Crown galls of grapevine (*Vitis vinifera*) host distinct microbiota

Running title: Crown galls of grapevine host distinct microbiota

Hanna Faist[a], Alexander Keller[b], Ute Hentschel[c,*], Rosalia Deeken[a]#

[a] Molecular Plant Physiology and Biophysics, Julius-von-Sachs-Institute for Biological Sciences, University of Wuerzburg, Wuerzburg, Germany

[b] Animal Ecology and Tropical Biology, University of Wuerzburg, Wuerzburg, Germany

[c] Ecophysiology and Vegetation Ecology, Julius-von-Sachs Institute for Biological Sciences, University of Wuerzburg, Wuerzburg, Germany

[*] GEOMAR Helmholtz Centre for Ocean Research, RD3 Marine Microbiology and Christian-Albrechts University of Kiel, Kiel, Germany

Corresponding author: Rosalia Deeken,

Address: Julius-von-Sachs-Platz 2, 97082 Wuerzburg, Germany

E-mail: deeken@botanik.uni-wuerzburg.de

Word count abstract: 241

Word count importance: 125

Word count text: 5897

ABSTRACT

Crown gall disease of grapevine is caused by virulent *Agrobacterium* strains and establishes a suitable habitat for agrobacteria and potentially, other bacteria. The microbial community associated with grapevine plants has not been investigated with respect to this disease, which frequently results in monetary losses. This study compares the endophytic microbiota of organs from grapevine plants with or without crown gall disease and the surrounding vineyard soil over the growing seasons of one year. Amplicon-based community profiling revealed that the dominating factor causing differences between the grapevine microbiota is the sample site, not the crown gall disease. The soil showed the highest microbial diversity, which decreased with the distance from the soil over the root, graft union of the trunk to the cane. Only the graft union microbiota was significantly affected by crown gall disease. The bacterial community of graft unions without a crown gall hosted transient microbiota with the three most abundant bacterial species changing from season to season. In contrast, graft unions with a crown gall had a higher species richness, which in every season, was dominated by the same three bacterial species (*Pseudomonas sp*., *Enterobacteriaceae sp*., and *Agrobacterium vitis*). For *in vitro* cultivated grapevine plantlets, *A. vitis* infection alone was sufficient to cause crown gall disease. Our data show that microbiota in crown galls are more stable over time than graft union microbiota of healthy trunks, and that the microbial community is not essential for crown gall disease outbreak.

IMPORTANCE

The characterization of bacterial populations in animal and human diseases using high throughput deep sequencing technologies such as 16S amplicon sequencing will sooner or later result in the identification of disease-specific microbiota. We analysed the microbiota of the crown gall

disease of grapevine, which is caused by infection with the bacterial pathogen *Agrobacterium vitis.* All other *Agrobacterium* species were found to be avirulent even though they lived together with *A. vitis* in the same crown gall tumour. As has been reported for human cancer, the crown gall tumour also hosted opportunistic bacteria that are adapted to the tumour microenvironment. Characterization of the microbiota in various diseases using amplicon sequencing may help in early diagnosis, so serve as a preventative measure of disease in the future.

50 **INTRODUCTION**

51 *Agrobacterium vitis* infects domesticated as well as wild grapevines (1) and is the most common
52 cause of crown gall disease in grapevine (2, 3). As well as *A. vitis*, other virulent *Agrobacterium*
53 species are known to induce grapevine crown gall development (4). *A. vitis* is known to persist in
54 debris from infested grapevine material in soil (5) and can enter grapevines via the root and move
55 through the xylem (6) to wounded parts of the plant where it transforms the cells (7-9). The
56 pathogen has been detected in the xylem sap of canes, so propagation material of grapevine
57 nurseries serves as an additional risk for distributing *A. vitis* (10, 11). Virulent *A. vitis* strains
58 harbour a tumour-inducing (Ti)-plasmid, which enables the transfer of T-DNA into the plant
59 genome, a process supported by virulence (Vir) genes of the Ti-plasmid (12, 13). The T-DNA
60 encoded genes express enzymes for opine and plant hormone production (3, 14, 15). Opines are a
61 nutrient source for virulent *A. vitis* and for other bacterial species that express opine-metabolizing
62 enzymes (16-20). The altered auxin and cytokinin levels at the transformation site induce
63 uncontrolled cell division and finally, crown gall development. Crown gall development gives
64 rise to an altered tissue morphology and physiology (21).
65 In nature, both abiotic and biotic factors influence the *A. vitis*-mediated infection process and
66 consequently, crown gall disease outbreak. Crown gall disease on grapevine occurs preferentially
67 in regions with cold winters, indicating that cold temperatures are beneficial for disease outbreak
68 (3). In addition, treatments that cause wounding such as farming devices and the grafting
69 procedure can also promote outbreak of the disease. Biotic factors that influence crown gall
70 disease in grapevine are both pathogenic and non-pathogenic bacteria. Antagonistic bacteria,
71 which are known as biocontrol agents (e.g., the *A. vitis* strain F2/5), prevent transformation of
72 grapevine cells by virulent *A. vitis* strains (22-24). Among the bacteria isolated from grapevine
73 xylem sap, *Pseudomonas sp.* for example showed inhibitory effects on crown gall growth (25).

74 In recent years, more and more research groups have studied the bacterial community and
75 structure of grapevine-associated microbiota, focusing on different aspects of viticulture and the
76 taste of the resulting wine. Techniques such as isolation of cultivable bacteria (26) in combination
77 with analysis of fluorescently-labelled terminal-restriction fragment analysis (27) and taxon or
78 genus specific real-time PCR (28) have been used. The 16S rRNA gene amplicon high
79 throughput sequencing technique provides a detailed overview of the microbiota and has been
80 employed to resolve bacterial communities to the species level (29). This technique has been used
81 to describe the above- and belowground microbiota of grapevines. (30-34). Grapevines from
82 vineyards in Europe (Italy (30, 31), Portugal (32)) and the USA (New York State (33), California
83 (34)) were sampled to analyse differences in the microbiota resulting from pest management
84 (fungicide vs. biocontrol (30), integrated vs. organic (31)), vegetative cycle (32), climate (34),
85 and edaphic factors (33). The leaf and grape microbiota correlate respectively with the vegetative
86 cycle of the plant and the temperature (32, 34). Moreover, the study on grapevine from Long
87 Island (Suffolk County, NY, USA) observed that the vineyard soil serves as a source for
88 grapevine- and grape must-associated microbiota (33). A better understanding of the microbiota-
89 plant interaction would help improve applications that promote plant growth and protection
90 against pathogens (35-37).
91 In the present study, we investigated the microbiota of grapevines with and without crown gall
92 disease because the microbiota of diseased and non-diseased grapevines have, to our knowledge,
93 have not yet been studied. Employing high throughput sequencing of 16S rRNA gene amplicons,
94 we analysed the microbiota of the soil, root and graft union of the trunk, and one-year-old canes
95 of grapevine plants with and without crown gall over the growing seasons of a year. We also
96 established an infection assay using *in vitro* cultivated grapevine plantlets. This assay allowed us

97 to investigate the capability of environmental *Agrobacterium* isolates to induce crown gall growth
98 and to analyse the role of the microbiota associated with crown gall disease.
99
100 **MATERIAL AND METHODS**
101
102 **Sample collection**
103 Grapevine material was collected from four individual plants (Fig. 1A) growing in one row in a
104 stretch of 22 meters located at a vineyard at Himmelstadt, Franconia, Germany (49°55´234.78N,
105 9°49`05.22O). The grapevines of the variety Carbernet Dorsa had been grafted on the rootstock
106 SO4, and planted in 2008 in loamy sand. Four different sampling sites of each grapevine plant
107 were analysed (Fig. 1B): (i) one-year-old cane (c), (ii) graft union of the trunk (g), (iii) root (r),
108 and (iv) soil (s) from the root environment. The samples were collected before noon on 2013-Oct-
109 30 (autumn), 2014-April-04 (spring), and 2014-July-23 (summer). At each time point, the
110 weather was sunny and the soil dry. Three replicates were harvested per sample site and season,
111 resulting in a total of 144 samples (Fig 1C). Roots were washed with tap water and the periderm
112 of all plant material was discarded. Half of the wooden grapevine material and the soil was stored
113 at -80°C for DNA extraction and the other half at 4°C for isolation of bacteria.
114
115 **DNA extraction and amplicon sequencing**
116 The frozen plant and soil samples (-80°C) were shredded in a ball mill MM2000 (Retsch, Haan,
117 Germany) and DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa
118 Ana, CA, USA). DNA extractions with the kit components and no added sample material served
119 as negative controls. For PCR of the 16S rRNA gene, the primers 515F and 806R including 2 x 8
120 bp multiplexing indices and Illumina Adapters attached to their 5' end were used to amplify the

