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PLANT MICROBE INTERACTIONS

Endophytes of Grapevine Flowers, Berries, and Seeds: Identification of Cultivable Bacteria, Comparison with Other Plant Parts, and Visualization of Niches of Colonization

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Abstract Endophytic bacteria can colonize various plants and organs. However, endophytes colonizing plant reproductive organs have been rarely analyzed. In this study, endophytes colonizing flowers as well as berries and seeds of grapevine plants grown under natural conditions were investigated by cultivation as well as by fluorescence in situ hybridization. For comparison, bacteria were additionally isolated from other plant parts and the rhizosphere and characterized. Flowers, fruits, and seeds hosted various endophytic bacteria. Some taxa were specifically isolated from plant reproductive organs, whereas others were also detected in the rhizosphere, endorhiza or grape inflo/infructescence stalk at the flowering or berry harvest stage. Microscopic analysis by fluorescence in situ hybridization of resin-embedded samples confirmed the presence of the isolated taxa in plant reproductive organs and enabled us to localize them within the plant. *Gammaproteobacteria* (including *Pseudomonas* spp.) and *Firmicutes* (including *Bacillus* spp.) were visualized inside the epidermis and xylem of ovary and/or inside flower ovules. *Firmicutes*, mainly *Bacillus* spp. were additionally visualized inside berries, in the intercellular spaces of pulp cells and/or xylem of pulp, but also along some cell walls inside parts of seeds. Analysis of cultivable bacteria as well as microscopic results indicated that certain endophytic bacteria can colonize flowers, berries, or seeds. Our results also indicated that some specific taxa may not only derive from the root environment but also from other sources such as the anthosphere.

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Introduction

In both managed and natural ecosystems, plants can interact with a wide range of bacteria, which can have pathogenic, neutral, or beneficial effects on their hosts. The majority of bacterial microsymbionts are epiphytic and are known to colonize the rhizosphere [9, 23]. A subset of the rhizosphere microflora can also enter and proliferate within plants as endophytes [17, 18]. Endophytes may also derive from other sources of colonization [8] and these kinds of microorganisms have been mostly isolated from endorhiza, stems and/or leaves [16, 29, 31]. Few studies have also demonstrated that part of the endophytic microflora can colonize plant reproductive organs [8, 10]. However, little

information is currently available, whether specific bacterial taxa are able to colonize plant reproductive organs. At present, very few studies have demonstrated that endophytic bacteria can colonize flowers, fruits or seeds (inside fruits) [4, 7, 21, 24, 26, 27], whereas in other studies endophytes were not found in these organs (reviewed in [16]). Some strains isolated from plant reproductive organs were also isolated from the rhizosphere or other plant parts under natural conditions, indicating a potential pathway of colonization (reviewed by [8]). In grapevine, inoculation of a bacterial endophyte involved in plant growth promotion resulted in systematic spreading of that strain in plant tissues [11] including plant reproductive organs [9, 10]. In addition, other endophytic bacteria other than the inoculant strain were detected inside plant reproductive organs. Nonetheless, our understanding of the ecology of bacteria colonizing reproductive organs of plants growing under natural conditions is still very limited. Moreover, if endophytic bacteria occur inside plant reproductive organs, niches of colonization should be also demonstrated by microscopic analysis to further proof the presence of these endophytes in these plant parts.

The objective of this study was therefore to analyze, which culturable bacteria colonize reproductive organs (flowers, fruits, and seeds), of grapevine cultivated under natural conditions, and to compare them with bacterial communities colonizing other plant parts and the rhizosphere. Plants were sampled at the flowering and berry stage. To further confirm the presence of endophytic bacteria in plant reproductive organs such as flowers, fruits, and seeds and to localize them in the various plant tissues, a cultivation-independent method, fluorescence in situ hybridization (FISH), was additionally employed.

Materials and Methods

Sampling of Flowers, Fruits, Seeds and Other Plant Parts

A vineyard grown on sandy soil, located in the Austrian National park of Illmitz and belonging to Helmut Gangl Wines was chosen for sampling. Phytosanitary products and microorganisms were never applied in this vineyard. The plants (SO4 as rootstock grafted with the cultivar Zweigelt clone GU9) were 7 years old.

Plant tissues, without any eye-visible injury, from three independent plants were sampled at the two different vegetation stages: at the beginning of the flowering (stage 61 according to the BBCH scale; [25]) and when berries were ready for harvest (stage 89 according to BBCH scale; [25]). Briefly, for each developmental stage, soil was carefully removed to locate roots. Roots were then sampled and then the remaining roots were covered with the same soil. For plant reproductive organs, inflorescences or infructescences were harvested with a clipper and flowers and berries with seeds were then separated of grape inflo/infructescence stalks immediately before preparation of samples for bacterial isolation or microscopy.

