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Black fungi and associated bacterial communities in the phyllosphere of grapevine

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ABSTRACT

In this study we investigate bacterial communities in association with an enriched black-fungal community in the plant phyllosphere to test whether these fungi create an environment for specific bacteria. Under organic conditions of agriculture, grapevine plants (*Vitis vinifera*) display an increased occurrence of the black fungi *Aureobasidium pullulans* and *Epicoccum nigrum*. Their enrichment agrees with the tolerance of these fungi to copper and sulphate, both used as main fungicides in organic viticulture. Both fungi also intrude the plant material to grow endophytically. Bacterial communities associated with black fungi of the plant surface and endosphere showed no differences compared to those found in conventionally managed *V. vinifera* plants. This suggests that despite an increase of these black fungi in organic practice, they do not shape bacterial diversity in grapevine plants. Nevertheless, dual cultures revealed a negative effect of *Aureobasidium* on the growth of certain bacilli, whereas growth of *Aureobasidium* was impeded by one *Pseudomonas* strain. Such singular effects are either not apparent in the natural black-fungal–bacterial community of the grape phyllosphere or are of rather localized effect.

Introduction

The cohabitation of fungi and bacteria is a complex story of repulse and affection. Both microbial groups compete for resources in similar microhabitats or cooperate for acquisition of nutrients. The outcome of fungal–bacterial relationships can be economically important, e.g., in mycorrhizal plants. Research on bacterial–fungal associations has focused on below-ground systems. Their positive effect on mycorrhiza formation is well established (Garbaye 1994), and it has been widely accepted that plants benefit from the bacterial involvement in the mycorrhizal functions. The 'helper effect' is not restricted to typical ectomycorrhiza, but is known also from arbuscular mycorrhiza (AM) (Bonfante & Anca 2009), and other types of mycorrhiza (Meena *et al.* 2010).

Negative effects of bacteria on fungi include inhibition of fungal ligno-cellulose degradation (de Boer *et al.* 2005) and inhibition of root infection by pathogenic fungi (Whipps 2001; Berg 2009). Niche differentiation between soil bacteria and fungi occurs during decomposition of plant-derived organic matter, as both microbial groups compete for simple plant-derived substrates and have developed antagonistic strategies. In some cases, there is evidence for specific mechanisms of selection acting in fungus-associated bacterial communities (de Boer *et al.* 2005). Moreover, the role of bacteria in the degradation of fungi, or mycophagy, has also been emphasized recently (Leveau & Preston 2008).

These and other works indicate that the presence of fungi can potentially alter the composition of bacterial communities and *vice versa*. Effects of the ectomycorrhizosphere on

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the *Pseudomonas fluorescens* populations select strains potentially beneficial to the symbiosis and to the plant (Frey-Klett *et al.* 2005). Such strains might be ubiquitous and widespread or, contrarily, highly specific for species or soil conditions (pH, humidity). Also the mycosphere, the zone below mushroom fruitbodies, hosts universal, and species-specific bacterial fungophiles (Warmink *et al.* 2008). However, the functionalities and variation of fungal–bacterial cohabitation are far from being fully described except in few model interaction systems (Tarkka *et al.* 2009).

Vitis vinifera L. is among the plants which have been cultivated since ancient times by humans, and which have a high economic importance still today, comprising an area of 7.44 million hectares grapevine culture worldwide (http://faostat.fao.org/). Concerns raised about the use of pesticides resulted in a strongly increasing proportion of organically managed viticulture (Willer 2008). However, the application of copper-containing products that forms a considerable aspect of organic management causes soil contamination and other environmental problems in a long run (Pietrzak & McPhail 2004). There is also little knowledge about the effects of copper treatment and other organic plant protection methods on plant-associated microorganisms. A recent study of the cultivar 'Sauvignon Blanc' from vineyards in Austria compared fungal communities of shoots, leaves, and grapes in conventionally and organically managed grapevine during 2 consecutive years (Schmid *et al.* 2011).

The copper-tolerant fungi *Aureobasidium pullulans* and *Epicoccum nigrum* were enriched in the communities of organically managed plants, suggesting a higher indigenous antiphytopathogenic potential. On the contrary, *Sporidiobolus pararoseus*, a basidiomycetous yeast occurred with higher abundance in conventionally managed cultures. In this paper, we studied the bacterial associates of ubiquitous black fungi, *A. pullulans* and *E. nigrum*, which dominate consortia found on grape (*V. vinifera*), compared the bacterial communities with those retrieved from conventionally managed grapevine, and conducted *in vitro* assays of fungal–bacterial interactions. In this work we are interested whether this increase also affects the bacterial communities of the grapevine phyllosphere, as an example for an aboveground fungal–bacterial community.

