Functional and Structural Microbial Diversity in Organic and Conventional Viticulture: Organic Farming benefits Natural Biocontrol Agents

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Statistically significant differences were found for the structure and function of above-ground grapevine-associated microorganisms from organically and conventionally managed vineyards. *Aureobasidium pullulans*, a copper detoxifying fungus and biocontrol agent, plays a key role to explain these differences: the black fungus was strongly enriched in the communities of organically managed plants and yielded into a higher indigenous anti-phytopathogenic potential.
Today’s farming systems undermine the well-being of communities in many ways: huge regions of natural habitats including their ecosystem services were destroyed, plant protection measures caused problems for human health and they are responsible for about 30% of greenhouse-gas-emission (15). An alternative to conventional agriculture is offered by organic farming, which aims to minimize the impact on the environment by practices using crop rotation, pathogen resistant cultivars, limited amount of chemical pesticides and organic manure instead of synthetic fertilizers. However, the benefit of organic agriculture on biodiversity in general and on plant-associated microorganisms in detail is still controversially discussed (6, 8, 9, 14).

*Vitis vinifera* L. is one of the oldest cultivated plants with high economic importance.

In viticulture, comprising an area of 7.4 million hectares grapevine plants worldwide (FAOSTAT, 2008), the proportion of organically cultivated grapes is strongly increasing (19). Although it was shown that taste and quality of organically produced wine is better than in conventional viticulture (20), plant protection using mainly copper-containing products is extremely problematic. The latter resulted in high soil contamination endangering plant health and fruit quality as well as in spread of copper- and antibiotic resistant microbes (4, 13). The impact of copper treatment and other organic plant protection methods on plant-associated microorganisms and their function in ecosystem service is still poorly understood. We hypothesise that the type of plant protection in conventional and organic vineyards has great impact on microbial and especially on the fungal community structure associated with grapevine.

To compare epiphytic and endophytic microbial communities of the phyllosphere of conventionally and organically managed grapevine, leaves, shoots and undamaged grapes of the cv. ‘Sauvignon Blanc’ were sampled 2006 and 2007 from vineyards in Schlossberg, Austria (46° 37' N, 15° 28' O; owner: Fachschule für Weinbau und Kellerwirtschaft und Weingut Silberberg) in the last week before harvest (on 3rd of October 2006 and 11th of...
September 2007 respectively). Half of the vineyards were managed conventionally, the other half organically. Chemical plant protection agents used in the conventional plots were: sulphur, paraffin oil, manganese-zinc ethylene bis(dithiocarbamate), proquinazid, iprovalicarb, folpet, pyrimethanil, mandipropanid, quinoxyfen, chlorpyrifos-methyl, boscalid and cyazofamid. Chemicals used in the organic parcel were sulphur, copper, Myco-Sin®, potassium water glass, Frutogard® and fennel oil. Four independent composite replicates for each habitat were investigated; the same microbial fraction isolated according to Berg et al. (2) was used for DNA- and cultivation based analysis.

To perform molecular analysis, total DNA was extracted from samples using FastDNA® Spin Kit for Soil (Qbiogene, Inc. Carlsbad, CA). Microbial fingerprints were performed using Single strand conformation polymorphism analysis (SSCP) according to Schwieger and Tebbe (16) using different primers (16, 18). Gels were analyzed with the program Gel Compare (Applied Maths, Kontrijk, Belgium) and statistically assessed (11). Gel slices containing single bands analyzed (16). Sequences obtained were submitted to EMBL: FN430614-FN430640. To quantify *Aureobasidium pullulans* primers ApuIIF1 (5’-GATCATTAAAGAGTAGCTCA-3’) and ApuIIR1 (5’-GCTCGCCTGGGACGAATC-3’), both developed by the National Exposure Research Laboratory (Cincinnati, OH 45268) were used. For quantification of *Sporidiobolus pararoseus*, primers Spa2f (5’-CCAATCTTTTCTTGATCG-3’) and Spa2r (5’-CCTTAATGAAGTTGGCCTC-3’) were designed as given in supplementary material (S1). Primer pair ITS1 and ITS2 (39) was used for quantification of total fungal ITS1 copies. 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. Each replicate was analysed three times. Significances in differences between conventional and organic treatment were calculated using unpaired Student’s t-test. Melting curves of Spa2f / Spa2r and ApuIIF1 /ApuIIR1 reactions resulted in one homogenous product.
To isolate grape-associated microorganisms, serial dilutions of the primary cell suspensions were plated out on R2A, Sabouraud-Dextrose-Agar (Roth, Karlsruhe, Germany) containing 100 µg ml\(^{-1}\) chloramphenicol, and Synthetic Low Nutrient agar (SNA) (2). From each habitat and replicate 18 yeast and 15 filamentous fungal isolates were selected randomly to test their antagonistic activity (1).

