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Microorganisms associated with grapevine (*Vitis vinifera* L.): antagonistic traits and influence of farming practices

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Abstract

Biological control, the application of plant beneficial microorganisms in order to suppress plant pathogens or strengthen plant health, is a promising tool in supporting sustainable agriculture. By this way a reduction of environmentally problematic conventional plant protection measures can be achieved. A basic requirement of effective biological control is the understanding of the interactions between biological control agents, host plants, target pathogens and the residing microbial community. One part of this work was dedicated to the investigation of the influence of farming practices on the grapevine phyllosphere associated microbial communities and their antagonistic potential towards *Botrytis cinerea*, a fungal pathogen causing severe damages in viticulture. Distinct differences in the abundances and distributions of microorganisms with antagonistic activity towards *B. cinerea* were evident between different microhabitats of organically and conventionally managed as well as uncultivated grapes. Especially organic grapes harboured a high proportion of antagonistic organisms. Molecular fingerprints of fungal communities of the phyllosphere revealed significantly different patterns between organically and conventionally managed plants. Amongst others, the yeast-like black fungus *Aureobasidium pullulans* was enriched in the organic samples, which was explained by the high copper and sulphur input in organic viticulture. Isolates of *A. pullulans* were antagonistic towards *B. cinerea in vitro* and accounted for the high antagonistic potential of communities from organic grapes. Interestingly the bacterial community was not affected by the different farming practices. As though *A. pullulans* inhibited growth of *Bacillus* strains isolated from the same habitat, no differences in the communities of *Bacillus* were found between the two farming practices by molecular fingerprints. Another part of the work dealt with the biological control of *B. cinerea*. One strain of *Pantoea ananatis*, isolated from grape berries was applied in a three year field trial and was effective in reducing disease incidence of *B. cinerea* on grapevine statistically significantly. Leaf assays, *in vitro* studies, and confocal laser scanning microscopy were conducted to further characterize the mechanisms of actions of this strain. Data suggest that *P. ananatis* BLBT1-08 acts via multiple mechanisms including occupation of possible infection sites on the leaves, inhibition of growth of *B. cinerea*, and competition for nutrients.

Zusammenfassung

Eine vielversprechende Alternative zu konventionellen Pflanzenschutzmaßnahmen ist die biologische Kontrolle, bei der Mikroorganismen zur Stärkung der Pflanzengesundheit eingesetzt werden. Ziel der biologischen Kontrolle ist es, den Einsatz von umweltschädlichen Pflanzenschutzmaßnahmen zu verringern. Um dies zu bewerkstelligen, ist es nötig die Interaktionen der eingesetzten Mikroorganismen mit den Wirtspflanzen, den Krankheitserregern sowie den bestehenden mikrobiellen Populationen, zu verstehen. Ein Teil dieser Arbeit beschäftigte sich mit dem Einfluss von landwirtschaftlichen Maßnahmen auf die Weinreben assoziierten, mikrobiellen Populationen sowie deren antagonistischer Wirksamkeit gegen *Botrytis cinerea*, einem wichtigen Schaderreger im Weinbau. Es konnten deutliche Unterschiede in den Abundanzen und Verteilungen von antagonistischen Mikroorganismen zwischen unterschiedlichen Mikrohabitaten von biologischen und konventionellen Weinreben, sowie unkultivierten Wildreben festgestellt werden. Besonders biologische Weinreben wurden von einem hohen Anteil an antagonistischen Mikroorganismen besiedelt. Molekulare Fingerprints der pilzlichen Populationen zeigten signifikante Unterschiede in den Phyllosphären-Populationen zwischen biologischen und konventionellen Weinreben. Unter anderem war an biologischen Reben der hefeartige, zu den „Black Fungi" gezählte Pilz *Aureobasidium pullulans* stark angereichert. Dies wurde auf den verstärkten Eintrag von Kupfer und Schwefel im biologischen Weinbau zurückgeführt und erklärte das an biologischen Reben erhöhte antagonistische Potential, da die isolierten *A. pullulans* Stämme *B. cinerea in vitro* hemmten. Die bakteriellen Populationen wurden durch die untersuchten Pflanzenschutzmethoden nicht signifikant beeinflusst. Obwohl *A. pullulans* in der Lage war, *Bacillus* Isolate aus demselben Habitat zu inhibieren, war kein Einfluss der *A. pullulans* Abundanz auf die *Bacillus* Populationen mittels molekularer Fingerprints zu beobachten. Ein weiterer Teil dieser Arbeit befasste sich mit der biologischen Kontrolle von *B. cinerea* an Weinreben. Ein von Trauben isolierter Stamm, welcher als *Pantoea ananatis* identifiziert wurde, reduzierte den *Botrytis*-Befall in einem dreijährigem Feldversuch signifikant. Mittels Blatt-Assays, *in vitro* Experimenten und „Confocaler Laser Scanning Mikroskopie" wurde der Stamm weiter charakterisiert. Daten aus diesen Experimenten lieferten Hinweise, dass die Wirkung von *P. ananatis* BLBT1-08 auf multiplen Mechanismen, darunter der Besetzung von möglichen Infektionsstellen an Blättern, der Inhibierung des Wachstums von *B. cinerea* und der Konkurrenz um Nährstoffe, beruht.

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1 Introduction

1.1 Grapevine (*Vitis vinifera* L.)

Cultivation of grapevine has a long history dating back to the Neolithic era (MULLINS *et al.* 1992). It is thought that the progenitor of nowadays cultivars, *Vitis vinifera* subsp. *sylvestris* (or also classified as *V. sylvestris*), was first cultivated in Transcaucasia leading to *Vitis vinifera* subsp. *vinifera*. The natural habitats of *V. vinifera* subsp. *sylvestris* are humid forests, where it grows on forest trees as a liana. Since its cultivation grapevine played an important role in all civilizations as known for example from Egypt, China or Greece (MULLINS *et al*. 1992). Today a total area of 7.6 million hectares is used worldwide for cultivation of grapes yielding approximately 70 million tons of grapes, which are processed to 27 million tons of wine (different types like e.g. table wine, sparkling wine, and fortified wine). The rest is used for table grapes, raisins, juice, distilled liquor and other products (FAOSTAT 2011; data from 2009). In Austria cultivation of grapes is mainly located at the eastern part with an area of 45,000 hectares, representing 3 % of total agronomic area. Grapes are processed exclusively to wine with an output of 235,000 tons in 2009 and an export volume of 638,000 hL (FAOSTAT 2011). Important cultivars in Austria are 'Grüner Veltliner', 'Welschriesling', which is important especially for Styria, and 'Müller-Thurgau', as well as 'Zweigelt' and 'Blaufränkisch'. 'Sauvignon Blanc' is a frequently grown cultivar of white wine in Styria.

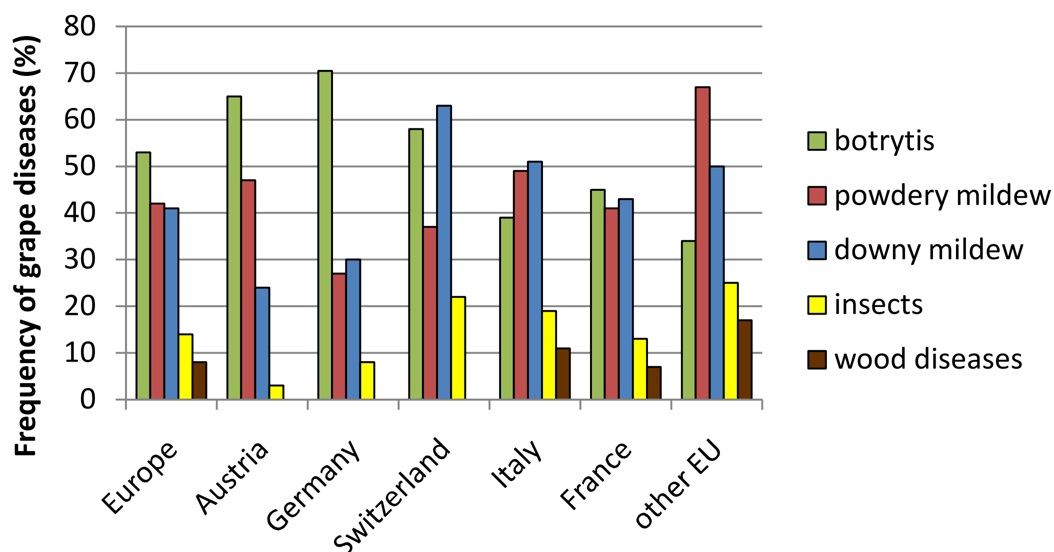

Figure 1-1. Ranking of the most important causes of grapevine diseases and pests in organic vineyards of European countries. Source: modified from Micheloni & Trioli 2006

Compared to other cultivated plants requirement of additional nutrients is relatively low in viticulture, but effort put in plant protection measures is high due to several severe diseases and pests endangering grapevine health and lowering yield and quality. The most frequently reported cause of grapevine diseases in Europe is *Botrytis* (Fig. 1-1). Among major pests all over Europe are grapevine moths (*Lobesia botrana*, *Eupoecillia ambiquella*), spider mites (*Panonychus ulmi, Tetranychus urticae*, *Calepitrimerus vitis, Colomerus vitis*), and leaf hoppers (*Empoasca vitis, Scaphoideus titanus, Hyalesthes obsoletus*) (TRIOLI & HOFMANN 2009). Probably due to climatic differences, problems caused by insect pests are lower in Austria and Germany than in other European countries like France, Italy, and also Switzerland (Fig 1-1). Pathogenic fungi outnumber insect pests as causative agents for diseases in Austria, being Botrytis (*Botrytis cinerea*), powdery mildew (*Erysiphe necator*), and downy mildew (*Plasmopara viticola*) the most important ones (MICHELONI & TRIOLI 2006).

1.2 *Botrytis cinerea* in viticulture

The genus *Botrytis* PERSOON belongs to the family Sclerotiniaceae WHETZEL within the order Helotiales of the Ascomycota. Fig 1-2 shows images of *B. cinerea* in pure culture and infecting grapevine leaves. Among the 22 species known in the genus *Botrytis*, the majority of species can infect only a narrow host range. In contrast to that the species *B. cinerea* can infect over 200 different eudicot plant species (MACFARLANE 1968; HENNEBERT 1973; YOHALEM *et al*. 2003). A recent study confirmed the phylogenetic relationships within the genus *Botrytis* based on molecular approaches using the genes *RPB2*, *G3PDH*, and *HSP60* and separated the genus into two clades, with *B. cinerea* belonging to clade 1 (STAATS *et al*. 2005). As members of *B. cinerea* show high intraspecies diversity, this group is thought to be a species complex. *Bc-hch*, *CYP51* and β-tubulin gene sequence comparison of 43 isolates (mainly from France) supported the division of *B. cinerea* into two groups, which seem to represent phylogenetic species (FOURNIER *et al*. 2005).

By forming sclerotia *B. cinerea* can endure throughout the winter as well as under adverse conditions. Furthermore it can survive without causing symptoms saprophytically in many parts of the vineyard, such as soil, grape vine prunings, necrotic plant tissue, and other host species (THOMAS *et al*. 1983; BISIACH *et al*. 1984; NAIR *et al*. 1995; FOWLER *et al*. 1999; SEYB 2004). Targets for infection are any succulent, stressed or senescent/dead tissues of plants. Important for viticulture are infections of inflorescent tissues from bunch closure until

harvest time (TRIOLI & HOFMANN 2009). Prior to infection conidia adhere to plant surfaces and via a sensing and recognition system germination is regulated (DOEHLEMANN *et al*. 2006; CHOQUER *et al*. 2007). Infection continues with penetration of the host surface, primary lesion formation, tissue maceration, and sporulation (VAN KAN 2006). Several genes and regulation mechanisms have been identified in these stages. Among these are mechanisms found in other plant pathogenic fungi, *Botrytis* specific mechanisms, but also strain specific mechanisms, which may explain the broad host range of this species. There are also mechanisms discussed by which *B. cinerea* is able to adapt on certain host species (CHOQUER *et al*. 2007). Conditions favourable for *Botrytis* infection are warm humid microclimate,

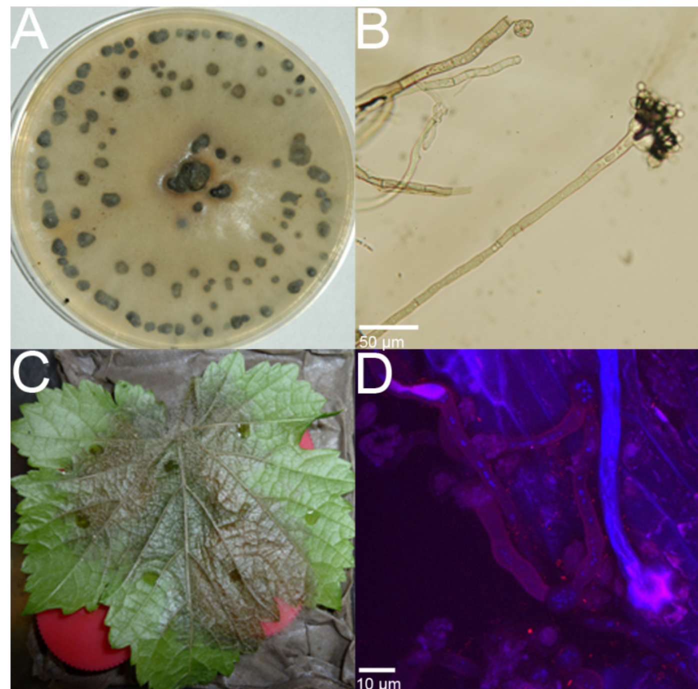

Figure 1-2. *Botrytis cinerea* on an agar plate developing typical sclerotia (A); mycelium and a conidiophore under the light microscope at 250 x magnification (B); grapevine leaf infected by *B. cinerea* (C); DAPI stained mycelium of *B. cinerea* on the surface of a grapevine leaf observed by confocal laser scanning microscopy (D)

frequent rainfalls, and damaged grape berries. Apart from high yield losses due to a disease called gray mould disease or bunch rot caused by *B. cinerea*, affection of berries with this fungus also produces chemical modifications of the must leading to undesired colour and flavour of wine (PEARSON & GOHEEN 1988). However, under special conditions infection of ripe berries by *B. cinerea* may also be desired. This so called noble rot leads to a relative increase of sugar and enables production of special sweet wines (TRIOLI & HOFMANN 2009).

The most important measures against *Botrytis* in viticulture are dedicated to prevention. Preventive measures in controlling *Botrytis* aim to create conditions, in which grape bunches dry out more quickly after rainfalls in order to avoid humidity. This can be

achieved by appropriate trellis system and pruning methods. But also selection of tolerant cultivars, avoiding of excess nitrogen and limiting of planting density are preventive measures (TRIOLI & HOFMANN 2009). In conventional viticulture several fungicides and botryticides are used for *Botrytis* control. They are tested according to European Union guidelines, but responsibility for authorization is up to the countries. In Austria products containing the following agents currently are licensed for control of *Botrytis* in viticulture: cymoxanil, dithianon, boscalid, mepanipyrim, pyrimethanil, cyprodinil, fludioxinil, fenhexamid, iprovalicarb, and folpet (Department for nutrition and agriculture, Austria, 2011; www.landnet.at). Although some of these substances inhibit growth of mycelium (e.g. fludioxonil), they are not effective in advanced infestations. Therefore also these agents have to be applied in a preventive manner and in order to minimize risk of development of resistances frequency and quantity of application is strongly regulated (ROßLENBROICH & STÜBLER 2000). In organic viticulture no specific measures against *Botrytis* are available, because application of chemical fungicides and botryticides is not allowed. Additional to aforementioned preventive measures silicates, equisetum extract, potassium bicarbonate and copper can be used to harden the cuticle of berries (TRIOLI & HOFMANN 2009). Furthermore biological control agents on the basis of beneficial microorganisms are available. The only biological control agent registered so far for viticulture in Austria is Botector® (Bio-Ferm GmbH, Tulln, Austria) based on *Aureobasidium pullulans*. Especially for organic viticulture the need for agents efficiently controlling *Botrytis* is urgent. But also in conventional viticulture alternatives to chemical botryticides, which often are under suspicion to be cancerogenic or toxic to humans, are of interest. Biological control agents may be promising contributions to these concerns and there are various products in development or already on the market (see section 1.4).

1.3 Microorganisms associated with the phyllosphere of grapevine

The aerial part of the plant is known as the phyllosphere. The phyllosphere is defined as the surface and the interior of aerial plant parts, whereas the phylloplane represents the surface of all upper plant parts (NEWTON *et al*. 2010). The latter is characterised by extreme conditions: changes between periods of drought and rainfall, rapid changes of temperature, high UV radiation and low nutrient availability. Microorganisms inhabiting the phylloplane

have developed several strategies to survive under these conditions, e.g. pigmentation, production of extracellular polymers for adhesion, oligotrophy, or mechanisms for draught stress endurance.

