

Microbial Diversity Inside Pumpkins: Microhabitat-Specific Communities Display a High Antagonistic Potential Against Phytopathogens

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Abstract Recent and substantial yield losses of Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) are primarily caused by the ascomycetous fungus *Didymella bryoniae* but bacterial pathogens are frequently involved as well. The diversity of endophytic microbial communities from seeds (spermosphere), roots (endorhiza), flowers (anthosphere), and fruits (carposphere) of three different pumpkin cultivars was studied to develop a biocontrol strategy. A multiphasic approach combining molecular, microscopic, and cultivation techniques was applied to select a consortium of endophytes for biocontrol. Specific community structures for *Pseudomonas* and *Bacillus*, two important plant-associated genera, were found for each microenvironment by fingerprinting of 16S ribosomal RNA genes. All microenvironments were dominated by bacteria; fungi were less abundant. Of the 2,320 microbial isolates analyzed in dual culture assays, 165 (7%) were tested positively for in vitro antagonism against *D.*

bryoniae. Out of these, 43 isolates inhibited the growth of bacterial pumpkin pathogens (*Pectobacterium carotovorum*, *Pseudomonas viridiflava*, *Xanthomonas cucurbitae*); here only bacteria were selected. Microenvironment-specific antagonists were found, and the spermosphere and anthosphere were revealed as underexplored reservoirs for antagonists. In the latter, a potential role of pollen grains as bacterial vectors between flowers was recognized. Six broad spectrum antagonists selected according to their activity, genotypic diversity, and occurrence were evaluated under greenhouse conditions. Disease severity on pumpkins of *D. bryoniae* was significantly reduced by *Pseudomonas chlororaphis* treatment and by a combined treatment of strains (*Lysobacter gummosus*, *P. chlororaphis*, *Paenibacillus polymyxa*, and *Serratia plymuthica*). This result provides a promising prospect to biologically control pumpkin diseases.

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Introduction

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a relatively new cultivar of Cucurbitaceae which arose in the nineteenth century: a specific mutation found in Styria (Austria) leads to characteristic dark green seeds with stunted outer hulls [1]. This unique cultivar is important not only in Austria, but also it has much wider growing areas, including several Southern European and African countries, China, Russia, and the USA. In the past decades, the dark-colored Styrian pumpkin seed oil with its intense nutty taste became internationally popular in gourmet cuisine. It is rich in polyunsaturated fatty acids and also contains vitamins, phytosterols, minerals, and polyphenols [1]. Studies suggest the usefulness of the oil in the prevention and treatment of benign prostatic hyperpla-

sia, in the prevention of arteriosclerosis, and the regulation of cholesterol level [2].

During the last decade, a dramatic increase of black rot in Styrian oil pumpkin has been observed [3]. The causal agent of black rot is *Didymella bryoniae* (Fuckel) Rehm, which is known to cause diseases on cucurbits all over the world [4, 5]. The ascomycetous fungus can infect any stage of the host plants and shows a variety of symptoms depending on the crop and stage concerned. Furthermore, it can be seed-borne, air-borne, or soil-borne [5]. In addition, Styrian oil pumpkins can be affected by several bacteria from the class Gammaproteobacteria, e.g., soft rot caused by *Pectobacterium carotovorum*. It was supposed that the bacterium is able to co-infect fruits synergistically with *D. bryoniae* [6]. Besides *P. carotovorum*, other bacteria such as *Pseudomonas viridiflava* and *Xanthomonas cucurbitae* can infest Styrian oil pumpkin plants as well [7]. Owing to the lack of effective fungicides and because a high proportion of oil pumpkin is cultivated under organic farming conditions, environmentally friendly and sustainable methods to protect pumpkins against microbial pathogens are desirable to control diseases.

Biocontrol using naturally occurring plant-associated microorganisms with beneficial effects on plant health provides promising perspectives for plant protection [8–10]. Especially, endophytes of the genera *Bacillus* and *Pseudomonas* can efficiently support the host plants by growth promotion and/or antagonism towards phytopathogens [11–13]. For application of antagonistic endophytes in biocontrol strategies, it is necessary to understand the microbial ecology of the host plant. Plant-associated microbial communities show a certain degree of plant specificity regarding species and cultivars [8], and different organs are often colonized by specific bacterial populations [14]. It is therefore essential to establish knowledge about pumpkin-specific endophytic microbial communities and its impact on plant growth and health.

The aim of this study was to analyze mainly endophytically living oil pumpkin-associated microbial communities with a special focus on the antagonistic potential towards the main oil pumpkin pathogens, the fungus *D. bryoniae*, and the bacteria *P. carotovorum*, *P. viridiflava*, and *X. cucurbitae*. Samples were obtained from three different field-grown pumpkin cultivars ‘Gleisdorfer Ölkürbis’, ‘Gleisdorfer Diamant’, and ‘Gleisdorfer Maximal’, at three plant developmental stages (young, flowering, senescent), from four different microhabitats: from seeds (spermosphere), roots (endorhiza), flowers (anthosphere), and fruits (carposphere). We used a multiphasic approach combining DNA-based studies, molecular fingerprints performed by single-stranded conformation polymorphism (SSCP) of 16S ribosomal RNA (rRNA) genes for *Bacillus* and *Pseudomo-*

nas and ITS genes for ascomycetous fungi, microscopic observations applying fluorescence in situ hybridization coupled with confocal laser scanning microscopy (FISH-CLSM), and a cultivation-dependent approach. On the basis of the latter, the functional diversity was studied and broad spectrum antagonists were selected. The effect of selected strains was characterized in more detail in a greenhouse experiment.

