.6 ml 1 N NaOH, 22 g agar. After autoclaving for 20 min, these antibiotics were added: 10 mg $l^{-1}$ chlortetracycline, 50 mg $l^{-1}$ dihydrostreptomycinsulfate, 100 mg $l^{-1}$ penicillin G. Plates were incubated for 5 days at 20°C, and colony forming units were counted to calculate the means of colonies ($log_{10}$ CFU) based on fresh weight. For further testing, a total of 48 bacterial isolates per location (KWL, HILP, SEL, SDK, AT, LV), sampling time (young plants, 90% of leaves cover the ground, harvestable size of sugar beets), and microenvironment (rhizosphere, endorhiza, phyllosphere, endosphere of sugar beet body and leaves) were randomly selected and subcultured on nutrient agar (NA). A total of 40 fungal isolates per parameter were selected and subcultured on Malt Agar (MA, Merck, Darmstadt, Germany). After incubation for 7 days at 20°C, the MA and NA plates were stored at 4°C. The bacteria (B) and fungi (F) isolated were encoded by using a combination of numbers and letters: The encoding includes: (a) location (AT = Attily, LV = La Veuve, HILP = Hilprechtshausen, SEL = Seligenstadt, KWL = Klein Wanzleben, SDK = Schoondijke), (b) microenvironment (R = rhizosphere, P = phyllosphere, RE = endorhiza, PE = endosphere), (c) consecutive number of the isolate per plant, (4) sampling time (1 = young plants, 2 = 90% of leaves cover the ground, 3 = harvestable size of sugar beets), (5) number of isolates (1–48).

For the isolation of endophytic bacteria, 5 g of roots were surface sterilized in a 20% sodium hypochlorite solution ($H_2O_2$) for 10 min and then washed in sterile water three times. The plant material was imprinted on NA as a sterility check. All samples were homogenized with mortar and pestle, serially diluted with sterile 0.85% NaCl and plated onto R2A medium (Difco, Detroit, MI). Plates were incubated for 5 days at 20°C. Colony forming units were counted and expressed as CFU per gram fresh weight. For each microenvironment, 48 bacterial and 40 fungal colonies were transferred to NA or MA, respectively. The isolates were purified and then stored at −70°C, the bacterial cultures in a liquid nutrient broth (NB) with glycerol (50% *v*/*v*) and the fungi in a liquid storage medium. The medium for fungi was prepared by blending the following sterile compounds: 60 ml glycerol, 20 ml glucose (50% *w*/*v*), 10 ml peptone (20% *w*/*v*), and 10 ml yeast extract (10% *w*/*v*).

*Screening for Fungi and Bacteria Antagonistic to Aphanomyces cochlioides, Phoma betae, Pythium ultimum, and Rhizoctonia solani.* The *in vitro* inhibition of the four plant pathogens was determined in a dual culture assay on different media. To prepare the mycelial suspension of *Phoma betae* (strain DSM63181), the fungus was homogenized with mortar and pestle. The suspension was diluted in sterile 0.85% NaCl and precultured for 7 days at 20°C in 200 ml of Czapec Dox Broth (Difco, Detroit, USA). Of the mycelia suspension, 200 µl was plated onto Waksman agar (WA) containing 5 g proteose–peptone (Merck, Darmstadt, Germany), 10 g glucose (Merck), 3 g yeast extract (Sifin, Berlin, Germany), 5 g NaCl (Merck), 20 g agar (Difco), filled up to 1 l with distilled water and adjusted to pH 6.8. A 5-mm-diameter agar plug was cut from each plate with the pathogens *A. cochlioides* (carrot medium), *P. ultimum* (V8 medium), and *R. solani* (PDA) and placed in the center of an agar plate. At the same time, the bacterial and fungal isolates were spotted on the plate in six locations. After 3–7 days of incubation at 20°C, zones of inhibition were measured according to Berg *et al.* [5]. The *R. solani* strains used to belong to the anastomosis groups four (AG 4). *A. cochlioides* was isolated of sugar beet seedlings and kindly provided by Sebastian Kiewnick (Bonn, Germany), and *P. ultimum* and *Rhizoctonia solani* were kindly provided by Rita Grosch (Großbeeren, Germany).

*Morphological Characteristics of Antagonistic Fungi.* Before the molecular characterization, all isolates were grouped by their morphology on MA. For this grouping, morphological characteristics such as colony morphology, production of pigments, conidiophores, or other morphological organs, which could be seen using light microscopy according to Domsch *et al.* [16] were used.

*Purification of DNA from Bacteria and Fungi.* Mycelia or colonies, grown on MA or NA for 3 to 7 days, were soaked with buffer (containing 10 mM Tris and 1 mM

EDTA) for 5 min (fungi). After removing the TE, equal amounts of sterile glass beads (Sigma, 0.25–0.5 mm) and 300 µl of extraction buffer [containing 200 mM Tris, 200 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS)] were added to the material. The mycelium/colonies were treated with a FastPrep™ instrument (Qbiogene BIO 101® systems, Karlsbad, USA) for 20 s at level 4. Of 3 M sodium acetate, 150 µl were added, and the samples were shaken with a vortexer. Then the samples were frozen for about 30 min. Centrifugation for 5 min at 13,000×*g*. Finally, the DNA (supernatant) was purified by phenol–chloroform extraction and precipi-tation by isopropanol. The resulting pellet was washed with 70% ethanol and dissolved in 50 µl TE buffer and stored at -20°C.