Preparation of Plant Samples for Bacterial Isolation

For surface sterilization, 1 g of plant tissues (flowers) or 5 g of roots, inflo/infructescence stalk, or berries with seeds were treated with 70% ethanol (5 min), followed by an immersion in 2.5% sodium hypochlorite solution (15 min roots, 2 min for flowers and 5 min for grape inflo/infructescence stalks, or for berries with seeds). Samples were then rinsed three times with sterile distilled water. In the case of berries, seeds were then extracted with pinces and sterilization was done again (70% ethanol, 5 min; 2.5% sodium hypochlorite solution 5 min, washes three times in distilled water). All the samples (roots, flowers, berries without seeds, and seeds) were then macerated independently with 10 mL sterile 0.85% NaCl using a mortar and pestle and further homogenized by vortexing for 60 s at high speed. The solutions were then used for further isolation of bacteria. In parallel, surfaces sterilization was checked by plating three times 100 μL of the last washing solution on R2A medium (Difco, Detroit, MI) and no bacterial colonies were shown growing on plates after 5 days of incubation at ambient temperature (25°C).

For the isolation of rhizosphere bacteria, rhizosphere soil was manually detached through gentle agitation of the roots sampled. Rhizosphere soils (5 g) were then mixed with 10 mL of sterile 0.85% NaCl solution and then homogenized by vortexing for 60 s at high speed. These suspensions were then used for the isolation of bacteria.

Isolation of Bacteria

To quantify bacterial populations, microbial suspensions were tenfold diluted with sterile 0.85% NaCl solution and then plated on R2A medium. Plates were incubated for 5 days at ambient temperature (25°C). Then, numbers of cultivable bacteria associated with different plant parts were counted and numbers of colony-forming units (log10 CFU) per gram fresh weight were calculated.

All isolates obtained by plating were additionally purified before storage at −80°C in sterile broth solution amended with 20% glycerol. Isolations were done three independent times from the different plant organs of the individual plants by selecting bacterial isolates based on their morphologies on plates. Up to 20 isolates were selected each time. In total, more than 50 isolates were obtained from plant reproductive organs and more than 340 bacterial isolates were selected from all plant parts analyzed.

DNA Isolation and Amplified rDNA Restriction Analysis (ARDRA)

Following bacterial isolation, DNA was isolated from isolates by using a Microbial DNA extraction kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturers' instructions. ARDRA analysis was then used to group isolates according to their taxonomy (genus/species levels). Briefly, 16S rRNA genes were PCR-amplified by using the primers 8for (5′-AGAGTTT GATCCTGGCTCAG-3′) [30] and 1520rev (5′-AAG GAGGTGATCCAGCCGGA-3′) [13] and the PCR conditions previously described [28], except that the number of amplification cycles was 24 instead of 30. PCR products were then digested with the restriction enzymes *Hae*III and *Rsa*I (Invitrogen) individually using 12.5 μL PCR product, 2 μL enzyme buffer (Invitrogen), 2.5 μL distilled water and 0.5 μL of enzyme (10 U/μL). Reactions were then incubated for 2 h at 37°C and the restriction products were analyzed on 2% agarose gels (AppliChem, Darmstadt, Germany).

Identification of ARDRA Groups of Bacterial Isolates

Four representative isolates of each ARDRA group of isolates were identified by partially sequencing the 16S rRNA gene. 16S rRNA genes were amplified as described above and sequencing was done by AGOWA (http://www.agowa.com/) using the 518Rev primer (5′-ATTACCGCGGCTGCTGG-3′) [20]. 16S rRNA gene sequences (500 bp) were then aligned with sequences of the NCBI sequence databases using the BLASTN algorithm as previously described [1].

Analysis of the 16S-23S rRNA Intergenic Spacer

To distinguish endophytes at the strain level and to compare if some stains detected in reproductive organs were the same that in other plant parts, analysis of the intergenic spacer (IGS) sequence was performed on all ARDRA groups in which some plant reproductive organs isolates were included. Briefly, the IGS region was amplified by PCR using the primers IGSfor (5′-TGCGGCTGGATCACCTCCT-3′) and IGSrev (5′-GGCT GCTTCTAAGCCAAC-3′) [24] using the same PCR conditions as described for the ARDRA analysis. PCR products (10 μL) were digested with 5 U *Hae*III and *Rsa*I (Invitrogen) at 37°C for 2 h and the resulting DNA fragments were analyzed by gel electrophoresis in 2% (*w*/*v*) agarose gels.