Methods

Experimental Design and Sampling

Plant samples were collected from a field located in Gleisdorf, Austria (47°05′23.96″ N, 15°43′45.71″ E, altitude 336 m). Samples were taken from three different Styrian oil pumpkin varieties: ‘Gleisdorfer Ölkürbis’, ‘Gleisdorfer Diamant’, and ‘Gleisdorfer Maximal’ (Saatzucht Gleisdorf, Gleisdorf, Austria) and from four different sites for each cultivar at three time points in 2009: 16th of June (young plants before flowering), 15th of July (flowering plants), and 26th of August (senescent plants with well-developed fruits). Four different microhabitats were investigated: seeds (spermosphere), roots (endorhiza), flowers (anthosphere), and fruits (carposphere). Seed-borne microorganisms were isolated from plants grown under gnotobiotic conditions because in seeds microorganisms are mostly in a dormant stage and difficult to cultivate [15]. Roots were collected at all three time points, whereas female flowers were sampled only at the second and fruits only at the third time point. For each sampling time, cultivar, and habitat, four independent composite samples were taken randomly from four different individual plants per site (number of root samples—36, flower samples—12, fruit samples—12, seed samples—5). These samples were used for DNA-dependent analysis as well as for cultivation.

Total Community DNA Isolation and Analysis

Roots were washed with tap water until soil particles were completely removed prior to surface sterilization in 4% NaOCl for 5 min and subsequently washed three times with sterile water, and sterility checks on culture media were performed according to Berg et al. [14]. Flowers were not surface-sterilized to preserve endophytes occurring in thin petals. Fruit pulp was cut out of the inner fruit under aseptic conditions. The different plant tissues were homogenized in sterile 0.85% NaCl with mortar and pestle. Suspensions used for cultivation-dependent analysis were centrifuged for 20 min at 10,000 × *g*. DNA was extracted from the pellets using the FastDNA[®] Spin Kit for Soil (MP Biomedicals, Irvine, USA).

Bacterial fingerprints of *Pseudomonas* and *Bacillus* from roots, flowers, and fruits were analyzed by SSCP analysis [16] using specific primers for *Pseudomonas*, *Bacillus*, and mainly ascomycetous fungi [17–19]. Because only flowers contained a considerable number of culturable fungi (data not shown), we prepared fungal fingerprints only of the anthosphere community. All DNA fragments were separated with a TGGE Maxi apparatus (Biometra, Göttingen, Germany) at 400 V and 26°C. Silver staining of gels was applied for visualization of the bands [20]. Dominant bands of gels were cut out and the sequences determined [16]. The closest matches of obtained sequences were found with BLASTn [21] as implemented in the database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

Microscopic Analysis of Bacterial Communities from the Anthosphere

Manually cut fragments of petals and pistils from female flowers (Gleisdorfer Opal) were washed in several steps with PBS buffer (pH 7.4). Samples were stored in 1:1 PBS/96% ethanol at –20°C until further processing. Prior to microscopic analysis, sections were placed on poly-L-lysine pre-coated microscopic slides (Thermo Fisher Scientific, Bremen, Germany). Samples were dried by using filter paper and a heating block (40°C) for 10 min. Fifty microliters of 0.5 mg ml⁻¹ lysozyme solution (Sigma-Aldrich, Steinheim, Germany) was added for 10 min before dehydration by ethanol series (50%, 80%, and 96% ethanol, 3 min incubation each one) and successive washing with PBS. FISH was performed as described by Lo Piccolo et al. [22] with modifications. Hybridization was carried out with 27 µl hybridization buffer (360 µl of 5 M NaCl, 40 µl of 1 M TRIS, pH of TRIS solution was set at pH 7.4, 300 µl of formamide (Sigma-Aldrich, Steinheim, Germany), 2 µl of 0.7 M SDS, filled up to 2 ml with ddH₂O) and 3 µl of labeled probes (50 ng µl⁻¹) at 45°C for 2 h in a dark humid chamber. Probe EUB338MIX (Cy3-labeled) was used for overall bacterial communities, Gammaproteobacteria were probed with GAM42a (Cy5-labeled), Alphaproteobacteria with ALF968 (Cy5-labeled), Firmicutes were probed with LGC354MIX-FITC, and probe BET42a (ATTO488-labeled) was used for Betaproteobacteria [23, 24]. All FISH probes were purchased from genXpress Service & Vertrieb GmbH, Wiener Neudorf, Austria. The hybridization mix was removed by filter paper and slides were placed in preheated (47°C) washing buffer (3.18 ml of 5 M NaCl, 1 ml of 1 M Tris at pH 7.4, filled up to 50 ml with ddH₂O) for 20 min. The slides with flower pieces were then dried before application of a few drops of cold ddH₂O on the flower pieces prior to drying with compressed air. Mounted with ProLong Gold antifadent (Molecular Probes, Eugene,

USA), slides were kept in the dark prior to CLSM. Stained samples were analyzed with a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with solid state and UV lasers. The software Imaris 7.0 (Bitplane, Zurich, Switzerland) was used for 3D rendering of confocal stacks and creation of isosurface-spot models.