121 variable region V4 of the 16S rRNA gene (38). The sequence of the forward primer was: 5' –
122 AAT GAT ACG GCG ACC ACC GAG ATC TAC ACX XXX XXX XTA TGG TAA TTG TGT
123 GCC AGC MGC CGC GGT AA – 3' and the reverse primer: 5' – CAA GCA GAA GAC GGC
124 ATA CGA GAT XXX XXX XXA GTC AGT CAG CCG GAC TAC HVG GGT WTC TAA T –
125 3'. XXX XXX XX indicate the index sequences.
126 Each sample was processed in three technical replicates to reduce random PCR effects (39). PCR
127 was performed in 10 µl reactions, each containing 5 µl 2 x Phusion® High Fidelity PCR Master
128 Mix (New England Biolabs, Ipswich, MA, USA), 0.33 µl of the 10 µM forward and reverse
129 primers (Eurofins MWG Operon, Huntsville, AL, USA), 3.34 µl PCR grade water, and 1 µl
130 template DNA. PCR conditions comprised an initial denaturation step at 95°C for 4 min, 35
131 cycles of denaturation at 95°C for 40 sec, annealing at 55°C for 30 sec, and elongation at 72°C
132 for 1 min, followed by a final extension step at 72°C for 5 min. We combined the three technical
133 PCR replicates to a 30 µl PCR pool. Successful amplification was verified with agarose gel
134 electrophoresis using 5 µl of the PCR pool. The remaining 25 µl were further processed using the
135 SequalPrep^{TM} Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA) to remove excess
136 primers and nucleotides as well as for normalizing the PCR product to quantities of 25 ng. 5 µl of
137 normalised DNA were used for pooling with the samples of other projects for parallel sequencing
138 (38). This final DNA pool was verified for DNA fragment size of the library with a High
139 Sensitivity DNA Chip (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) and
140 quantified with the dsDNA High Sensitivity Assay (Life Technologies GmbH, Darmstadt,
141 Germany). The final DNA pool was diluted to 2 nM, and 3 µl of each of the custom sequencing
142 and indexed primers were added to the cartridge of a 2 x 250 bp v2 paired-end sequencing MiSeq
143 sequencing kit (Illumina, San Diego, CA, USA). 16S rRNA amplicons were sequenced according

to the manufacturer's protocol for the Illumina MiSeq instrument using a 2 x 250 bp v2 paired-end sequencing run.

Amplicon sequencing data analysis

The quality of the sequences was analysed using FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The program fastq-join v.1.8.0 (https://code.google.com/p/ea-utils/wiki/FastqJoin) was used to join forward and reverse reads. The reads were filtered with USEARCH v8 (40), which included quality filtering according to the phred score (> Q20) and the sequence length (> 250bp). Clusters of operational taxonomic units (OTUs) were built, chimeras removed and taxonomically classified using the UCLUST (40) and UCHIME (41) algorithms, as implemented in USEARCH (42) v.7.0.1090. Using the Ribosomal Database Project, RDP-classifier v.2.2 (43), we assigned the taxonomy for each OTU. A phylogenetic tree was calculated using FastTree (44) v.2.1.3. Plastids and mitochondrial 16S rRNA gene sequences were removed from the OTU table before continuing the analyses. The R script of the following downstream analyses, using the packages phyloseq (45) and vegan (http://CRAN.R-project.org/package=vegan) are provided in the supplemental material. Using the OTU table without any normalization (46), bacterial community dissimilarities between each of the individual samples were estimated using the Bray Curtis distance and the resulting beta diversity was visualized through non-metric multidimensional scaling (NMDS). Four outliers were excluded according to the NMDS. The various influential factors (sample site, season and crown gall disease) were fitted onto the ordination axes, representing the differences in the microbiota, so as to identify significant correlations. A general linear model with the coefficients, soil, root, graft union, cane, and the scores was generated and the relevance of this model for our data was tested using an ANOVA statistical test (NMDS axis one). Fold changes of the sample

types were calculated using the R package EdgeR (46, 47). Significant fold changes had a False Discovery Rate (FDR) <0.001. Only OTUs with a mean abundance ≥20 sequences per sample in at least one group were considered for analysis.

We determined the bacterial species richness as raw counts of the OTUs and calculated the alpha diversity using the Shannon Index (48) based on the OTUs. Significant differences in the alpha diversity and bacterial species richness between sample types were calculated using the Wilcoxon test (49). For taxonomic analysis of the microbiota, all samples from one site and all OTUs of the same taxonomic rank were merged. To calculate the relative abundance of a taxonomic rank, the sequences of a taxonomic rank were divided by all sequences of one sample site. We merged the taxonomic ranks that were less abundant than 0.5% to one group called "other". The relative abundance of each OTU within one sample was calculated by using Random Forest (50), a supervised learning analysis, with 1,500 decision trees. The relative sample counts of the OTUs were used as predictors with season, sample site or crown gall disease as class labels. The percentage of calculated and actual sample class labels resulted in the out-of-bag error (OOB). A small OOB indicates distinctive microbiota according to the class labels. The VennDiagram package (51) of the R software was used to calculate shared OTUs between the sample sites of galled and non-galled grapevines. Within each season, we randomly paired a galled and a non-galled grapevine plant for a paired Wilcoxon test. This allowed us to calculate any significant differences between the amounts of shared OTUs between, for example, canes and graft unions of plants with and without a crown gall. This calculation was repeated for soils and roots.

Isolation and PCR screening of agrobacteria

We isolated bacteria from the graft union material used in this study for amplicon sequencing. The wooden parts of the grapevine material were shredded using a ball mill (Retsch, Hannover,

192 Germany). Purified water (RotisolV HPLC Gradient Grade, Roth) was added to 300 mg of the
193 processed grapevine material or soil. After 2 h at 28°C, the supernatant was used for tenfold
194 serial dilutions and 100 µl were incubated for five days on agar plates supplemented with 213 µM
195 cycloheximide (CHX, Sigma-Aldrich, St. Louis, USA) to prevent growth of fungi. Either YEB-
196 CHX agar plates [0.5% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 0.5% (w/v) Sucrose, 1.23%
197 (w/v) $MgSO_4$ (AppliChem, Darmstadt, Germany), 1.5% (w/v) Agar-Agar Kobe I (Carl-Roth,
198 Karlsruhe, Germany)] or LB-CHX agar plates [1% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 1%
199 (w/v) NaCl, (AppliChem, Darmstadt, Gerany), 1.5% (w/v) Agar-Agar Kobe I, (Carl-Roth,
200 Karlsruhe, Germany)] were used for bacterial growth. Single colonies with an *Agrobacterium*-
201 like morphology were sub-cultured on YEB-CHX or LB-CHX agar plates. PCR-based screening
202 for *Agrobacterium* colonies was performed as follows: (i) Two different fragments of the 16
203 rRNA gene was amplified to identify *Agrobacterium*, (ii) a RecA gene fragment (52) served for
204 differentiation between *Agrobacterium vitis* and other *Agrobacterium* species, and (iii), a
205 fragment of the VirD2 was PCR amplified to screen for the presence of the Ti-plasmid (11). The
206 following primer sequences were used: (i) 16S rRNA gene (27F: 5' AGR GTT YGA TYM TGG
207 CTG AG 3' and 1492R: 5' GGY TAC CTT GTT ACG ACT T 3'; or 515F: 5' GTG YCA GCM
208 GCC GCG GTA A 3' and 806R: 5' GGA CTA CNV GGG TWT CTA AT 3'), (ii) A. spp.
209 specific RecA (F8360: 5' AGC TCG GTT CCA ATG AAA 3'; F8361: 5' GCT TGC GCA GCG
210 CCT GGC T 3'), *A. vitis* specific RecA (G0004F: 5' GAT ATC GCG CTC GGC ATT GGT 3';
211 G0005R: 5' CCT TCG ATT TCA GCT TTC G 3') (52), and (iii) virD2 (virD2F: 5' TTG GAA
212 TAT CTG TCC CGG AAG 3'; virD2R: 5' CTT GTA CCA GCA GGG AAG CTT A 3') (11). A
213 50 µl PCR reaction mixture contained: 1 x HF-buffer (New England BioLabs, Ipswich, USA), an
214 experimentally determined amount of custom-made Phusion Polymerase (53), 0.2 µM of each
215 primer, 400 µM of dNTPs (Fermentas, Waltham, USA), and 2 µl of a bacterial colony

resuspended and boiled in 100 µl HPLC-grade water (RotisolV HPLC Gradient Grade, Roth) for 10 minutes. The 16S rRNA gene sequences of the PCR-products were analysed by Sanger sequencing (GATC, Konstanz, Germany) followed by a nucleotide search using the BLAST algorithm (54) from the NCBI database (55).