Several structures present on leaves provide colonization sites for leaf associated microorganisms (Fig 1-3). Hotspots of microbial colonization are the bases of trichomes, stomata, epidermal cell wall junctions, grooves of veins, depressions in the cuticula, and other specific structures (BEATTIE & LINDOW 1999). Nevertheless some species occur distributed over the whole surface (this work, manuscript 1). Whereas some microorganisms are restricted to the surface of leaves, some establish subsurface and endophytic colonization (SABARATNAM & BEATTIE 2003). Investigation of leaf surface populations is difficult due to spatial and temporal dynamics in population size and structure (COLLINS *et al.* 2003).

The main source of nutrients in the phylloplane are leachates - substances released by the plant tissue. They contain sugars (sucrose, glucose, and fructose), amino acids, organic acids, vitamins, growth regulators, alkaloids and phenols (TUKEY 1970). The amount and the composition of leachates are influenced by many factors, such as leaf age, light intensity, plant nutritional status, temperature, and humidity (TUKEY *et al*. 1957; TUKEY 1970). Utilisation of leachate compounds is an important factor for the coexistence of phylloplane microorganisms. The more similar the nutrient utilisation spectrum of a certain species is with another species the lower is their level of coexistence, and *vice versa*. This process is called nutrient resource partitioning (WILSON & LINDOW 1994).

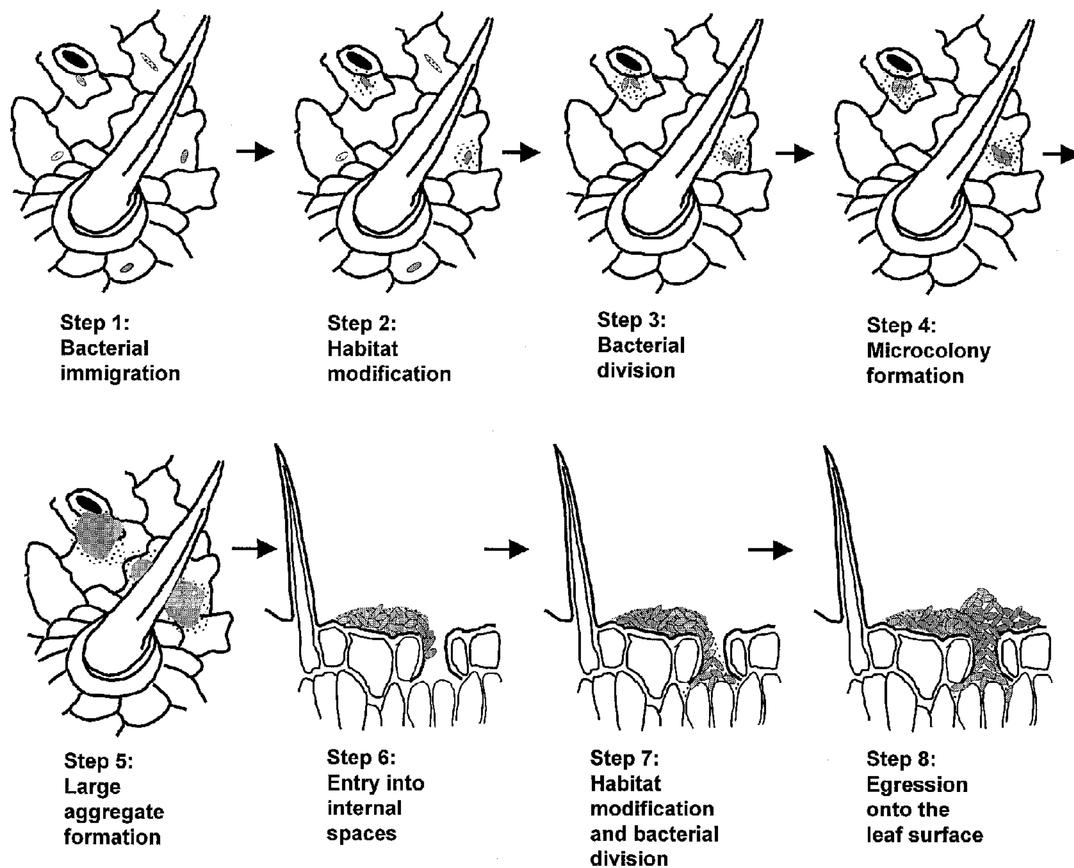

Figure 1-3. Colonization behaviour of leaf associated bacteria at stomata, trichomes and leaf wounds. Source: Beattie & Lindow 1999

1.3.1 Leaf associated microorganisms

Complexity of phylloplane microorganism communities was underestimated for a long time (YANG *et al.* 2001). Today the microbial community is recognised as a dynamic and diverse population of microorganisms, which play important roles for the health of their host plants and are of interest for biotechnology due to their biochemical and physiological traits. Frequently found fungi in the phylloplane are basidiomycetous yeasts like *Cryptococcus* and *Sporidiobolus*, the yeast like fungus *Aureobasidium pullulans* and filamentous fungi like *Aspergillus* (WAHAB 1975; DICKINSON 1982; ANDREWS *et al.* 1994; SPENCER & SPENCER 1997; NAKASE 2000; ANDREWS & BUCK 2002). Among typical phylloplane bacteria are *Bacillus*, *Micrococcus*, *Staphylococcus*, and *Enterobacteriaceae* (RUINEN 1974; DICKINSON 1982; LIN *et al.* 1992). Grapevine phylloplane microbial communities also contain these typical microorganisms. *Aureobasidium pullulans*, *Cladosporium*, *Fusarium*, *Alternaria*, *Cryptococcus*, *Sporidiobolus*, and *Epicoccum* were found on leaves and berries of grapevine

(SCHMID *et al*. 2011). Dominating bacteria were *Bacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea*, *Sphingomonas* and *Pseudomonas* (GRUBE *et al*. 2011). Endophytic colonization patterns of grapevine tissue resemble the phylloplane communities to a certain degree. Fungi found in the endosphere of grapevine are *Aureobasidium*, *Cystofilobasidium*, *Rhodotorula*, *Sporobolomyces*, *Alternaria*, and *Pleospora* (SCHWEIGKOFLER & PRILLINGER 1999). In leaves enterobacteria (especially *Pantoea*), *Pseudomonas*, *Curtobacterium*, *Bacillus* and *Micrococcus* were found using different approaches (BELL *et al*. 1995; BULGARI *et al*. 2009; LO PICCOLO *et al*. 2010). *Xylella fastidiosa* can be found in the xyleme from plants suffering the Pierce´s disease, but also from symptom free plants (HOPKINS 1983).

1.3.2 Grape berry associated microorganisms

An important role in producing wine play berry associated microorganisms as they are present also in the fermentation broth and thus influence fermentation process and product. *Saccharomyces cerevisiae* is the most abundant species at the later stages of the fermentation process and is the main producer of ethanol present in the product. However, it can be found only in minor abundances on grapes. Detectable abundances of *S. cerevisiae* were found in approximately one out of 1000 berries (MORTIMER & POLSINELLI 1999). Other, and more abundant, yeast species present on grapes and thus at the beginning of the fermentation are *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, and *Zygosaccharomyces* (BARNETT *et al*. 1972; DAVENPORT 1974; ROSINI *et al*. 1982; YANAGIDA *et al*. 1992; MARTINI *et al*. 1996; KURTZMANN & FELL 1998). Whereas abundances of these yeasts may increase upon damages of the berries, *Aureobasidium pullulans* is the most dominant species on undamaged grape berries (PRAKITCHAIWATTANA *et al*. 2004). Among filamentous fungi several opportunistic pathogens can be found on undamaged grapes, such as *Botrytis*, *Uncinula*, *Alternaria*, *Plasmopara*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Oidium* and *Cladosporium* (EMMETT *et al*. 1988; FUGELSANG 1997; FLEET 2001). Bacterial species found on grapes belong to the *Lactobacillales*, *Gluconobacter*, *Pseudomonas*, *Burkholderia*, γ-Proteobacteria, and *Bacillus* (RENOUF *et al*. 2007). *Bacillus*, *Pseudomonas*, *Curtobacterium*, and *Streptomyces* could be identified as endophytes of grape berries using gas chromatography of fatty acid methyl esters (WEST *et al*. 2010). As soon as fermentation starts certain microorganisms are enriched. At the beginning of alcoholic

fermentation *Hanseniaspora*, *Torulopsis*, and *Saccharomyces*, as well as lactic acid bacteria (*Pediococcus*, *Leuconostoc*, *Oenococcus*, *and Lactobacillus*) can be found as dominant microorganisms. With the rising of ethanol concentration during fermentation only *S. cerevisiae* is left as dominant microorganism in the fermentation broth (FLEET *et al*. 1984; MILLS *et al*. 2008).

1.3.3 The role of phyllosphere associated microorganisms

Microorganisms residing on plant surfaces are not only of importance for quality of food products. They also play a crucial role for plant health. For ecosystems in general it is established that a higher biodiversity is positively correlated with disease suppression, productivity, and sustainability (TILMAN *et al*. 1996; HOOPER *et al*. 2005; BRUSSAARD *et al*. 2007). This is also shown for biodiversity of soil microbial communities (van BRUGGEN & GRUNWALD 1996; GARBEVA *et al*. 2004). It is shown by Crowder *et al*. that biodiversity indices need not necessarily be a crucial factor for the health status of an ecosystem. Experiments in potato field plots revealed that, with a larger evenness of predators and (insect) pests, biomass production and mortality of pest species increased (CROWDER *et al*. 2010). On the level of microorganisms such holistic approaches are difficult to conduct as total microbial diversity cannot be fully monitored or controlled and interactions between microorganisms are very complex (who are the predators, who are the pathogens?). In the phylloplane nutrient limitation is a limiting factor for many processes, such as germination of fungal spores. Saprophytic bacteria residing on leaves compete for nutrients leached out from plant tissue and spores with germinating spores thus lowering the germination rate of pathogenic fungi (Figure 1-4; BLAKEMAN & FRASER 1971). Plant associated microorganisms are known to confer systemic resistance against phytopathogenic microorganisms to the plant (KUC 1982). Whereas colonisation of roots with certain rhizospheric bacteria leads to induced systemic resistance (ISR) and is mediated through jasmonate or ethylene, systemic acquired resistance (SAR) can be induced locally or systemically on aerial plant parts and is mediated through salicylic acid (WALTERS *et al*. 2005). For example controlled treatment of cucumber with *Colletotrichum lagenarium* led to an increased resistance against this fungus later on (KUC & RICHMOND 1977). Suspensions of *Pantoea agglomerans* restored the protective effect of compost sprays against anthracnose in cucumber (caused by *Colletotrichum orbiculare*) and bacterial speck in *Arabidopsis* (caused by *Pseudomonas syringae*) after autoclaving

(ZHANG *et al*. 1998). Furthermore many microorganisms elaborate antimicrobial substances in order to suppress growth of potential competitors. For example strains of *Pantoea agglomerans* are known to produce antibiotics and bacteriocines – antimicrobial compounds with a very narrow spectrum of activity, mostly towards closely related species (Huang & English 1996; Wright *et al*. 2001). Especially endophytic bacteria can lead to plant growth promotion (JAMES & OLIVARES 1998; BERG *et al*. 2004). In grapevine the plant growth promoting endophytic bacterium *Burkholderia phytofirmans* PsJN is well characterised (COMPANT *et al*. 2008). Phylloplane associated microorganisms also can produce substances, which are used by the plant. Methylotrophic bacteria can positively influence the

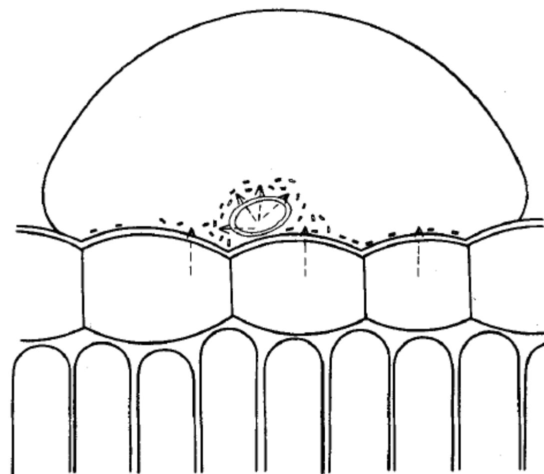

Figure 1-4. Schematic visualisiation of nutrient leaching in a water droplet on a leaf surface. Nutrients are leaching out of a conidia of *Botrytis cinerea* and epidermal plant cells by diffusion. Saprophytic bacteria present consume these nutrients and by this way enhance the leaching effect. This causes higher nutrient loss for the plant, but it also leads to an inhibition of germination of fungal conidia. Source: Blakeman & Fraser 1971

biosynthesis of flavour compounds by contributing precursors of certain compounds (ZABETAKIS 1997). Application of *Methylobacterium extorquens* leads to an increased biosynthesis of furanone in greenhouse trials (VERGINER *et al*. 2010). Thus for the plant it is of advantage to harbour a defined microbial consortium in the phyllosphere.

As leaf leachates are the main nutrient source in the phylloplane, factors influencing the composition of the leachates subsequently will affect the residing microbial community (see page 7). But also anthropogenic factors can influence the phylloplane microbial community. Fungicides and other pesticides affect target and non-target microorganisms. In most cases fungicides reduce microbial abundances in the phylloplane (HISLOP & COX 1969; BAINBRIDGE and DICKINSON 1972; JENKYN & PREW 1973; FOKKEMA *et al*. 1975). Different

management strategies can influence the community qualitatively. For example *Aureobasidium pullulans* was enriched in organically treated grapevine phyllosphere, whereas relative abundance of *Sporidiobolus pararoseus* was reduced compared to conventionally managed plants (SCHMID *et al*. 2011). Phylloplane microbial communities can be influenced by the amendment of straw to the soil (RODGERS-GRAY & SHAW 2001). Maize plants receiving either organic manure or mineral fertilizer differed in their endophytic community structure of type I methanotrophs (SEGHERS *et al*. 2003). Low tillage systems in winter wheat showed a lower incidence of eyespot compared to high tillage systems (BROOKS & DAWSON 1968).

1.4 Biological control of *Botrytis cinerea*

The need for effective protection measures against *Botrytis* especially in organic viticulture, concerns about safety of conventional botryticides with respect to human and environmental health, and problems with development of resistances made evident the need for alternatives. Biological control, the application of plant beneficial microorganisms in order to control diseases and promote plant health, is a promising alternative. Mechanisms, by which biological control agents act, include promotion of plant growth, making available of nutrients (e.g. phosphate), induction of systemic resistance of the plant, and antagonism towards plant pathogens (BERG 2009). Antagonistic activity of microorganisms can be elaborated by the production of antimicrobial compounds (e.g. antibiotics, cell wall degrading enzymes), parasitism, occupation of colonisation sites, and competition for nutrients. Many microorganisms effective against *Botrytis in vitro* have been found, several of them were tested under field conditions, and some of them were developed into products. A survey about current products and promising antagonists is given by ELMER & REGLINSKI (2006). Examples for products developed for grapevine are Botector® on the basis of *Aureobasidium pullulans*, Sonata® (*Bacillus pumilus*), Biocure® (*Candida saitoana*), Shemer® (*Metschnikowia fructicola*), Saccharopulvin® (*Saccharomyces chevalieri*), Sentinel® (*Trichoderma atroviride*), and Botry-Zen® (*Ulocladium oudemansii*). A frequently observed mechanism of action against *Botrytis* is the competition for nutrients and space, as described for *Pseudomonas fluorescens*, *P. syringae*, *Candida oleophila*, *Pichia guillermondii*, *Rhodotorula glutinis*, and *Sporobolomyces roseus* (WISNIEWSKI *et al*. 1991; MERCIER & WILSON 1994; FILONOW 1998; LIMA *et al*. 1998; ELAD & STEWART 2004). Several *Bacillus* strains induce

systemic resistance in the host plant (FERREIRA 1990). *Brevibacillus* and *Bacillus subtilis* are known to produce inhibitory compounds, and *Pichia membranifaciens* acts via killer toxins (ELLIS 1996; SANTOS & MARQUINA 2004; TOURÉ *et al*. 2004). Mycoparasitism is the mechanism of action in *Pythium* and *Trichoderma* (DUBOS *et al*. 1982; PAPAVIZAS 1985; PAUL 2004). Probably many organisms do not suppress *Botrytis* via a single mechanism, but rather via cooperation of several mechanisms. For example *Pantoea ananatis* reduced disease incidence in field trials by producing antimicrobial substances, efficient colonization of plant surfaces and competition for nutrients (this work, manuscript I). *Aureobasidium pullulans* has good wound colonisation traits, induces systemic resistance, competes for nutrients and synthesizes aureobasidins (CASTORIA *et al*. 2001). Biocontrol agents against *Botrytis* need not necessarily be applied on the aerial parts of the plant. A strain of *Pseudomonas aeruginosa* was able to confer induced systemic resistance against *Botrytis* to bean plants when colonizing the rhizosphere (DE MEYER & HÖFTE 1997).