Fingerprints from the fungal community obtained by ITS-PCR SSCP and their statistical analysis resulted in a clear separation of samples from conventional and organic management (Fig. 1). P-values showing statistically significant differences between organic and conventional samples were < 0.001, both for ectosphere and grape berries (Fig. 1a) and for endosphere samples (Fig. 1b). Altogether, the fingerprints from organically managed plants were more homogenous and showed a higher similarity between each other than those from conventionally managed plants. From SSCP gels, differing and dominant bands were identified by sequencing. The strongest band (Fig. 1a, b) in samples from the conventional treatment corresponded to *Sporidiobolus pararoseus*, a yeast already described associated with grapes (12, 20). Conversely, bands for *Aureobasidium pullulans* were stronger from organic treatment. *A. pullulans* is a cosmopolitan yeast-like (black) fungus and ubiquitously associated with plants including grapevine (5, 8). *Cladosporium sp.* and *Alternaria tenuissima* were common species found in all samples. DNA from a different *Alternaria*, which could not be identified on species level, was detected in ectosphere and, to a greater extent, in grape samples only from conventionally managed plants.

Due to the dominance of *A. pullulans* and *S. pararoseus* bands and their discriminative character in fingerprints of fungal communities, both fungi and total fungal ITS copy numbers were determined using quantitative PCR. In general, fungal ITS copy number was higher in samples from organic than from conventional agriculture (data not shown). The relative quantification of *A. pullulans* ITS copy numbers showed a higher abundance of this species in organically compared to conventionally managed plants (Fig. 2a). This result was statistically
significant for all investigated samples from both years, with only one exception (grapes in 2006, p=0.43). Significant higher relative amounts of *S. pararoseus* DNA were present in grapes and endosphere of conventionally managed plants (Fig. 2b). A tendency of higher *S. pararoseus* ITS copy numbers was to be seen for ectospheres from conventionally managed plants. Results were confirmed by analysing samples from 2006 (data not shown).

Above-ground microhabitats of grapes were highly colonized by microorganisms; up to $5.7 \log_{10} \text{CFU}$ were calculated for bacteria, $5.2$ for yeasts and $5.3$ for fungi. Statistically significant differences in CFUs between samples of organically and conventionally managed plants were found, e.g. on/in organically managed plants higher abundances of filamentous fungi and yeasts were isolated. The anti-phytopathogenic potential, estimated by *in vitro* antagonism towards *Botrytis cinerea* (1), from organically managed plants was higher than from conventionally managed plants (Fig. 3a). As $33$ out of $34$ *A. pullulans* isolates showed antagonistic activity against *B. cinerea in vitro* (data not shown), we assessed the contribution of the fungus to the total indigenous antagonistic potential. In addition to results of quantitative PCR, cultivation of *A. pullulans* yielded in a higher abundance in samples from organically managed plants in comparison with conventional treated plants (Fig. 3b). All results support the key role of the yeast-like fungus *A. pullulans* to explain the structural as well as functional differences in both agricultural systems. Interestingly, *Aureobasidium* can utilize inorganic sulphur (10) and is able to absorb, and by this way detoxify copper (7). These properties can explain the enrichment under organic farming conditions, which was also reported for apples under storage conditions (8). Furthermore, *A. pullulans* is a well-studied potent antagonist against several fungal pathogens; mechanisms of *A. pullulans* antagonism against fungi include competition for nutrients and space and production of cell wall degrading enzymes (5). Interestingly, typical flavour components of wine were detected as being produced by *A. pullulans* (17), which can explain the better taste of organically produced wine (20).
In addition, two well-known dominant groups of plant-associated bacteria were analysed to compare bacterial fingerprints: *Pseudomonas* and Firmicutes (1, 3). Although cultivable cell numbers of bacteria differed between the two treatments, no significant difference was found in the community profiles of both bacterial groups. Results led to the conclusion, that vineyard management has no influence on the bacterial, at least the *Pseudomonas* and *Firmicutes* community. This can be explained with the fact, that for plant protection in both managements mainly antifungal substances are used.