Infection assay of *in vitro* cultivated grapevine

Four to eight-week-old *in vitro* cultivated grapevine plantlets (variety Mueller Thurgau and 5BB) provided by the vine nursery Steinmann, Sommerhausen, Germany, were used in a virulence assay (Fig. S1). *In vitro* plantlets originate from one-year-old cane pieces with one node of cuttings from environmental grapevine plants from the year 2000. The cane pieces were surface sterilized (ethanol 96%, sodium hypochloride 6%) and after root and shoot induction, the plantlets were sub-cultivated every 8 to 12 weeks in plastic boxes filled with 3 cm of a grapevine-specific agar growth medium. Plantlets were incubated in a growth chamber with a 14 h photoperiod (light, 23°C; dark, 21°C) and a light intensity of 180 µmol s^{-1} m^{-2} using universal white lamps (L 36W/25, Osram, Munich, Germany). *Agrobacterium* isolates were inoculated to induce crown gall development at the second or third internode of the grapevine stems using a sterile needle dipped into a colony. The known virulent *Agrobacterium vitis* strain S4 (13) and the non-virulent disarmed *Agrobacterium tumefaciens* strain GV3101 (56) served as positive and negative controls respectively. At least eight plantlets were inoculated with the same *Agrobacterium* strain/isolate. We visually screened the plantlets for the appearance of crown galls on a weekly basis for up to eight weeks. Four-week-old infection sites were used for 16S rRNA gene amplicon sequencing as described in the paragraphs “DNA extraction and amplicon sequencing” and “Amplicon sequencing data analysis”.

240 **Data availability**

241 Raw 16S rRNA gene sequencing data are deposited at the European Nucleotide Archive (ENA),
242 http://www.ebi.ac.uk/ena, accession number PRJEB12040.

243

244 **RESULTS**

245

246 **Each sampling site harbours a distinct microbiota**

247 Material of two grapevines with and two without a crown gall (Fig. 1A) from four sampling sites
248 each were collected at three different time points over a year (Fig. 1B), resulting in 144 samples
249 (Fig. 1C). A total of 4,572,415 16S rRNA gene sequences were analysed. After removing the
250 sequences belonging to chloroplasts and mitochondria, 1,201,593 sequences remained. These
251 were grouped into 8,674 different operational taxonomic units (OTUs). The non-metric
252 multidimensional scaling (NMDS) ordination shows that the structural differences in the
253 microbial community composition were determined first and foremost by the sample site (Fig. 2,
254 environmental fit: r^2=84%, p-value≤0,001). The calculation of a general linear model of the
255 values of NMDS1 resulted in a significant influence of each sampling site on the microbiota (soil:
256 t-value=32, p-value<0.001; root: t-value=12, p-value<0.001; graft union: t-value=8, p-
257 value<0.001; cane: t-value=-18, p-value<0.001). The ANOVA test for the general linear model
258 (F= 357, p-value<0.001, residuals degrees of freedom= 136) and Random Forest analysis (Table
259 S1) illustrates that the sample site accounts for the main difference in microbial community
260 composition.

261

262 **Microbial structure of the sampling sites**

263 In terms of richness, the soil microbiota harboured a greater diversity of bacterial taxa than the
264 other sampling sites (Fig. 3A, avg±SD richness=2712±673). The richness in bacterial taxa
265 decreased with the distance from the soil over the root (richness=253±170), to the graft union
266 (richness=166±50), and the cane (richness=76±41). Similarity analyses of the microbiota from
267 the different sites showed that 410 OTUs (5%) were identical in the soil, root, graft union, and the
268 cane. Each sample site shared most of the OTUs with the soil (root: 88%, graft union: 82%, and
269 cane: 79%). The alpha biodiversity (Shannon index) also changed with the distance from the soil
270 (Fig. 3B) in that the microbiota of the soil was most diverse (mean±sd: Shannon Index=6.8±0.2),
271 followed by the root (mean±sd: Shannon Index=4.0±0.7), graft union (mean±sd: Shannon
272 Index=3.2±0.9), and the cane (mean±sd: Shannon Index=3.0±1).
273 A detailed analysis of the bacterial phyla composition revealed that the relative number of
274 *Proteobacteria* sequences increased with the distance from the soil (Fig. 3C). Proteobacterial
275 sequences comprised 22% of all OTUs in the soil, 62% in the root, 89% in the graft union, and
276 89% in one-year-old cane samples. In contrast, *Actinobacteria* decreased along the plant axis
277 from 20% in root, 5% in graft union, with 2% in cane samples. Sequences of the class
278 *Acidobacteria* were only present in the soil (17%) and root (2%) microbiota. In addition,
279 *Bacteroidetes* (soil: 9%, root: 9%), *Planctomycetes* (soil: 6%, root: 1%), and *Verrucomicrobia*
280 (soil: 5%, root: 1%) were more represented in soil and root samples than in the graft union and
281 cane microbiota. At the genus level (Fig. 3D), *Pseudomona*s dominated the aboveground
282 microbiota (graft union: 46%, cane: 72%), while in the soil it was "*Nitrososphaera*" (24%), and
283 in the root *Methylobacterium* (12%). *Agrobacterium*-related sequences were mainly present in
284 roots (2%) and graft unions (17%, Fig. 3D), while in soil (0.3%) and canes (0.7%), the relative
285 sequence numbers were very low. Taken together, the sample site-specific grapevine-associated

286 microbiota changed with the distance from the soil in diversity, richness, shared operational
287 taxonomic units, composition, and structure.
288
289 **Impact of the seasons and crown gall disease on the microbiota**
290 We next analysed the amplicon data of each sample site with respect to the seasons (Fig. 4).
291 Separate NMDS ordinations for each sample site (Fig. 4A) demonstrated the effects of the season
292 on the microbiota of soil (environmental fit: r^2=29%, p-value<0.001), graft unions
293 (environmental fit: r^2=52%, p-value<0.001), and canes (environmental fit: r^2=65%, p-
294 value<0.001). The seasons had no significant influence on the root microbiota (root,
295 environmental fit: r^2=13%, p-value≤ 0.067). Computable classification by Random Forest of the
296 samples taking the seasons into account revealed the highest error rate for the root samples (Table
297 S2, out-of-bag estimated error, OOB: 21%) followed by the soil (OOB: 11%) and finally the
298 aboveground samples, graft union (OOB: 8%) and cane (OOB: 9%). Both, the NMDS
299 ordinations and the Random Forest classifications indicated a greater influence of the seasons on
300 aboveground than on belowground microbiota.
301 With respect to the presence/absence of crown gall disease, the data showed neither a significant
302 effect on the soil microbial community composition (Fig. 4B, environmental fit: r^2=0%, p-
303 value≤0.91), on the root (B: r^2=1%, p-value≤0.76) nor on the cane (D: r^2=4%, p-value≤0.23).
304 However, the microbiota differed significantly between the graft unions with a crown gall and
305 those without (Fig. 4B, environmental fit, r^2=25%, p-value<0.001). Computable classification
306 using Random Forest revealed the smallest out-of-bag estimated error rate in the microbiota of
307 the graft unions (Table S3, OOB, graft unions= 8%) as compared to the soil (OOB: 40%,), root
308 (OOB: 59%), and cane (OOB: 37%).