1.5 Organic Farming *versus* conventional farming in viticulture

Organically cultivated areas in viticulture increased from 43000 ha in 1998 to 95000 ha in 2006 (WILLER 2008). This reflects the demand for more sustainable production methods. For plant protection in organic viticulture the application of copper, sulphur, plant extracts, mineral and paraffin oil, potassium bicarbonate, silicates, biocontrol agents and other natural substances is allowed (TRIOLI & HOFMANN 2009). Especially the application of copper, which is much more extensive in organic than in conventional management, over a longer period leads to soil contamination endangering plant health, fruit quality, and ground water (PIETRZAK & MCPHAIL 2004). In some vineyards in France, which show a long history of treatment with Bordeaux mixture, soil copper concentrations are elevated up to 500 mg/kg. In comparison to that, normal agricultural soil has concentrations of 5-30 mg/kg copper (BRUN *et al*. 1998). To face this problem modern copper based fungicides contain copper salts of lower solubility than copper sulphate, like for example Cu_2O, $Cu(OH)_2$ and $CuCl_2 \cdot 3Cu(OH)_2$ (RICHARDSON 1997). Furthermore regulations regarding copper applications are getting more vigorously with the aim to reduce the amount of copper brought into the soil (European Union Council Regulation (EEC) 2092/91, Annex II). Biological control measures based on beneficial microorganisms are promising alternatives to copper based compounds.

Whether organic farming benefits ecological diversity and ecosystem health has been under investigation in many studies. As mentioned in section 1.3.3. A higher biodiversity is regarded commonly as an indication for high ecosystem health and therefore higher resistance towards pathogen outbreaks. A survey conducted by Hole *et al*. showed that the diversity of non-crop plants, earthworms, and insects is higher in organic plots than in conventional plots (HOLE *et al*. 2005). Several studies have compared soil microbial communities of organically and conventionally managed fields. Comparisons of soil biological parameters from farms differing in management system and other factors often led to the conclusion that type of soil has the most significant influence on microbial activity/biomass, whereas influence of management type only had a little effect (BOSSIO *et al*. 1998; GIRVAN *et al*. 2003). But results from long term comparative studies suggest significant differences in microbial biomass, microbial enzyme activity, and biodiversity in favour to organic practices, mostly due to organic manure treatment (GUNAPALA & SCOW 1998; MÄDER *et al*. 2002; ESPERSCHÜTZ *et al*. 2007; FLIEßBACH *et al*. 2007). A higher species richness of arbuscular mycorrhizal fungi (AMF) was found in organically managed fields in a comparison of 13 pairs of organically and conventionally managed fields (VERBRUGGEN *et al*. 2010). Studies investigating the influence of farming practices on the phyllosphere microbial communities are scarce. Significant differences in the bacterial community of apple phyllosphere were found between organically and conventionally managed plants based on cultivation and 16S rRNA gene clone libraries (WAIPARA *et al*. 2002; OTTESEN 2009). Fungal colonisation of stored apples differed among conventional and organic production (GRANADO *et al*. 2008). A study comparing species richness, community composition and abundance of plants, spiders and grasshoppers in Swiss vineyards did not find differences between organically and conventionally managed vineyards (BRUGGISSER *et al*. 2010).

With the focus on insect pests it was shown that organically managed potato fields had a higher evenness of species in general, as well as a higher evenness of pest predators and of pathogens (CROWDER *et al*. 2010). This shows that functions of organisms, whose abundances are changed by certain treatments, are important to consider when doing ecological studies. For example it was shown for grapevine that upon organic management *Aureobasidium pullulans* was enriched. Isolates belonging to this species had antagonistic activity against *Botrytis cinerea in vitro*, which also could affect the overall indigenous resistance of organically managed grapevine plants against *Botrytis* (SCHMID *et al*. 2011).

2 References

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3 Essences of the Doctoral Thesis

I. Populations of antagonistic microorganisms differ between organically and conventionally managed grapes. Microorganisms with antagonistic activity against *Botrytis cinerea* were differently abundant and distributed throughout different phyllosphere microhabitats between organically and conventionally managed grapes of the same location (Publication 1).

II. Abundances of certain fungal species are altered in the phyllosphere of organically managed grapes. Fungal community structures assessed by genetic community fingerprints were significantly different between organically and conventionally managed grapes. Abundances of *Aureobasidium pullulans* and *Epicoccum nigrum* were elevated in organic samples, whereas abundances of *Sporidiobolus pararoseus* were elevated in conventional samples. High amounts of copper and sulphur applied in the organic plot may contribute to these differences (Publication 2 and Manuscript 2).

III. Organic farming measures and elevated abundance of *A. pullulans* did not alter the communities of *Bacillus* and *Pseudomonas*. In organic samples with high abundances of *A. pullulans* genetic microbial fingerprints of *Bacillus* and *Pseudomonas* did not differ from conventional samples. As though isolates of *A. pullulans* inhibited growth of *Bacillus* isolates from the same habitat, no difference in *Bacillus* communities was observed between organic and conventional samples (Publication 3).

IV. The phyllosphere colonizing strain *Pantoea ananatis* BLBT1-08 is efficient in controlling *Botrytis cinerea* infection *ad planta* and in the field. *P. ananatis* BLBT1-08 reduced statistically significantly disease incidence of *B. cinerea* on grapes in a three year field trial. This strain is efficient in colonizing the leaf surface, has amino acid dependent antimicrobial activity *in vitro*, and does not inhibit conidia germination. Occupation of infection sites, competition for nutrients and inhibition of growth of mycelium contribute to its mechanisms of biocontrol activity towards *B. cinerea* (Manuscript 1).

4 Appendix

Publication 1 .... p. 24

Grapevine-associated microorganisms: Antagonistic potential towards *Botrytis cinerea* varies between habitats, cultivation methods and grapevine species.

Schmid F and Berg G (2009) *IOBC/WPRS Bulletin* **43**: 354-451

Publication 2 .... p. 28

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

Schmid F, Moser G, Müller H, Berg G (2011) *Applied and Environmental Microbiology* **77**: 2188-2191

Publication 3 .... p. 35

Black fungi and associated bacterial communities in the phyllosphere of grapevine.

Grube M, Schmid F, Berg G (2011) *Fungal Biology*. In Press
doi:10.1016/j.funbio.2011.04.004

Manuscript 1 .... p. 48

Successful introduction of *Pantoea ananatis* in the phyllosphere of grapevine to control grey mould disease caused by *Botrytis cinerea*.

Schmid F, Cardinale M, Schildberger B, Berg G

Manuscript 2 .... p.68

Organic versus conventional agriculture: A Review from a microorganism's point of view.

Schmid F and Berg G

I. Publication 1

Grapevine-associated microorganisms: Antagonistic potential towards *Botrytis cinerea* varies between habitats, cultivation methods and grapevine species

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***Botrytis cinerea* is a serious fungal pathogen of grapevine all over the world. To develop effective strategies in biological control it is important to understand the microbial ecology of grapevine. Dual culture plate assays were conducted to find out the distributions of the antagonistic potential in different habitats and different plant varieties. In this study, the abundances of antagonists in cultivated grapevine, organically and conventionally treated, were compared to wild varieties of *Vitis vinifera sylvestris* and *V. riparia*. Significant differences of the occurrence and composition of bacterial and fungal antagonists between the different habitats and plant varieties were found.**

Key words: biological control, *Vitis*, fungal and bacterial antagonists

The ascomycetous fungus *Botrytis cinerea* Pers. is on the one hand widely used in the production of sweet wines but on the other hand comprises a dangerous pathogen of grapevine correlated with high losses in harvest. Due to the problem of resistances and for ecological reasons, the interest in alternatives to chemical pesticides rises. Throughout the decades, a lot of microorganisms with activity against this pathogen have been isolated and characterised (rev. in Elmer and Reglinski, 2006) and some also have been shown beneficial effects on plants (Barka et al., 2002). The use of naturally occurring microorganisms is a safe, environmentally friendly and effective way to control microbial pathogens (Whipps, 2001). In order to maintain an ecologically healthy and therefore robust microbial flora in agricultural systems, studies on biological control aim to find native microorganisms with antagonistic potential against certain pathogens (Zachow et al., 2007). Thus, it is important to understand the role and occurrence of microorganisms present in a habitat to gain the maximum effect of biocontrol organisms. The aim of this study was to get an overview of the occurrence of antagonistic microorganisms in different habitats of grapevine.

MATERIAL AND METHODS

Isolation of bacteria, yeasts and fungi. Wild varieties of *Vitis vinifera sylvestris* and *Vitis riparia* were sampled both in October 2007 in the botanical garden of the Karl-Franzens University, Graz. Latter is a clone of an individuum grown in the Danube floodplain forest around Marchegg, former originating from Grosseto, Toskana. Samples were taken 2006 and 2007 from Sauvignon Blanc from Schlossberg, Leutschach, Austria in the last week before harvest

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(October and September respectively). Sampling was carried out by pooling samples from 4 plants. Four independent replicates were investigated. For isolation of microorganisms from stem and leave ectosphere, a certain amount of the sample was treated in a Stomacher bag with 0.85% NaCl according to Berg et al. (2002). Serial dilutions were plated out on R2A (Roth)-, Sabouraud-Dextrose (Roth)-, and SNA-agar for determination of CFU and isolation of bacteria, yeasts and fungi respectively. Endophytic microorganisms were isolated by crushing plant material in 0.85% NaCl after surface sterilisation with 4% NaOCl for 5min and washing in 50 ml H2O three times. Grapes were crushed as a whole under sterile conditions in 1x PBS. Serial dilutions of endosphere and grape suspensions plated out as described above. Single isolates for antagonism assays were selected randomly from the respective Petri dishes.

Dual culture plate assays. Antagonistic potential towards *Botrytis cinerea* PERS. (DSM 5145) of single isolates was tested using a dual culture plate assay. Four 5 mm diameter agar plugs covered with mycelium of *B. cinerea* were placed in equal distances to each other on a fresh PDA plate (Roth). Material of 4 test isolates per plate was stroked out in between these *Botrytis* plugs. Plates were incubated for 3-4 days at 25°C. Isolates were identified as antagonists, when an inhibition zone was formed around the growing area of the test strain. When testing fungal isolates, only one *Botrytis* plug was placed in the centre of the PDA plate and 4 agar plugs covered with the test strains were placed in the surrounding of the *Botrytis* plug.

RESULTS AND DISCUSSION

Abundances of microorganisms. Abundances of bacteria, yeasts and fungi in the different habitats (grapes, ectosphere of stems and leaves, endosphere of stems and leaves) were analysed in cultivated Sauvignon Blanc (conventionally and organically treated) and wild varieties of grapevine (*Vitis vinifera sylvestris* and *Vitis riparia*) (see Table 1). In all cases the ectosphere microbial population exceeded the endophytic communities by approximately 10^2 to 10^3 cfu/g freshweight, which fits well with values found in literature (Bell et al., 1995). Significantly more microorganisms could be found on leaves of organically treated Sauvignon Blanc in comparison to conventionally treated grapevine. On the other hand, the bacterial and yeast flora of grapes was to be found richer in conventionally treated Sauvignon Blanc than in organically treated one and also richer than in the wild variety of grapevine. Interestingly, more endophytes could be found in organically treated grapevine compared to all the other plants. The reason for this could be the fact that leaves of organically treated grapevine have a higher exposure to fungi (and also pathogens) than conventionally treated and also wild variety grapevine. This leads to a lower rate of assimilation, and therefore, production of fresh leaves and stems is aborted whereas plant growth is continued in the other plant varieties (Thünauer, pers. communication).

Table 1. Abundances of microorganisms colonising grapevine. Comparison of different species and cultivation methods taking into account the cell numbers of microorganisms present in different parts of the plant (numbers in cfu/g fresh weight).

Plant	Organisms	Grape	Stem ectosphere	Leave ectosphere	Stem endosphere	Leave endosphere
Vitis vinifera sylvestris (wild variety)						
	Bacteria	$2x10^4$	$2x10^5$	$4x10^5$	$<10^2$	$2x10^2$
	Yeasts	$5x10^3$	$2x10^4$	$9x10^4$	$<10^2$	$<10^2$
	Fungi	$9x10^3$	6x103	$1x10^4$	$<10^2$	$2x10^2$
Vitis riparia (wild variety)						
	Bacteria	*)	$5x10^5$	$5x10^5$	$2x10^2$	$2x10^2$
	Yeasts	*)	$4x10^4$	$5x10^4$	$<10^2$	$<10^2$
	Fungi	*)	$1x10^4$	$3x10^4$	$<10^2$	$<10^2$
Sauvignon Blanc organically treated						
	Bacteria	$1x10^4$	$2x10^5$	$5x10^5$	$3x10^3$	$2x10^3$
	Yeasts	$9x10^3$	$6x10^4$	$2x10^5$	$8x10^2$	$5x10^2$
	Fungi	$7x10^3$	$1x10^4$	$2x10^5$	$5x10^2$	$6x10^2$
Sauvignon Blanc conventionally treated						
	Bacteria	$1x10^5$	$1x10^5$	$7x10^4$	$5x10^2$	$<10^2$
	Yeasts	$5x10^4$	$3x10^4$	$4x10^4$	$3x10^2$	$<10^2$
	Fungi	$7x10^3$	$8x10^3$	$1x10^4$	$<10^2$	$<10^2$

*) No grapes available as the wild variety of *Vitis* are dioecious plants and in this case pollination was not possible

Distribution of antagonists. Using dual culture plate assays, the antagonistic activity of approximately 3600 isolates against *Botrytis cinerea* was determined. For each habitat and each plant variety 72 bacterial and yeast strains and 60 fungal strains were tested. From endosphere habitats only 50 strains of each group of organisms were tested.

Yeasts play a major role as antagonists throughout all habitats in organically treated Sauvignon Blanc (see Figure 1). In contrast, in conventionally treated Sauvignon Blanc yeasts are only dominant as antagonists in grape berries. Furthermore, in the latter variety fungi on leaves and bacteria on grape berries show a high proportion of antagonists.

Interestingly, the overall amount of antagonists found in the wild type variety *Vitis vinifera sylvestris* was significantly lower than in organically and conventionally treated cultivated grapevine (5% versus 10% and 9% respectively). The lower exposure to *Botrytis* and probably other fungal pathogens in the natural habitat can be one explanation for this observation. As also to be seen from Figure 1, the majority of antagonists can be found among bacteria and fungi in both, *V. vinifera sylvestris* and *V. riparia*.

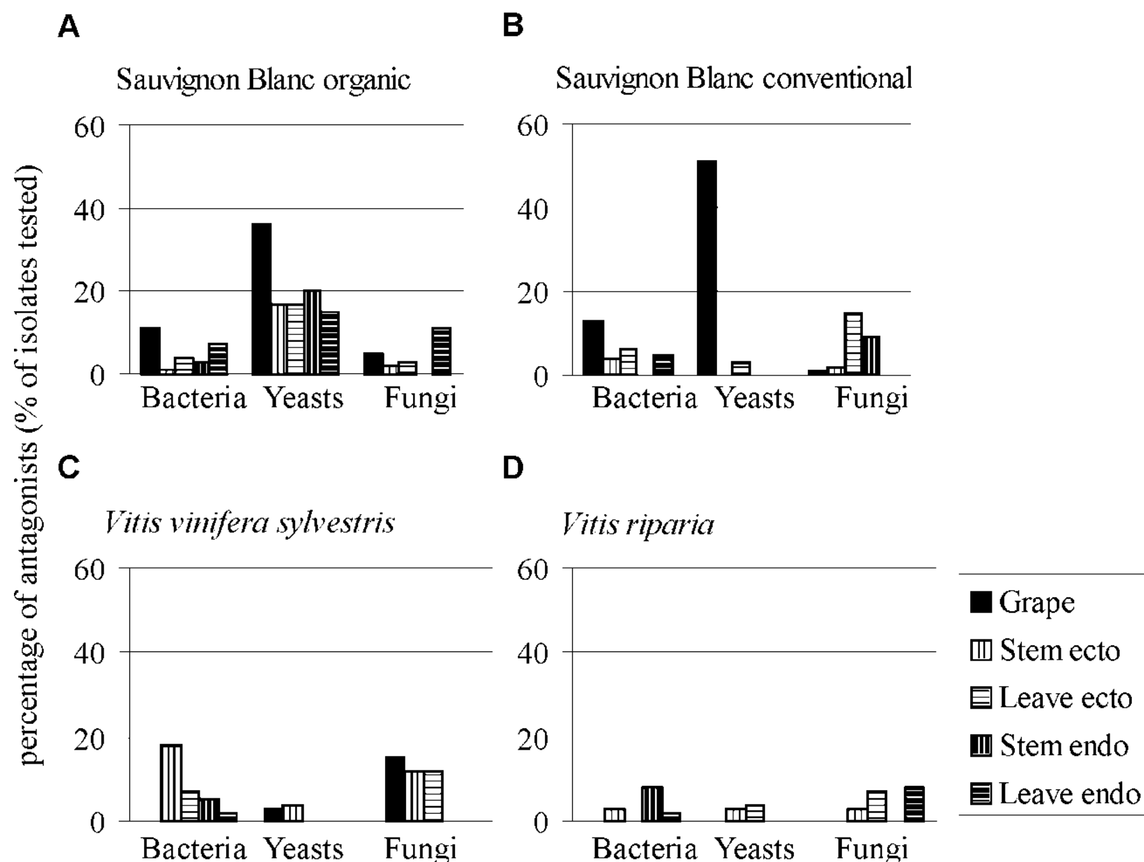

Figure 1. Distributions of *in vitro* antagonists in the different habitats of the examined species. Dual culture plate assays were conducted to define the percentage of antagonists against *B. cinerea* in the different habitats and plant variations.