Isolation of Microorganisms from Styrian Oil Pumpkin Plants

Microorganisms were isolated from suspensions obtained from plant materials on R2A medium for bacteria (Roth GmbH, Karlsruhe, Germany) and SNA medium for fungi (Spezieller Nährstoffarmer Agar) containing 1 g KH₂PO₄, 1 g KNO₃, 0.5 g MgSO₄ × 7 H₂O, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, and 22 g agar per l (pH 6.5). After autoclaving for 20 min, the following antibiotics were added: 10 mg l⁻¹ chlorotetracycline, 50 mg l⁻¹ dihydrostreptomycin sulfate, and 100 mg l⁻¹ penicillin G. After 5 days of incubation at 20°C, colony-forming units (CFU) were determined and representative colonies were transferred onto Luria–Bertani (LB) medium for bacteria and potato dextrose agar (PDA) medium for fungi (all media from Roth).

In Vitro Assays to Characterize Antagonistic Activity of Isolated Microorganisms

The following pathogenic strains were used in dual culture assays: *D. bryoniae* A-220-2b, *P. carotovorum* subsp. *atrosepticum* 25–2, *P. viridiflava* 2 d1, and *X. cucurbitae* 6 h4. The fungal strain is from our own collection [6], whereas bacterial pathogenic strains were obtained from the Göttinger Sammlung Phytopathogener Bakterien: GSPB, University of Göttingen, Germany. Altogether, 1,748 bacterial isolates (152 from spermosphere, 930 from endorhiza, 336 from anthosphere, and 330 from carposphere) were streaked out on nutrient agar (Sifin, Berlin, Germany) together with mycelium fragments of *D. bryoniae*. Mycelium fragments of fungal isolates (47 from spermosphere, 200 from endorhiza, 304 from anthosphere, and 21 from carposphere) were placed on PDA plates together with *D. bryoniae*. Antagonistic activity was assessed after 5 days of incubation at 20°C according to Berg et al. [14]. *D. bryoniae* antagonists were also tested for their broad spectrum activity against the bacterial pathogens *P. carotovorum* subsp. *atrosepticum* 25–2, *P. viridiflava* 2 d1, and *X. cucurbitae* 6 h4. Bacterial suspensions grown overnight in tryptic soy broth (TSB; Roth) at 30°C were mixed with LB agar (containing 1.2% agar). Subsequently, bacterial isolates were streaked out on these treated plates, whereas mycelium fragments were

placed on the agar surface. After 5 days of incubation at 20°C, the presence or absence of clearing zones (due to growth inhibition of pathogens) surrounding the test strains was assessed. Isolates exhibiting antagonistic potential against at least two of the three tested bacterial phytopathogens were designated as broad spectrum antagonists.

Genotypic Characterization and Identification of Broad Spectrum Antagonists

Bacterial DNA was isolated according to Berg et al. [14]. In the first step, strains were characterized taxonomically by Amplified Ribosomal DNA Restriction Analysis (ARDRA) using the restriction endonuclease *HhaI* (MP Biomedicals, Eschwege, Germany) and by a more discriminative method based on whole genome—BOX PCR fingerprints [14, 25]. Representative strains were selected according to their ARDRA and BOX patterns and their partial 16S rRNA genes were sequenced and analyzed by BLASTn analysis [21].

Evaluation of Mode of Action of Broad Spectrum Antagonists and Their Effects Ad Planta

The capacity of antagonists to inhibit the growth of *D. bryoniae* A-220-2b via the production of bioactive volatile organic compounds was tested with dual culture assays comprising two-compartment petri dishes (side 1—50 µl from overnight cultures in LB or TSB; side 2—a 0.3-mm mycelium fragment of *D. bryoniae* on PDA). After 3 days of incubation, diameters of *D. bryoniae* colonies were measured. Furthermore, the production of antimicrobial compounds against *D. bryoniae* was studied by analysis of supernatants of growth media in which antagonists were cultivated. Supernatants of 3-day-old liquid cultures of antagonists were filtered (0.45 µm pore size) and mixed with PDA (1.5%) in a ratio 1:3. In the control treatments, sterile media were mixed with PDA. Mycelium fragments of *D. bryoniae* A-220-2b were placed on top of the supernatant-containing agar and diameters of colonies were measured after 4 days of growth at 20°C. Both experiments were conducted in triplicate.

The effects of antagonists on disease severity (ds) caused by *D. bryoniae* on oil pumpkin were evaluated in a greenhouse experiment. Seeds of the cultivar ‘Gleisdorfer Opal’ were treated for 13 h with suspensions at 6.9×10^9 CFU ml⁻¹ of all six antagonists separately or with a mix of suspensions of strains *Lysobacter gummosus* L101, *Serratia plymuthica* S13, *Pseudomonas chlororaphis* P34, and *Paenibacillus polymyxa* PB71 (one representative for each bacterial genus) in 0.85% NaCl. For the control treatment, seeds were only treated with 0.85% NaCl. Seeds were planted into peat moss substrate with clay (Gramoflor,

Vechta, Germany). For each treatment, always three 5-l pots with five plants per pot were studied. Plants were grown under artificial illumination (12 h per day) at 26°C. Thirteen days after sowing, 50 µl of a conidial suspension (2.3×10^5 ml⁻¹ of *D. bryoniae* SP2) was injected into leaf stems of first and second leaves [6]. Plants were covered with plastic bags to produce humid conditions, which support the infection. After 12 days, disease severity was observed on primary and secondary leaves and scored using the following numbers: 1 = no infestation on leaf, 2 = approx. 25% of leaf area infested, 3 = 50% of leaf area infested, 4 = 75% of leaf are infested, and 5 = leaf was completely infested.