*Characterization of Bacterial Antagonists by Amplified Ribosomal DNA Restriction Analysis.* The 16S ribosomal DNA of bacterial antagonists was PCR amplified with the universal eubacterial primer EubI (5′ GAG TTT GAT CCT GGC TCA G 3′) or 907r (5′ CCG TCA ATT C(AC)T TT(AG) AGT TT 3′), respectively, and EubII (5′ AGA AAG GAG GTG ATC CAG CC 3′). The PCR conditions consist of an initial denaturing cycle (95°C, 5 min), 9 amplification cycles (95°C, 30 s; 52°C 30 s; 72°C 1 min 40 s), 19 amplification cycles (95°C, 30 s; 52°C, 30 s; 72°C, 1 min 30 + 10 s), and a final elongation cycle (72°C, 5 min). The restriction enzyme chosen was *Hha*I. The enzymatic reactions were digested for 3 h at 37°C in 20 µl volumes containing 15 µl of the PCR product solution, 2 µl of commercially supplied incubation buffer, 2.55 µl of water, 0.2 µl of 100× BSA, and 0.25 µl (20 U/µl) of *Hha*I. Restriction products were run on a 2% Agarose gel (AppliChem, Darmstadt, Germany) in a 1× tris–borate–EDTA buffer for 5 h at 100 V/m. The resulting bands were made visible with ethidium bromide. Isolates showing the same molecular band pattern were arranged to form a category. Characteristic isolates were chosen to identify the groups by sequencing partially the 16S rRNA.

*Identification of Plant-Associated Bacteria and Fungi.* All bacterial antagonistic isolates were identified by sequencing about 700 bases of the 16S rRNA gene. The sequences obtained were aligned with the reference 16S rRNA gene sequence using BLAST algorithm according to Altschul *et al.* [2].

*Data Analysis and Statistics.* All data (CFU, % of sugar beet antagonists) were analyzed for significance using the confidence interval ($P \leq 0.05$) and were statistically analyzed using the Tukey's procedure with $P = 0.05$ with one-way analysis of variance (ANOVA) by Statistical Product and Service Solutions for Windows, Rel. 9. 0. 1. (SPSS Inc., Chicago, USA). The silver-stained SSCP gels were scanned transmissively (Epson Perfection™ 4990 scanner, Meerbusch, Germany) to obtain digitized gel images that could be processed using GelCompar® software (Applied Math, Kortrijk, Belgium). After gel normalization, which allowed the band patterns of different gels to be compared, and background subtraction, similarity matrices were calculated using the Dice Correlation coefficient. Finally, the band patterns representing the similarity of the microbial communities were hierarchically clustered to produce dendrograms using the unweighted pair group method with arithmetic averages (UPGMA). The actual distances calculated and the distances shown on the dendrogram were compared by Pearson product-moment correlation (cophenic correlation) to ensure that the dendrogram gave an accurate representation of the data [11]. Computer-assisted pattern analyses of molecular fingerprints of the communities resulted in a correlation matrix, which were subjected to test for significance by applying permutation test with $10^5$ random permutations of sample elements [30]. The relationship between samples taken over the season and those taken from different microenvironments were investigated.

*Nucleotide Sequence Accession Numbers.* Bacterial sequence accession numbers for sequences submitted to the EMBL nucleotide sequence database are AJ969075 to AJ969092 and AM048839 to AM048842.

## Results

*Cultivation-independent Analysis of Sugar Beet-associated Microbial Communities.* To study the diversity and dynamics of the dominant bacterial and fungal communities, samples from all sugar beet-associated microenvironments, sampling times and sites were analyzed by SSCP. In all analyzed samples, an impressive diversity of bacteria and fungi, expressed as various bands in the gel, was found. For example, Fig. 1 shows the different SSCP patterns obtained for 16S rDNA genes from rhizosphere samples taken at the second sampling time in different fields in Europe. According to the cluster analysis, site-specific SSCP patterns were found. They differed significantly ($P \leq 0.1$) from each other. Furthermore, there was an influence of the sampling time on the composition of bands; the number of bands increased during the sampling times (data not shown). Again, this effect was statistically significant ($P \leq 0.1$). In addition, microenvironment-specific SSCP patterns were detected. There were significant differences between the endophytic and the ectophytic microenvironments (Fig. 2). In general, the analyses of samples from ectophytic habitats generated more molecular bands than endophytic samples, and rhizosphere samples generated more bands than phyllosphere samples. Analysis of endorhiza samples