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II. Publication 2

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

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Statistically significant differences in the structure and function of above-ground grapevine-associated microorganisms from organically and conventionally managed vineyards were found. *Aureobasidium pullulans*, a copper-detoxifying fungus and biocontrol agent, plays a key role in explaining these differences. The black fungus was strongly enriched in the communities of organically managed plants and yielded a higher indigenous antiphytopathogenic potential.

Contemporary farming systems undermine the wellbeing of communities in many ways. Huge regions of natural habitats, including their ecosystem services, have been destroyed; plant protection measures have caused human health problems, and they are responsible for about 30% of greenhouse gas emissions (15). An alternative to conventional agriculture is offered by organic farming, which aims to minimize its impact on the environment by using crop rotation, pathogen-resistant cultivars, limited amounts of chemical pesticides, and organic manure instead of synthetic fertilizers. However, the beneficial effect of organic agriculture on biodiversity in general and plant-associated microorganisms in particular is still controversial (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 of grapevine plants worldwide (FAOSTAT, 2008), the proportion of organically cultivated grapes is strongly increasing (19). Although it was shown that the taste and quality of organically produced wine are better than those of wine produced by 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 the 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 hypothesize that the type of plant protection used in conventional and organic vineyards has a great impact on the microbial, and especially the fungal, community structure associated with grapevine plants.

To compare epiphytic and endophytic microbial communities of the phyllosphere of conventionally and organically managed grapevines, we sampled leaves, shoots, and undamaged grapes of the cultivar Sauvignon Blanc in vineyards in Schlossberg, Austria (46°37' N, 15°28' E; owned by the Fachschule für Weinbau und Kellerwirtschaft

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und Weingut Silberberg), in the last week before the harvest (on 3 October 2006 and 11 September 2007, respectively). Half of the vineyards were managed conventionally, and the other half were managed organically. The chemical plant protection agents used in the conventional plots were sulfur, paraffin oil, manganese-zinc ethylene bis (dithiocarbamate), proquinazid, iprovalicarb, folpet, pyrimethanil, mandipropamid, quinoxyfen, chlorpyrifosmethyl, boscalid, and cyazofamid. The chemicals used in the organic parcel were sulfur, 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 analyses.

To perform molecular analysis, total DNA was extracted from samples using the Fast DNA Spin Kit for Soil (Qbiogene, Inc., Carlsbad, CA). Microbial fingerprinting was performed by single-strand conformation polymorphism analysis (SSCP) according to Schwieger and Tebbe (16) with different primers (16, 18). Gels were analyzed with the program GelCompare (Applied Maths, Kortrijk, Belgium) and statistically assessed (11). Gel slices containing single bands were analyzed (16). To quantify *Aureobasidium pullulans*, primers ApuIIF1 (5'-GATCATTAAAGAGTAAGGGTGCTCA-3') and ApuIIR1 (5'-GCTCGCCTGGGACGAATC-3'), both developed by the National Exposure Research Laboratory (Cincinnati, OH) were used. For quantification of Sporidiobolus pararoseus, primers Spa2f (5'-CCAATCTTTTCTTGTAATCG-3') and Spa2r (5'–CCTTAATGAAGTTGGCTC-3') were designed as described in the supplemental material. Primers ITS1 and ITS2 (18) were used for quantification of total fungal internal transcribed spacer 1 (ITS1) copies. The calculated copy number was corrected by PCR efficiency in a sample matrix, which was determined by measurement of serial dilutions of standard fragments in a DNase I-digested sample matrix. Each replicate was analyzed three times. Significances of differences between conventional and organic treatments were calculated by using the unpaired Student t test. Melting curves of Spa2f/Spa2r and ApuIIF1/ApuIIR1 reactions resulted in one homogeneous 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 each replicate 18 yeast and 15 filamentous fungal isolates were selected randomly to test their antagonistic activity (1).

Fingerprints from the fungal community were obtained by ITS PCR and SSCP, and their statistical analysis resulted in a clear separation of samples from the conventional and organic management techniques (Fig. 1). P values showing statistically significant differences between organic and conventional samples were < 0.001, both for ectosphere samples and grape berries (Fig. 1A) and for endosphere samples (Fig. 1B). Altogether, the fingerprints from organically managed plants were more homogeneous and showed a higher similarity to each other than those from conventionally managed plants. From SSCP gels, differing and dominant bands were identified by sequencing. The strongest band (Fig. 1A and B) in samples from the conventional treatment corresponded to *S. pararoseus*, a yeast already described as associated with grapes (12, 20). Conversely, bands for *A. pullulans* were stronger from organic treatment samples. *A. pullulans* is a cosmopolitan

yeastlike (black) fungus and is ubiquitously associated with plants, including grapevines (5, 8). *Cladosporium* sp. and *Alternaria tenuissima* were common species found in all of our samples.

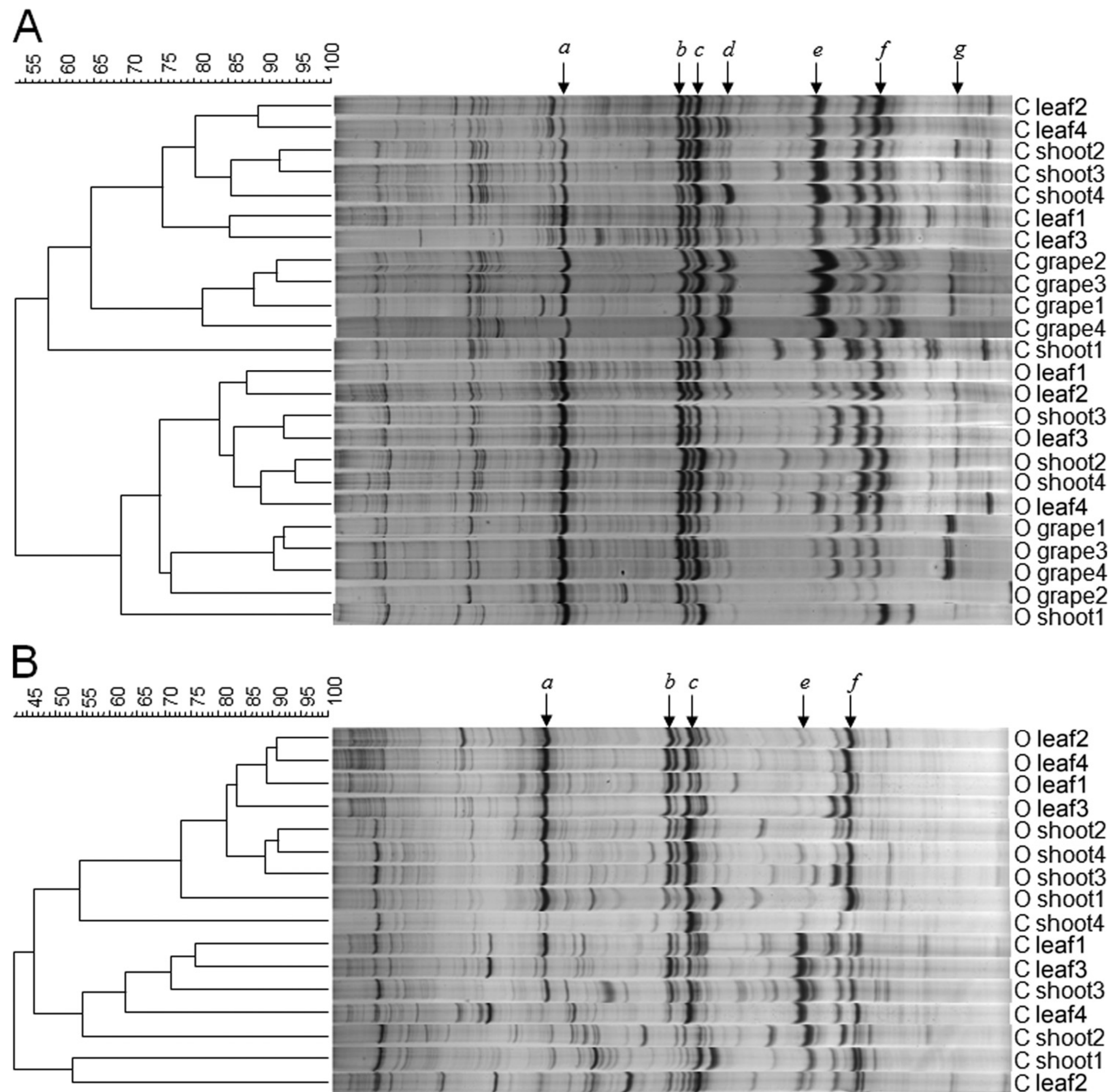

FIG. 1. Clustering of the SSCP profile of the fungal community of grapevine plants showing a clear difference between conventionally (C) and organically (O) managed plants. Samples from shoots, leaves, and grapes were taken 1 week prior to harvest. Community DNA was extracted from the respective plant parts, followed by amplification of the fungal ITS region using general primers. The PCR product was subjected to SSCP analysis. Band patterns were compared and clustered by the unweighted-pair group method using average linkages. Sequences of single bands were obtained and compared to those in the GenBank database using the BLAST algorithm, giving hits for *A. pullulans* (*a*), *Cladosporium* sp. (*b*), *A. tenuissima* (*c*), *Alternaria* sp. (*d*), *S. pararoseus* (*e*), *Epicoccum nigrum* (*f*), and *Cryptococcus flavescens* (*g*). (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 accessio numbers FN430614 to FN430640. Data shown are for samples from 2007.

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DNA from a different *Alternaria* that could not be identified to the species level was detected in ectosphere and to a greater extent in grape samples only from conventionally managed plants.

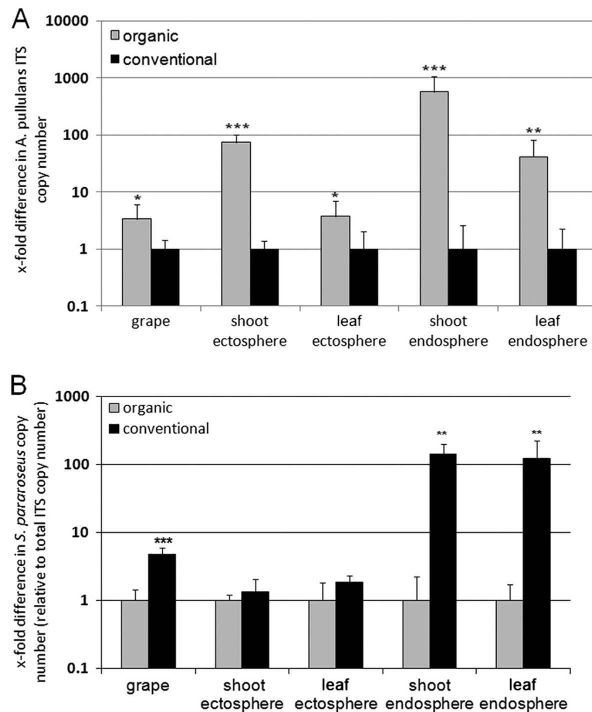

FIG. 2. ITS copy numbers per gram of fresh weight determined by quantitative PCR. (A) Specific primers were used for determination of *A. pullulans* ITS1 copy numbers in samples from organically and conventionally managed plants. (B) ITS2 copy numbers of *S. pararoseus* determined using specific primers relative to total fungal ITS copy numbers determined with 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 deviations, and asterisks indicate significances of differences with respect to management type (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Data shown are for samples from 2007.

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, the fungal ITS copy number was higher in organic than in conventional agriculture samples (data not shown). The relative quantification of *A. pullulans* ITS copy numbers showed a higher abundance of this species in organically than in 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). Significantly larger relative amounts of *S. pararoseus* DNA were present in the grapes and endosphere samples of conventionally managed plants (Fig. 2B). A tendency toward higher *S. pararoseus* ITS copy numbers was seen in the ectosphere samples of conventionally managed plants. Results were confirmed by analyzing samples from 2006 (data not shown).

Above-ground microhabitats of grapes were highly colonized by microorganisms; up to 5.7 log10 CFU were calculated for bacteria, 5.2 log10 CFU were calculated for yeasts, and 5.3 log10 CFU were calculated for fungi. Statistically significant differences in CFU counts 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 antiphytopathogenic potential of fungal isolates, estimated by *in vitro* antagonism toward *Botrytis cinerea* (1), on organically managed plants was greater than that on 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 a higher abundance in samples from organically managed plants than in those from conventionally treated plants (Fig. 3B). All of our results support a key role for the yeastlike fungus *A. pullulans* in explaining the structural and functional differences between the two agricultural systems. Interestingly, *Aureobasidium* can

utilize inorganic sulfur (10) and is able to absorb, and in 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 wellstudied potent antagonist of 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 flavor components of wine were detected as being produced by *A. pullulans* (17), which can explain the better taste of organically produced wine (20).

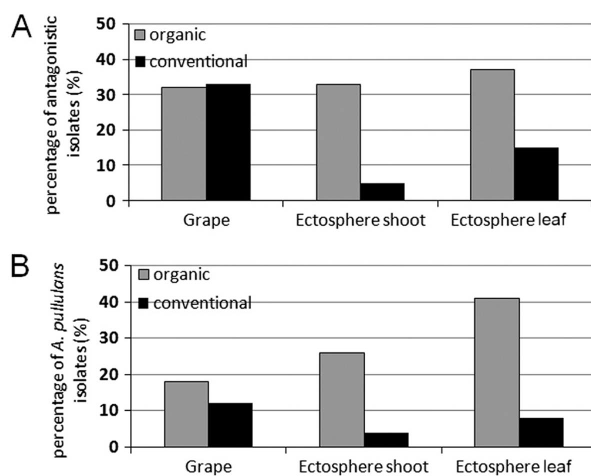

FIG. 3. Correlation of the antagonistic potential of different plant parts and plant protection management systems with the relative abundance of *A. pullulans*. Yeasts and filamentous fungi were isolated from samples of organically (gray bars) and conventionally (black bars) managed Sauvignon Blanc grapevines. (A) Antagonistic potential of isolates assayed by using dual-culture plate assays determining the percentage of isolates with antagonistic activity against *B. cinerea*. (B) Percentage of *A. pullulans* isolates among isolates determined by morphological characterization. Each value was calculated from a total of 132 isolates (72 yeast and 60 filamentous fungal isolates). Data are for samples from 2007.

In addition, two well-known dominant groups of plant-associated bacteria were analyzed to compare bacterial fingerprints: *Pseudomonas* and *Firmicutes* (1, 3). Although cultivable cell numbers of bacteria differed between the two treatments, no significant difference between the community profiles of the two bacterial groups was found. 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 by the fact that mainly antifungal substances are used for plant protection in both management systems.

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 but also the function of the fungal community was affected. 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 not direct evidence for a biocontrol effect on grapes, there are hints at this beneficial interaction. *A. pullulans* was described as a potent *ad planta* antagonist (5), and biocontrol products against *Botrytis* based on this fungus are already on the market (Botector; bio-ferm, Tulln, Austria). Biological control comprises the application of naturally occurring antagonists as biocontrol agents, as well as management of the indigenous antagonistic potential (3). Our study was an unexpected but interesting example of the latter and showed that basic knowledge of the structure and function of plant-associated microbial communities is essential for the development of environmentally friendly strategies for plant protection.

Nucleotide sequence accession numbers. The sequences obtained in this study were submitted to EMBL and assigned accession no. FN430614 to FN430640.

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SUPPLEMENTAL MATERIAL

S1 Firmicutes specific primers from this study were selected upon an alignment containing sequences of different *Bacillus* species (AL009126.3, NC_009725.1, NC_005945.1, NC_004722.1, NC_006582.1, NC_002570.2, NC_006322.1, NC_009848.1, NC_005957.1, NC_010184.1), *Staphylococcus aureus* (NC_002745.2), *Lactobacillus plantarum* (NC_004567.1), *Clostridium difficile* (NC_009089.1), *Acholeplasma laidlawii* (NC_010163.1), *Streptomyces cattleya* (AB045871.1), *Escherichia coli* (U00096.2), and *Vitis vinifera* chloroplast (NC_007957.1) from GenBank database. Primers Bspez3f and Bspez6f were selected due to their exclusivity for chloroplast sequences and their specificity for Firmicutes sequences (verified by BLAST search using the query Bacteria [ORGN] NOT Firmicutes [ORGN] in the NCBI genomes database).

Primer pair Spa2f – Spa2r was designed upon an alignment of *Sporidiobolus pararoseus* sequences AF417115.1, FJ999721.1, AB030338.1, AF444604.1, AY015429.1, FJ755245.1, EU409803.1, FJ758388.1, EF505797.1, EF505785.1, EU002958.1, sequences from other *Sporidiobolus* species (EF592111.1, AB030337.1, FJ515195.1, EU276010.1, EF592138,.1, AB073255.1) and from relative genera (AB073230.1, FJ515206.1, AB073255.1, AF444510.1, FJ345357.1, AY069991.1, FJ591128.1, AY070005.1, DQ640071.1). Sequences with specificity for *S. pararoseus*, which did not match sequences from other *Sporidiobolus* species or relative genera were selected for the primer pair. BLAST analysis showed a high degree of specificity for *S. pararoseus*, but also sequences from *Sporobolomyces* species were found among the hits (*S. ruberrimus*, *S. phaffi*, *S. patagonicus*).