Fluorescence In Situ Hybridization

Fixation, Dehydration, Embedding and Slicing

Following sampling, flowers, fruits or seeds (inside berries), from same samples used for cultivation of micro-organisms, were used for microscopic analysis. Samples were however not surface-sterilized, cut in small sections (0.5 cm; excepted for flowers and seeds), and immediately fixed overnight at 4°C in a 4% paraformaldehyde solution in sterile phosphate buffer. Then, they were washed three times in sterile phosphate buffer for 5 min. Following this step, samples were dehydrated in an ethanol gradient series (25% to 99.9%), before to be treated with a Technovit 7100® resin and embedded in capsules at 4°C during 3 days. Plant samples were then sliced with a glass microtome into sections of 2–3 μm. A slicing series of more than ten slices for plant parts samples (except for seeds: three slices) was prepared to remove first parts. Following this step, embedded tissues were continuously sliced and slices were placed on microscope slides previously washed with ethanol 70%. Each time 20 slices were put on slides. Six independent slides were further done for each three independent plant parts and then used for FISH analysis. Due to the difficulty in cutting seeds with glass knives, only ten sections were analyzed on five seeds from three plants.

Probe Labeling and Hybridization

FISH analyses were done on all slices with general or specific probes labeled with specific dyes. Probes (Table 1) targeting most bacteria or specific bacteria as well as a control probe (NONEUB), were used with an primary amine at the 5′ position (Genecust) and labeled with dylight 488 dye (EUB338, EUB338II, EUB338III, NONEUB) or dylight 549 (specific probes; Table 1). Fluorescence hybridizations of slices with labeled probes were then done according to [2] with formamide concentrations depending on the probes (detailed in [22]). Post-hybridization were done according to [2] before that slides were rinsed twice with sterile distilled water. Microscope slides containing general probes and a specific probe were then analyzed under an inverted microscope (Axiovert 200 M, Carl Zeiss, Germany) equipped with a fluorescence lamp and different wavelengths (UV, blue light, and green light). Due to the combination of green-labeled probes targeting all bacteria and red-labeled probes targeting a specific group, all specific groups appeared as yellow-stained cells under blue light (470 nm), whereas bacteria not targeted by the specific probe appeared as green-stained cells. Non-hybridized slices or slices hybridized with NONEUB probe were additionally used as negative controls.

All colored bacteria were photographed with a camera, except fluorescent bacteria having a halo of fluorescence because of their artifact results (described in [10]) and because control treatments (without hybridization or by hybridizing with NONEUB probes) may also yield fluorescent bacteria with a halo of fluorescence.

Table 1 Name, accession numbers and targets of probes used for FISH

Probes names	Accession numbers	Target	References
EUB338	pB-00189	Most bacteria	[2]
EUB338II	pB-00160	*Plantomycetes*	[12]
EUB338III	pB-00161	*Verrucomicrobia*	[12]
NONEUB	pB-00243	Control probe complementary to EUB 338	[29]
LGC	pB-01040	Firmicutes (low G+C Gram-positive bacteria)	[19]
REX72	pB-01069	*Bacillus* spp.	[14]
GAM42a	pB-00174	*Gammaproteobacteria*	[23]
Pae997	pB-00371	*Pseudomonas* spp.'	[3]

Statistical Analysis

Analyses of bacterial isolates were done three independent times with different samples taken from three independent plants. Statistical analyses of CFU were tested for significance using the student *t* test. For FISH analyses, analysis with individual sections and different probes were done once, but with different sections and slides. Each time general probes and a specific probe were used for color combinations.

Nucleotide Sequence Numbers

The 16S rDNA sequences of plant reproductive organs isolates determined in this study were submitted to the GenBank database with the accession Nos. HQ432807–HQ432815. The 16S rDNA sequences of at least one isolate of each ARDRA group of this study can be also found in the GenBank database with the accession Nos. JF683255-JF683314.

Results

Numbers of Cultivable Bacteria Associated with Flowers, Fruits, and Seeds and Comparison to Other Plant Parts

In flowers 2.77±1.08 log10 CFU g^{-1} were detected as endophytic bacteria (Fig. 1a). In comparison, 9.55±0.83 log10 CFU g^{-1} were isolated from rhizosphere soil, 6.89±0.25 log10 CFU g^{-1} from the root interior, and 3.43±0.98 log10 CFU g^{-1} bacteria from inside grape inflorescence stalks at the flowering stage (Fig. 1a).

At berry harvest, 2.87±2.2 log10 CFU g^{-1} endophytes were found in the pulp of berries and 1.44±1.44 log10 CFU g^{-1} in seeds of berries (Fig. 1b). In comparison, 7.73±0.4 log10 CFU g^{-1} were found as rhizobacteria, 5.92±1.43 log10 CFU g^{-1} as root endophytes and as 3.69±0.1 log10 CFU g^{-1} in grape infructescence stalks at berry harvest (Fig. 1b).

Analysis of Endophyte Isolates

At flowering, three ARDRA groups of cultivable bacteria were detected in flowers, whereas 28 were found among rhizosphere isolates, ten among root endophytes, and two within grape inflorescence stalks (Fig. 1c). Some ARDRA groups (e.g., representing *Bacillus pumilus* relatives) were detected not only in flowers but also in the rhizosphere of grapevine at flowering stage of development. IGS analysis showed that some strains detected in flowers were identical to the ones found in the rhizosphere whereas others were not. Other groups, such as those representing bacteria related to some *Pseudomonas* sp., and *Bacillus cereus* group (*B. thuringiensis*; Tables 2 and 3), were specifically isolated from flowers at the flowering stage, but these ARDRA groups were found at the berry stage in the rhizosphere, endorhiza, grape infructescence stalk (Table 2). IGS analysis confirmed that several isolates belonging to these groups found in the different plant parts were identical.