Methods

Experimental design and sampling

Leaves, shoots, and undamaged grapes of the *Vitis vinifera* subsp. *vinifera* cultivar 'Sauvignon Blanc' were sampled 2006 and 2007 from vineyards in Schlossberg, Austria (46°37′N, 15°28′E; owner: Fachschule für Weinbau und Kellerwirtschaft und Weingut Silberberg) in the last week before harvest (on 3rd of Oct. 2006 and 11th of Sep. 2007 respectively). Half of the vineyards were managed conventionally, the other half organically. In the conventional parcel 22 kg ha^{-1} sulphur and the preparations Dithane Neo Tec® (active component manganese-zinc ethylene bis(dithiocarbamate)), Talendo® (proquinazid), Melody® combi (iprovalicarb and folpet), Scala® (pyrimethanil), Pergado® (folpet and mandipropamid), Legend® (quinoxyfen), Reldan® (chlorpyrifos-methyl), Cantus® (boscalid), and Mildicut® (cyazofamid) were used for plant protection. In the organic parcel 32 kg ha^{-1} sulphur, 8 L Cuprozin (460.6 g L^{-1} copper hydroxide) as well as the products Myco-Sin® (Dr. Schaette, Germany) and Frutogard® (Spiess-Urania Chemicals, Germany) were used in each growing season. Details of the treatments are given in Schmid *et al.* (2011). Wild populations of *Vitis* are located in Austria: tissues from *V. vinifera* subsp. *sylvestris* were sampled 2007 in Marchegg, Austria (48° 17′ N, 16° 54′ O) and 2008 in the Botanical Garden Graz, Austria. For each tissue type four replicates were sampled.

The microbial fraction from leaf and shoot ectosphere (5–10 g) was isolated by treating in a Stomacher Bag according to Berg *et al.* (2002). Grapes were crushed in 1 mL 1× PBS buffer (8 g L^{-1} NaCl, 1.4 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KCl, 0.24 g L^{-1} KH_2PO_4; pH 7.4) under sterile conditions. The liquid part was transferred into 2 mL tubes and spun down at 10 000×*g* for 20 min at 4 °C. Endosphere leaves and shoots were surface sterilised in 50 mL tubes using 4 % NaOCl (Roth, Karlsruhe, Germany) for 5 min. After removing of NaOCl, material was washed three times with 50 mL of sterile A. dest. To check success of surface sterilisation plant material was whisked onto a nutrient agar plate (Nutrient Agar II, Sifin, Berlin, Germany) after the third washing step. After removal of the plant material the plate was incubated at 22 °C for 4 d. Sterilised material was crushed in 2 mL 1× PBS, liquid part was transferred to 2 mL tubes and spun down under above mentioned conditions. Pellets from each preparation were stored at −70 °C. Total DNA was extracted from prepared samples using Fast DNA® Spin Kit for Soil (Qbiogene, Inc. Carlsbad, CA) according to manual instructions. Dilutions of sample preparation were plated out on Sabouraud Dextrose Agar (Roth) containing 100 μg mL^{-1} chloramphenicol (Roth) and R2A agar (Roth). For each tissue type 132 fungal isolates (from Sabouraud Dextrose Agar) and 72 bacterial isolates were chosen randomly.

Genetic fingerprints of bacterial isolates (BOX patterns) and sequencing

Whole genomic DNA was extracted from bacterial isolates by bead beating and subsequent chloroform–phenol extraction. Box fingerprinting was carried out using primer BoxA1R (McManus & Jones 1995). PCR fragments were separated on 1.5 % agarose gels in TBE buffer (54 g L^{-1} Trishydroxymethylaminomethane, 27.5 g L^{-1} boric acid, 10 mM EDTA), stained with ethidiumbromide and recorded under UV light. Normalization and cluster analysis of band patterns, evaluated on band intensity, was carried out with the program Gel Compare (Applied Maths, Kontrijk, Belgium). Background correction was applied for each track. The Pearson's correlation index for each pair of lanes within a gel was calculated as a measure of similarity between the fingerprints. Finally, cluster analysis was performed by applying the unweighted pair group method using average linkages (UPGMA) to the matrix of similarities obtained.

PCR was conducted from isolated DNA with primers 27f (5′-AGAGTTTGATCMTGGCTCAG) and 1492r (5′-TACGGY TACCTTGTTACGACTT) (Lane 1991) in a reaction containing 1× Taq-&GoTM Mastermix (BIO101® Qbiogene) and 0.5 μM of each primer with initial denaturing of 5 min at 95 °C following 30 cycles of 30 s at 95 °C, 30 s at 56 °C and 90 s at 72 °C. Final elongation step of 72 °C for 10 min. PCR products were purified using

Promega Wizard® SV Gel and PCR Clean up system (Promega Corporation, Madison, USA) and sequenced at the sequencing core facility ZMF, Medical University of Graz, Austria.