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III. Publication 3

Black fungi and associated bacterial communities in the phyllosphere of grapevine

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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.

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] and [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-

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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 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.

MATERIAL AND 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′-TACGGYTACCTTGTTACGACTT) (Lane 1991) in a reaction containing 1 x 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 x Taq-&GoTM Mastermix (Qbiogene) with 0.5 µM of each primer, 0.5 x 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 x 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).

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 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 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.

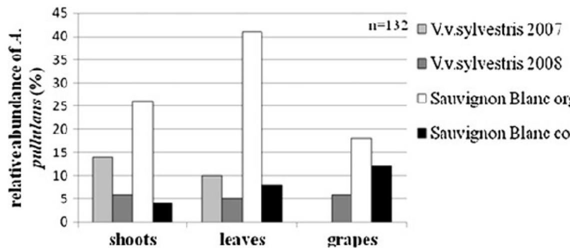

Fig 1 - Abundance of *A. pullulans* relative to total amount of fungal isolates. Fungal isolates were selected randomly from isolation plates and relative abundance of *A. pullulans* was assessed upon morphological characterisation.

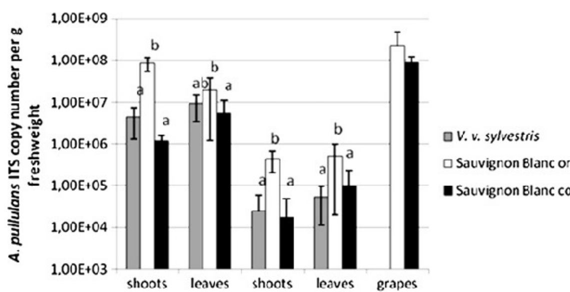

Fig 2 - Abundance of *A. pullulans* ITS copy numbers determined by quantitative PCR in samples of shoots, leaves shoots endosphere, leaves, endosphere, and grapes from *Vitis vinifera* subsp. *sylvestris* (sampling 2007), and organically and conventionally managed Sauvignon Blanc. No grape samples were 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.

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 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.

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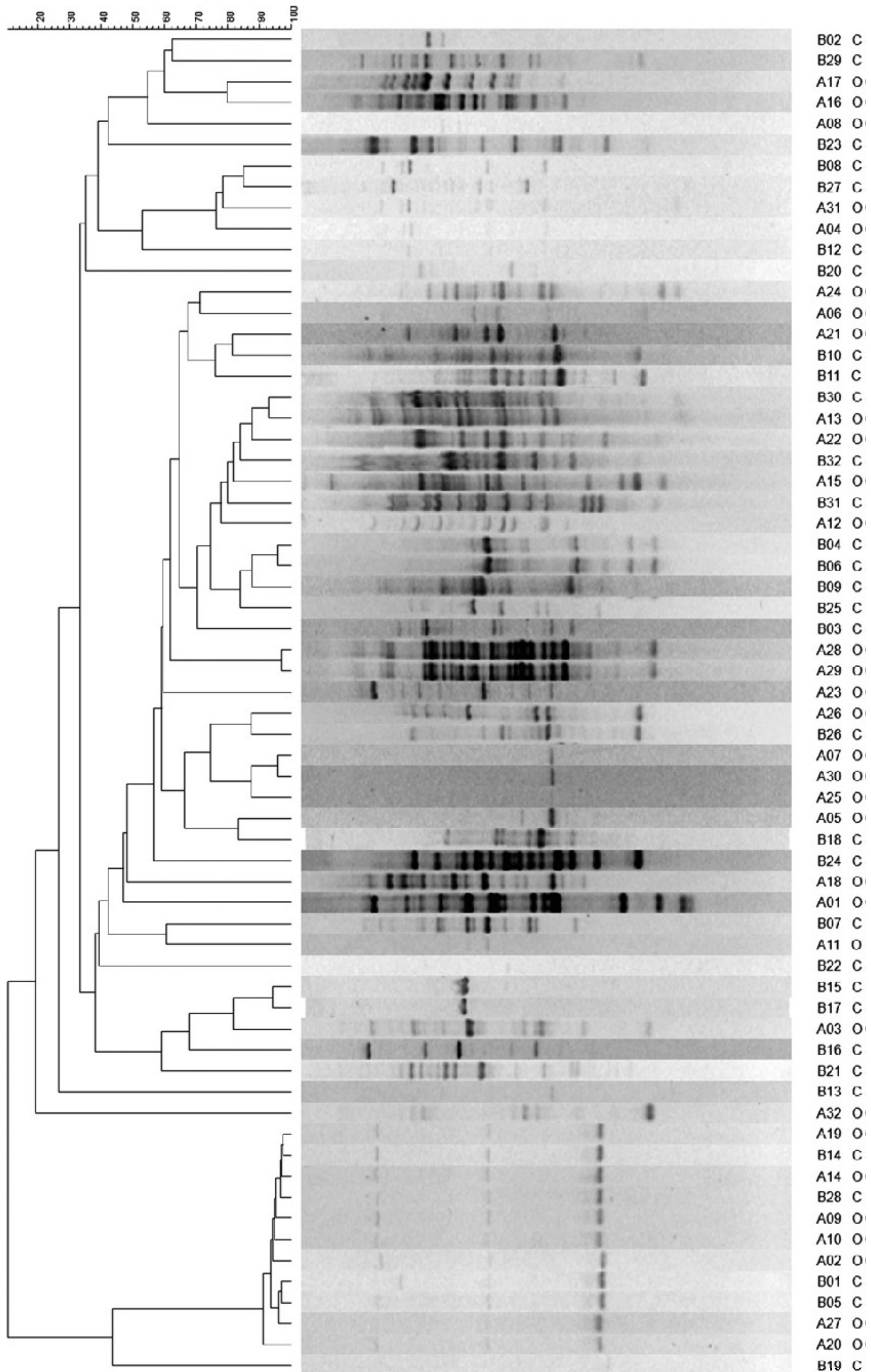

Fig 3 - Genetic fingerprints of bacterial isolates associated with tissues from organically (O) and conventionally (C) managed grapevine. Whole genomic DNA from isolates was extracted and used as template for BOX-PCR. Fragments were separated on agarose gel and clusters were calculated from band patterns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

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[1]	species[2]	inhibition by *A. pullulans*[3]	antagonistic against *A. pullulans*[4]	origin[5]	EMBL Acc. number
A05	*Staphylococcus sp.*	-	-	O	FR717258
A06	*Micrococcus sp.*	-	-	O	FR717259
A07	*Staphylococcus sp.*	-	-	O	FR717260
A12	*Bacillus sp.*	+	-	O	FR717261
A15	*Sphingomonas sp.*	-	-	O	FR717262
A22	*Sphingomonas sp.*	-	-	O	FR717263
A28	*Pseudomonas sp.*	-	+	O	FR717264
A31	*Bacillus weihenstephanensis*	+	-	O	FR717265
A32	*Micrococcus sp.*	-	-	O	FR717266
B06	*Frigoribacterium sp.*	-	-	C	FR717267
B11	*Pantoea agglomerans*	-	-	C	FR717268
B16	*Bacillus sp.*	+	-	C	FR717269
B19	*Staphylococcus epidermidis*	-	-	C	FR717270
B21	*Micrococcus sp.*	-	-	C	FR717271
B23	*Micrococcus sp.*	-	-	C	FR717272
B24	*Pantoea agglomerans*	-	-	C	FR717273
B25	*Micrococcus luteus*	-	-	C	FR717274
B27	*Chryseobacterium sp.*	-	-	C	FR717275
B31	*Sphingomonas sp.*	-	-	C	FR717276
B32	*Pantoea agglomerans*	-	-	C	FR717277

[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.

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).

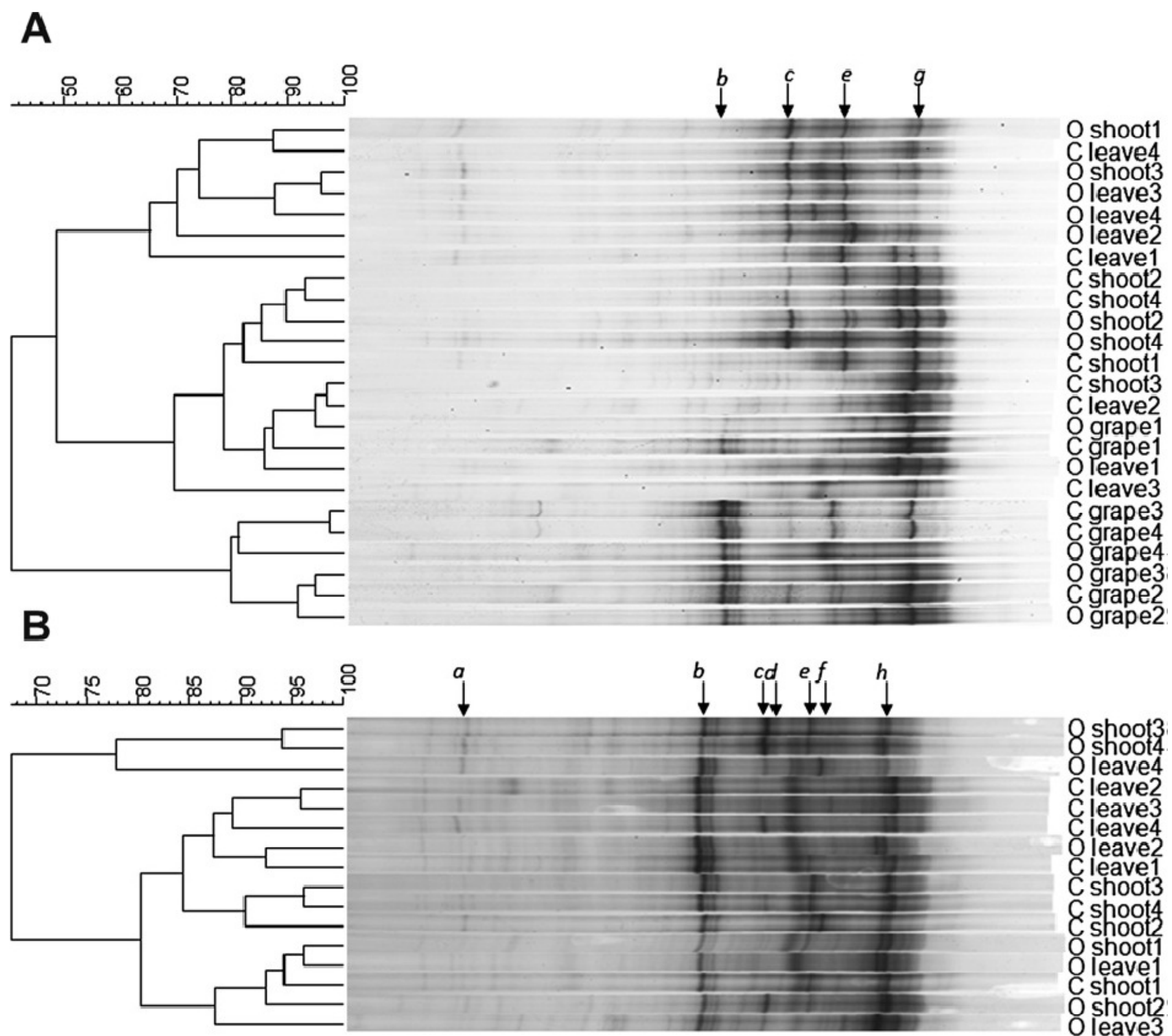

Fig 4 - Clustering of the SSCP profile of the *Pseudomonas* community of grapevine plants. Only minor differences are discernible between conventionally (C) and organically (O) managed plants. Shoots, leaves, and grapes were sampled 1 week prior to harvest. Community DNA was extracted from the respective 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.

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

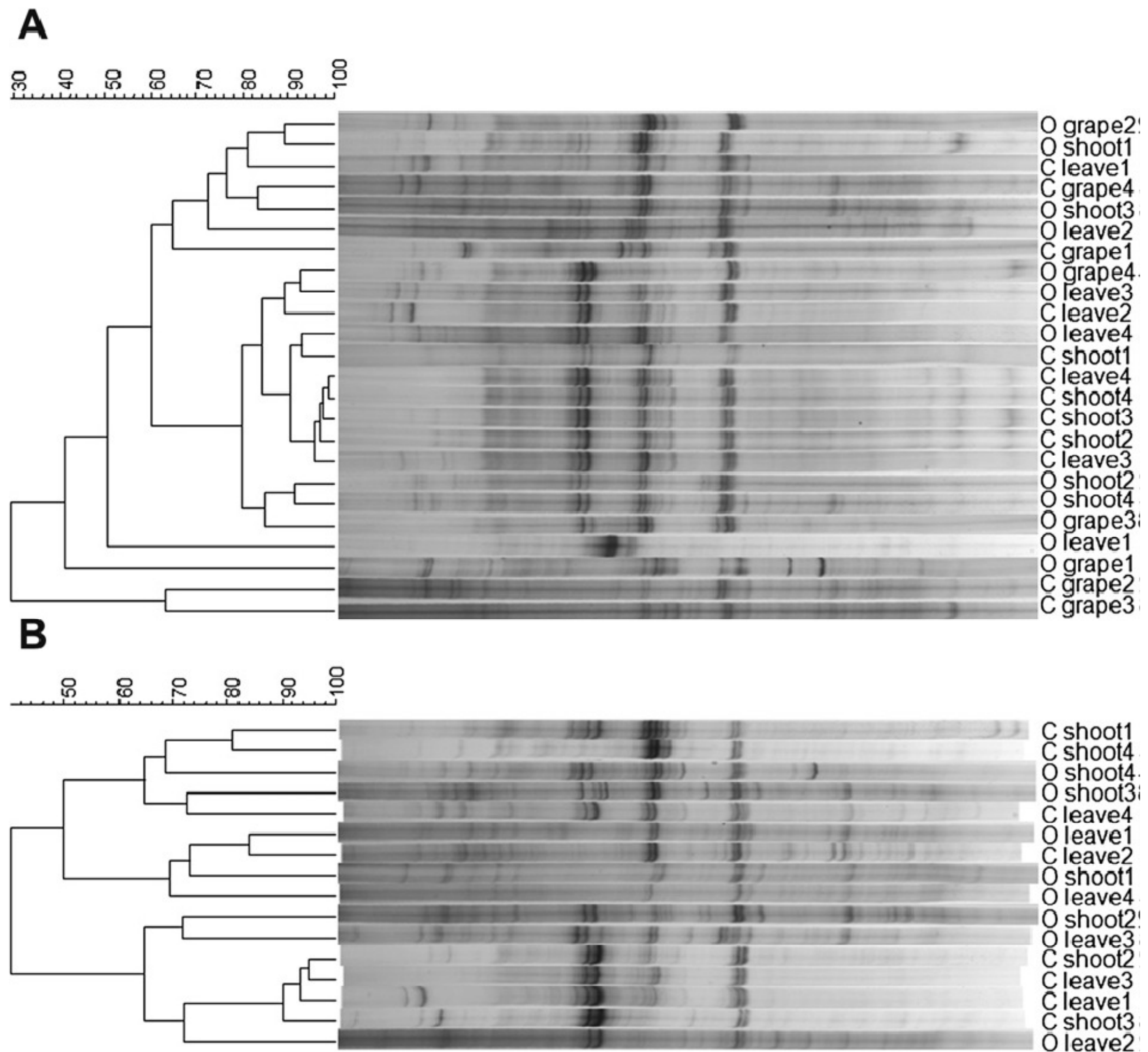

Fig 5 - Clustering of the SSCP profile of the *Firmicutes* community of grapevine plants. No significant differences are discernible between conventionally (C) and organically (O) managed plants. Samples from shoots, leaves, and grapes were taken 1 week prior to harvest. Community DNA was extracted from 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.

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], [Turian, 1977], [Gorbushina et al., 2005] and [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] and [Chi et al., 2009]). Mechanisms of action include competition for nutrients and production of cell wall degrading enzymes ([Andrews et al., 1983] and [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] and [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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IV. Manuscript 1

Successful introduction of *Pantoea ananatis* in the phyllosphere of grapevine to control grey mould disease caused by *Botrytis cinerea*

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Abstract

Botrytis cinerea is a plant pathogenic fungus causing severe diseases in a large number of plant species including species of agronomic importance such as tomatoes, strawberries and grapevine. We used grapevine as a model system, where *B. cinerea* is the causative agent of grey mould disease, which is a major risk factor in viticulture. Especially in organic viticulture effective measures against this disease are lacking. Biological control, the application of beneficial microorganisms in order to control plant pathogenic organisms, is a promising tool to overcome this problem and to reduce the amount of pesticides applied in agriculture. We assessed the efficacy of the bacterial strain *Pantoea ananatis* BLBT1-08 in reducing *Botrytis* infection on grapes and characterised possible modes of actions. This strain was isolated from grape berries and had *in vitro* antagonistic activity against *B. cinerea*.