Statistical Analysis

Banding patterns obtained from single isolates (ARDRA, BOX PCR) as well as from community analyses (SSCP) were normalized and subjected to cluster analysis based on the unweighted pair group method using average linkages to the matrix of similarities obtained using the Gel ComparII software (Version 5.1, Applied Maths, Kortrijk, Belgium). Similarity matrices were analyzed with R software version 2.12.1 (The R Foundation for Statistical Computing, ISBN 3-900051-07-0) to perform a permutation test ($P < 0.05$) in order to find significant differences between SSCP band patterns. Data from *Pseudomonas* and *Bacillus* fingerprints were additionally analyzed by detrended correspondence analysis (DCA) with the Canoco software (Version 4.52, Biometris, Wageningen, The Netherlands). Analysis of variance (ANOVA) in addition with Fisher’s least significant difference test (LSD; $P < 0.05$) was performed to compare mean diameters of *D. bryoniae* measured in the antibiosis assays (for soluble and volatile compounds). Duncan’s multiple range test ($P < 0.1$) was used to compare mean values for ds in ad planta tests. ANOVA and post hoc tests were performed with Predictive Analysis Software (PASW, Version 18.0.0).

Nucleotide Sequence Accession Numbers

Obtained sequences were deposited in GenBank under accession numbers HQ163897 to HQ163900, HQ163902 to HQ163914, HQ661145 to HQ661152, and JF796744 to JF796748.

Results

Abundances of Oil Pumpkin-Associated Microorganisms

Bacterial abundances from different field-grown Styrian oil pumpkin cultivars and microhabitats were in a range

between 2.83 and 7.03 \log_{10} CFU g^{-1} fresh weight of plant material (Table 1). The anthosphere exhibited the highest number of cultivable bacteria (6.99–7.03 \log_{10} CFU g^{-1}) followed by the endorhiza (4.19–4.21 \log_{10} CFU g^{-1}), carposphere (2.83–3.58 \log_{10} CFU g^{-1}), and spermosphere (under the detection limit of <2.0 \log_{10} CFU g^{-1}). Fungal abundances above the detection limit were only found in the anthosphere (2.36–3.08 \log_{10} CFU g^{-1}).

Microbial Fingerprints of Oil Pumpkin-Associated Communities

In order to characterize pumpkin-associated communities, SSCP fingerprints of *Pseudomonas* and *Bacillus* were analyzed; both genera represent dominant inhabitants and antagonists of plants [9, 10, 13]. For all microenvironments, a high diversity for both genera was found. *Bacillus* and *Pseudomonas*-specific patterns were detected in the investigated microenvironments (Fig. S1). Statistically significant differences between different microenvironments were confirmed by permutation tests ($P<0.001$). Furthermore, significantly different *Pseudomonas* and *Bacillus* communities were found for the endorhiza at different plant developmental stages ($P<0.001$). In contrast, only a minor, statistically insignificant influence of the cultivars on *Bacillus* and *Pseudomonas* species composition was found (data not shown). Differences between microbial communities in the microenvironments and at different plant stages were illustrated by DCA (Figs. 1 and 2), which clearly show the dependence on biotic parameters.

Dominant bands from SSCP fingerprints were sequenced: the *Pseudomonas* species *Pseudomonas oryzae*, *Pseudomonas putida*, *Pseudomonas syringae*, *P. viridiflava*, and *Pseudomonas fluorescens* were identified (Table 2). For *Bacillus*, *Bacillus weihenstephanensis*, *Bacillus flexus*, *Bacillus psychrodurans*, *Bacillus siralis*, *Bacillus indicus*, *Bacillus subtilis*, *Bacillus gibsonii*, and *Bacillus firmus* (only 96% 16S rRNA gene sequence match) were detected in different microhabitats of Styrian oil pumpkin. *B. flexus* was found in endorhizas and anthospheres, whereas *B. subtilis* was found in all three

observed microhabitats. *B. weihenstephanensis*, *B. psychrodurans*, and *B. siralis* could only be detected in endorhizas, whereas *B. gibsonii*, *B. indicus*, and *B. firmus* were found only in anthospheres (Table 3).

For fungal fingerprints, only the anthosphere was investigated. The other microenvironments were not considered because fungi were under the detection limit in the cultivation approach. If the amount of fungal DNA is too low, it is not possible to perform reliable fingerprints. Again, an influence of the cultivar on structure was observed but this impact was not significant for all samples (data not shown). The following members of ascomycetes were identified: *Plectosphaerella cucumerina* (AJ246154.1), *Phoma herbarum* (FN868459.1), uncultured *Oidiodendron* (GQ338892.1), *Capnobotryella* sp. (AJ972854.1), and *Pleosporaceae* sp. (EF060611.1).

Microscopic Analysis of Bacterial Communities from the Anthosphere

According to the high abundances and diverse fingerprints found in the anthosphere, bacterial colonies on petals and pistils were observed in more detail by FISH-CLSM. Female flowers were densely colonized by bacteria (Fig. 3a–d). Petals were colonized on their inner surface by diverse micro-colonies (Fig. 3a). On pistils, Beta- and Gammaproteobacteria and diverse unclassified bacteria were detected, and all of them were found separately as micro-colonies (Fig. 3c). Furthermore, it was visualized that pollen grains on pistils were densely colonized predominantly by Gammaproteobacteria (Fig. 3b, d). The Gammaproteobacteria formed dense patches, whereas members of Betaproteobacteria were found in micro-colonies both on the surface of pollen grains.