Quantitative PCR

10 μL reactions were conducted in 1× Taq-&GoTM Mastermix (Qbiogene) with 0.5 μM of each primer, 0.5× SYBR® Green (Invitrogen, Carlsbad, CA, USA) and 1 μL template. Rotor Gene 6000 (Corbett Research) cycler was used for quantification of fluorescence. Primers ApuIIF1 (5′-GATCATTAAAGAGTAAGGGTGCTCA) and ApuIIR1 (5′-GCTCGCCTGGGACGAATC), both developed by the National Exposure Research Laboratory (Cincinnati, OH 45268) were used for quantification of *Aureobasidium pullulans* ITS1 copies. For absolute quantification the respective PCR fragments were cloned into a pGEM®-Teasy Vector (Promega, Madison, WI, USA). Serial dilutions of PCR fragments generated with the primers usp (5′-GTAAAACGACAACCAGT) and rsp (5′-CAGGAAACAGCTATGACC), which specifically bind to sides flanking the multi cloning side of pGEM®-Teasy, were used as standard for calculation of copy number. The calculated copy number was corrected by the PCR efficiency in sample matrix, which was determined by measurement of serial dilutions of standard fragments in DNase I digested sample matrix. Concentrations determined by absolute quantification were calculated to copy number/g fresh weight. Each replicate was analysed three times. Mean values of each habitat were compared among different plant types and significance of differences were analysed with Tukey's Honestly Significant Difference (HSD) multiple range test ($p = 0.05$) using SPSS for Windows vers. 11.5.1. (SPSS Inc.).

SSCP analysis of 16SrDNA fragments from community DNA

All PCR reactions were, if not stated otherwise, conducted in 1× Taq-&GoTM Mastermix (BIO101® Qbiogene) with 0.5 μM of each primer, 1 μL template for ectosphere and grape samples or 2 μL template for endosphere samples in a Tpersonal Thermocycler (Biometra, Göttingen, Germany). Each PCR program had an initial denaturation step of 95 °C for 5 min and a 72 °C final extension step for 10 min. Analysis of the *Pseudomonas* community was modified and adjusted for SSCP analysis from Milling *et al.* (2004). Template DNA was amplified in a reaction containing 0.15 mg mL^{-1} bovine serum albumin (New England Biolab) and no additional $MgCl_2$ added. 2 ng of PCR product from this PCR were used in a second reaction using the universal primer pair unibac-II-515f and unibac-II-927rP used in Berg *et al.* (2005). A semi-nested PCR approach was used for analysis of the *Firmicutes* community. A first amplification was carried out with primer pair Bspez3f (5′-AGACTGGGATAACTCCG, this study) and BACr833P (Nechitaylo *et al.*2009)with a 5′ phosphorylation in a 20μL reaction with 30 cycles of 95°C 45 s, 54 °C 30 s and 72 °C 45 s. 1 μL of PCR product from the first PCR was used as template in a 60 μL reaction using the primer pair Bspez6f (5′-CGACCTGAGAGGGT, this study) and BACr833P. The reaction contained 0.15 mg mL^{-1} bovine serum albumin (NEB) and was conducted with the same program as the first reaction.

Single strand conformation polymorphism analysis (SSCP) was carried out according to Schwieger & Tebbe (1998) using a 9 % polyacrylamide gel running for 17 h for fungal community analysis and a 8 % polyacrylamide gel running for 26 h for *Pseudomonas* and *Firmicutes* community analysis both at 400 V. Gels were scanned transmissively (Epson perfection 4990 Photo, Nagano, Japan) to obtain digitized gel images. Analysis of band patterns was done as described above. Significances of differences between clusters were calculated with permutation analysis of pair-wise similarities using permtest package of R statistics (R: Copyright, 2005; The R Foundation for Statistical Computing Version 2.1.1).

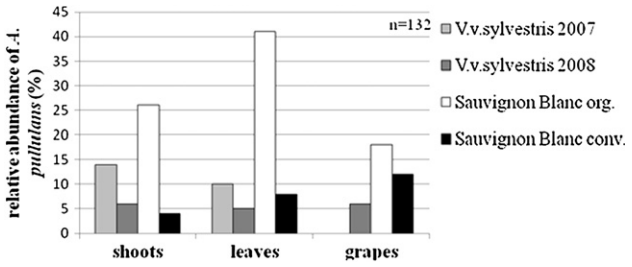

Fig 1 – Abundance of *A. pullulans* relative to total amount of fungal isolates. Fungal isolates were selected randomly from isolation plates and relative abundance of *A. pullulans* was assessed upon morphological characterisation.

Extraction and sequencing of DNA from single bands of SSCP gels

Gel slices containing single bands were frozen at −70 °C and DNA was extracted following the protocol of Schwieger & Tebbe (1998). DNA was resolved in 20 μL A. dest. and cleaned up with Geneclean® Turbo Kit (Qbiogene) according to manual instructions. The fragment was amplified by PCR as described under SSCP analysis procedure. PCR product was sequenced at the sequencing core facility ZMF, Medical University of Graz, Austria with the Applied Biosystems 3130l Genetic

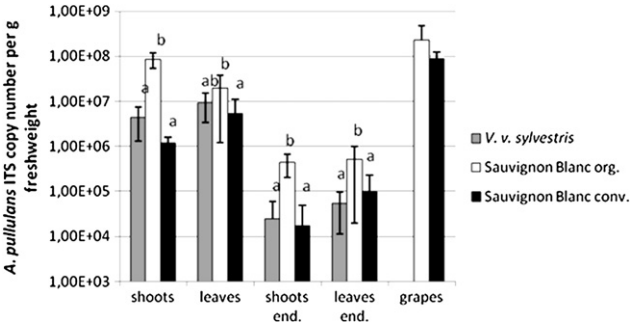

Fig 2 – Abundance of *A. pullulans* ITS copy numbers determined by quantitative PCR in samples of shoots, leaves shoots endosphere, leaves, endosphere, and grapes from *Vitis vinifera* subsp. *sylvestris* (sampling 2007), and organically and conventionally managed Sauvignon Blanc. No grape samples were available for *V. vinifera* subsp. *sylvestris*. Different letters indicate significant differences calculated with Tukey's HSD multiple range test at a level of significance of 0.05.