In a three year field trial, as well as in leaf and *in vitro* assays the efficacy of the strain in reducing the disease was tested. Confocal laser scanning microscopy (CLSM) using a fluorescence labelled variant of BLBT1-08, quantitative PCR of a *Botrytis* specific gene, reisolation and physiological assays were performed to study the mode of action and colonisation abilities on the leaf.

The strain was able to reduce statistically significantly disease incidence on the field and in leaf assays. *Botrytis* abundance was not reduced in comparison to non-treated, symptom free leaves.

In order to provide prerequisites for optimisation of the biological control agent mode of action was investigated. Antimicrobial activity against *Botrytis* was dependent partially on the presence of amino acids. CLSM revealed dense colonization of the strain on

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the leaf surface, but not in the inner parts of the plant tissue. No direct interaction with conidia, germ tubes or mycelium of *B. cinerea* was observed on BLBT1-08 treated leaves and germination was not inhibited in plate assays.

Synthesis and applications. We found *Pantoea ananatis* as a promising biological control agent against *B. cinerea* and highly effective in reducing disease incidence.

Introduction

Botrytis cinerea is an ascomycetous fungus, which infects over 200 different species of plants (Elad *et al*. 2004). It causes pre- and postharvest diseases in crops like for example tomatoes, strawberries and grapevine. *Botrytis* is hard to control due to its ability to develop resistances rapidly, to survive in alternative host plants or in latent states and to infect via different mechanisms (Williamson *et al*. 2007). In viticulture *Botrytis* is an important risk factor being the causative agent of grey mould disease. It has deleterious effects on yield and on quality of wine (Pearson & Goheen 1988). Especially in organic viticulture, existing measures against this disease are often not sufficiently effective (Trioli & Hofmann 2009). Furthermore high amounts of copper input lead to enrichment of this heavy metal in the soil causing severe danger for ground water, ecosystem and human health (Pietrzak & McPhail 2004). Biological control is a promising measure to handle problematic plant pathogens and to reduce negative environmental impacts (Berg 2009). For this end plant beneficial microorganisms, which promote plant growth, enhance the systemic resistance of plants or antagonize plant pathogens, are applied. Mechanisms involved in microbial antagonism are synthesis of pathogen inhibiting compounds, parasitism and competition for space and nutrients (Berg 2009). In a previous study we analysed the ecology of grapevine-associated microorganisms with antagonistic activity towards *B. cinerea* in different grapevine phyllosphere habitats (Schmid & Berg 2009). Results from these studies revealed a higher abundance of antagonistic isolates in samples from organically managed grapes (Schmid *et al*. 2011). *Pantoea ananatis* BLBT1-08 was isolated from organically managed grape berries and selected according to i) its high antagonistic activity and ii) to its safety regarding human pathogenicity.

Pantoea ananatis, formerly known as *Erwinia ananatis* Serrano or *E. uredovora* Dye is a Gammaproteobacterium frequently found in association with aerial plant parts (Coutinho & Venter 2009). Several adaptations enable this bacterium to colonize leaves and fruits with

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high efficiency: production of the phytohormone indole acetic acid (IAA), pigments, homoserine lactones, and extracellular polysaccharides (Beattie & Lindow, 1999; Coutinho & Venter, 2009). Due to these adaptations to plant associated habitats and its ability to produce substances with antimicrobial and plant growth stimulating properties *P. ananatis* has been subject of studies concerned with biological control. Strains with activity against the postharvest disease *Penicillium expansum* Link on pome fruits, against several plant pathogenic fungi on leaves of tomato, and against *Xanthomonas axonopodis* Starr & Garces on pepper are described (Torres *et al*. 2005; Enya *et al*. 2007; Kang *et al*. 2007). Currently no detailed studies are available on the biocontrol activity of *P. ananatis* against *B. cinerea*. Also other members of the genus *Pantoea* are effective in controlling plant diseases, like for example strains of *P. agglomerans* Ewing & Fife and *P. dispersa*, which are used in the control of fire blight, caused by *Erwinia amylovora* Burrill (Beer, Rundle & Norelli 1984; Johnson *et al*. 1993). Frequent reports about involvement of *P. agglomerans* strains in human diseases led to concerns about the safety of *P. agglomerans* as biocontrol agent (Cruz, Cazacu & Allen 2007). In contrast to that there is only one report about the occurrence of *P. ananatis* in a human infection (De Baere *et al*. 2004). So far little is known about the mechanisms of antimicrobial activity of *Pantoea ananatis ad planta*. Knowledge about the modes of action of biocontrol agents is important for optimization and application to achieve consistent effects under field conditions.

Diseases caused by the fungus *Botrytis cinerea* lead to high yield losses all over the world, but environmental friendly measures against the fungus are lacking (Elad *et al*. 2004). The objective of this work was to assess the efficacy of the bacterium *P. ananatis* in controlling grey mould disease using grapevine as a model system and to elucidate major mechanisms of its mode of action. In this study we (i) demonstrated the reduction of disease incidence by application of *P. ananatis* in a field trial and (ii) under controlled conditions in leaf assays, and (iii) identified mechanisms involved in the biocontrol activity *ad planta* and *in vitro*, which enables optimization of the application of *P. ananatis* as a biological control agent.

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Material and Methods

Strain descriptions, culture conditions and plant material

Pantoea ananatis BLBT1-08 was isolated from ripe healthy grape berries (*Vitis vinifera vinifera* cv. Sauvignon Blanc) in 2007 from an organically managed vineyard in Schlossberg, Styria, Austria (46° 37' N, 15° 28' E; owner: Fachschule für Weinbau und Kellerwirtschaft und Weingut Silberberg). Identification was carried out by 16S rRNA gene sequencing (EMBL acc. nr. FR865900). If not stated otherwise BLBT1-08 was cultivated in nutrient broth II or on nutrient agar II (both Sifin, Berlin, Germany) at 30°C. The DsRed labelled derivate of BLBT1-08, Panred4, was cultivated on the same media supplemented with tetracycline at a concentration of 40 µg/mL. *Botrytis cinerea* KNB was isolated from grape berries in Klosterneuburg, Lower Austria, Austria (48° 18' N, 16° 20' E) in 2007 using *Botrytis* selective medium according to Edwards & Seddon (2001) and cultivated on potato extract dextrose agar (PDA, Roth, Karlsruhe, Germany) at room temperature in the dark. The strain was identified morphologically and by sequencing ITS region between 18S and 28S rRNA gene (EMBL acc. nr. FR872755). Production of conidia was induced by incubation of one week old cultures on PDA under 12 h light at conditions also used for plant growth (see below). Cuttings from *Vitis vinifera vinifera* cv. Sauvignon Blanc were obtained from the Research Centre for Agriculture Haidegg, Department 10A in Graz, Austria and planted in 5 L pots containing standard compost soil (Gramoflor, Vechta, Germany) and 1 % v/v quartz sand (Quartzwerke, Melk, Austria). Plants were grown under 12 h light provided by halogen bulb lamps (43 kLux at a distance of 60 cm) at 22 – 24 °C.

Field trial

BLBT1-08 was grown in a 2 L flask with 500 mL culture medium for 24 h shaking at 180 rpm to a cell density of approximately 2 x 10^9 cfu/mL. Culture medium was removed by centrifugation at 4500 x g for 15 min and cell material was resuspended in water to a final volume of 5 L prior to application. The suspension was applied to grapes (*V. vinifera vinifera* cv. Rheinriesling) located in Klosterneuburg, Lower Austria, Austria (48° 18' N, 16° 20' O) by spraying the berry zone. 5 L of suspension were used for 40 grape plants and applied three times per growing season beginning with flower cap fall and ending one month prior to harvest. Effect on *Botrytis* affection was determined by visual rating. For each rating 100

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grape bunches were classified according to their percentage of infected grape berries (0: no infection, 1: 0.1 – 5 % infected berries, 2: 5.1 – 10 %, 3: 10.1 – 20 %, 4: 20.1 – 40 %, 5: 40.1 – 100 %). Index of infection was calculated by dividing the sum of the products of class and respective number of bunches by the overall number of rated bunches. Degree of infestation was the mean of rating values of infected bunches. Significance of differences between values of treated and non-treated variants was assessed by Two Independent Sample Wilcoxon Rank Sum test. Frequency of infestation was the percentage of infested bunches in 100 grape bunches.

Detached leaf assays

Three weeks old leaves from Sauvignon Blanc plantlets were gently washed under deionised water and the abaxial side was sprayed with suspensions of bacteria or *Botrytis* conidia. Suspensions of BLBT1-08 or Panred4 were obtained by spinning down aliquots of overnight liquid cultures for 10 minutes at 4500 x g and resuspending the pellet in sterile deionised water to a calculated optical density at 600 nm of 0.1. This corresponded to a density of 1.5×10^8 colony forming units per mL. Conidia of *B. cinerea* were washed from plates with 4 mL of Tween 80 (10 % w/v), spinned down at 13000 x g for 2.5 min, washed with sterile deionised water and finally diluted to a concentration of 2×10^5 conidia per mL. Concentration was assessed with a counting chamber under light microscope. Treated leaves were sprayed each with 2 mL of bacterial and conidia suspensions respectively. Non-treated control leaves were sprayed each with 2 mL of sterile water and conidia suspensions respectively. Leaves were incubated with the abaxial side upwards in plastic boxes at 22 °C in the dark for 5 days with wetted filter paper. Area of necrosis was assessed by counting and measuring spots of necrosis on each leaf. Leaves were either used immediately for confocal laser scanning microscopy or lyophilized at 8×10^{-2} mbar and -50°C for three days for subsequent DNA isolation.

Quantitative PCR

DNA was isolated from 20 mg of lyophilized and homogenized leaf material using the FastDNA® SPIN Kit for Soil and the FastPrep Instrument® (MP Biomedicals, Santa Ana, CA, USA) according to the manual. DNA was eluted in 50 µL of DNase free water and used in 1:10 dilutions. For quantification of *Botrytis* DNA primer pair CG11/CG12, which specifically binds

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to cutinase A of *Botrytis cinerea*, was used (Gachon & Saindrenan 2004). Detection was carried out in a Rotor Gene 6000 (Corbett Research, Mortlake, Australia) thermocycler using 0.4 x SYBR® Green I (Invitrogen, Eugene, Oregon, USA) in a 10 µL reaction containing Taq-& GOTM Mastermix (MP Biomedicals), 0.5 pmol/µL of each primer, and 1 µL of diluted template. One cycle consisted of a 30 s denaturation step at 95 °C, a 40 s annealing step at 59 °C, and a 30 s step of 72 °C. Absolute quantification was based on a standard row of 1:5 dilutions of target fragments ranging from 1 x 10^4 to 80 copies / µL. The target fragment was obtained by cloning the CG11/CG12 PCR product into pGEM®-Teasy Vector (Promega Corporation, Madison, USA). Target fragment was amplified from this construct using the primers usp (5'-GTAAAACGACAACCAGT-3') and rsp (5'-CAGGAAACAGCTATGACC-3'). Due to high fluctuations below 80 copies / µL the quantification limit was determined at 80 copies / µl leading to an experimental quantification limit of 2 x 10^6 copies / g dry weight. Ct values of non template controls were always higher than Ct values from the standard solutions containing 80 copies / µL. The standard curve was linear in the range used (R2 ≥ 0.986). Efficiency calculated from the slope of the standard curve was between 1.12 and 1.23. Amplification efficiencies calculated from the slope of the exponential phase of the fluorescence history of each sample using Rotor Gene software were between 1.84 and 2.04.

Physiological assays

Production of C4-C6 acyl homoserine-lactones (AHLs) was tested by a plate assay according to McClean *et al*. (1997). The test strain was cross-streaked against the reporter strain *Chromobacterium violaceum* CV026, an AHL deficient mutant, on nutrient agar II (Sifin). Development of purple colour indicating violacein production by *C. violaceum* was evaluated after 24 h incubation at 30°C. Ability of synthesis of indole-3-acetic acid (IAA) was tested by a colorimetric assay in liquid culture according to Sarwar & Kremer (1995). Growth conditions and mode of detection are described in Gasser *et al*. (2011). Chitinase and α-Glucanase activity were tested on minimal medium according to the protocol according to Chernin *et al*. (1995) and Rasche *et al*. (2006) respectively. Clearing zones or blue haloes were detected five days after incubation at 20 °C. Production of volatile compounds (VOCs) with activity against *Botrytis cinerea* was tested using two compartment split plates (Sarstedt, Nümbrecht, Germany). Agar discs grown with *B. cinerea* mycelium were set on one half of the plate containing PDA. On the other half, containing nutrient agar II (Sifin), a suspension

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of the test strain was streaked in a defined area. The plates were sealed with parafilm and incubated for four days at 18 °C. Diameter of fungal colonies was measured and compared to a control without test strain. The test was considered positive, if significance of difference in diameters between test strain and control was < 0.05 on behalf of Student´s t-test.

Antibiosis tests

Growth inhibition of mycelium of *B. cinerea* KNB on rich medium was tested on PDA (Roth). Agar discs grown with mycelium of *B. cinerea* were placed on the centre of a plate and test strain was streaked out radially to that. The impact of amino acids on the inhibition of *B. cinerea* by *P. ananatis* was tested on synthetic low nutrient agar (SNA, Berg *et al*. 2005a). Fungal mycelium was inoculated on the centre of the plates as described above. Cell material of strain BLBT1-08 grown on plate over night at 30°C was transferred to the minimal medium by stamping with the tip of an inoculation loop, bent horizontally. Influence of *P. ananatis* on growth of *Escherichia coli* MG1655 was assessed by plating out dilutions of *E. coli* cultures grown over night in nutrient broth on minimal medium according to Winkelmann, Lupp & Jung *et al*. (1980). Cell material of *P. ananatis* was inoculated with an inoculation loop as described above. If required amino acids or casamino acids were supplemented in a concentration of 5 mM or 0.2 % respectively prior to autoclaving. Plates were incubated for five days in case of *B. cinerea* and for three days in case of *E. coli* at room temperature and inhibition was quantified by measuring the radius of the inhibition zone. Significances of differences between sizes of inhibition zones were calculated with Scheffe procedure at a level of significance of 0.001 using PASW Statistics 18 (SPSS Inc.).

Construction of a DsRed labelled derivate of P. ananatis *BLBT1-08*

Pantoea ananatis BLBT1-08 was transformed by electroporation with plasmid pME6031-DsRed2 (constructed by J. Fatehi, unpublished data) according to the procedure described by Zachow *et al*. (2010) leading to strain Panred4. The resulting transformant was resistant to tetracycline and showed a red fluorescent signal under the microscope after cultivation on both selective and non selective media for four days at room temperature. Box PCR genomic fingerprints according to Rademaker and De Bruijn (1997) were done using colony PCR according to the protocol of Berg *et al*. (2005b). The obtained fingerprints were identical to

that from BLBT1-08. Physiological traits, *in vitro* antimicrobial activity, and biocontrol activity in detached leaf assays were identical to the wild type.

Reisolation of P. ananatis *Panred4 from leaves*

Leaves were inoculated with Panred4 as described above and incubated for five days. As a control not inoculated leaves (sprayed with water instead of bacterial suspension) were used. Microorganisms were washed from leaves in bags containing 25 mL PBS + Tween (8 g NaCl, 1.42 g Na_2HPO_4, 0.2 g KCl, 0.24 g KH_2PO_4, all per litre; 0.05 % w/v Tween 80; pH 7.4) for 90 s using a BagMixer® (Interscience, St. Nom, France). Serial dilutions of the suspensions were plated on R2A Agar (Roth, Carlsruhe, Germany) containing 40 µg/mL tetracycline (Roth) and on R2A agar (Roth). Colony forming units were counted after incubation over night at 30°C.

Confocal laser scanning microscopy (CLSM)

Material from the intervein area was cut from grapevine leaves with a sterile scalpel. Microscopical specimens were prepared using Slow Fade® Gold antifade reagent (Invitrogen, Eugene, Oregon, USA) or Slow Fade® Gold antifade reagent with DAPI in order to stain conidia and hyphae. As staining of conidia with DAPI led to a faint signal in most of the cases, localization of conidia was assessed with bright field and marked in the image. Cover glasses were sealed with nail polish and observation was carried out with a TCS SPE confocal microscope (Leica Microsystems, Germany). DsRed was visualized with an excitation of 532 nm and a detection range of 550-620 nm. Autofluorescence of plant tissue was visualized with a laser at 405 nm and a detection range of 420-600 nm. Confocal stacks were generated with a Z-step size of 0.17 µm. Three-dimensional models, based on spots and isosurfaces, were created with the software Imaris 7.3 (Bitplane, Zurich, Switzerland). Figures were assembled in Adobe Photoshop CS3, version 10.0.1 (Adobe Systems Inc., USA).

Germination assays

Aliquots of 100 µL of suspensions of conidia (2 x 10^5 conidia/mL) were plated on PDA (Roth), SNA, and SNA with 0.2 % casamino acids. Cell material of test strains was streaked on the plates and plates were incubated for two or three days at room temperature. Germination

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of conidia in the vicinity of *P. ananatis* colonies was observed using a stereo magnifier at 16x and 40x magnification.