In berries, one ARDRA group was detected in the pulp, three in seeds, whereas at this stage 18 groups were identified among rhizosphere isolates, six among root endophytes, and one among isolates obtained from grape infructescence stalks (Table 2). Identification of ARDRA group members showed that bacteria closely related to the *B. cereus* group (*B. weihenstephanensis*) were specifically detected in pulp. Relatives of *Bacillus altitudinis*, *B. simplex*, *B. thuringiensis*, *Paenibacillus amylolyticus*, as well as *Staphylococcus aureus* were found in seeds (Tables 2 and 3). Some of these groups (e.g., *S. aureus*) were not isolated from other plant parts at the berry harvest stage (Table 2). IGS analyses showed that the strains found in seeds and other plant tissues (relatives of *Bacillus* spp. and *P. amylolyticus*) were identical. Again, some ARDRA groups isolated from berries were not only isolated at the berry harvest stage but also at flowering (Table 2). The different strains within groups isolated from pulp and seeds were also detected in plant parts sampled at the flowering stage as revealed by IGS analysis (*B. cereus* group members, *Bacillus* spp. and *P. amylolyticus*).

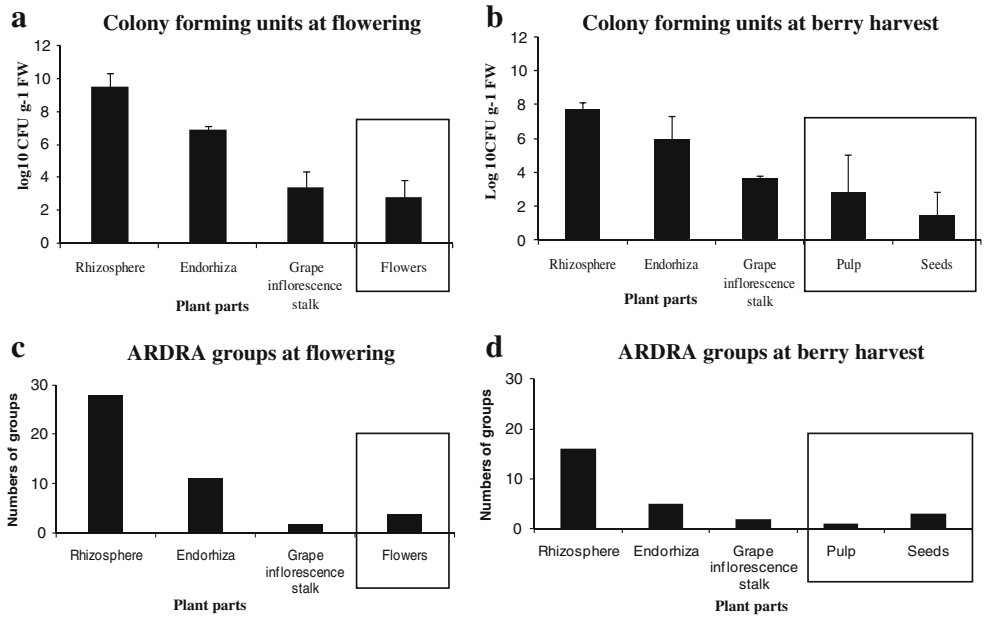

Figure 1 Colony-forming units obtained from various plant parts (**a**–**b**) and numbers of ARDRA groups (**c**–**d**) at the beginning of flowering (**a** and **c**) or at berries harvest (**b** and **d**). Boxes indicate plant reproductive organs results

FISH Analysis

Visualization of Endophytes Inside Flowers

In flowers, some *Gammaproteobacteria* together with other bacteria colonizing epidermal cells as well as xylem vessels were observed (Fig. 2a–c). Gammaproteobacterial members such as *Pseudomonas* spp. were also detected inside flowers, particularly inside epidermis cells in close contact to other bacteria as well as inside the xylem of ovaries (Fig. 2d–e). Furthermore, *Firmicutes* including *Bacillus* spp. were visualized inside flowers. They were detected inside epidermal cells, xylem vessels of ovaries (Fig. 2f–g). In some sections up to 5 *Firmicutes* were visualized, further demonstrating the abilities of these *Firmicutes* to colonize ovules (Fig. 2h). Some *Bacillus* spp. were additionally shown to thrive as endophytes inside xylem elements of ovaries as well as inside epidermal cells at pedicel level (Fig. 2i–j) in close contact to other bacteria (Fig. 2j).