We then compared the microbiota of the two types of graft unions (without and with a crown gall) from each season (spring, summer, autumn) to each other (Fig. 5). The bacterial richness was higher in the graft unions with a crown gall in spring (p-value≤0.065), summer (p-value≤0.092), and autumn (p-value≤0.065) compared to those without (Fig. 5A). Furthermore, the higher richness in the microbiota of the graft unions with a crown gall did not change significantly over the seasons. In contrast, in graft unions without a crown gall, the richness was significant lower in autumn compared to spring and summer (Wilcoxon test, spring-autumn: p-value≤0.004, summer-autumn: p-value≤0.005). The richness analysis indicates that the microbial community in graft unions with a crown gall contains additional bacterial taxa and is more stable over the seasons than those without. The alpha diversity (Shannon index) did not change prominently in graft unions without a crown gall over the seasons. In contrast, the alpha diversity differed significantly between the seasons in graft unions with a crown gall (Fig. 5B, Wilcoxon-test, p-value: spring-summer≤ 0.002, summer-autumn≤ 0.002) and was highest in summer.

Bacterial taxa that are affected by the crown gall disease

We recovered 23 *Agrobacterium* isolates from the grapevine and soil material used for amplicon sequencing. The screening for agrobacterial virulence resulted in identification of six virulent *Agrobacterium vitis.* The remaining 17 non-*A. vitis* isolates were classified as non-virulent agrobacteria. *A. vitis* isolates were only found in crown galls and roots of the diseased grapevine plants together with non-virulent *Agrobacterium* species. According to the RDP classifier, *A. vitis* is also one of the three most abundant OTUs in graft unions with a crown gall, the others being *Pseudomonas sp.,* OTU_0005 and *Enterobacter,* OTU_0008. In graft unions with a crown gall, these three most abundant OTUs (*A. vitis*, OTU_0003; *Pseudomonas sp.,* OTU_0005; *Enterobacter,* OTU_0008) amounted to 53% and 58% of all sequences in spring and autumn,

333 respectively, although in summer this dropped to 19% of all obtained sequences (Fig. 5C).
334 Nevertheless, these three OTUs still remained the most abundant ones in summer. In contrast, the
335 three most abundant OTUs in graft unions without a crown gall differed in every season (Fig.
336 5C): in spring three *Pseudomonas* species (OTU_0055, OTU_2368, OTU_4255); in summer
337 *Pseudomonas sp*. (OTU_0005), *Sphingomonas sp*. (OTU_0052), and *Curtobacterium sp.*
338 (OTU_0011), and in autumn, *Pseudomonas sp*. (OTU_0055), *Ralstonia sp.* (OTU_0021), and
339 *Erwinia sp.* (OTU_7832).
340 To record the bacterial taxa that are significantly affected by the crown gall disease, we
341 calculated the fold changes of the sequence numbers for the OTUs detected in graft unions with
342 and without a crown gall separately for each season (EdgeR, FDR<0.001, Table S4). Of the 28
343 different OTUs with significant changes in sequence numbers, 24 increased in graft unions with a
344 crown gall compared to those without. Among the latter, nine OTUs comprised zero sequences in
345 graft unions without a crown gall; hence, these were exclusively present in graft unions with a
346 crown gall. Of the four OTUs of which the sequence numbers decreased in graft unions with a
347 crown gall, three (OTU_0005, OTU_0011, OTU_0052) were less abundant in summer. At this
348 time of the year, two other OTUs showed a significant increase: an unknown member of the
349 *Proteobacteria* phylum (OTU_3436) and *A. vitis* (OTU_0003). These two were significantly
350 enriched in all seasons and are part of the core microbiota in graft unions with a crown gall. Four
351 additional OTUs contributed to the core microbiota of crown galls: OTU_0005 (Pseudomonas
352 sp.), OTU_0007 (*Burkholderiales*), OTU_0008 (*Enterobacteriales*), and OTU_0032
353 (*Agrobacterium sp.*). These represented more than 20 sequences per sample in at least 80% of the
354 graft union samples with a crown gall. In graft unions without a crown gall, no OTU met this
355 definition; in graft unions of healthy trunks, the microbiota were more fluctuating.
356

Crown gall induction without core microbiota

We also analysed an amplicon-sequencing data set of *in vitro* cultivated grapevine plantlets to address the question as to whether crown gall development requires a core microbiota and if this in turn profits from the crown gall environment. The *in vitro* cultivated plantlets were inoculated with either the virulent *A. vitis* S7, an isolate from a grapevine crown gall of the same vineyard used for sampling in this study (Fig. S1A and B), or with the disarmed *Agrobacterium tumefaciens* strain GV3101. Uninoculated plantlets served as controls (Fig. S1C). Altogether, amplicon sequencing was performed on 18 samples (Fig. S1D), resulting in a total of 568,855 sequences. After removing the plastid and mitochondrial related 16S rRNA gene sequences, 42,700 sequences remained and were grouped into 612 OTUs. In non-inoculated *in vitro* cultivated grapevines, no OTU was detected with more than 15 amplicon sequences, suggesting an extremely low abundance of bacteria. The stems inoculated with the avirulent *A. tumefaciens* GV3101 contained an enriched number of sequences of this strain (OTU_0507), another *Agrobacterium* (OTU_0032) and *Curtobacterium* (OTU_0011; Table 1, EdgeR, FDR<0.001). In crown galls of the plantlets inoculated with the virulent *A. vitis* S7 strain (OTU_0003), no other OTU was significantly increased (Table 1). This experiment indicates that the virulent *A. vitis* S7 can induce crown gall disease on grapevine without any requirement of a core microbiota.

Bacterial taxa shared between crown galls and the other sample sites

To identify the source of the additional bacterial taxa found in native crown galls, we analysed the OTUs of the graft unions shared with the other sample sites (soil, root, cane) separately for diseased and non-diseased native grapevines. We randomly paired a diseased with a non-diseased plant sample from the same season using a paired Wilcoxon test. The microbiota of the graft unions with a crown gall shared significantly more bacteria with the root (p-value≤0.024) and the

soil (p-value≤0.003) than with the healthy graft unions without a crown gall. In contrast, the latter shared more OTUs with the cane (p-value≤0.009). Thus, the soil and root rather than the cane serve as a source for bacteria in grapevine crown galls.

DISCUSSION

To understand the infection ecology of the crown gall disease, we investigated the endophytic microbial community of grapevines with and without a crown gall. Amplicon-based community profiling revealed a distinct microbial community for each of the sample sites (soil, root, graft union of the trunk, cane). Distinct microbiota have previously been published for grapevines from vineyards in Long Island (Suffolk county, NY, USA) for soil, root, leaf, flower, and grape berry (33) and from Lussac St. Emillion (Gironde, France) for soil, bark, leaf, and grape berry samples (27). *Proteobacteria*, *Acidobacteria*, *Actinobacteria,* and *Bacteroidetes* dominated the microbiota in our soil samples and in those from Long Island, while in the samples from Lussac St. Emillion, no *Bacteroidetes* colonies were isolated. Furthermore, our root samples and those from Long Island (33) as well as our cane samples and those from Trentino, Italy (26), were similar on the phylum level. The microbiota of our sample sites, those from Long Island (33), and Lussac St. Emillion (27) all showed a gradient from belowground to aboveground. In all cases, the structure of the soil microbiota was most complex (highest richness and alpha diversity) with that of the aboveground least complex (lowest richness and alpha diversity). This gradient in the microbial structure and composition is most likely the result of changing environmental factors such as humidity, distance from the soil, organic substrates, and UV exposure (57-60). The factor season had an additional impact on the microbial structure, which was stronger on our aboveground than belowground samples. Not only do the seasons have an impact on the microbial composition of the leaf microbiota of grapevine (28, 32), but the time of year also influences endophytic bacteria