Statistics

In vitro assays were done in four replicates and repeated independently at least two times. Leaf assays were done in six replicates and repeated three times. For quantitative PCR each sample was analyzed in two independent runs each in four replicates.

Results

Application of P. ananatis *BLBT1-08 decreases* Botrytis *infection in a field trial*

In a three year field trial efficacy of *P. ananatis* BLBT1-08 against *Botrytis* infection was evaluated. Suspensions of BLBT1-08 were applied by spraying the berry zone of grape plants. In all three years, the index of infection, calculated as the sum of rating classes and respective number of grape bunches divided by the number of rated bunches, was reduced statistically significantly in comparison to a non-treated control (Table 1). Also the percentage of infected grape bunches (frequency of infestation) was lower in the BLBT1-08 treatments in all three years. Degree of infestation among infested grape bunches did not differ significantly from the control. In 2008, difference in degree of infestation among infested grape bunches was highest with a significance of P = 0.08. Infection pressure was lower in 2008 than in 2009 and 2010 in both, treated and non-treated plants. Within each season, index of infection from treated and non-treated grapes rose with ripening state of grape berries (data not shown).

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Table 1. Visual rating of *Botrytis* infection of grapes in a three year field trial using *P. ananatis* BLBT1-08. Percentage of *Botrytis* infected grape berries in 100 grape bunches was assessed visually one week prior to harvest and index of infection, degree of infestation, and frequency of infestation was calculated. Three applications of suspensions of *P. ananatis* BLBT1-08 were conducted in the time between cap-fall and one month prior to harvest in each year by spraying.

	2008		2009		2010	
	control	treated[1]	control	treated[1]	control	treated[1]
index of infection	0.56	0.27**	2.48	1.71**	2.62	1.90**
degree of infestation (%)[2]	11.3	7.2	34.5	29.4	33.4	28.2
frequency of infestation (%)[3]	29	17	77	59	82	66

[1]) Significance of differences of index of infection and degree of infestation between treated and control samples is indicated by asterisks (* p < 0.05; ** p < 0.005)
[2]) Degree of infestation was calculated as the mean of infestation from infested bunches only
[3]) No significance of differences was determined for frequency of infestation

Inhibition of Botrytis *infection by* P. ananatis *BLBT1-08 in leaf assays*

To verify efficacy of BLBT1-08 in decreasing disease incidence under controlled conditions, detached leaf assays were conducted. Detached grapevine leaves were sprayed with suspensions of BLBT1-08 and conidia of *Botrytis* (treated) or with water and suspensions of conidia (non-treated control). Within all leaf assays conducted BLBT1-08 treated leaves produced no symptoms of infection after five days of incubation (Fig. IV-1). Among non-treated controls, 13 out of 18 leaves showed necrosis as a symptom of *Botrytis* infection. *Botrytis* was re-isolated from such leaves (data not shown). Quantification of *Botrytis* DNA was done by quantitative PCR measuring the copy number of cutinase A gene (Gachon & Saindrenan 2004). Area of necrosis correlated with cutinase gene copy number in a linear manner (R^2 = 0.835) within symptomatic leaves. There was no statistically significant difference in cutinase gene copy number between treated and non-treated but symptom free leaves (P = 0.335). Leaves sprayed with BLBT1-08 alone did not develop any symptoms.

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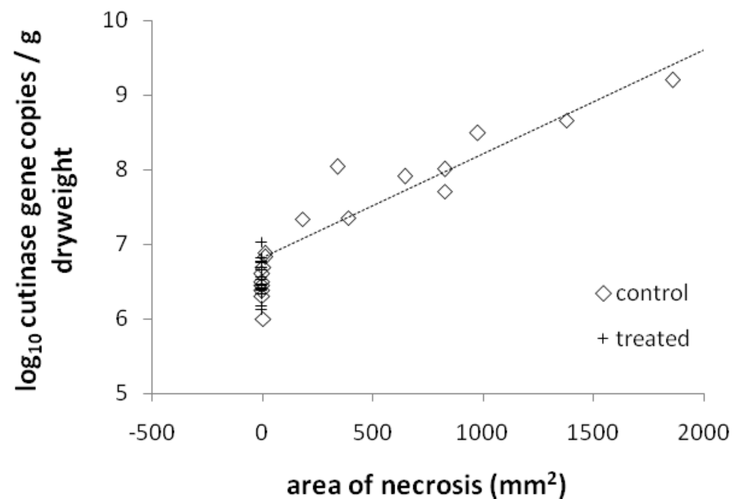

Figure IV-1. Inhibition of *Botrytis* infection of detached leaves by *Pantoea ananatis* BLBT1-08. Grapevine leaves were sprayed with suspensions of *P. ananatis* and conidia (treated) or with water and conidia (control). Area of necrosis was measured in symptomatic leaves and copy number of cutinase gene was determined by quantitative PCR. The dotted line represents the least-square line calculated from data from symptomatic leaves.

Physiological traits with relevance for biocontrol

In order to elucidate the mechanisms, by which BLBT1-08 reduces *Botrytis* infection incidence, *in vitro* tests were performed. *Pantoea ananatis* BLBT1-08 was able to produce the plant growth hormone indole acetic acid (IAA) in a concentration of 20-30 µg/mL in the cell free supernatant when grown in a tryptophan amended liquid medium. Furthermore, it produced at least one type of quorum sensing autoinducer stimulating violacein biosynthesis in an autoinducer negative mutant of *Chromobacterium violaceum*. No production of the fungal cell-wall degrading enzymes chitinase and glucanase was observed on minimal medium. *Pantoea ananatis* BLBT1-08 was not able to inhibit growth of *B. cinerea* by volatile compounds.

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In vitro antimicrobial activity of P. ananatis *BLBT1-08*

On nutrient-rich medium such as potato dextrose agar, *P. ananatis* reduced growth of *B. cinerea* mycelium. When streaked radially on the plate, an inhibition zone was formed at the outer part of the plate (Fig. IV-2A). This inhibition was strongest when incubated at 18°C and 12°C. At higher temperatures (20°C and 25°C) the inhibitory effect of BLBT1-08 was lower but still present. Growth of *B. cinerea* KNB was inhibited at 30°C. A clear inhibition zone was formed in dual culture with BLBT1-08 on SNA (synthetic low nutrient agar) at all temperatures tested (Fig. IV-2B and Table 2). Addition of casamino acids reduced inhibition zone significantly (Fig. IV-2C). Leucine, isoleucine, methionine, tryptophan, threonine, and ornithine mitigated inhibition completely or to a level comparable with or lower than that observed with casamino acids. Most of the other amino acids tested reduced the inhibition zone, but not to as great an extent as casamino acids. Only tyrosine, lysine, and arginine did not reduce the size of the inhibition zone (Table 2 and Figs IV-2D & IV-2E). BLBT1-08 formed a clear inhibition zone with a diameter of 14 ± 2 mm on bacterial lawns of *Escherichia coli* MG1655 on minimal agar plates, which also was prohibited by addition of casamino acids (Figs IV-2F & IV-2G). The inhibition zone also was absent in medium containing the single amino acids isoleucine, methionine, and threonine (Table 2 and Fig. IV-2H). Glutamine and ornithine reduced inhibition zone statistically significantly, whereas other amino acids tested did not affect the size of the inhibition zone. Interestingly alanine, tyrosine, and lysine led to larger inhibition zones than observed in minimal medium without amino acids.

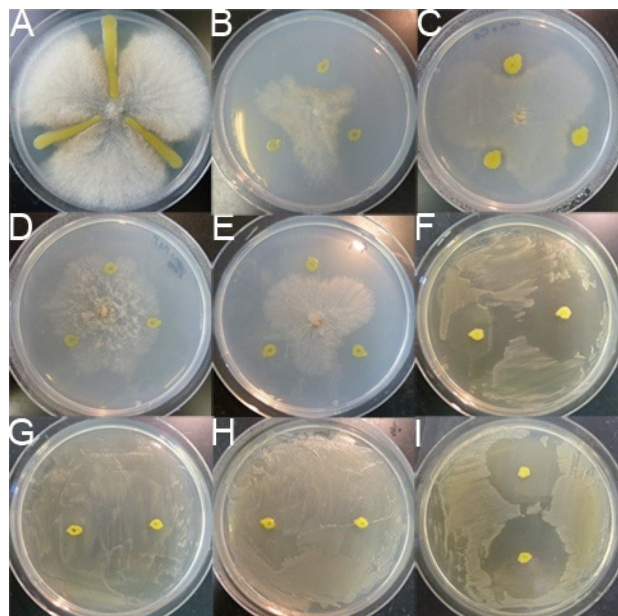

Figure IV-2. In vitro antimicrobial activity of *Pantoea ananatis* BLBT1-08 against *Botrytis cinerea* and *Escherichia coli* under different conditions. *P. ananatis* and *B. cinerea* on potato dextrose agar (A), synthetic low nutrient agar (B), SNA and 0.2 % casamino acids (C), SNA and 5 mM methionine (D), SNA and 5 mM glutamine (E); *P. ananatis* and *E. coli* on minimal medium (F), minimal medium and 0.2 % casamino acids (G), minimal medium and 5 mM threonine (H), minimal medium and 5 mM serine (I). PDA was incubated at 18°C, all other plates were incubated at room temperature.

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Table 2. Inhibition of *Botrytis cinerea* and *Escherichia coli* by *Pantoea ananatis* BLBT1-08 in the presence of casamino acids or single amino acids[1]

P. ananatis versus B. cinerea						*P. ananatis versus E. coli*					
SNA[2]	7.2^{bcd}	Pro	3.4^{ab}	Cys	2.6^{ab}	MM[2]	13.9^{bc}	Pro	12.4^{bc}	Cys	10.6^{bc}
CA[3]	1.2^{a}	Phe	3.2^{ab}	Lys	8.7^{bcd}	CA	0.0^{a}	Phe	10.4^{bc}	Lys	15.8^{c}
Gly	4.2^{abc}	Trp	0.2^{a}	Arg	9.3^{bcd}	Gly	10.6^{bc}	Trp	13.4^{bc}	Arg	10.9^{bc}
Ala	2.3^{ab}	Ser	3.8^{abc}	His	4.1^{abc}	Ala	15.5^{c}	Ser	14.1^{bc}	His	14.6^{bc}
Val	3.3^{ab}	Thr	0.0^{a}	Orn	0.1^{a}	Val	n.d.[5]	Thr	0.0^{a}	Orn	9.2^{b}
Leu	0.5^{a}	Asn	3.8^{abc}	Asp	n.d[4]	Leu	12.8^{bc}	Asn	14.1^{bc}	Asp	13.8^{bc}
Ile	1.6^{a}	Gln	4.3^{abc}	Glu	n.d	Ile	0.0^{a}	Gln	9.25^{b}	Glu	13.9^{bc}
Met	0.0^{a}	Tyr	7.0^{bcd}			Met	0.0^{a}	Tyr	15.4^{c}		

[1]) Radius of inhibition zone formed by *P. ananatis* was measured (mm). Amino acids were added in a concentration of 5 mM. Different letters indicate significance differences calculated with Scheffe procedure ($\alpha = 0.001$)
[2]) Synthetic low nutrient agar (SNA) or minimal medium (MM) without additional amino acids
[3]) Casamino acids 0.2 %
[4]) Influence of aspartic acid and glutamic acid was not determined because lowered pH prohibited polymerization of agar
[5]) Influence of valine was not assessed because growth of *E. coli* MG1655 is inhibited by valine in minimal medium (Tatum 1946)

Colonization of grapevine leaves by P. ananatis *Panred4*

Confocal laser scanning microscopy (CLSM) was performed to study the colonization behaviour of *P. ananatis*. Therefore, a strain labelled with the red fluorescent protein DsRed was constructed. This strain was referred to as *P. ananatis* Panred4. Furthermore, re-isolation experiments were conducted in order to quantify colonization efficacy. Five days after spraying leaves with bacterial suspensions with an optical density of 0.1, Panred4 was re-isolated on tetracycline containing plates in a quantity of $\log_{10}$ 4.98 ± 0.28 CFU/g fresh weight. All colonies growing on tetracycline containing plates had the same appearance and resembled Panred4 morphologically. No tetracycline resistant bacteria were isolated from leaves not inoculated with Panred4. Total number of bacteria isolated from inoculated leaves was $\log_{10}$ 6.97 ± 0.25 cfu/g fresh weight. Number of total bacteria isolated from not inoculated leaves was slightly but not significantly lower than from inoculated leaves (6.77 ± 0.29; $P = 0.099$). CLSM revealed a dense colonization of the leaf surface by Panred4. Cells of Panred4 were scattered as single cells or micro-colonies on the uppermost layer of the abaxial surface (Figs IV-3A to IV-3D) and also colonized trichomes (Fig. IV-3E). Three dimensional rendering of the images revealed localization of single cells along epidermal cell wall junctions (Figs IV-3H & IV-3I). Panred4 was not detected in the leaf endosphere. No signal of DsRed was detected in not inoculated leaves.

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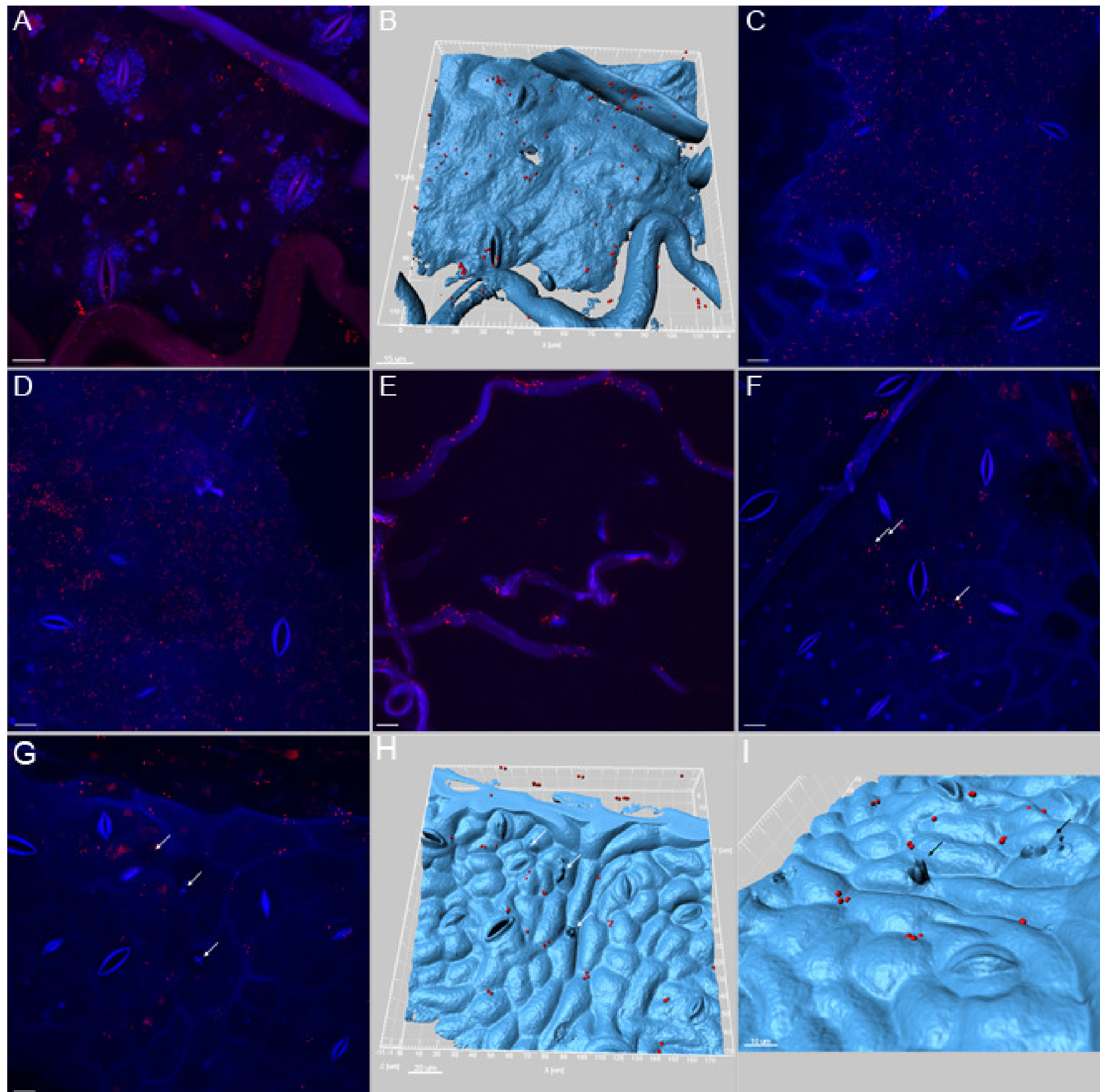

Figure IV-3. Confocal laser scanning microscopy of *Pantoea ananatis* on grapevine leaves. Strain BLBT1-08 was transformed with a DsRed carrying plasmid enabling fluorescence microscopy. Leaves were sprayed with the transformed strain (Panred4) (A-E) or with Panred4 and *Botrytis* conidia (F-I). Red signal represents fluorescence of DsRed. Three dimensional models were created with Imaris software (B, H, I). Scale bars are 10 µm. Arrows indicate localization of *Botrytis* conidia.