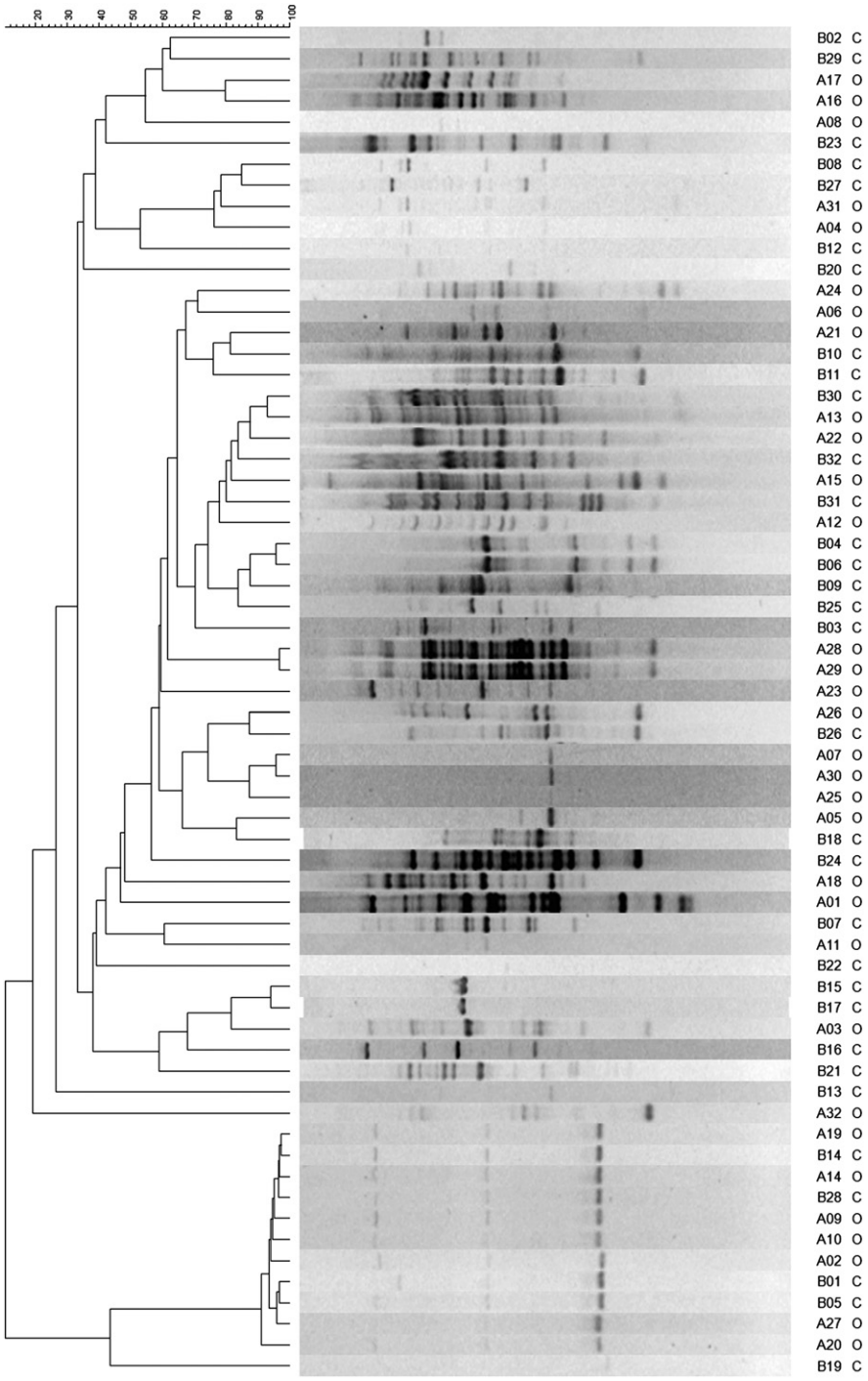

Fig 3 – Genetic fingerprints of bacterial isolates associated with tissues from organically (O) and conventionally (C) managed grapevine. Whole genomic DNA from isolates was extracted and used as template for BOX-PCR. Fragments were separated on agarose gel and clusters were calculated from band patterns using UPGMA. Identification of strains see Table 1.

Analyser sequencer Data Collection v. 3.0, Sequencing Analysis v. 5. (Foster City, USA). Obtained sequences were aligned with reference gene sequences from GenBank using BLAST algorithm. Sequences obtained were submitted to EMBL. Sequences of uncultured fungal species: FN430614-FN430640. Sequences of uncultured *Pseudomonas* species: FN430641-FN430652.