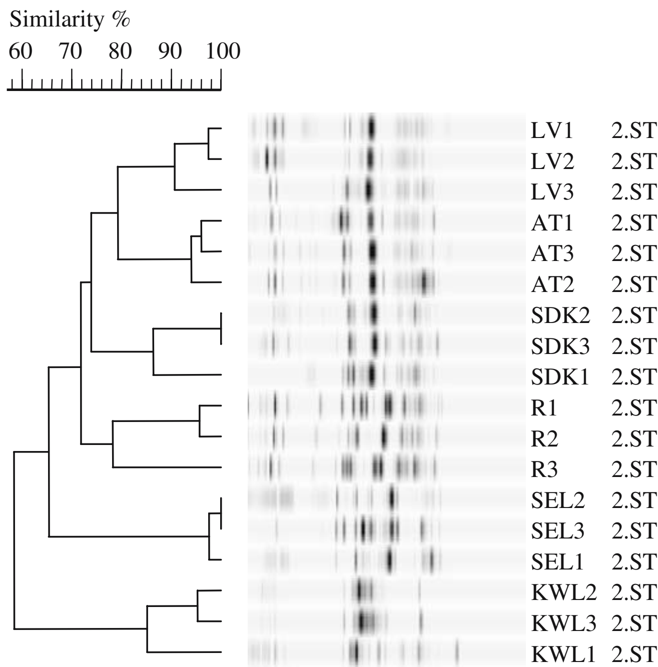


**Figure 1.** SSCP patterns obtained from single-stranded DNA products amplified by eubacterial PCR from rhizosphere samples at different locations of second sampling time (2. ST). The *three lanes* of each location represent individual replicates ($n=3$, each consists of six plants). UPGMA dendrogramm obtained by Gelcompar®, Dice coefficient. France: *AT* Attily, *LV* La Veuve; The Netherlands: *SDK* Schoondijke; Germany: *R* Hilprechtshausen, *SEL* Seligenstadt, *KWL* Klein Wanzleben. Cophenic correlation coefficient = 85.5%.

showed close correlations with internal samples from sugar beets; they also showed significant differences ($P \leq 0.1$) from ectophytic habitats. Also for the investigated cultivars Philippa, Laetitia, and Dorena, slight differences were found (data not shown).

In contrast to the bacterial rhizosphere community patterns, the fungal fingerprints included a larger quantity of smaller bands. For example, SSCP community profiles of fungal communities of the rhizosphere of different sites are shown in Fig. 3. Again, the patterns were specific for each site ($P \leq 0.1$). Like for the bacteria communities, an influence of the sampling time was found (data not shown). Using the primers for 18S/ITS region, at least for the endophytic microenvironments, no PCR product was obtained.

*Culturable Bacteria and Fungi from Sugar-beet Associated Habitats.* At all sampling times, the average bacterial density yielded significantly four orders of magnitude higher on average ($\log_{10}$ 8.0 ± 0.5 $g^{-1}$ fresh weight = fw) than those for fungi ($\log_{10}$ 4.5 ± 0.4 $g^{-1}$ fw). Fungi were detected only in the rhizosphere ($\log_{10}$ 5.3 ± 0.8 $g^{-1}$ fw) and phyllosphere ($\log_{10}$ 4.5 ± 0.4 $g^{-1}$ fw), but not in the endophytic microenvironments. The bacterial density determined for rhizosphere samples were the highest of all habitats ($\log_{10}$ 8.1 ± 0.3 $g^{-1}$ fw), followed by the phyllosphere ($\log_{10}$ 7.2 ± 0.3 $g^{-1}$ fw), the endorhiza ($\log_{10}$ 5.1 ± 0.4 $g^{-1}$ fw), the bacteria living inside the sugar beet body ($\log_{10}$ 4.0 ± 1.2 $g^{-1}$ fw), and the endosphere ($\log_{10}$ 3.0 ± 0.4 $g^{-1}$ fw). The bacterial abundances of different locations varied from $\log_{10}$ 7.9 ± 0.3 $g^{-1}$ fw (Klein Wanzleben) to $\log_{10}$ 8.7 ± 0.9 $g^{-1}$ fw (La Veuve) $g^{-1}$ fresh weight whereas the fungal CFU ranged from $\log_{10}$ 4.3 ± 2.1 $g^{-1}$ fw (Klein Wanzleben) to $\log_{10}$ 5.7 ± 0.4 $g^{-1}$ fw (Seligenstadt) $g^{-1}$ fresh weight. Furthermore, bacterial and fungal abundances at the different stages of the plant development do not show statistically significant differences.