Cultivation-Based Screening for Antagonists

Representative bacterial and fungal strains were selected from each microhabitat and cultivar and screened in a first step for their in vitro activity towards *D. bryoniae*. Out of the 2,230 tested bacterial and fungal isolates, 128 (=7%) and 37 (=6.5%) strains, respectively, showed in vitro

Table 1 Abundances in different microenvironments from the Styrian oil pumpkin cultivars ‘Gleisdorfer lkrbis’, ‘Gleisdorfer Diamant’, and ‘Gleisdorfer Maximal’

Microhabitat	Gleisdorfer lkrbis		Gleisdorfer Diamant		Gleisdorfer Maximal	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
Endorhiza	4.19 (± 0.44)	<2.00	4.21 (± 0.39)	<2.00	4.20 (± 0.37)	<2.00
Anthosphere	6.99 (± 0.22)	2.36 (± 0.37)	7.01 (± 0.27)	3.08 (± 0.34)	7.03 (± 0.57)	2.94 (± 0.39)
Carposphere	3.58 (± 0.27)	<2.00	3.47 (± 0.32)	<2.00	2.83 (± 0.11)	<2.00

Mean values of \log_{10} -transformed colony forming units (CFUs) of isolated microorganisms per gram plant material are presented; values in brackets indicate standard deviations

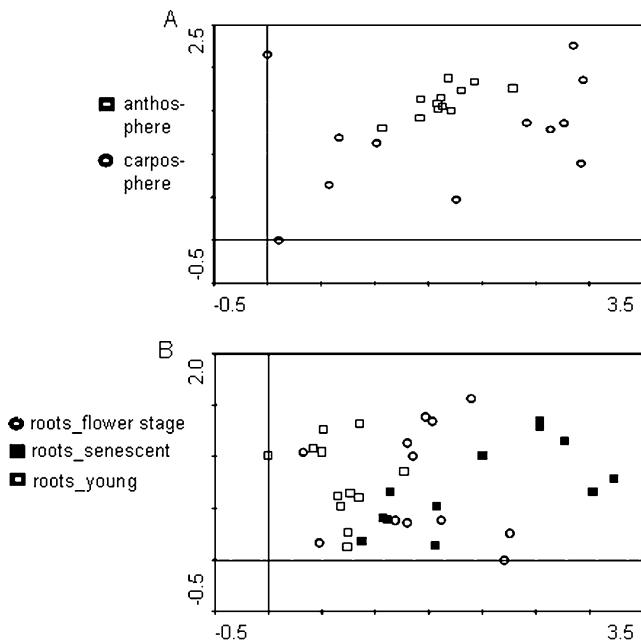


Figure 1 Detrended correspondence analysis based on *Pseudomonas*-specific patterns on SSCP gels originated from the **a** anthosphere and carposphere and **b** endorhizas (roots) at different plant developmental stages from Styrian oil pumpkin

antagonism against *D. bryoniae* (Fig. S2). In general, the highest number of antagonists was found in the endorhiza, in contrast to low amounts of antagonists in the anthosphere and carposphere (Fig. 4). The highest proportion of bacterial antagonists was found in the spermosphere (16%) and for fungal antagonists in the endorhiza (16%).

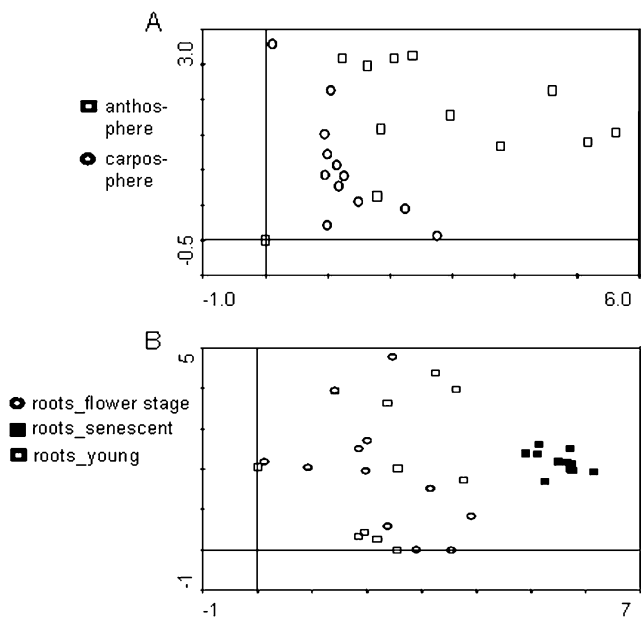


Figure 2 Detrended correspondence analysis based on *Bacillus*-specific patterns on SSCP gels originated from the **a** anthosphere and carposphere and **b** endorhizas (roots) at different plant developmental stages from Styrian oil pumpkin

The antifungal antagonists were screened against the bacterial pathogens of pumpkin *P. viridiflava* 2 d1, *P. carotovorum* subsp. *atrosepticum* 25–2, and *X. cucurbitae* 6 h4. Out of 128 bacterial and 31 fungal isolates, 49% and 32%, respectively, showed antagonistic activity against at least one of the bacterial pathogens; 34% of the analyzed bacterial isolates exhibited growth inhibition of at least two of the observed strains, and 6% of the bacterial antagonists inhibited growth of all three pathogenic bacteria. None of the tested fungi demonstrated antagonism against more than one bacterial strain. A significant portion of bacteria and fungi (40% and 29%) were effective against *P. viridiflava*, followed by 30% and 3%, which suppressed growth of *X. cucurbitae* 6 h4. Altogether, 19% of the bacterial strains could suppress *P. carotovorum*, whereas no fungal isolate showed inhibition of this pathogen. The 43 bacterial isolates exhibiting antagonistic potential against *D. bryoniae* and two bacterial pathogens were classified as broad spectrum antagonists and further characterized. In contrast, no fungal broad spectrum antagonist was selected due to the missing activity of the fungal strains against bacterial pathogens.