In vitro *antagonism tests*

For assessment of antibacterial activity of *Aureobasidium pullulans* suspensions of bacterial test strains were inoculated on potato extract dextrose agar (Roth). *Aureobasidium pullulans* strains LBPek3-10 and LBPen1-1, which were isolated from

Table 1 – Interactions between *A. pullulans* and associated bacterial isolates. Inhibition of growth of bacterial isolates by *A. pullulans* and antagonistic activity of bacterial isolates towards *A. pullulans* was assessed by *in vitro* plate assays.

Strain[a]	Phylogenetic affiliation[b]	Inhibition by *A. pullulans*[c]	Inhibition of *A. pullulans*[d]	Origin[e]	EMBL Acc. number
A05	*Staphylococcus* sp. (FR750268.1, 100)	–	–	O	FR717258
A06	*Micrococcus* sp. (HQ622519.1, 99)	–	–	O	FR717259
A07	*Staphylococcus* sp. (HQ246299.1, 99)	–	–	O	FR717260
A12	*Bacillus* sp. (HQ727953.1, 99)	+	–	O	FR717261
A15	*Sphingomonas* sp. (HM224453.1, 100)	–	–	O	FR717262
A22	*Sphingomonas* sp. (HM224453.1, 100)	–	–	O	FR717263
A28	*Pseudomonas* sp. (AJ970168.1, 99)	–	+	O	FR717264
A31	*Bacillus* sp. (FJ215792.2, 100)	+	–	O	FR717265
A32	*Micrococcus* sp. (HQ622519.1, 99)	–	–	O	FR717266
B06	*Frigoribacterium* sp. (HM640282.1, 99)	–	–	C	FR717267
B11	*Pantoea agglomerans* (FJ756356.1, 99)	–	–	C	FR717268
B16	*Bacillus* sp. (FJ215792.2, 100)	+	–	C	FR717269
B19	*Staphylococcus epidermidis* (HQ908728, 99)	–	–	C	FR717270
B21	*Micrococcus* sp. (GQ246727.1, 99)	–	–	C	FR717271
B23	*Micrococcus* sp. (FR750272.1, 97)	–	–	C	FR717272
B24	*Pantoea agglomerans* (HM130689.1, 100)	–	–	C	FR717273
B25	*Micrococcus* sp. (GQ246727.1, 98)	–	–	C	FR717274
B27	*Chryseobacterium* sp. (DQ279361.1, 100)	–	–	C	FR717275
B31	*Sphingomonas* sp. (GU980219.1, 100)	–	–	C	FR717276

a Strain number according to BOX patterns in Fig 2.
b Identification by comparison of 16S rRNA gene fragment sequence with NCBI database using BLAST. Phylogenetic affiliation, accession number, and maximum identity (%) of best hit are shown. In case of multiple best hits with same identity values one hit is shown exemplarily. Query coverage was 100 % in all cases.
c Development of inhibition zone around *A. pullulans* colony on potato dextrose agar plated with test strain.
d Inhibition of growth of *A. pullulans* in dual culture plate assay.
e Isolate's origin from samples of organically (O) or conventionally (C) managed plants.

organically managed grapevine, were striked out on these plates. Plates were incubated for 3 d at 22 °C and antibacterial activity was determined by measuring inhibition zones. Antagonistic activity of bacterial isolates against *A. pullulans* was determined by dual culture plate assays as described previously (Berg *et al.*2002).

Results

Abundance of Aureobasidium pullulans *associated with* Vitis vinifera *subsp.* sylvestris *and subsp.* vinifera

Isolation of fungal isolates from grapevine tissues revealed frequent occurrence of *A. pullulans* in all investigated samples (Fig 1). *Aureobasidium pullulans* was mostly abundant in samples from organically managed plants, where 26 %, 41 %, and 18 % of all fungal isolates of shoots, leaves, and grapes respectively were identified as *A. pullulans*. Compared to these figures, the relative abundances of *A. pullulans* in tissues from conventionally managed grapevine and from the wild *Vitis* populations (subsp. *sylvestris*) in Austria ranged from 4 % to 14 %. The elevated abundance of *A. pullulans* associated with organically managed grapevine was supported by quantitative PCR results. *Aureobasidium pullulans* ITS copy number in samples from organically managed grapevine was significantly higher in all samples studied when compared to conventionally managed plants and *V. vinifera* subsp. *sylvestris* (Fig 2). The highest number of ITS copies associated with organically managed plants was found in grapes ($2.3 \times 10^8 \pm 2.4 \times 10^8$) followed by $8.6 \times 10^7 \pm 3.1 \times 10^7$, and $1.2 \times 10^7 \pm 1.9 \times 10^7$ in phyllosphere samples from shoots and leaves respectively. In the endosphere of shoots and leaves a copy number of $4.4 \times 10^5 \pm 2.3 \times 10^5$ and $5.2 \times 10^5 \pm 5.0 \times 10^5$ was determined.