*Proportion of Antagonistic Bacteria and Fungi Toward Phytopathogens.* A total of 1952 bacteria and 1344 fungi were randomly selected from all locations, microenvironments, cultivars, and stages of plant development and were tested for *in vitro* antagonism toward plant pathogens. All selected bacterial and fungal isolates were screened for their antagonistic ability to suppress the growth of *A. cochlioides*, *P. betae*, *P. ultimum*, and *R. solani* in dual culture systems (Table 1). Altogether, a total of 885 bacterial (= 45%) and 437

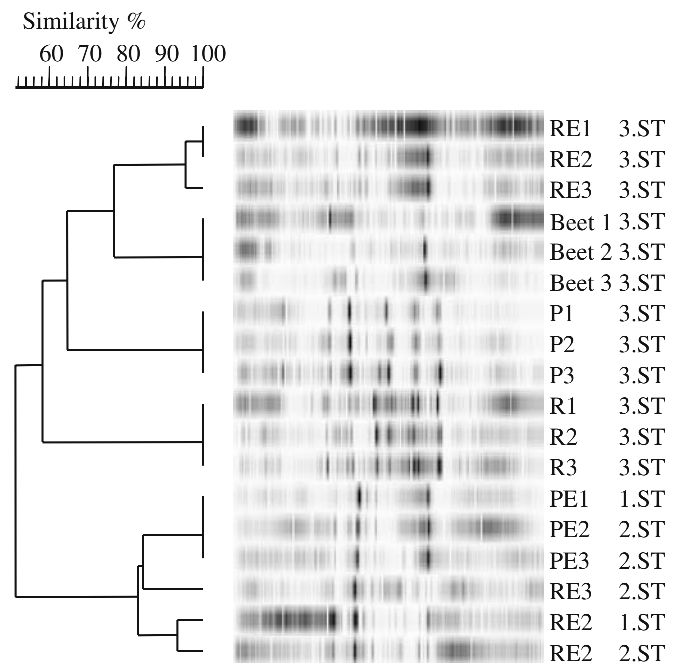


**Figure 2.** SSCP patterns obtained from single-stranded DNA products amplified by eubacterial PCR from different micro-enviroment samples on Hilprechtshausen of different sampling times (ST). The *three lanes* of each location represent individual replicates ($n=3$, each consists of six plants). After comparison of fingerprints using Dice similarity coefficient, clustering was carried out using the UPGMA method of Gelcompar®. *R* Rhizosphere, *RE* endorhiza, *P* phyllosphere, *PE* endosphere, *Beet* internal parts of the sugar beet body. Cophenic correlation coefficient = 86.0%.

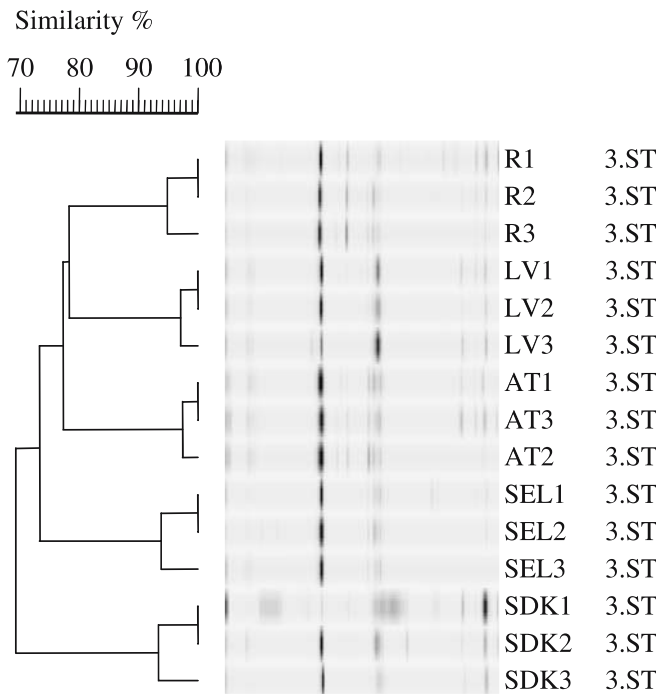


**Figure 3.** SSCP patterns and UPGMA dendrogram generated from the SSCP community profiles of fungal communities of the rhizosphere of different location at the third sampling time with Gelcompar®, Dice coefficient. France: *AT* Attily, *LV* La Veuve; The Netherlands: *SDK* Schoondijke; Germany: *R* Hilprechtshausen, *SEL* Seligenstadt. The *three lanes* of each location represent individual replicates ($n = 3$, each consists of six plants). Cophenic correlation coefficient = 84.4%.

fungal (= 33%) isolates demonstrated antagonistic potential against one, two, three or all plant-pathogens. It is surprising to note that only four bacteria and seven fungi were antagonistic toward all four pathogens tested, whereas 664 bacteria (= 75%) and 256 fungi (= 59%) showed antagonistic activity against only one of these pathogens. For all model pathogens, an antagonistic potential was found, but the proportion varied depending on the pathogenic species. In general, more bacterial isolates were antagonistic toward *P. ultimum* (599 isolates = 31.8%) than toward *R. solani*, *A. cochlioides*, and *P. betae* (13.2, 11.4, 10.5%). In contrast, the highest proportion of antagonistic fungal isolates (17.0%) was found for *A. cochlioides* and *R. solani* followed by antagonists against *P. betae* (= 12.6%) and *P. ultimum* (= 6.0%). Furthermore, the proportion of antagonists varied depending on the sampling time. Whereas the proportions of bacterial antagonists varied between 3.5 and 39.0%, the proportions of fungal antagonists varied between 3.5 and 25.2%. In both microbial groups, at the second sampling time (plant stage: 90% of leaves cover the ground), the highest proportion of antagonists was found. All microenvironments of plants were colonized with microorganisms with antagonistic properties including the endophytic ones (Table 2). Again,