Genotypic Characterization of Broad Spectrum Antagonists

To characterize the 43 antagonists at the genotypic level and to select unique strains for the biocontrol strategy, two fingerprinting methods were applied. In the first step, ARDRA analysis was used to differentiate four groups (I–IV). In the second step, BOX PCR was used to characterize the strains within each ARDRA group at the population level. The ARDRA groups could be subdivided into one to five groups with similar patterns. For example, ARDRA group IV, which was the largest group with a high intraspecific diversity later identified as *Lysobacter*, clustered into five different BOX PCR fingerprint patterns (Fig. 5). Finally, six broad spectrum antagonists with unique BOX patterns were chosen for identification: one representative of ARDRA groups I–III and three of ARDRA group IV. Strains were identified as *L. gummosus*, *L. antibioticus*, *P. polymyxa*, *P. chlororaphis*, and *S. plymuthica* (Table 4).

Mode of Action and Biocontrol Effect

The majority of broad spectrum antagonists produced soluble and/or volatile antibiotics in vitro (Table 4). The only exception was *L. antibioticus* strain L175. Secretion of soluble antimicrobial compounds was observed for *L. gummosus* L101, *L. antibioticus* L169, *P. chlororaphis* P34, and *P. polymyxa* PB71, whereas emission of antimicrobial volatiles was found for *L. gummosus* L101, *S. plymuthica* S13, *P. chlororaphis* P34, and *P. polymyxa* PB71.

Table 2 Taxonomic characterization of Styrian oil pumpkin-associated *Pseudomonas* strains based on partial 16S rRNA gene sequences obtained from SSCP gels

Microhabitat	Clone	Taxonomic affiliation based on BLAST matches (accession numbers)	Similarity in %
Endorhiza	PR1	<i>P. oryzihabitans</i> (EU977742.1)	100
	PR2	<i>P. putida</i> (CP002290.1)	100
	PR3	<i>P. syringae</i> (DQ294981.1)	97
Anthosphere	PB1	<i>P. viridiflava</i> (HM190229.1)	100
	PB2	<i>P. syringae</i> (HQ267378.1)	100
Carposphere	PF1	<i>P. putida</i> (HQ658765.1)	97
	PF2	<i>P. fluorescens</i> (HM016864.1)	100
	PF3	<i>P. syringae</i> (HQ267378.1)	100

To study the biocontrol activity of selected broad spectrum antagonistic bacteria, oil pumpkin seeds were soaked in cell suspensions of the selected antagonists, grown in pots for 13 days and artificially infected with *D. bryoniae*. Reduction of disease severity was evaluated by comparison with a nontreated control (Table 4). Strain *L. antibioticus* L169 showed no antagonistic activity ad planta, whereas the highest reduction in disease severity compared to the control treatment was observed for *P. chlororaphis* P34 with 20.4% (Table 4). Considerable reduction (19.4%) of disease severity compared to the control was exerted by a mixture of antagonists including *L. gummosus* L101, *P. polymyxa* PB71, *S. plymuthica* S13, and *P. chlororaphis* P34.

Discussion

Our study about the ecology of pumpkin-associated microorganisms revealed bacteria dominating the microbial communities. The community structure was influenced by plant stage and organ but not by the cultivar. The spermo-

sphere and anthosphere were discovered as important new reservoirs for antagonistic bacteria. Six efficient bacterial antagonists against pathogens tested were selected and evaluated under greenhouse conditions. In contrast, fungi were less abundant and no broad spectrum antagonist could be isolated from the fungal community.

Virtually, all plant species are colonized by microorganisms including bacteria and fungi, which are neutral or show positive plant–microbe interaction. For pumpkin, we found bacteria-dominated microbial communities, except for the anthosphere. In general, various biotic and abiotic parameters such as plant age, vegetation time, or pH influence the plant-associated microbial communities [8]. We found an impact of plant stage and organ on the composition of *Bacillus* and *Pseudomonas* communities. The importance of both factors is also known from studies with other plants [8, 26]. Interestingly, analysis of flower-associated microbial communities in *Saponaria officinalis* and *Lotus corniculatus* showed also a strongly different community structure for leaves and petals: Enterobacteriaceae dominated here the floral communities [27]. In contrast to other studies [8], we found no influence of the

Table 3 Taxonomic characterization of Styrian oil pumpkin-associated *Bacillus* strains based on partial 16S rRNA gene sequences obtained from SSCP gels

Microhabitat	Clone	Taxonomic affiliation based on BLAST matches (accession numbers)	Similarity in %
Endorhiza	R1	<i>B. weihenstephanensis</i> (AB021199.1)	100
	R2	<i>B. flexus</i> (AB021185.1)	100
	R3	<i>B. psychrodurans</i> (AJ277984.1)	99
	R4	<i>B. soralis</i> (NR_028709.1)	98
	R6	<i>B. subtilis</i> (AJ276351.1)	100
	Anthosphere	B1	<i>B. indicus</i> (AJ583158.1)
B2		<i>B. gibsonii</i> (X76446.1)	98
B3		<i>B. flexus</i> (AB021185.1)	98
B4		<i>B. firmus</i> (D16268.1)	96
B5		<i>B. subtilis</i> (AJ276351.1)	99
Carposphere	F1	<i>B. subtilis</i> (AJ276351.1)	99