Bacterial community associated with organically and conventionally managed grapevine

Genetic fingerprints of bacterial strains (as BOX patterns) isolated from organically and conventionally managed plants were generated with PCR using primer BoxA1R in order to assess the influence of a higher abundance of *Aureobasidium pullulans* on the bacterial community. As shown in Fig 3, a high number of different BOX groups were retrieved suggesting an overall high diversity of bacteria associated with the investigated habitats. In the large BOX group consisting of *Bacillus* sp. representatives from both organically and conventionally managed grapevine plants were present. Five out of seven strains with BOX patterns similar to *Staphylococcus* sp. and two isolates forming a distinct cluster, of which one was identified as *Pseudomonas* sp. (A028), were isolated from organically managed plants. Four strains with similar BOX patterns to B06 (*Frigoribacterium* sp.) were isolated from conventionally managed plants. Interaction between *A. pullulans* and associated bacterial representatives of each BOX group were selected for *in vitro* interaction studies. A small proportion of isolates was antagonised by *A. pullulans* isolates LBPek3-10 and LBPen1-1 (Table 1). The inhibition zones formed by strain LBPek3-10 (LBPen1-1) on bacterial isolates were 3 (4) mm for

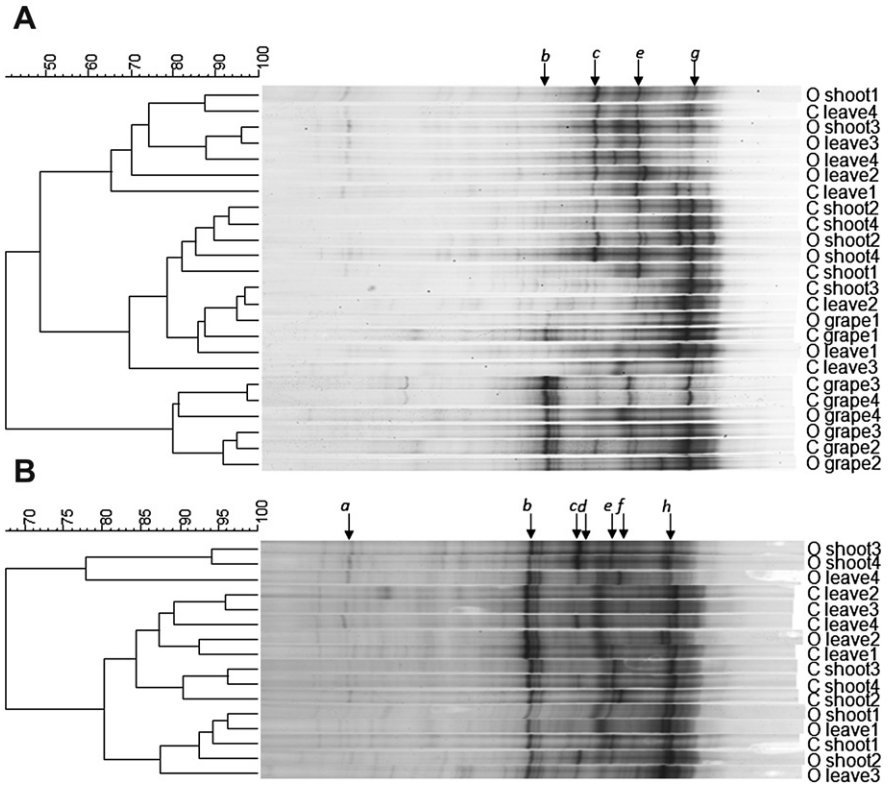

Fig 4 – Clustering of the SSCP profile of the *Pseudomonas* community of grapevine plants. Only minor differences are discernible between conventionally (C) and organically (O) managed plants. Shoots, leaves, and grapes were sampled 1 week prior to harvest. Community DNA was extracted from the respective plant parts followed by *Pseudomonas* specific amplification of 16S rRNA gene fragments and SSCP analysis. Band patterns were compared and clustered by UPGMA method. A: Community profile of ectosphere samples of leaves, shoots and grapes. B: Community profile of endosphere samples of leaves and shoots. (a) Uncultured bacterium, (b) Chloroplast, (c) *P. graminis/lutea*, (d) *P. syringae/fluorescens*, (e) *P. rhizosphaerae/abietaniphila*, (f) *P. fragi*, (g) *Pseudomonas* sp., (h) *P. tolaasii/poae/oryzihabitans/plecoglossicida*. For sequences refer to EMBL numbers FN430641-FN430652. Data shown for samples from 2007.

isolate A31, 4 (3) mm for isolate A17 and 4 (4) mm for isolate B16. All isolates susceptible to inhibition by *A. pullulans* were identified as species belonging to the genus *Bacillus*. One bacterial isolate (A28) tested was able to suppress growth of *A. pullulans*. This strain was identified as a *Pseudomonas* species.