**Table 1.** Proportions (%) of bacterial and fungal isolates antagonistic toward *Aphanomyces cochlioides*, *Phoma betae*, *Pythium ultimum*, and *Rhizoctonia solani* in the rhizosphere at different stages of plant development

| | | | *Aphanomyces cochlioides* | | *Phoma betae* | | *Pythium ultimum* | | *Rhizoctonia solani* | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Microenvironment* | *Group* | *Sampling time* | *Number of isolates* | *Percentage of antagonists (%)* | *Number of isolates* | *Percentage of antagonists (%)* | *Number of isolates* | *Percentage of antagonists (%)* | *Number of isolates* | *Percentage of antagonists (%)* |
| Rhizosphere | Bacteria | 1. ST | 281 | $11.9 \pm 5.5^{a}$ | 562 | $11.5 \pm 5.1^{a}$ | 469 | $22.3 \pm 5.3^{a}$ | 556 | $11.7 \pm 2.9^{a}$ |
| | | 2. ST | 535 | $10.9 \pm 2.9^{a}$ | 524 | $14.6 \pm 3.4^{b}$ | 456 | $39.0 \pm 5.4^{b}$ | 516 | $19.0 \pm 4.4^{b}$ |
| | | 3. ST | 501 | $11.1 \pm 3.5^{a}$ | 502 | $3.5 \pm 2.0^{ab}$ | 502 | $34.0 \pm 5.1^{bc}$ | 503 | $7.6 \pm 2.8^{a}$ |
| | Fungi | 1. ST | 303 | $15.2 \pm 4.3^{a}$ | 441 | $8.8 \pm 2.9^{a}$ | 429 | $3.5 \pm 2.5^{a}$ | 454 | $25.2 \pm 5.0^{ab}$ |
| | | 2. ST | 368 | $18.9 \pm 4.6^{a}$ | 424 | $16.2 \pm 4.0^{b}$ | 395 | $12.4 \pm 4.9^{b}$ | 405 | $17.0 \pm 5.8^{a}$ |
| | | 3. ST | 440 | $12.5 \pm 4^{a}$ | 440 | $11.0 \pm 3.1^{a}$ | 440 | $2.1 \pm 1.3^{ab}$ | 440 | $6.0 \pm 2.6^{a}$ |

The average was determined of the locations in France, Germany, and The Netherlands. Means followed by the same letters indicate no significant differences according to Tukey's test.
*ST* Sample time

microorganisms with an endophytic life style showed a high degree of pathogen-specificity. However, in all endophytic microenvironments, a high antagonistic potential against *P. ultimum* was found in general (1.7–42.0%). In addition, the proportion of antagonistic microorganisms varied at the different sampling sites. In general, the highest percentages were found for the French locations (Table 3). In contrast, in Germany, the lowest proportions of antagonists were found. There was no statistically significant difference between the three investigated sugar beet cultivars Philippa, Laetitia, and Dorena.

*Characterization of Sugar Beet-associated Antagonistic Bacteria.* To analyze the diversity at genotypic level, bacterial isolates which demonstrated the highest antagonistic activity against each of the four model pathogens and caused inhibition zones of more than 10 mm (+++) were chosen for further investigations. According to this method, 19 bacteria showed antagonistic activity toward *A. cochlioides*, and 23 antagonists to *P. betae*, four to *P. ultimum*, and 53 to *R. solani*. A total of 75 bacterial isolates was characterized by 16S rRNA restriction fragment length polymorphism (RFLP) and could be assigned to six ARDRA types (1–6). The biggest ARDRA group (group 1) included the majority (= 70) of the isolates analyzed. The ARDRA patterns of the groups two to six were represented by only one isolate. These isolated and representative strains from ARDRA group 1 were identified by sequencing the partial 16S rRNA gene (Table 4). All strains of the ARDRA group 1 belong to the *Pseudomonas* genus. Bacteria with unique ARDRA patterns showed a high similarity to *Bacillus subtilis*, *Variovorax paradoxus*, *Flavobacterium* sp., and *Lysobacter gummosus* isolates. In addition, strains with an interesting antagonistic spectrum obtained from the endophytic microenvironments were identified. Again, the majority of strains belonged to *Pseudomonas* and could be identified only on genus level. However, also some special antagonists were found like strain 20 and 21: *Subtercola pratensis* and *Microbacterium testaceum*.