Visualization of Endophytes Inside Pulp and Seeds of Berries

Firmicutes were visualized inside berries, particularly inside intercellular spaces of pulp with some other bacteria (Fig. 3a–b), xylem vessels (Fig. 3c) and inside seeds (Fig. 3d). Moreover, among *Firmicutes*, *Bacillus* spp. were visualized in the intercellular spaces of pulp with other bacteria (Fig. 3e–f). Few *Bacillus* spp. cells were additionally visualized in xylem vessels of pulp. Analysis of seeds showed that *Bacillus* spp. can be present along the cell walls of some cells inside seeds (Fig. 3h) and in some sections some non *Bacillus* spp. were additionally visualized (Fig. 3g). However in some sections analyzed, no bacteria were visualized, suggesting that *Bacillus* spp. may colonize only some parts of seeds.

Discussion

As endophytes of reproductive organs have been rarely addressed, we analyzed bacterial endophytes in flowers, berries and seeds of grapevine plants by cultivation and compared them with bacteria isolated from other plant parts such as the root interior, grape inflorescence/infructescence stalks and the rhizosphere. Endophytes were found in all plant reproductive organs as well as in other tissues, although their cell densities were low as compared to other plant parts and more endophytic cells were present in flowers and in the pulp of berries than in seeds. This is in agreement with previous studies demonstrating that endophytic populations decrease from underground parts to the above-ground parts (reviewed in [16, 29]). In this study, we consistently found bacterial endophytes in plant reproductive organs by cultivation and confirmed their presence by microscopic analysis. In flowers, fruits or seeds we, however, detected a rather low diversity of endophytic bacteria in comparison to other plant parts as revealed by ARDRA groups, IGS and 16S rDNA analysis. This is most probably due to the fact that only a sub-population of endophytes colonizing the below-ground plant tissues, i.e., the root interior, can translocate to above-ground plant parts

Table 2 Comparison of ARDRA groups and bacterial species isolated from flowers and berries to rhizosphere and other endophytic bacteria

ARDRA group	No isolates	Closest related species[a]	Origin flowering/Berry harv.	
A1	59	*Pseudomonas fluorescens*	RHF[28]	RHB[28], EB[3]
		Pseudomonas sp.		
A2	15	*Pseudomonas cannabina*	RHF[1], EF[5]	RHB[6], EB[3]
		Pseudomonas sp.		
A3	6	*Pseudomonas fulgida*	SF[6]	
		Pseudomonas sp.		
A4	6	*Pseudomonas* sp.[b]	F*[3]	SB[3]
B1	5	*Bacillus* sp.	RHF[5]	
B2	39	*Bacillus pumilus*[b]	RHF[13], F*[21]	RHB[5]
B3	35	*Bacillus cereus*[b]	F*[9]	
		Bacillus[b] *weihenstephanensis*		RHB[10], SB[3], PuB*[9]
		Bacillus samanii		RHB[2]
		Bacillus sp.		EB[2]
B4	15	*Bacillus* sp.[b]	RHF[2], EF[1]	RHB[6], EB[2], SeB*[4]
B5	1	*Bacillus* sp.	RHF[1]	
B6	1	*Bacillus shackletonii*	RHF[1]	
C1	2	*Paenibacillus lautus*	RHF[2]	
D1	3	*Pseudomonas* sp.	RHF[3]	
D2	1	*Pseudomonas* sp.	RHF[1]	
E1	4	*Arthrobacter* sp.	RHF[1]	RHB[3]
E2	2	*Arthrobacter* sp.	RHF[2]	
E4	10	*Arthrobacter* sp.	RHF[1]	RHB[9]
F1	9	*Pantoea agglomerans*	EF[9]	
G1	1	*Variovorax paradoxus*	RHF[1]	
H1	3	*Paenibacillus* sp.	RHF[1]	RHB[2]
H2	8	*Paenibacillus amylolyticus*	EF[1]	RHB[1], EB[2], SeB*[4]
I1	2	*Pseudomonas* sp.	RHF[2]	
J1	1	*soil bacterium*	RHF[1]	
K1	1	*Rhodococcus* sp.	RHF[1]	
K2	1	*Rhodococcus* sp.	RHF[1]	
L1	29	*Bacillus megaterium*	RHF[9]	RHB[10], EB[10]
		Bacillus sp.		
L2	1	*Bacillus* sp.	RHF[1]	
M1	1	*Pseudomonas* sp.	EF[1]	
N1	14	*S. phaechromogenes*	RHF[10]	RHB[4]
		Streptomyces sp.		
N2	1	*Streptomyces lavendulae*	RHF[1]	
O1	1	*Agrobacterium rhizogenes*	EF[1]	
P1	3	*Enterobacter* sp.	EF[3]	
Q1	1	*Massilia* sp.	RHF[1]	
R1	7	*Agrobacterium tumefasciens*	RHF[1], EF[6]	
S1	2	*Rhizobium* sp.	EF[2]	
T1	1	*Arthrobacter* sp.	RHF[1]	
U1	1	*Bacillus* sp.	RHF[1]	
V1	31	*Pantoea* sp.	EF[21]	
		Pantoea agglomerans		
V2	1	Soil bacterium	EF[1]	
W1	3	*Xanthomonas* sp.		
X1	3	*A. calcoaceticus*	RHF[3]	