405 in woody material, as shown in this study. Thus, we conclude that grapevines have similar phyla
406 compositions in distinct locations and that the crown gall disease does not substantially affect the
407 microbial structure of the soil, root, and cane.
408 Crown gall disease affected the microbiota only in graft unions. The microbiota of graft unions
409 with a crown gall contained a higher number of different bacterial species in all seasons
410 compared to graft unions without a crown gall. *Agrobacterium vitis* together with eight other
411 bacterial species caused the difference in the microbial community between graft unions with and
412 without a crown gall. These were exclusively found in graft unions with a crown gall. Likewise,
413 *Arabidopsis* leaves infected with the fungus *Albugo* significantly enriched a subset of bacterial
414 endophytes (61). This suggests that both pathogens (*A. vitis* and *Albugo*) promote colonization
415 with certain endophytic microbes at the infection site. Compared to healthy graft unions, crown
416 galls share more bacteria with the belowground microbiota and fewer with canes. Therefore, the
417 source for the invasive bacteria in crown galls seems to be the soil and root. This finding supports
418 the idea of the soil as a microbial seed bank for grapevine-associated microbiota, as previously
419 postulated (33).
420 In graft unions with a crown gall, three OTUs, *A. vitis* (OTU_0003), *Pseudomonas sp.,*
421 (OTU_0005), and *Enterobacteriaceae sp.* (OTU_0008) were most abundant in every season.
422 These three, together with three additional OTUs, were present in 80% of graft union samples
423 with a crown gall, indicating that the crown gall microbiota is relatively stable. In summer the
424 percentage of the three most abundant bacterial species, which included *A. vitis*, decreased in
425 crown galls, thereby increasing the species evenness in the bacterial communities at this time of
426 year. Other studies have reported that in summer, the colony forming units of *A. vitis* are reduced
427 in grapevines (62) and that the isolation of *A. vitis* from grapevine samples is more difficult (7). A
428 reason for the reduction in species abundance in summer could be higher temperatures and

429 drought stress. It is known from the model plant *Arabidopsis* (63, 64) and *Ricinus communis* (65)
430 that crown gall growth is affected by drought stress. In graft unions without a crown gall, the
431 diversity of the bacterial species was only marginally affected by the season. The three most
432 abundant OTUs encompassed 57% of all sequences in summer, which was only marginally
433 higher than the three in spring (42%) and autumn (36%). However, the three most abundant
434 bacterial species varied between the seasons, indicating that, unlike in graft unions with a crown
435 gall, no core microbiota exists in graft unions without a crown gall. Thus, crown gall disease
436 seems to stabilize the bacterial composition over the seasons, as previously reported for
437 phytoplasma infected grapevine leaves (28). Nonetheless, the striking decrease in abundance of
438 bacterial species in graft unions with a crown gall in summer seems to be specific for crown gall
439 tissue.
440 The bacterial species exclusively found or enriched in graft unions with a crown gall may profit
441 from the crown gall environment. Indeed, it is well known from the literature that this habitat
442 provides opines and other accumulating metabolites, as well as additional living space (14, 66). It
443 has been shown that opines serve as common nutrients and cause an increase in the local
444 population of opine metabolising bacteria (19). This has also been demonstrated by transgenic
445 opine-producing legume species, which harboured an altered bacterial composition, including an
446 increase in opine degrading *Pseudomonas* (67). We also found a *Pseudomonas* strain
447 (OTU_0005) clearly enriched in spring and autumn in crown galls. *Pseudomonas* is able to cause
448 wounds by producing ice crystals (68, 69). Wounds induce *Agrobacterium*-mediated processes
449 such as plant cell transformation, production of opines, and phytohormones (70). For example,
450 indole-3-acetic acid (IAA) is involved in plant and crown gall developmental processes, enriched
451 in crown galls, and can serve as a source of carbon for *Pseudomonas putida* 1290 (71).
452 Furthermore, *Pseudomonas sp.* (72, 73), *Enterobacteriaceae sp*. and many other endophytic

453 grapevine bacteria (26) are able to produce IAA. Interactions of non-pathogenic with pathogenic
454 bacteria are known for tumours of olive trees, induced by *Pseudomonas savastanoi* (*pv.*
455 *Savastanoi*), which host a non-pathogenic *Erwinia* species (74). An *Erwinia* species (OTU_7832)
456 was enriched in graft unions with a crown gall and it seems to profit from the crown gall
457 environment.
458 We used *in vitro* cultivated grapevine plantlets to investigate the mechanisms of the infection
459 process and development of crown gall disease. This infection assay demonstrated that *A. vitis*
460 and no other *Agrobacterium* species of the environmental isolates, including *A. tumefaciens*,
461 caused crown gall disease. This indicates that in grapevines, *A. vitis* retains its virulence
462 machinery. This finding is in accordance with a high throughput isolation study of agrobacteria
463 from crown galls of herbaceous and woody hosts (75). In this study, only seven out of 5419
464 isolates became non-virulent-mutants after being inoculated into host plants to induce crown
465 galls. Furthermore, induction of crown gall growth on *in vitro* cultivated grapevine plantlets
466 proved that *A. vitis* does not require a microbial community for disease outbreak. This
467 observation suggests that the crown gall-specific bacterial species and those that strongly
468 multiply in crown galls appear to benefit from the crown gall environment provided for them by
469 *A. vitis* infection.
470
471 **CONCLUSION**
472 Grapevine organs and the vineyard soil harbour a distinct microbial community, which is not
473 affected by crown gall disease, except at the site of graft union and gall formation. Graft unions
474 with a crown gall stabilise core microbiota and host opportunistic bacteria over the seasons.
475 These however, are not essential for the induction of crown gall growth. Our *in vitro* assay
476 showed that induction of crown gall growth requires no other bacterium than *Agrobacterium*

vitis. This finding suggests that none of the invasive endophytic bacteria including *A. tumefaciens* is obligate for crown gall development. Nonetheless, a supportive role in the performance of crown gall development cannot be excluded and will be addressed in future studies. The invasive bacterial species most likely profit from the crown gall environment in that they have an advantage, nutritional or otherwise, by living within crown gall tissues. Unravelling the role of the opportunistic bacteria in crown gall performance may help to support disease management in the future.

FUNDING INFORMATION

This work was funded by the DFG Graduiertenkolleg GK1342 "Progress in Lipid Signalling" (TPs A8, U. Hentschel and A5, R. Deeken) and by the development grant of the Chamber of Industry and Commerce 2012, Schweinfurt-Wuerzburg, Germany to U. Hentschel and R. Deeken. The funders had no role in study design, data collection and interpretation, or decision to submit the work for publication.

ACKNOWLEDGEMENTS

Special thanks go to Peter Schwappach (Bavarian Regional Office for Viticulture and Horticulture, Veitshoechheim, Germany) for providing grapevine plants from the vineyard. We are very grateful to Gabriele Brendel (Vine Nursery Steinmann, Sommerhausen, Germany) for providing *in vitro* cultivated grapevine plantlets, as well as for ongoing discussions and practical advice. Many thanks go also to Wiebke Sickel, Gudrun Grimmer, and Lisa Walther (University of Wuerzburg, Germany) for support in the laboratory and Hannes Horn (University of Wuerzburg, Germany) for support in bioinformatics. We also acknowledge Anne Müller and Lorenz Hoffmann who performed their Master thesis (2012) and Diploma thesis (2013) at the University of Würzburg, respectively, on this topic. Finally, we thank Rainer Hedrich (University of Wuerzburg, Germany) for his support during this study and Tracey A. Cuin (University of Wuerzburg, Germany) for critical reading the manuscript.

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715

716 **FIGURE LEGENDS**

717 **FIG 1** Grapevine plants and sampling procedure used for comparison of grapevine microbiota.
718 (A) Graft unions of the trunk of the two grapevine plants with (1, 2) and two without (3, 4) a
719 crown gall. (B) Illustration of the sampling sides; c, one-year-old cane, g, graft union, r, root, and
720 s, soil. (C) Scheme of the experimental setup.

721 **FIG 2** Distribution of the 144 grapevine associated microbiota within a non-metric
722 multidimensional scaling (NMDS) ordination. Analysis is based on the Bray Curtis distance. The
723 factor sample site explains 84% (R^2) of the variation among the microbiota. Significance (p-
724 value) was calculated using a permutation test. Colours indicate sample sites.