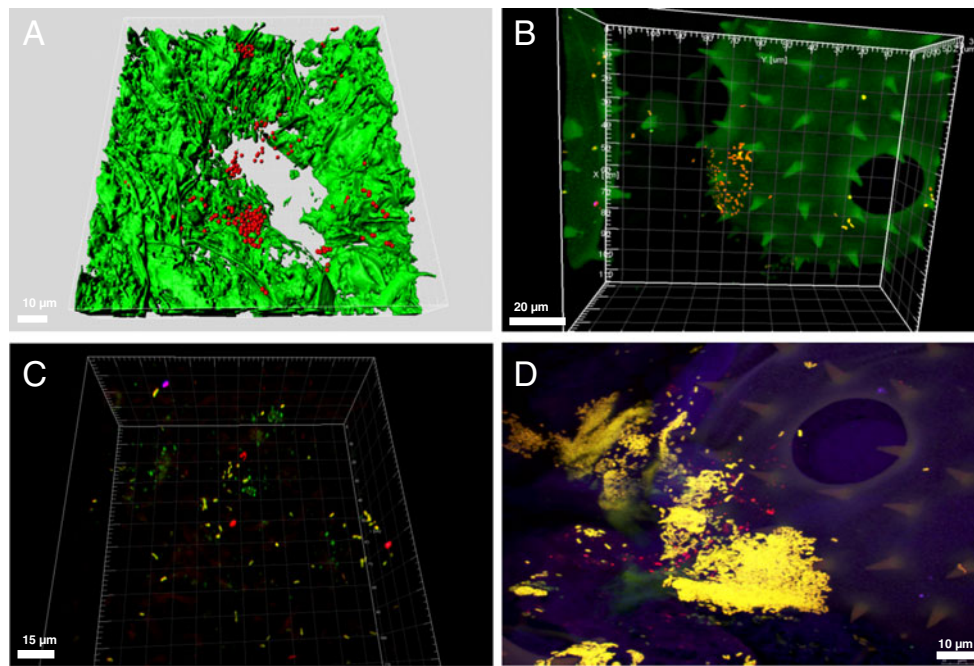


Figure 3 Visualization of FISH-labeled bacteria inhabiting female oil pumpkin flower parts by CLSM. **a** 3D rendered image (Imaris software) of overall bacterial communities (in red) on petals labeled with EUB338MIX-Cy3. **b** Alphaproteobacteria (in yellow), not taxonomically classified bacteria (in red) and Firmicutes (pinkish) labeled with ALF968-Cy5, EUB338MIX-Cy3, and LGC354MIX-FITC on pollen grains located on pistils. **c** Gammaproteobacteria (in

yellow), Betaproteobacteria (pinkish), and not taxonomically classified bacteria (in red) on pistils labeled with GAM42a-Cy5 and BET42a-ATTO488, respectively, with EUB338MIX-Cy3. **d** Gammaproteobacteria (in yellow) and not taxonomically classified bacteria (in red) on pollen grains on pistils labeled with GAM42a-Cy5 and EUB338MIX-Cy3

cultivar. Genetic differences between pumpkin cultivars are relatively low in comparison to other crops because a special breeding program for oilseed pumpkin exists in Styria only for 15 years. The plant microhabitat contributes to specific differences in the functional roles of the culturable fraction. The endorhiza is confirmed here as one of the main reservoirs for antagonistic strains. In addition, we discovered the spermosphere and the anthosphere as potential habitats for antagonists. This merits further study in other plants, especially since both organs were till now regarded rather as reservoirs for pathogens,

e.g., the anthosphere for *Erwinia amylovora* or *Botrytis cineria* and the spermosphere for diverse seed-borne pathogens. By cultivation-independent approach, we also identified not only potential beneficial but also strains known as pathogens. *P. syringae* is well known as an important plant pathogen and *P. oryzihabitans*, present in the endorhiza, is an opportunistic pathogen of humans [28]. Although the identification of potential pathogens is only a hint, which needs further evidence and experiments, our findings support the view of the rhizosphere as a reservoir of opportunistic or emerging pathogens [29].

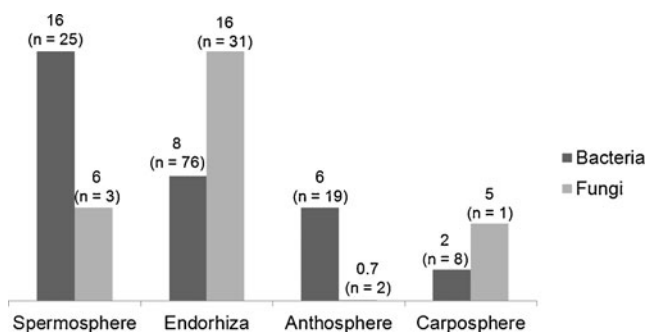
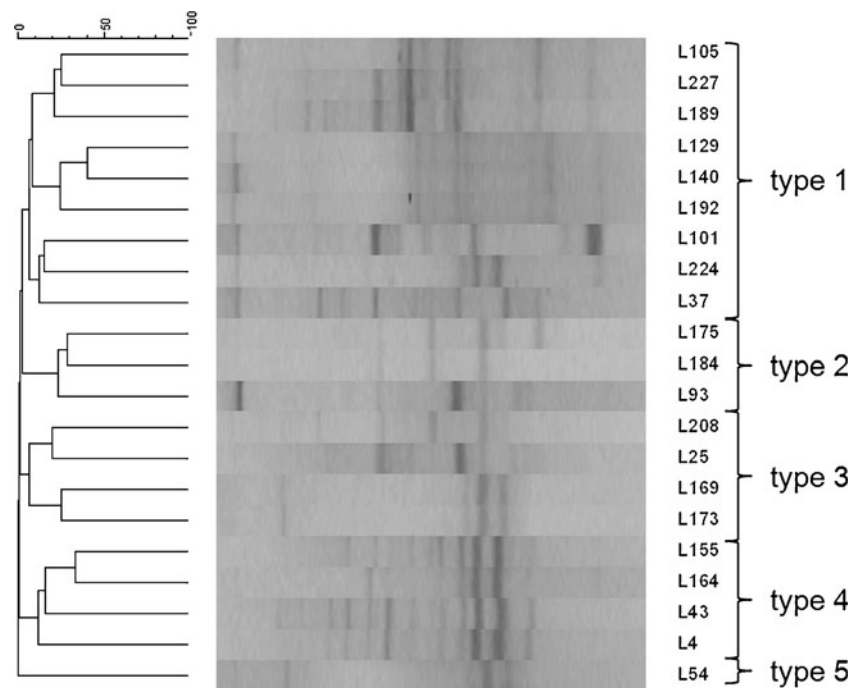