Table 2 (continued)

ARDRA group	No isolates	Closest related species[a]	Origin flowering/Berry harv.	
Y1	1	*Pseudomonas fulgida*	SF[1]	
Z1	5	*Microbacterium* sp.		RHB[5]
Z2	1	*Microbacterium* sp.		RHB[1]
γ1	2	*Flavobacteriaceae*		RHB[2]
γ2	1	*Flavobacterium* sp.		RHB[1]
γ3	1	*Flavobacterium* sp.		RHB[1]
σ1	2	*Exiguobacterium sibiricum*		RHB[2]
α1	2	*Staphylococcus aureus*[b]		SeB*[2]

RHF rhizosphere at flowering, *RHB* rhizosphere at berry harvest, *EF* endorhiza at flowering, *EB* endorhiza at berry harvest, *F** flowers, *SF* (inflorecence) stalk of flowers, *SB* (infructescence) stalk of berries, *PuB** pulp of berries, *SeB** seeds of berries

[a] Identification based on partial sequence analysis of the 16S rRNA gene

[b] Bacterial species isolated from reproductive organs (flowers, pulp and seeds). When similar species were isolated from reproductive organs and other plant parts, IGS was used to distinguish potentially different strains. All species that have been found in reproductive organs and other plant parts possess some identical strains

and to reproductive organs and proliferate in these tissues as discussed in Compant et al. [8].

Bacteria, which could be isolated from flowers, included some *Gammaproteobacteria* (relatives of *Pseudomonas* sp.), and *Firmicutes* (relatives of *B. pumilus*, *B. cereus* group members). Members of these taxa were mostly detected also in other plant parts. To confirm our results by cultivation and to further precisely localize endophytes, fluorescence in situ hybridization was applied on resin-embedded samples. Inside flowers, *Gammaproteobacteria*, in particular *Pseudomonas* spp., and *Firmicutes*, including *Bacillus* spp. were visualized, indicating not only the presence of these bacterial groups but also that they are in an active state to be detectable by FISH. *Gammaproteobacteria* and *Firmicutes* were mainly visualized inside the epidermis layer of flowers as well as inside xylem of ovaries, demonstrating two different niches for colonization within flowers. Xylem vessels colonization may be explained by spreading of endophytes derived from the rhizosphere [8]. However, in the case of epidermal cell layer colonization, endophytes may have derived from the anthosphere and bacteria may have entered via stomata of flowers that can be present on ovaries [15]. This pathway of colonization may potentially also contribute to the transfer of some specific microorganisms from the external environment to reproductive organs and to other internal tissues (discussed in [8]). However, anthosphere-restricted bacteria have not been analyzed in this study by cultivation

Table 3 ARDRA groups, bacterial species and strains isolated from flowers and berries and their 16S rDNA identities

ARDRA group	Strain	Plant organ	Plant stage	Accession number	Closest related species	% Sequence identity	Taxonomic grouping
A4	F29	Flowers	Flowering	HQ432807	*Pseudomonas fulgida* (DQ122321)	99	*Gammaproteobacteria*
B2	F3	Flowers	Flowering	HQ432808	*Bacillus pumilus* (EF010673)	99	*Firmicutes*
B3	F9	Flowers	Flowering	HQ432809	*Bacillus thuringiensis* (AB560665)	99	*Firmicutes*
B3	P2	Pulp	Berries harvest	HQ432810	*Bacillus weihenstephanensis* (GU213138)	99	*Firmicutes*
B4	Se1	Seed	Berries harvest	HQ432811	*Bacillus altitudinis* (HM582688)	99	*Firmicutes*
B4	Se2	Seed	Berries harvest	HQ432812	Bacillus simplex (HM480356)	99	*Firmicutes*
B4	Se10	Seed	Berries harvest	HQ432813	*Bacillus thuringiensis* (AB560665)	99	*Firmicutes*
H2	Se9	Seed	Berries harvest	HQ432814	*Paenibacillus amylolyticus* (FJ487574)	99	*Firmicutes*
α1	Se8	Seed	Berries harvest	HQ432815	*Staphylococcus aureus* subsp. *aureus* (CP002114)	99	*Firmicutes*

The letters represent the different ARDRA patterns. Arabic numerals represent isolate numbers