725 **FIG 3** Comparison of the microbial communities from the soil, the grapevine roots, graft unions,
726 and canes. (A) Number of bacterial taxa (richness) and (B) Shannon Index (α-diversity) for each
727 of the sample sites. P-values are calculated according to Wilcoxon rank sum test. (C) Percentages
728 of phyla and (D) genera in the microbial community for each sample site. Phyla or genera with a
729 relative abundance lower than 0.5% in the microbial community are combined into the group
730 "other".

731 **FIG 4** Factors determining the differences between the microbial communities of the sample
732 sites. Non-metric multidimensional scaling (NMDS) ordinations for (A) the factors seasons and
733 (B) graft unions without or with a crown gall. The percentage of variation among the microbiota
734 of a sample site was correlated with the factor seasons or crown gall disease (R^2). Significance
735 was calculated using permutation test (p-value).

736 **FIG 5** Comparison of the microbial communities of the graft unions without and with crown gall
737 disease. (A) Number of bacterial taxa (richness) and (B) including abundance within each
738 bacterial taxa (α-diversity), calculated according to Shannon for each of the sample sites. P-
739 values are calculated according to the Wilcoxon rank sum test. (C) Percentage of the three most

abundant operational taxonomic units (OTU) in the microbial community for each sample site. All OTUs with a relative abundance lower than 0.5% were merged, forming the group "other".

TABLES

TABLE 1 Operational taxonomic units (OTUs) with significant differences in 16S rRNA gene sequence numbers from stems without and with a crown gall of *in vitro* cultivated grapevine plantlets. Stems were inoculated with the virulent *Agrobacterium vitis* isolate S7 (OTU 0003) and the disarmed *Agrobacterium tumefaciens* strain GV3101 (OTU 0507) four weeks before analysis. Displayed are mean sequence numbers in the samples with and without a crown gall calculated according to the EdgeR package in R. Log2 fold changes (logFC) and log2 counts per million (logCPM). P-values are adjusted to multiple testing according to Benjamin-Hochberg (FDR, false discovery rate <0.001).

	16S sequence numbers				**adjusted p-value**
bacterial identity	**without**	**with**	**logFC**	**logCPM**	**FDR**
Agrobacterium tumefaciens GV3101 (OTU 0507)	3591	78	5.5	16.5	6.29E-63
Agrobacterium (OTU 0032)	124	1	5.8	11.8	1.03E-64
Agrobacterium vitis isolate S7 (OTU 0003)	6	2758	-8.6	16.1	8.09E-33
Curtobacterium (OTU 0011)	30	1	4.1	10.1	3.45E-07

SUPPLEMENT LEGENDS

FIG S1 Infection procedure of *in vitro* cultivated grapevine plantlets. (A) Inoculation of agrobacteria into grapevine stems using a needle. (B) Grapevine stems with a crown gall and (C) mock-inoculated stems without a crown gall. Scale bar distance in B and C is 1 mm. (D) Experimental setup and number of samples used for amplicon sequencing.

760 **TABLE S1** Classification of the grapevine-associated microbiota attained by performing a
761 supervised learning analysis according to the factor sampling site (Random Forest). The predicted
762 sample site classifications (soil, root, graft union, cane; horizontal) were compared to the known
763 classifications of the sample sites (vertical). The percentage of wrongly classified samples within
764 one sample site is called class error while the percentage of wrongly classified samples of all
765 classified samples is termed the out-of-bag error (OOB: 2%).
766 **TABLE S2** Grapevine-associated microbiota were used to perform a supervised learning analysis
767 (Random Forest) according to the factor season. Predicted classifications of the samples into
768 spring, summer and autumn (horizontal) were compared to the known classifications in spring
769 summer and autumn (vertical). The percentage of wrongly classified samples within one season is
770 called class error while the percentage of wrongly classified samples within one sample site (soil,
771 root, graft union, cane) is called out-of-bag error (OOB).
772 **TABLE S3** Classification of the grapevine-associated microbiota according to the factor crown
773 gall from performing a supervised learning analysis (Random Forest). The predicted
774 classification of the sample sites from grapevines with and without a crown gall (horizontal) are
775 compared to the known sample site classifications of grapevines with and without a crown gall
776 (vertical). The percentage of wrongly classified samples of grapevines with or without a crown
777 gall is called class error while the percentage of wrongly classified samples within one sampling
778 site (soil, root, graft union, cane) is termed an out-of-bag error (OOB).
779 **TABLE S4** Operational taxonomic units (OTUs) with significant differences (FDR < 0.001) in
780 the mean number of 16S rRNA gene amplicon sequences. Differences between graft unions with
781 'A' and without 'B' crown gall disease in spring, summer, and autumn. Fold changes are given as
782 log2FC and log2CPM (average log2 counts per million). Statistics analysis was performed using

783 a two-sided test for calculation of the p-values. The false discovery rate (FDR) gives an adjusted

784 p-value for multiple testing according to Benjamin-Hochberg.

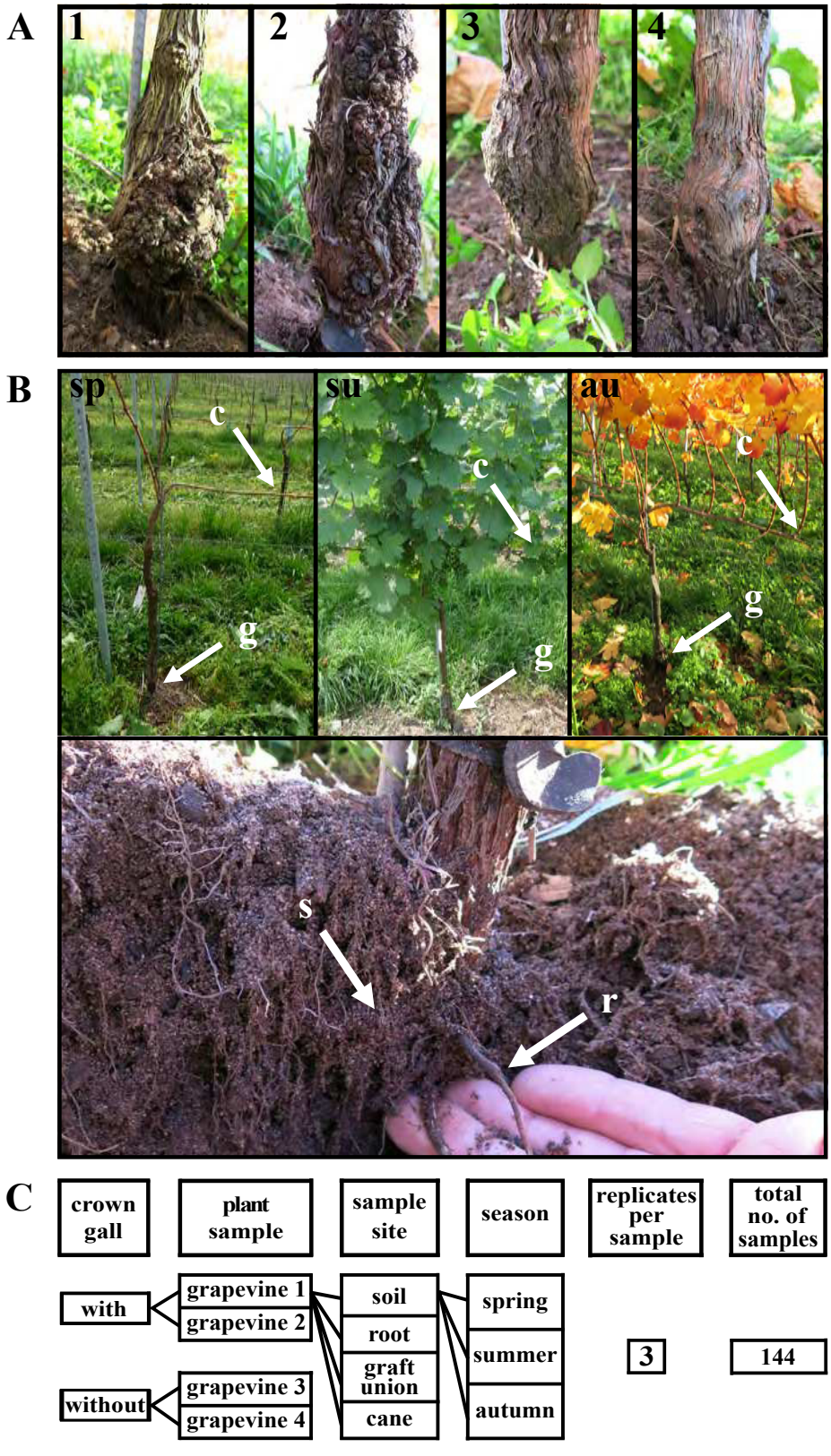

FIG 1 Grapevine plants and sampling procedure used for comparison of grapevine microbiota. (A) Graft unions of the trunk of the two grapevine plants with (1, 2) and two without (3, 4) a crown gall. (B) Illustration of the sampling sides; c, one-year-old cane, g, graft union, r, root, and s, soil. (C) Scheme of the experimental setup.