In our study we showed that plant protection in conventional and organic vineyards influenced grape-associated microorganisms. The most interesting fact was, that not only the structure of the fungal community was affected, also the function. In/on organically managed grapevine plants, the number of *in vitro* antagonists was enhanced, due to an enrichment of *A. pullulans*. Despite the fact that this is no direct evidence for a biocontrol effect on grapes, there are hints for this beneficial interaction: *A. pullulans* was described as potent *ad planta* antagonist (5), and biocontrol products against *Botrytis* based on this fungus are already on the market (Botector®, bio-ferm, Austria). Biological control comprises the application of naturally occurring antagonists as biocontrol agents as well as the management of the indigenous antagonistic potential (3). Our study was an unexpected but interesting example for the latter and showed that basic knowledge about the structure and function of plant associated microbial communities is essential for the development of environmentally friendly strategies for plant protection.

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Legends to the Figures

FIG. 1 Clustering of the SSCP profile of the fungal community of grapevine plants showing a clear difference 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 amplification of the fungal ITS region using general primers. The PCR product was subject to SSCP analysis. Band patterns were compared and clustered by UPGMA method. Sequences of single bands were obtained and compared to GenBank database using the BLAST algorithm giving the following hits: a) Aureobasidium pullulans, b) Cladosporium sp., c) Alternaria tenusissima, d) Alternaria sp., e) Sporidiobolus pararoseus, f) Epicoccum nigrum, g) Cryptococcus flavescens. A: Community profile of ectosphere samples of leaves, shoots and grapes. B: Community profile of endosphere samples of leaves and shoots. For sequences refer to EMBL numbers FN430614-FN430640. Data shown for samples from 2007.

FIG. 2 ITS copy number per gram fresh weight determined by quantitative PCR. A: Specific primers were used for determination of A. pullulans ITS 1 copy number in samples from organically and conventionally managed plants. B: ITS 2 copy number of S. pararoseus using specific primers relative to total fungal ITS copy number determined by general fungal primers. The relative copy number of samples from conventionally managed plants is shown in relation to the copy number of samples from organically managed plants. Error bars indicate standard variations, asterisks indicate significances of differences with respect to management type (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001). Data shown for samples from 2007.
FIG. 3 Correlation of antagonistic potential of different plant parts and plant protection management types with *Aureobasidium pullulans* relative abundance. Yeasts and filamentous fungi were isolated from samples of organically (grey bars) and conventionally (black bars) managed Sauvignon Blanc. Antagonistic potential of isolates was assayed using dual culture plate assays determining the percentage of isolates with antagonistic activity against *Botrytis cinerea* (B). Percentage of *A. pullulans* isolates among isolates was determined by morphological characterisation (A). Each value was calculated from a total number of 132 isolates (72 yeast and 60 filamentous fungal isolates). Data from sampling in 2007.
FIG. 2

A

x-fold difference in *A. pullulans* ITS copy number

B

x-fold difference in *S. pararoseus* copy number (relative to total ITS copy number)
FIG. 3