F flowers, *P* pulp of berries, *Se* seeds of berries

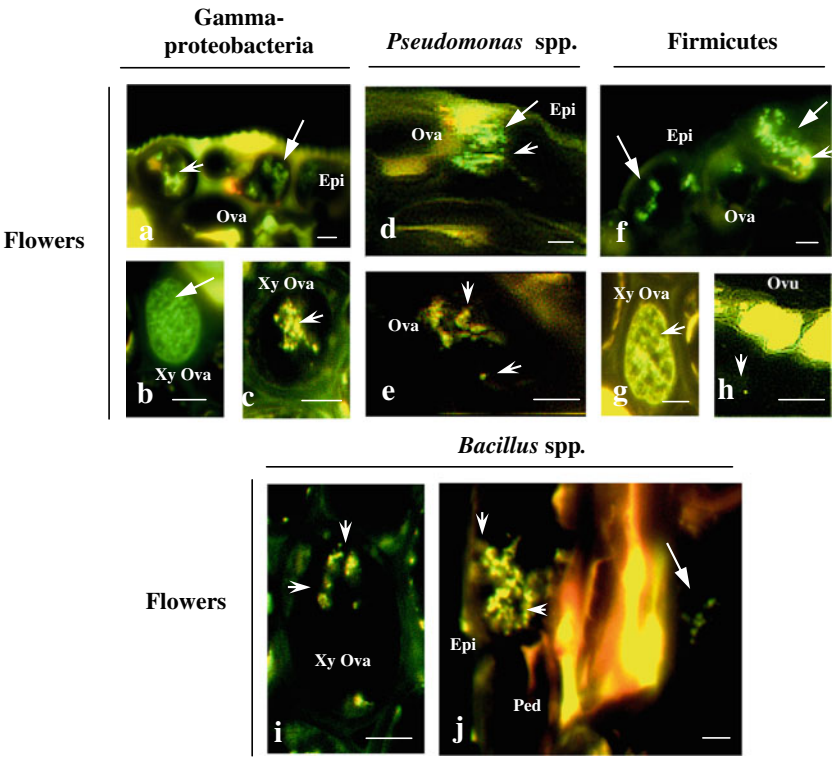

Figure 2 Analysis of endophytic bacteria in flowers by FISH analysis. *Gammaproteobacteria*, *Pseudomonas* spp., *Firmicutes*, as well as *Bacillus* spp. were visualized inside flowers embedded in resin showing their niches of colonization in ovary and xylem of ovaries or ovules. Universal probes EUB338, EUB338II, EUB338III coupled with dylight 487 dye were used for the different plant parts to detect all kind of bacteria as green fluorescent microorganisms. Specific probes for microbial taxa were also used but were coupled with dylight 549 that result in yellow fluorescent detectable bacteria. Each hybridization with specific probes was done independently of the others on different slices, resulting each time of yellow fluorescent color when the specific taxon was detected (*arrow heads*) or strong green fluorescence for other bacteria (*arrows*). *Epi* Epidermis, *Ova* ovary, *Ovu* ovule, *Xy* xylem. *Scale bars* 10 μm

approaches, and thus comparison with those residing inside flowers could not be done. It is also possible that some woundings caused by potent microbes from the surfaces and or dead tissues may have supported the entry of surface-colonizing bacteria, but tissues with injuries were not detected by microscopy analysis on the sections analyzed. Microscopic analysis further showed the presence of *Gammaproteobacteria* and *Firmicutes* members inside several epidermal cells in the same sections. Some of the bacteria were also visualized in plant tissues other than xylem vessels, suggesting an alternative way of colonization, which does not involve xylem vessels for flower endophytes. In flowers, few bacterial cells were visualized additionally inside ovules of flowers, especially *Firmicutes*, suggesting that these bacteria could be later colonizers of seeds.

In berries, i.e., pulp and seeds, only *Firmicutes* (relatives of *Bacillus weihenstephanensis*, *Bacillus* spp., *P. amyloliticus* and *S. aureus*) were found by isolation. As for flower endophytes, some of these bacteria were also found in other plant parts, whereas others were not. Microscopic analysis confirmed the presence of *Firmicutes*, especially *Bacillus* spp. inside pulp, in the intercellular space as well as inside xylem vessels of embedded tissues. However, no bacteria were detected inside the epidermal cell layer of pulp, suggesting that endophytic bacteria did not derive from the carposphere or that these endophytes colonized via stomata at flowering and then continued to spread inside plant reproductive organs. As for flowers, however, carposphere bacteria were not analyzed by cultivation approaches and compared to endophytes from berries to further confirm this hypothesis. Nevertheless, no bacteria were visualized at all inside epidermal cells of non-damaged berries by microscopic analysis and without surface sterilization, suggesting that endophytes isolated from the interior of berries could not derive from the carposphere.