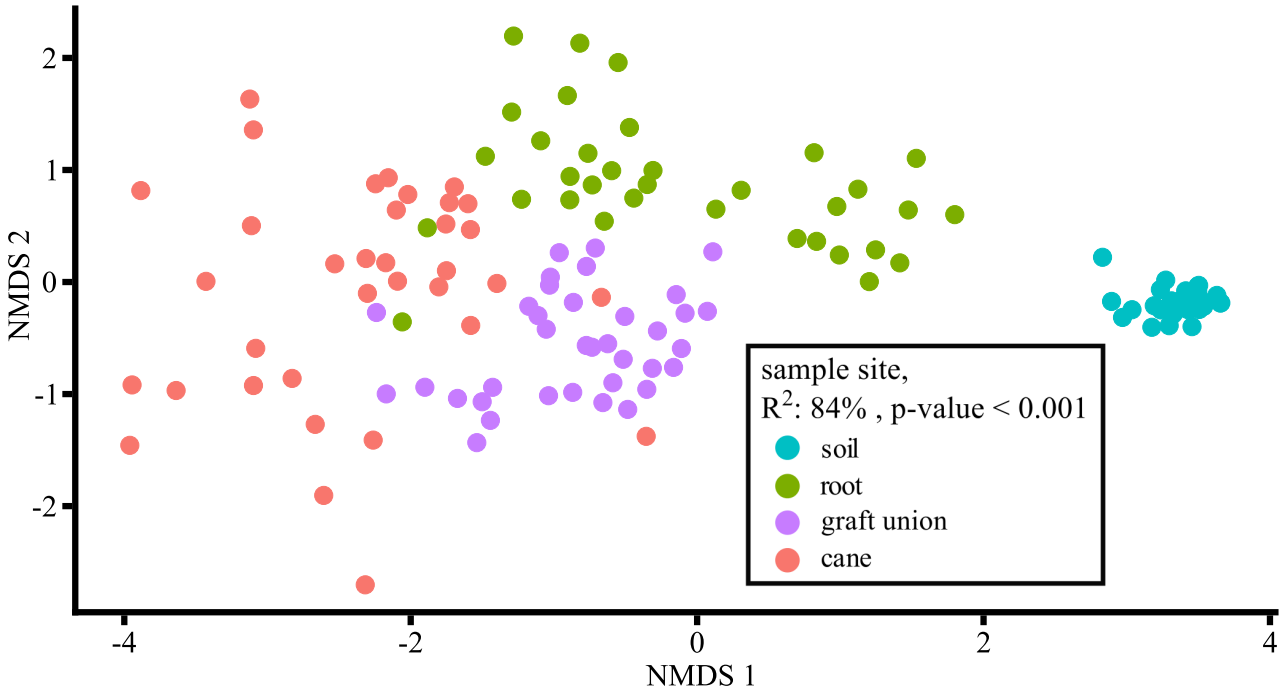

FIG 2 Distribution of the 144 grapevine associated microbiota within a non-metric multidimensional scaling (NMDS) ordination. Analysis is based on the Bray Curtis distance. The factor sample site explains 84% (R2) of the variation among the microbiota. Significance (p-value) was calculated using a permutation test. Colours indicate sample sites.

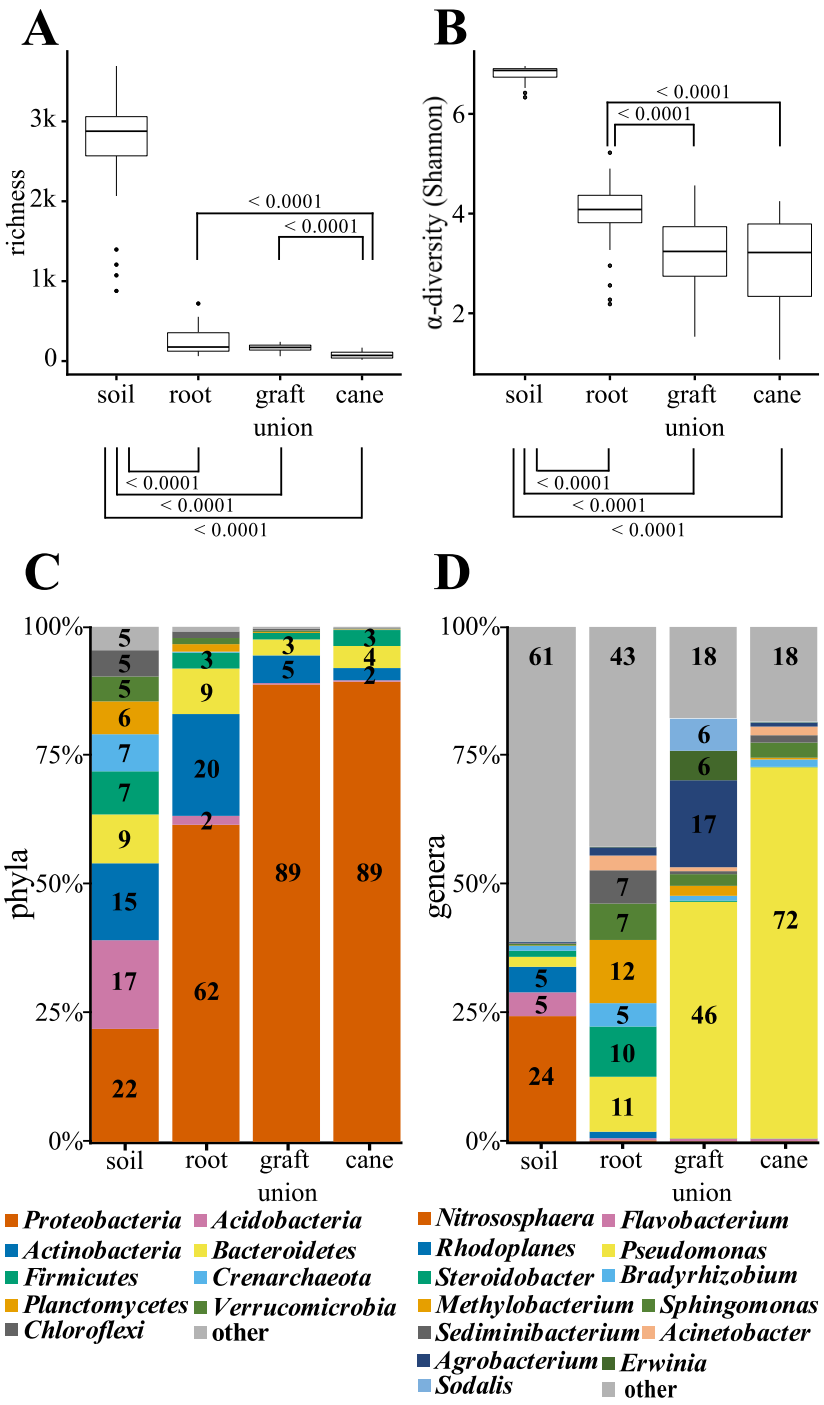

FIG 3 Comparison of the microbial communities from the soil, the grapevine roots, graft unions, and canes. (A) Number of bacterial taxa (richness) and (B) Shannon Index (α-diversity) for each of the sample sites. P-values are calculated according to Wilcoxon rank sum test. (C) Percentages of phyla and (D) genera in the microbial community for each sample site. Phyla or genera with a relative abundance lower than 0.5% in the microbial community are combined into the group "other".