*Characterization of Sugar beet-associated Antagonistic Fungi.* All antagonistic fungi with antagonistic activity were grouped based on their phenotype. An impressive diversity of fungal colonies was obtained. For example, the mycelium was white, gray, black, green, yellow, pinkish, or brown, and sometimes, some red or black color was diffusing into the agar. In addition, some colonies of a typical odor, e.g., fruity, aromatic like coco, perfume, or solvents like acetone were found. Most of the fungi produced different anamorphs, whereas teleomorphs were not detected. The morphological characterization of 437 antagonistic fungi resulted in 39 morphological groups. *Penicillium* was the most dominant genus (22%) followed

**Table 2.** **Proportions (%) of bacterial and fungal isolates antagonistic toward *Aphanomyces cochlioides*, *Phoma betae*, *Pythium ultimum*, and *Rhizoctonia solani* in the rhizosphere over the growing season**

| | | | *Aphanomyces cochlioides* | | *Phoma betae* | | *Pythium ultimum* | | *Rhizoctonia solani* | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Microenvironment* | *Group* | *Sampling time* | *Number of isolates* | *Percentage of antagonists (%)* | *Number of isolates* | *Percentage of antagonists (%)* | *Number of isolates* | *Percentage of antagonists (%)* | *Number of isolates* | *Percentage of antagonists (%)* |
| Rhizosphere | Bacteria | Σ 1.–3. ST | 628 | 12.1 ± 2.9 | 773 | 8.5 ± 2.3 | 691 | 33.0 ± 4.4 | 759 | 11.6 ± 2.6 |
| | Fungi | Σ 1.–3. ST | 542 | 16.2 ± 4.2 | 698 | 11.3 ± 2.8 | 665 | 3.5 ± 1.8 | 709 | 13.9 ± 4.6 |
| Phyllosphere | Bacteria | Σ 1.–3. ST | 83 | 14.4 ± 8.5 | 129 | 10.0 ± 9.3 | 133 | 42.0 ± 17.1 | 129 | 19.4 ± 13.4 |
| | Fungi | Σ 1.–3. ST | 110 | 6.0 ± 4.4 | 125 | 5.8 ± 3.6 | 111 | 1.7 ± 2.1 | 125 | 11.0 ± 5.4 |
| Endosphere | Bacteria | Σ 1.–3. ST | 36 | 8.3 ± 14.9 | 94 | 35.5 ± 20.1 | 88 | 32.5 ± 17.7 | 84 | 12.7 ± 10.3 |
| Endorhiza | Bacteria | Σ 1.–3. ST | 120 | 12.5 ± 6.4 | 174 | 12.8 ± 6.8 | 154 | 39.1 ± 15.0 | 172 | 7.5 ± 6.6 |
| Sugar beets | Bacteria | Σ 1.–3. ST | 56 | 1.7 ± 2.8 | 56 | 5.4 ± 3.1 | 56 | 23.9 ± 11.4 | 57 | 7.1 ± 0.7 |

The average and confidence was determined of the three sampling times (Σ 1.–3. ST) and the locations in France, Germany, and The Netherlands.
*ST* Sample time

**Table 3. Proportions (%) of bacterial and fungal isolates obtained from different locations of sugar beet plants**

| | *Bacteria* | | *Fungi* | |
|---|---|---|---|---|
| *Location (country)* | *Number of isolates tested* | *Percentage of antagonistic isolates (%)* | *Number of isolates tested* | *Percentage of antagonistic isolates (%)* |
| Attily (F)(ab) | 156 | 47.7 ± 8.1(ab) | 120 | 37.5 ± 11.8[a] |
| La Veuve (F)(a) | 144 | 55.6 ± 8.9(a) | 120 | 42.5 ± 9.6[a] |
| Schoondijke (NL)(ab) | 150 | 38.5 ± 10.1(ab) | 123 | 30.8 ± 14.3[a] |
| Hilprechtshausen (G)(ab) | 150 | 30.9 ± 11.3(ab) | 123 | 23.7 ± 10.9[a] |
| Klein Wanzleben (G)(ab) | 156 | 34.2 ± 6.9(ab) | 124 | 23.9 ± 9.7[a] |
| Seligenstadt (G)(b) | 154 | 29.2 ± 6.5(b) | 120 | 35.8 ± 12.3[a] |

The average was determined of the three sampling times (Σ 1.–3. ST). Means followed by the same letters in parentheses indicate no significant differences according to Tukey's test.

*F* France, *NL* The Netherlands, *G* Germany.

by *Paecilomyces* (4%) and *Monographella* species (4%), which are also important genera with antagonistic activity.

## Discussion

The results of our study reveal an extraordinary high indigenous antagonistic potential of bacteria and fungi in and on sugar beets. The antagonistic potential was influenced by the location, the stage of plant development, and the microenvironments of sugar beet plants. Whereas several studies identified different biotic and abiotic factors influencing microbial communities (reviewed in Garbeva *et al.* [21]), this study demonstrated the influence of these parameters on the functional group of antagonists. We showed for the first time that the antagonistic potential of microorganisms, determined in dual culture assays, is highly specific for each plant pathogen. Remarkably, the majority of the microorganisms were specialized to the pathogens and showed activity against only one of the four pathogens tested. Only very few isolates harbor a broad host range. They belong to the well-studied antagonistic genera *Pseudomonas* and *Trichoderma* [25, 34, 49]. Furthermore, antagonists never reported in the literature, e.g., *Subtercola pratensis* or *M. testaceum*, were found in the internal part of the sugar beet body.