Inside seeds, some *Firmicutes* and *Bacillus* spp. were detected along the cell walls of the seed endosperma. *Firmicutes* and in particular endospore-producing genera such as *Bacillus* were consistently found inside flower ovules as well as in the pulp and inside seeds of berries. Endospore formation might be an important characteristic for seed-colonizers as they can be protected during mature seed formation for further development, although FISH analysis indicated the presence of some active bacterial cells. However for some images concerning some seeds

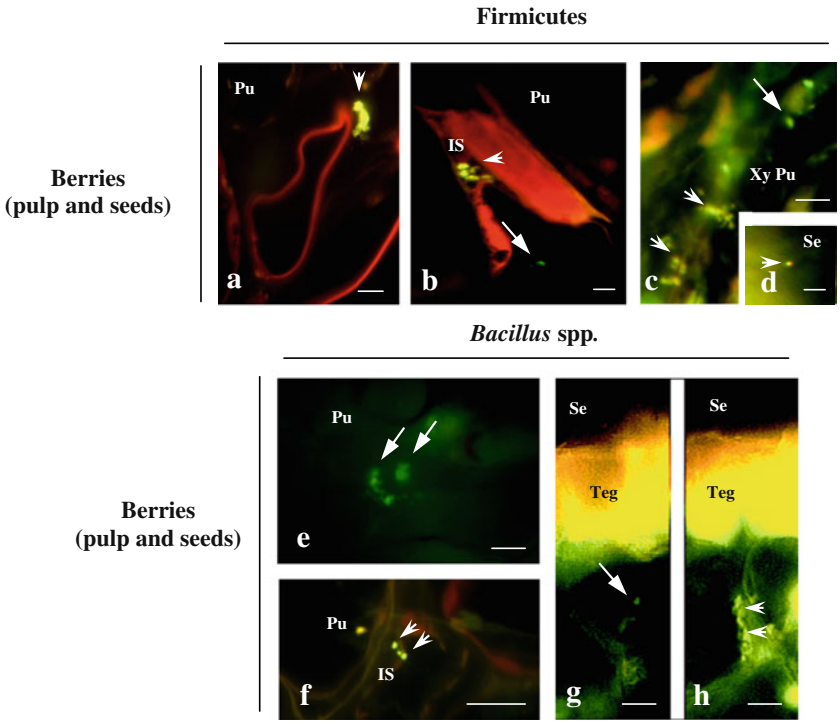

Figure 3 Analysis of endophytic bacteria in pulp and seeds of berries by FISH analysis. *Firmicutes*, as well as *Bacillus* spp. were visualized inside berries (pulp and/or seeds embedded in resin). Universal probes EUB338, EUB338II, EUB338III coupled with dylight 487 dye were used for the different plant parts to detect all kind of bacteria as green fluorescent microorganisms. Specific probes for microbial taxa were also used but were coupled with dylight 549 that result in yellow fluorescent detectable bacteria. Each hybridization with specific probes was done independently of the others on different slices, resulting each time of yellow fluorescent color when a specific taxon was detected (*arrow heads*) or strong green fluorescence for other bacteria (*arrows*). *IS* Intercellular space, *Pu* pulp, *Se* seed, *Teg* tegument, *Xy* xylem. *Scale bars* 10 μm

samples no bacteria were visualized. It is possible that microbes inhabiting these plant tissues are confined to some parts of seeds or that they are inactives. Due to the difficulty to fix and to cut seeds inside the resin with glass microtome, only some parts of seeds could be examined. It may be therefore possible that other microbes can be inhabitants of seeds of grapevine. Metagenomic analysis of bacterial communities residing inside seeds could help to determine them. Moreover, low number of bacteria has been isolated from flowers, fruits, and seeds, and microscopic analysis via FISH allows demonstrating that consistent communities of *Gammaproteobacteria* and *Firmicutes* inhabit flower internal tissues, berries and/or seeds. Again metagenomic analysis such a using T-RFLP or DGGE could help to determine all the viable but non-cultivable bacteria that were visualized inside plant reproductive organs.

In previous studies, the diversity and plant growth-promotion properties of grapevine endophytes, derived from stems or leaves, were assessed [5, 6, 32]. In this study, we showed therefore that also reproductive organs such as flowers, berries and seeds can be colonized by various endophytes under natural conditions. Particularly, *Firmicutes* were prominently found in these tissues. FISH analysis indicated that the major bacterial taxa were isolated and confirmed that cells were living and active. Although we cannot exclude the presence of other non-culturable taxa that can be detected by other cultivation-independent analyses, microscopic analysis revealed the precise location of these endophytes. Furthermore, FISH analysis indicated that bacteria colonizing plant reproductive organs such as flowers, fruits and seeds may not only derive from the rhizosphere but may also derive from other sources such as the anthosphere. Taking into account the previous concept of colonization of endophytes inside plants colonizing the root system and spreading inside plants via xylem vessels, this study therefore shows and confirms an old hypothesis that endophytes inside plants may also derive from other sources and cannot therefore be exclusively soil-derived, especially for plant reproductive organs. Indeed, some bacteria could use xylem elements to spread inside plants, whereas other may have derived from some specific plant surfaces and colonized endophytically plant reproductive organs.

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