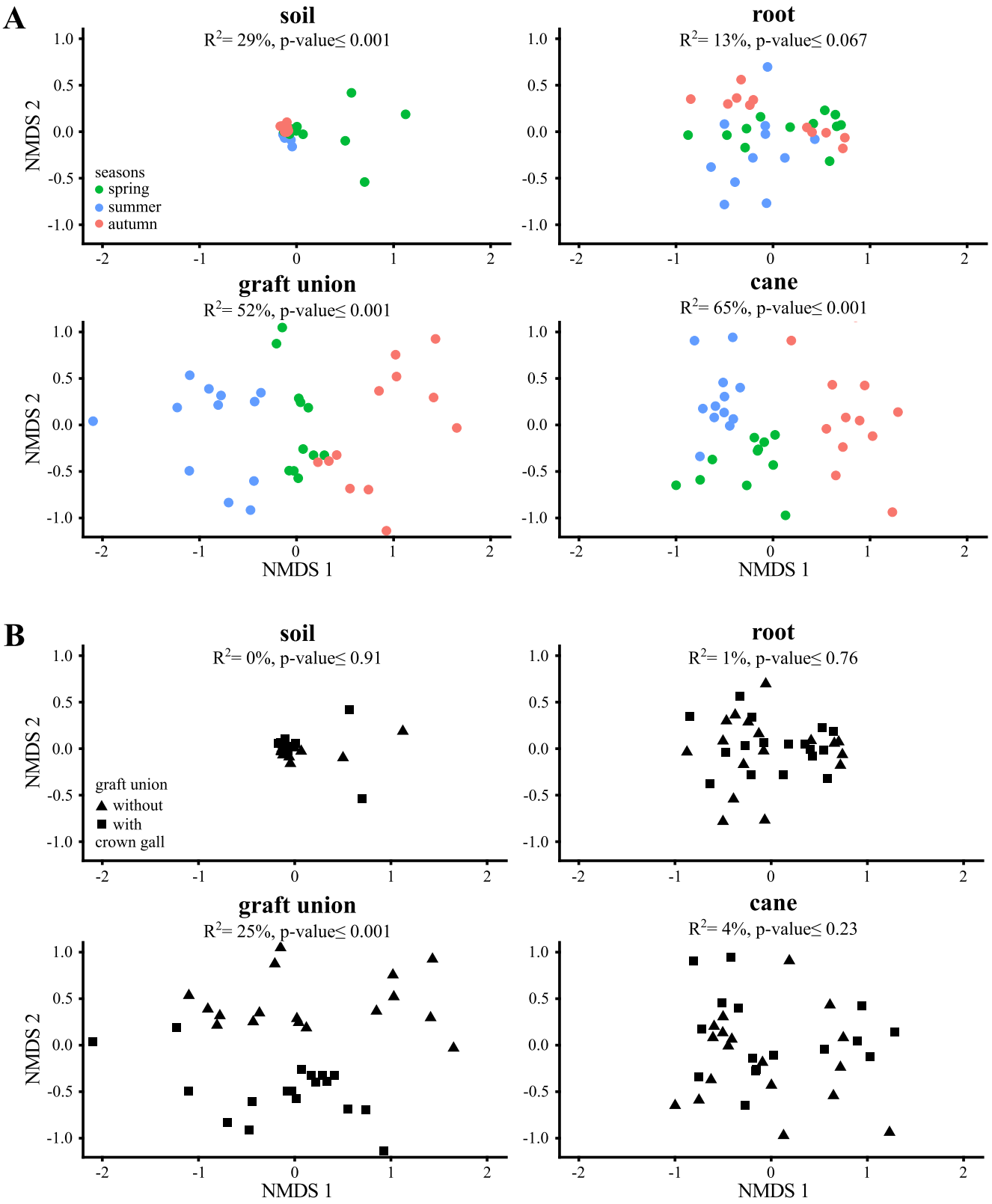

FIG 4 Factors determining the differences between the microbial communities of the sample sites. Non-metric multidimensional scaling (NMDS) ordinations for (A) the factors seasons and (B) graft unions without or with a crown gall. The percentage of variation among the microbiota of a sample site was correlated with the factor seasons or crown gall disease (R2). Significance was calculated using permutation test (p-value).

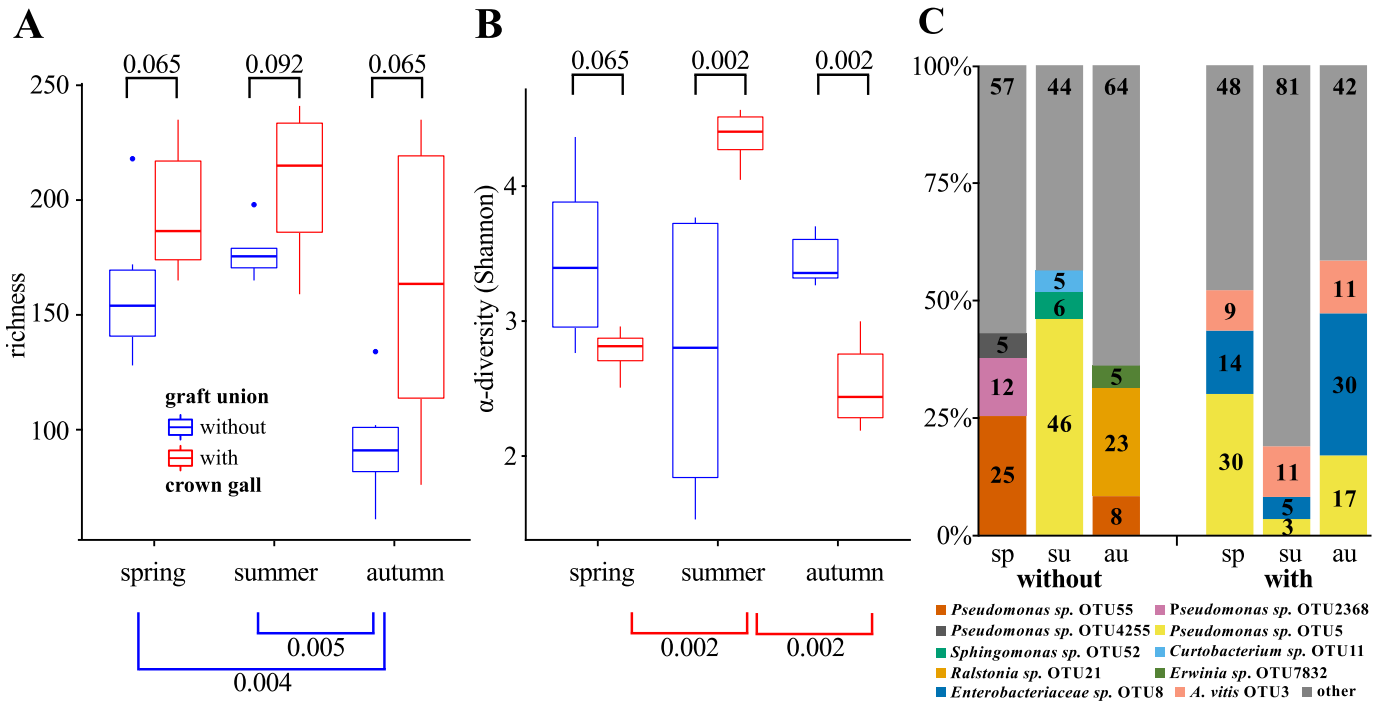

FIG 5 Comparison of the microbial communities of the graft unions without and with crown gall disease. (A) Number of bacterial taxa (richness) and (B) including abundance within each bacterial taxa (α-diversity), calculated according to Shannon for each of the sample sites. P-values are calculated according to the Wilcoxon rank sum test. (C) Percentage of the three most abundant operational taxonomic units (OTU) in the microbial community for each sample site. All OTUs with a relative abundance lower than 0.5% were merged, forming the group "other".

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