Comparison of the bacterial community fingerprints

As *Aureobasidium pullulans* was shown to interact in an antagonistic way against certain bacterial groups (*Bacillus*) a cultivation independent approach was used to investigate the bacterial community of organically and conventionally managed grape plants. Two well-known dominant groups of plant-associated bacteria, *Pseudomonas* (Haas & Défago 2005) and *Firmicutes* (Smalla *et al.* 2001), were analysed to assess bacterial fingerprints of black fungi enriched communities in organically grown cultures and to compare those of conventional vine cultures. Although cultivable cell numbers of bacteria differed between the two treatments, no significant difference was found in the community profiles of these bacterial groups. Pseudomonads were present in conventionally and organically managed plants likewise (Fig 4A and B). Sequences matching *Pseudomonas tolaasii* and *Pseudomonas poae* (99 % identity to sequences HQ660061.1 and GU188956.1) as well as *Pseudomonas oryzihabitans* and *Pseudomonas plecoglossicida* (100 % identity to sequences EU977742.1 and EU977739.1) were found in all endosphere samples (*h* in Fig 4b). Sequences showing high similarity to *Pseudomonas graminis* and *Pseudomonas lutea* (100 % identity to GU585128.1 and EU184082.1) as well as to *Pseudomonas rhizosphaerae* and *Pseudomonas abietaniphila* (99 % identity to GU585129.1 and AJ011504.1) were present frequently but neither restricted to conventional nor to organic treatment. Another sequence, which could not be assigned to a distinct species, was found in all ectosphere and grape samples (*g* in Fig 4a; 100 % identity to FN555446.1).

Similarly, no apparent differences were found in the *Firmicutes* fractions of the bacterial communities among conventionally and organically cultivated grapevine plants (Fig 5A and B). Permutation analysis of the similarity matrices of the gels showed no statistical significance between organic and conventional samples of $p = 0.9$ for ectosphere and grape samples and $p = 0.08$ for endosphere samples. Specificity of the primers was confirmed by sequencing selected bands from the SSCP gels. This revealed only *Bacillus* and *Staphylococcus*

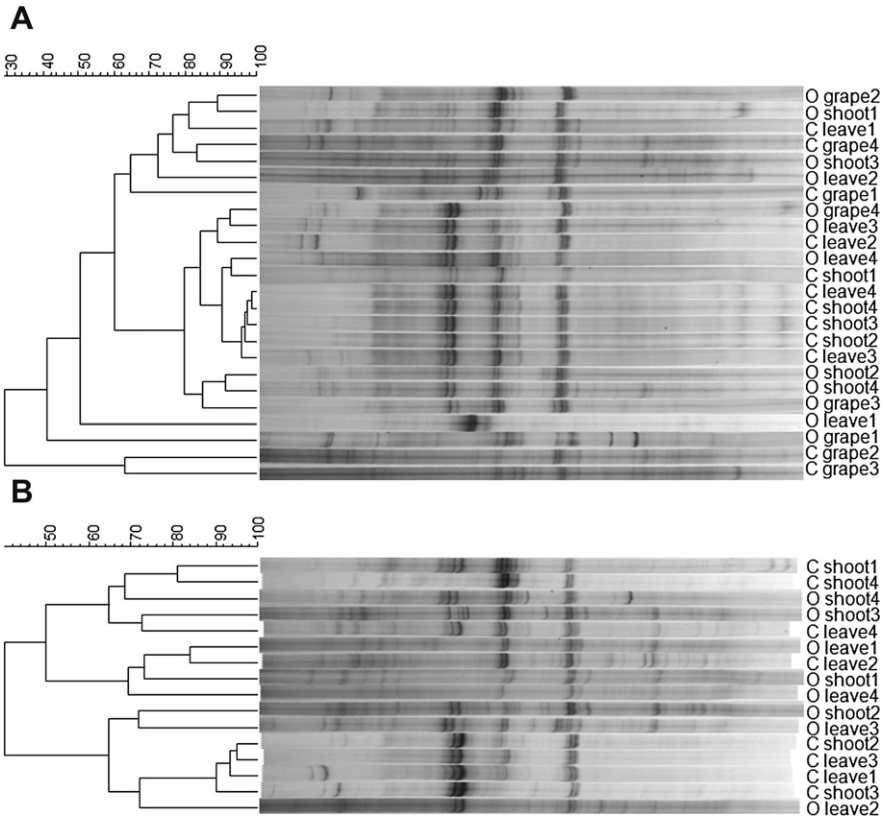

Fig 5 – Clustering of the SSCP profile of the *Firmicutes* community of grapevine plants. No significant differences are discernible between conventionally (C) and organically (O) managed plants. Samples from shoots, leaves, and grapes were taken 1 week prior to harvest. Community DNA was extracted from the respective plant parts followed by *Firmicutes* specific amplification of 16S rRNA gene fragments and SSCP analyses. Band patterns were compared and clustered by the UPGMA method. A: Community profile of ectosphere samples of leaves, shoots, and grapes. B: Community profile of endosphere samples of leaves and shoots. Data shown for samples from 2007.

species but sequences obtained were too short to assign bands to certain species.

Discussion

We did not find evidence for an influence of elevated black fungi populations on the diversity of plant-associated bacterial communities. *Aureobasidium pullulans* and *Epicoccum nigrum* were previously found to be associated with grapevines (Martini *et al.* 2009), and recent work indicated their enrichment in organically managed *Vitis vinifera* L. (Schmid *et al.* 2011). This pattern agrees well with copper and sulphur tolerance of these fungi. For its biotechnological importance we have then concentrated on *Aureobasidium*, and confirmed the higher abundance also by qPCR. We then compared the associated bacterial communities in conventionally managed and black-fungal-enriched vine cultures. Although cultivable cell numbers of bacteria differed among the two management practices (data not shown), no significant difference in the taxonomic structure was found in the community profiles of *Firmicutes* and pseudomonads. The latter displayed a ubiquitous presence of sequences matching *Pseudomonas fulva* (*Pseudomonas putida* group) and frequent presence of those related to the *Pseudomonas graminis* group, irrespective of management type. Sequencing selected bands of *Firmicutes* from the SSCP gels revealed only *Bacillus* and *Staphylococcus* species. The latter finding was intriguing since antagonistic effects of *Aureobasidium* and *Epicoccum*, or their products, against isolates of these bacilli have previously been reported (e.g., Burge *et al.* 1976; Berg *et al.* 2000; Abdel-Lateff *et al.* 2009). Although no distinct effect on the composition of *Firmicutes* was observed according to the molecular fingerprints, our dual culture experiments also revealed that significant antagonistic effects exist against *Firmicutes* strains *in vitro*. The antagonistic effect was present only against few strains, suggesting rather specific principles of activity.