The cultivation-independent SSCP analyses demonstrate the influences of the biotic and abiotic parameters investigated on the microbial communities. The microbial communities at different sites in Europe differ greatly. Different soil textures and types could be one reason, but also the climate (temperature, rainfall) can have an influence on the structure of microbial communities. The different microenvironments of sugar beet plants also affect the microbial constitutions to a large degree. In more detail, a high difference was found between the endophytic and the ectophytic microenvironments. The theory that only a subset of the plant-associated microflora is able to invade into the plant [26] was found for potato [6, 28] and was supported by the finding that more molecular bands of bacterial communities occurred in the ectophytic than in the endophytic habitats. Moreover, the results present specific bacterial and fungal communities at different sampling times, an effect which was described in many studies analyzing plant-associated communities, and explained by changing root exudates and morphology [41, 43]. Using the molecular techniques, it was not possible to detect fungi in the endophytic microenvironments of sugar beets. Although it is well-known that endophytic fungi are widely distributed in many plant species, we could not find fungi neither by cultivation-dependent nor by -independent methods. Our experiments were carried out under commercial conditions, and fungicides were applied to the sugar beet seeds and to the plants in the field.

The cultivation-dependent analysis revealed an extremely high indigenous antagonistic potential of sugar beet-associated microorganisms. Nearly half of the culturable bacteria (45%) showed antagonistic activity. The highest proportion of antagonistic bacteria reported in literature was found on bryophytes with 35% [38], whereas 3 to 9% were found in the rhizosphere of *Verticillium* host plants [5], 16% in the rhizosphere of oilseed rape [3], and 18% in the rhizosphere of various weeds [29]. The highest proportion of antagonists was found for the two French locations, and the lowest proportion was found in the rhizosphere samples from German locations independent of soil type. The higher temperature could be the reason for the generally higher proportion of antagonists in France. Furthermore, in addition to the unusual high proportion of antagonists, the molecular fingerprints by ARDRA release an unusual low diversity of the bacteria, which were selected according to their high antagonistic potential against the different plant pathogens *in vivo*. We identified 92% of these antagonists as different *Pseudomonas* species, e.g., *P. fluorescens* or *P. aurantica*. In a field study, Berg *et al.* [6] determined a proportion of 77% of antagonistic *Pseudomonas* strains in the strawberry rhizosphere. In contrast, potato and oilseed rape-associated bacteria showed a lower abundance combined with a higher diversity, and much more bacterial species belonging to

**Table 4.** List of selected bacterial antagonistic species isolated from different microenvironments of sugar beet with different antagonistic properties

| Number | Strain number | Microenvironment | ARDRA group | Percent SI[a] (%) | Closest database match (EMBL number) | Antagonistic activity toward[b] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *A. cochlioides* | *P. betae* | *P. ultimum* | *R solani* |
| 1 | AT2-2-8 | Rhizosphere | 1 | 99 | *Pseudomonas* sp. Fa2 (AJ969085) | + | ++ | − | +++ |
| 2 | Dooh1-2-25 | Rhizosphere | 6 | 95 | *Flavobcterium* sp. BH12 (AJ969088) | + | − | +++ | − |
| 3 | Dooh2-3-5 | Rhizosphere | 1 | 99 | *Pseudomonas fluorescens* (AM048839) | ++ | +++ | ++ | + |
| 4 | DoSt2-3-2 | Rhizosphere | 1 | 99 | *Pseudomonas fluorescens* (AM048840) | +++ | ++ | + | ++ |
| 5 | PhSt1-2-1 | Rhizosphere | 2 | 100 | *Variovorax paradoxus* (AJ969086) | − | − | ++ | +++ |
| 6 | PhSt2-2-12 | Rhizosphere | 1 | 100 | *Pseudomonas aurantica* (AM048842) | +++ | + | + | + |
| 7 | KWL2-3-7 | Rhizosphere | 3 | 99 | *Lysobacter gummosus* (AJ969090) | − | +++ | + | + |
| 8 | KWL2-1-27 | Rhizosphere | n. d. | 78 | *Pseudomonas fluorescens* (AJ969089) | − | n. d. | ++ | + |
| 9 | LV1-2-14 | Rhizosphere | 1 | 99 | *Pseudomonas fluorescens* (AM048841) | ++ | + | + | +++ |
| 10 | LV2-2-10 | Rhizosphere | 1 | 87 | *Pseudomonas* sp. SBW25 (AJ969079) | − | − | + | − |
| 11 | LV2-2-11 | Rhizosphere | 4 | 98 | *Bacillus subtilis* (AJ969080) | + | n. d. | + | +++ |
| 12 | SDK2-2-6 | Rhizosphere | 1 | 98 | *Pseudomonas fluorescens* (AJ969083) | ++ | ++ | + | +++ |
| 13 | SEL2-1-4 | Rhizosphere | 1 | 99 | *Pseudomonas* sp. MFY 107 (AJ969084) | +++ | − | + | ++ |
| 14 | P1-3-17 | Phyllosphere | 1 | 99 | *Pseudomonas* sp. TB2-10-II (AJ969077) | − | − | + | +++ |
| 15 | P1-3-18 | Phyllosphere | 1 | 99 | *Pseudomonas* sp. TB2-10-II (AJ969087) | − | − | + | +++ |
| 16 | P1-3-20 | Phyllosphere | 1 | 99 | *Pseudomonas* sp. E2.2. (AJ969091) | ++ | − | + | +++ |
| 17 | PE1-2-6 | Endosphere | 1 | 99 | *Pseudomonas* sp. (AJ969081) | − | + | ++ | − |
| 18 | PE1-2-15 | Endosphere | 1 | 96 | *Pseudomonas* sp. Fa2 (AJ969082) | − | + | ++ | − |
| 19 | RE1-2-18 | Endorhiza | 5 | 100 | *Pantoea agglomerans* (AJ969092) | − | − | ++ | − |
| 20 | RE1-2-24 | Endorhiza | n. d. | 86 | *Pseudomonas* sp. K1 (AJ969078) | − | − | + | − |
| 21 | Beet1-3-7 | Internal part of sugar beet body | n. d. | 100 | *Subtercola pratensis* (AJ969075) | − | − | + | − |
| 22 | Beet1-3-26 | Internal part of sugar beet body | n. d. | 99 | *Microbacterium testaceum* (AJ969076) | − | − | + | − |