Figure 4 In vitro antagonists against *D. bryoniae* isolated from different microhabitats of Styrian oil pumpkin presented as proportion in percentage of tested bacterial and fungal strains

Plants acquire their microorganisms from the environment, primarily from the soil [8]. Recent investigations suggested also a vertical transmission of bacteria from the parent plant [30]. Also for mosses, the phylogenetically oldest land plants, a transmission of the core microbiome from the gametophyte to the sporophyte and vice versa was discovered. This moss-specific microbiome was found to be modified by abiotic factors (nutrient richness, pH) (Bragina et al., under revision). In the present study, we found hints that bacteria are transferred with pollen, as suggested by microscopy and by shared occurrence of several strains in the anthosphere and carposphere. This should be not only considered in the interpretation of floral traits, it is also important for pollination and microbial ecology [27]. Many

Figure 5 Grouping of BOX PCR band patterns obtained from broad spectrum antagonists belonging to *Lysobacter* spp. (ARDRA group IV)



reports have been published on the use of honey and bumble bees to disseminate biocontrol agents to flowers especially against *B. cineria*. To use specifically adapted bacteria from the anthosphere for this purpose is an interesting objective.

Although the proportion of fungal antagonists against *D. bryoniae* was similar to those of the bacteria in general, at last only bacterial strains were selected. The reason for this selection was the missing activity of the fungal strains against the bacterial pathogens. This agrees with former studies, where a high antifungal but a low antibacterial potential within the indigenous plant-associated communi-

ties was found [31, 32]. All bacteria of our collection belong to well-known antagonistic species [33–35]. However, the extent of antagonistic activity is highly specific for each of these strains. This was found for the two *L. antibioticus* strains investigated: L175 showed no production of antibiotics in vitro but was able to reduce the disease index of *Didymella*, while L169 produced antibiotics but showed no disease-suppressive activity. Members of the genus *Lysobacter* are well-known disease-suppressive bacteria with strain-specific effects [35]. Recent investigations of evolutionary genomics show that species are defined by the paleome (core genes, which allow basic

Table 4 Taxonomic affiliation of broad spectrum antagonists including their abilities to produce bioactive soluble and volatile organic compounds and to decrease disease severity (ds) on Styrian oil pumpkin caused by *D. bryoniae*

Strain	Origin	Identification	Synthesis of inhibitory substances against <i>D. bryoniae</i>		Interaction with plants Decrease of ds ^b [%]	
			Volatile	Soluble		
		Taxonomic affiliation ^a [accession numbers]	Similarity [%]			
L101	Endorhiza	<i>Lysobacter gummosus</i> (HQ163910.1)	98	+	+	8.6
L169	Endorhiza	<i>Lysobacter antibioticus</i> (HQ163912.1)	98	–	+	0
L175	Endorhiza	<i>Lysobacter antibioticus</i> (HQ163913.1)	99	–	–	9.7
S13	Anthosphere	<i>Serratia plymuthica</i> (HQ163914.1)	98	+	–	18.2
P34	Endorhiza	<i>Pseudomonas chlororaphis</i> (HQ163911.1)	97	+	+	20.4*
PB71	Spermosphere	<i>Paenibacillus polymyxa</i> (HQ163909.1)	99	+	+	11.8

+ signify significant lower diameters of *D. bryoniae* colonies compared to control treatment (LSD, $P < 0.05$)

^a of partial 16S rRNA genes by BLAST analysis

^b percent reduction of disease severity compared to control treatment based on mean values of grade sums, stating degrees of infestation of infected leaves

* $P < 0.1$, significant different to control treatment (ANOVA, Duncan's multiple range test)

metabolism), whereas strains are characterized by their cenome (genes, which allow cells to live in and explore a particular niche). The latter genomic content is made responsible for strain-specific properties [36]. This is important for the characterization of biocontrol strains: each strain of one species can have their specific antagonistic properties and can show different biocontrol effects in the field [13, 31]. Therefore, a selection strategy considering these strain-specific effects is necessary. Our strain-specific consortium is therefore optimally suited to suppress various pathogens which frequently co-occur in pumpkin disease. Diseases caused by cooperation of various pathogens are on the rise also due to climate change [6], for which biological control based on synergistic biocontrol agents needs to be further developed.

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