Members of the *A. pullulans* complex are extremely versatile in their ecology and include also extremotolerant strains. The phylogenetic relationships of the *A. pullulans* group have been revised recently by Zalar *et al.* (2008), who found considerable genetic variation. Using primarily material from arctic habitats, they distinguished several, phenotypically supported varieties and found yet unnamed lineages as well. However, another variety – distinguished in its metabolic capabilities (Yurlova & de Hoog 1997) – was not phylogenetically separate from *A. pullulans* s.str. in Zalar *et al.* (2008). This suggests that genetic variation in the presently used markers

does not predict physiological distinctiveness of *A. pullulans* strains. Our strains from grapevine all belong to *A. pullulans* s.str. (data not shown), but we have not tested their capacity to produce compounds that are antagonistic against specific strains of bacilli. Given the phenotypic plasticity of *A. pullulans* (Slepecky & Starmer 2009), variation in production of metabolites cannot be excluded. Moreover, extrapolation of effects in dual cultures to the activities of the fungus *in vivo* is difficult. Copper influences the growth patterns of *Aureobasidium* (Gadd & Griffiths 1980), and moisture was shown to influence antagonistic patterns on artificial leaves (McCormack *et al.* 1995). The specific effects found in dual cultures could be interesting for discovery of strain-specific antibacterial compounds. One pseudomonad with antagonistic activity against *Aureobasidium* was isolated in this study (A28), but this activity had apparently little impact on the general abundance of *Aureobasidium* on organically managed grapes.

Specific antagonistic activities are a selective force that influences the composition of microbial communities. This is also found in associations of black fungi with bacteria. A recent study of aquatic leaf litter revealed inhibition of *Cladosporium herbarum* by *Ralstonia pickettii* in co-cultures, and *Chryseobacterium* lowered lytic activities of this fungus in microcosms (Baschien *et al.* 2009). A role of black yeast–bacterial interaction has also been suggested in leaf cutting ant symbioses (Little & Currie 2008). *Phialophora* strains associate with a *Pseudonocardia* strain that is carried on the thorax of the ants. The black fungus compromises the antibiotic activity of this bacterial strain against a detrimental fungus in the ant's nests, thereby playing an important role for the functional network of the whole symbiotic community (Little 2010). Recent evidence further suggests that bacteria can also be present inside black-fungal hyphae (Hoffman & Arnold 2010). One *A. pullulans* isolate was reported to harbour a strain of *Burkholderiaceae* (*Betaproteobacteria*), whereas another strain contained a *Pasteurellaceae* (*Gammaproteobacteria*). The ecological meaning of these phenomena are not yet clear, and frequent loss of bacteria following subculturing was interpreted by Hoffman & Arnold (2010) as an indication of facultative interactions. Also, mutualistic effects of microorganisms with black fungi in extreme habitats should be studied more carefully. On rocks, black fungi may grow adjacent to cyanobacteria (Sterflinger 2006) or reside in extremotolerant lichens (Harutyunyan *et al.* 2008). Associations of black fungi with algae have been reported (Turian 1975, 1977; Gorbushina *et al.* 2005; Brunauer *et al.* 2007).

Better knowledge of black-fungal interactions with fungi and bacteria is also interesting from an applied point of view. *Aureobasidium pullulans* has the potential to support plant protection in agricultural systems and could reduce application of problematic chemicals. Products on the basis of the biocontrol agent *A. pullulans* are already on the market (Chi *et al.* 2009). Efficacy of *A. pullulans* in controlling postharvest diseases of fruits caused by moulds has been shown by several studies (Castoria *et al.* 2001; Dimakopoulou *et al.* 2008; Chi *et al.* 2009). Mechanisms of action include competition for nutrients and production of cell wall degrading enzymes (Andrews *et al.* 1983; Castoria *et al.* 2001). Due to their plant surface colonisation traits, *A. pullulans* strains also showed positive effects on diseases of plant leaves and stems caused by moulds (Andrews *et al.* 1983; Dik *et al.* 1999). Antagonistic effects were also reported against fire-blight bacteria (*Erwinia amylovora*) in co-culture experiments, but these have not been confirmed *ad planta* (Seibold *et al.* 2004). The finding of low influence of *A. pullulans* on the phyllosphere bacterial communities could, however, help to develop new biocontrol formulations which include antagonistic bacteria in addition to the black fungus *A. pullulans*.

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