[a] SI Similarity index. For isolates identified by 16S rDNA sequencing, values range from 0 to 100%.
[b] + 0–5 mm, ++ 5–10 mm, +++ >10 mm radius of zone of inhibition in dual culture assay, − no suppression

other genera were identified [8]. It is interesting to note that in a comparative study using wheat cultivars, Germida and Siciliano [23] found out that the modern cultivars are more colonized by fast-growing, antibiotic-producing pseudomonads than the ancient ones. In addition, Lambert *et al.* [31] found fast-growing pseudomonads on the root surface of young sugar beet plants. However, the genus *Pseudomonas* is known for their beneficial association with their host plants and for their antagonistic activity against plant pathogens [23, 25]. Related to the work of Lilley *et al.* [33] with mature sugar beet plants, three of the most common genera (*Bacillus*, *Pseudomonas*, and *Microbacterium*) were found in the rhizosphere. Observations made in a long-term study of sugar beet phyllosphere suggested that pseudomonads organized in highly specialized genotypes, whose occurrence was determined by abiotic factors [18].

During this study, basic information about sugar beet-associated microorganisms with an antagonistic activity toward *A. cochlioides*, *P. betae*, *P. ultimum*, and *R. solani*, factors influencing their antagonistic proportion and molecular diversity, was gathered. This knowledge is important for optimizing biological control applications. Successful and consistent biological control requires a good knowledge of the dynamics and composition of antagonistic communities. We could show that inside and outside the sugar beets, impressive autochthonous antagonistic potential was available. This potential was specific for each microenvironment, and in particular, for each pathogen. The high degree of pathogen specificity was never described before and was not expected by the authors. Mechanisms responsible for antagonistic activity include (a) inhibition of the pathogen by antibiotics, toxins, and bio-surfactants (antibiosis), (b) competition for colonization sites, nutrients, and minerals, (c) parasitism that may involve the production of extracellular cell wall-degrading enzymes such as chitinase and β-1,3 glucanase, and (d) mycophagy [10, 20, 39]. Furthermore, the importance to recognize and adhere to plant roots or fungal hyphae for all plant-associated bacteria was pointed out in many biocontrol studies [27]. A first step in the generation of a plant–bacterium interaction is the attachment of cells to plant roots, in which, for example, fimbriae and cell-surface proteins are involved [17]. For the colonization of plant roots, flagella, O-antigen of lipopolysaccharides (LPS), the growth rate, and the ability to grow on root exudates are important [34]. These factors may be also responsible for plant and pathogen specificity. A detailed study of the host–pathogen–antagonist interaction is important for successful biocontrol effects.

In the literature, some biocontrol studies analyzing pathosystems of sugar beet are reported. For example, Georgakopoulos *et al.* [22] found out that strains belonging to *Pseudomonas* were the best candidates to control damping-off caused by *P. ultimum* in sugar beet. In addition, Tilcher [43] described a *Pseudomonas* treatment around the sugar beet seeds which resulted in a better protection against damping-off than a *Bacillus* treatment. However, there is no product to control plant diseases on sugar beets available on the market. In our study, we detected on and especially in sugar beets, specific antagonists and antagonists with a broad host range. In addition, some of the very efficient *in vitro* antagonists originated from the endosphere. The endorhiza and endosphere are the major sources for antagonists. The high potential of endophytes was suggested by many authors as reviewed by Berg and Hallman [4] and could be also confirmed in field studies [24, 44].

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