Interaction of P. ananatis *with* B. cinerea ad planta

To assess whether *P. ananatis* interacts with *B. cinerea ad planta* leaves sprayed with both microorganisms were observed using CLSM. Presence of conidia or germinating conidia on the leaves did not alter the colonization pattern of *P. ananatis* Panred4. Panred4 did not co-localize with or adhere to *Botrytis* conidia or germ tubes on grapevine leaves (Figs IV-3E & IV-3F). Germinating conidia were found after 1, 2 and 3 days in both, treated and non-treated

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leaves likewise. In plate assays on potato dextrose agar, SNA and SNA with 0.2 % casamino acids *P. ananatis* BLBT1-08 and Panred4 did not suppress germination of *B. cinerea* conidia. Germination of conidia and development of first hyphae occurred evenly distributed throughout the plates and also in the vicinity of *P. ananatis* colonies on all media tested (Figs IV-4A & IV-4B). Whereas mycelial growth continued on PDA and SNA with casamino acids, further growth was suppressed in the vicinity of *P. ananatis* colonies on SNA (Figs IV-4C and IV-4D).

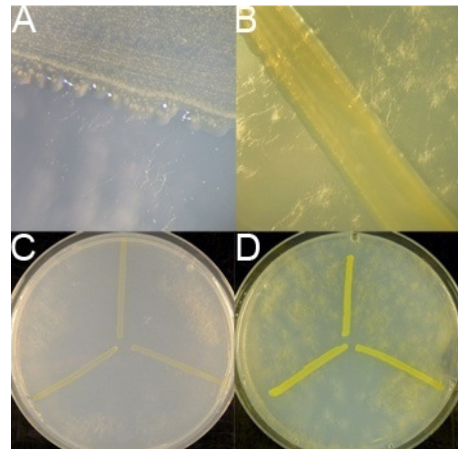

Figure IV-4. Germination of *B. cinerea* conidia on agar plates in coculture with *P. ananatis*. Suspensions of conidia were plated out on different media and cell material of *P. ananatis* was streaked out. After two days of incubation germination of conidia was assessed on SNA (A, 40x) and on SNA with 0.2 % casamino acids (B, 16x) with a stereo magnifier. After three days of incubation further growth of mycelium was assessed visually on SNA (C) and SNA with 0.2 % casamino acids (D).

Discussion

A three year field trial and detached leaf assays demonstrated efficient control of *Botrytis cinerea* by *P. ananatis* BLBT1-08. In both field trials and leaf assays a lowered incidence of *Botrytis* infection was present rather than a mitigation of infection severity. Mycelial growth was inhibited in an amino acid dependent manner, but germination of *Botrytis* conidia was not affected in the presence of *P. ananatis*.

The most surprising result was that equal amounts of *Botrytis* DNA were detected in *Pantoea*-treated and non-treated but symptom free leaves. This indicates that the strain acts by preventing *Botrytis* from infection. Bonaterra *et al*. (2003) found a direct interaction of a strain of *P. agglomerans* on *Botrytis* and inhibition of germination of conidia. In this study no evidence for inhibition of germination of conidia was found under the conditions studied. Data from plate assays suggest that inhibition of amino acid biosynthesis was involved in the antimicrobial activity towards *B. cinerea*. Interestingly, more than one amino acid was able to restore the inhibiting effect of *P. ananatis,* when added to the medium separately: methionine, threonine, tryptophan, leucine, and ornithine. Two of these amino acids,

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namely methionine and threonine also restored the inhibition of *E. coli* growth on minimal medium. Some amino acid reversible antibiotics are known from strains of *P. agglomerans*. For example *P. agglomerans* EH318 produces the histidine reversible pantocin A as well as the arginine reversible pantocin B (Brady *et al*. 1999; Wright *et al*. 2001). A351, a peptide antibiotic isolated from *P. agglomerans* Eh351 is arginine reversible, and other histidine reversible substances, namely mccEh252 from strain Eh252 and herbicolin O from strain C9-1, are known (Ishimaru, Klos & Brubaker 1988; Vanneste, Yu & Beer 1992; Jin 2003). Herbicolin A and B are peptides produced by *P. ananatis* A111 (formerly known as *Erwinia herbicola* A111) inhibitory to fungi, but the effect of amino acids on their function has not been investigated (Winkelmann, Lupp & Jung 1980; Greiner & Winkelmann 1991). Weak acids like acetate are known to inhibit growth of *E. coli* in a multifactorial manner (Booth 1985; Russell & Diez-Gonzalez 1998; Stratford & Anslow 1998). This inhibition can be restored by addition of methionine and glycine (Han, Kong & Lim 1993; Roe *et al*. 2002). Also growth inhibition caused by other stress factors like high temperatures can be restored by methionine (Ron & Davis 1971). As we observed restoration of *Botrytis* and *E. coli* growth by addition of more than one amino acid separately, we exclude the possibility that *P. ananatis* BLBT1-08 acts directly on amino acid biosynthesis pathways exclusively. Further investigations are necessary to elucidate the nature of the substances involved in the observed inhibition. *Pantoea ananatis* BLBT1-08 inhibited growth of *Botrytis* also on rich medium suggesting further mechanisms independent of presence of amino acids.

In this work no evidence for plant pathogenicity of *P. ananatis* BLBT1-08 was found. Leaves treated with BLBT1-08 alone did not develop any symptoms even after prolonged incubation (data not shown). Some strains of *P. ananatis* have been found to be connected with plant diseases for example in melons, onions and pineapples (Wells *et al*. 1987; Gitaitis & Gay 1997; Coutinho & Venter 2009). Conversely *P. ananatis* is isolated frequently from asymptomatic plants and elicits antagonistic traits against plant pathogenic bacteria and fungi, thereby conveying protection to their host plants (Vantomme *et al*. 1989). Further studies are required to identify factors, which influence the behaviour of *P. ananatis* strains towards their host plants.

Biocontrol activity of *P. ananatis* BLBT1-08 *ad planta* is of multifactorial nature. One important prerequisite for an effective biological control agent is its ability to colonise plant tissues. Re-isolation data and confocal laser scanning microscopy revealed efficient

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colonization of grapevine leaves by *P. ananatis* in this study. Cells were distributed singularly and in microcolonies throughout the leaf surface. Similar observations were done for *P. agglomerans* on leaves of maize and bean (Sabaratnam & Beattie 2003). By occupation of possible infection sites, *P. ananatis* BLBT1-08 lowers the possibility of *Botrytis* infection as was observed in this study. Whether inhibitory effects found under *in vitro* conditions also play a role in reduction of *Botrytis* infection *ad planta* requires further investigations. However in an oligotrophic environment as represented by leaf surfaces, imposing microbial competitors to amino acid dependency can be of advance for *P. ananatis*.

This study characterizes *Pantoea ananatis* strain BLBT1-08 as an efficient agent in controlling the plant pathogen *Botrytis cinerea* both in leaf assays and in a three year field trial. We suggest that its mode of action is multifactorial as BLBT1-08 has good plant colonizing traits but also shows antimicrobial activity *in vitro*.

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V. Manuscript 2

Organic versus conventional agriculture: A Review from a microorganism's point of view

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Abstract

As though yields from organically managed production systems in general are lower than those from conventionally agricultural systems, there are advantages in organic farming in terms of lower energy, fertilizer, and pesticide inputs, optimized nutrient utilization, better soil quality, and a lower environmental impact. Higher faunal and floral biodiversity levels have been found in organically managed farms, but for microbial biodiversity controversial data is available. One reason for that are problems and differences in methods used for determination of microbial diversity. Beside qualitative differences in microbial community structures most of the studies show higher microbial biomass, higher microbial species richness, and higher biodiversity based on diversity indices in organic systems, whereas evenness is not significantly different. As though many plant associated microorganisms are known to have plant beneficial traits, little is known about the direct association between a higher microbial biodiversity and plant health or plant growth. Results from some studies indicate an enhancement of plant beneficial microorganisms by organic farming.

Introduction

Microbial communities are adapted to certain environmental changes or stress factors. Extreme changes accomplished within a short period of time, as seen in the conversion of natural ecosystems into arable land, will favour dominance of few microorganisms, thus lowering species richness and evenness (Torsvik *et al*. 2002). Only in a few exceptions the conversion into arable land increases microbial diversity, e.g. if desert soil is converted into agricultural soil in a process called desert farming (Köberl *et al*. 2011). But in the most cases agricultural measures lead to a decrease in biodiversity. Stress factors like pH changes, high salinity, or heavy metal contamination as well as disturbances in humidity decrease catabolic evenness of crop soil microbial communities stronger than that of communities from a comparable pasture soil (Degens *et al*. 2001). The process of diversity decrease has negative

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implications for agriculture, because a lowered biodiversity is negatively correlated with plant disease suppression, productivity and sustainability of an agricultural system (Tilman *et al*. 1996; Hooper *et al*. 2005; Brussaard *et al*. 2007). This correlation is also true for biodiversity of soil microorganisms (van Bruggen & Grunwald 1996; Garbeva *et al*. 2004).

The aim of organic farming is to minimize the impact of agricultural measures on the environment by practices such as crop rotation, growing of pathogen resistant cultivars, limited use of chemical pesticides and the use of organic manure instead of synthetic fertilizers (Council Regulation of the European Commission No 834/2007 and No 889/2008). By this way it is expected also to approach biodiversity conditions present in natural ecosystems. From an economic point of view organic farming requires 10-20 % more labour input and retrieves lower yields, depending on the crop, than conventional farming in European countries. Nevertheless profits and success of organic farming equal conventional farming due to higher prices for organic products and due to support payments (Offermann & Nieberg 2000). Costs for corn and soybean equal after some time in both farming systems, but high value crops can be grown less frequently due to organic crop rotation (Hanson *et al*. 1997). As though yields from organic farming are lower in general, there are differences between crops. Yields for cereals from organic farming are only 60-70 % of yields from conventional farming, yields for pulses are 20 % lower in organic farming, and yields for vegetables are mostly the same as conventional ones. High variations in yields are found for potatoes (Offermann & Nieberg 2000). These values are in accordance with results from other studies (Mäder *et al*. 2002; Taylor & Cormack 2002; Cooper *et al*. 2007; Mäder *et al*. 2007). Cooper *et al*. (2007) correlated yields of winter barley with the factors crop protection and fertilization management, both organic and conventional, and found that conventional crop protection and conventional fertilization led to higher yields than organic treatments. A higher incidence of leaf blotch disease was a crucial factor for the lower yields in organic treatments, whereas a lower incidence of lodging was observed in organic treatments (Cooper *et al*. 2007). Incidence of *Septoria spp.* in wheat was enhanced by organic fertilization (Cooper *et al*. 2006). However, some studies show no significant differences in yields from organic and conventional treatments. For example corn yields were not significant different in a comparison between a conventional, an organic manure fertilized and an organic legume fertilized system conducted in a long term study in the USA (Drinkwater *et al*. 1998). Yields and disease incidence in apple production were the same in a

five year comparison between an organic, an integrated and a conventional system (Reganold *et al*. 2001). Advantages of organic farming are lower inputs in terms of energy, fertilizer and pesticide requirements, lower N losses, better soil quality, and a lower environmental impact (Drinkwater *et al*. 1998; Reganold *et al*. 2001; Mäder *et al*. 2002; Kramer *et al*. 2006). Energy and fertilizer input was reduced 34-53 % and pesticide input even 97 % in comparison to conventional plots in the long term field trial DOK (Mäder *et al*. 2002). Soil from an organic apple orchard showed lower nitrogen losses in comparison to soil from a conventional orchard due to five times lower rates of nitrate leaching in the organic orchard. As though denitrification rates were higher in the organic orchard, levels of N_2O were not significantly altered (Kramer *et al*. 2006). Higher leaching of nitrate also was observed in a long term trial conducted by the Rodale Institute in the US (Drinkwater *et al*. 1998).

Methods for determining microbial biodiversity

A detailed review about methods used for determining microbial biodiversity in environmental samples is given by Kirk *et al*. (2004). Biochemical methods are plate counts, physiological profiling of communities, and fatty acid methyl ester analysis. Molecular techniques commonly used are measurement of GC content, reassociation and hybridization of nucleic acids, microarrays, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (t-RFLP), and ribosomal intergenic spacer analysis (RISA). Additionally microscopic approaches can be used to study microbial diversity. With unspecific staining methods like for example DAPI staining total cell counts can be obtained independently of cultivation (Shannon *et al*. 2002). More detailed information about structure and colonization patterns of microbial communities can be obtained using labelled oligonucleotides specific for certain groups or domains and subsequent laser scanning microscopy, a technique called fluorescence in situ hybridization (Moter & Göbel 2000). Microbial communities of several habitats have been investigated using FISH, like for example lichens (Grube *et al*. 2009), root associated habitats (Mogge *et al*. 2000; Watts *et al*. 2004), mosses (Bragina *et al*. 2011) and leaf surfaces (Li *et al*. 1997). Fluorescence labelling with genetic markers and subsequent confocal laser scanning

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microscopy is a powerful instrument for investigating ecological behaviour of species or strains of interest. For example colonization patterns of rhizospheric microorganisms have been investigated by that way (Zachow *et al*. 2010; Gasser *et al*. 2011). Physiological functions of microorganisms can be elucidated *in situ* with stable isotope probing (SIP) (Lueders *et al*. 2004). New sequencing technologies have enabled deep insights into the microbial communities of a variety of ecosystems, but so far no study has elucidated differences in community structure or microbial diversity produced by organic or conventional treatment using these techniques. Metagenomics and amplicon sequencing, favoured by a rapid development of new sequencing techniques in the last years, are likely to enable more detailed insights into community structures and will probably provide more accurate ecological data in forthcoming studies. For comparing species richness, diversity indices or evenness estimated from amplicon pyrosequencing it is important to consider experimental methodology, because amplicon length and choice of primer pairs influence amplification efficiency and thus diversity estimates (Engelbrektson *et al*. 2010).

Investigating microbial diversity comes up with several methodical limitations. The majority of microorganisms present in environmental samples cannot be cultivated by common methods. In soil samples typically the proportion of yet-to-be-cultured bacterial cells lies between 95 and 99.9 % (Fægri *et al*. 1977; Bakken 1985). Reasons for this discrepancy are contributed to cells, which are in a physiological state called viable, but not culturable, and to species, which are not cultivated due to special metabolic requirements or due to long generation times under laboratory conditions (Kirk *et al*. 2004). The problem of low culturability of microorganisms can be overcome by using molecular techniques based on PCR and subsequent analysis of fragments (e.g. DGGE, t-RFLP). However, estimation of diversity indices from PCR based methods may not reflect properly the actual diversity present in a sample. This has been shown for tRFLP analysis of artificial communities, especially when number of species used was high (Blackwood *et al*. 2007). Other problems cannot be solved alone by alternative techniques. One problem is the spatial heterogeneity and the diversity of microhabitats, which raises questions about sample size and sample number (Trevors 1998; Franklin & Mill 2003). Another difficulty is the definition of phylogenetic groups. A critical point in PCR or nucleic acid based approaches is the extraction of nucleic acids from community samples. For example extraction of DNA from

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spores or gram positive bacteria requires harsher conditions than that of gram negative bacteria (Prosser 2002; Kirk *et al*. 2004).

Microbial diversity under organic management

The term 'organic farming' implies a variety of measures which aim to reduce the negative impact of agricultural practices on the environment. Studying the impact of organic farming practices on the microbial diversity can be done either by comparing the sum of all measures with a conventionally managed control or by varying single parameters. Within the long term field trial DOK, running since 1978 in Therwil, Switzerland, the parameters fertilization and crop protection are varied within five different systems in a 7-year crop rotation (Mäder *et al*. 2002). In this experimental setup fertilization with farmyard manure had the strongest impact on microbial biomass and biodiversity, whereas type of the previous crop did not have a significant influence (Hartmann *et al*. 2006). However, type of crop rotation does have significant impacts on the microbial diversity (Orr *et al*. 2011). In comparisons including sites from different regions soil type turned out to have the strongest impact on microbial biomass, regardless of management type (Bossio *et al*. 1998; Girvan *et al*. 2003). Thus experimental setup has to be considered when comparing findings of influences on microbial communities.

A survey of literature conducted by Hole *et al*. (2005) has shown that biodiversity of e.g. non crop plants, earthworms, spiders, and beetles is higher in organically managed than in conventionally managed farms. Despite of methodical drawbacks in quantitative assessment of microbial diversity, several studies have compared microbial communities of organically and conventionally managed fields. Along with the frequently observed fact that microbial biomass is increased in organic systems, also species richness, diversity indices and evenness tend to be larger. Microbial biomass in soil was shown to be increased due to application of organic manure in several studies using plate counts, microscopy, PLFA extraction and measurement of DNA and organic C contents (Fraser *et al*. 1988; Wander *et al*. 1995; Bossio *et al*. 1998; Gunapala & Scow 1998; Shannon *et al*. 2002; Hartmann *et al*. 2006). Also the phylloplane is colonized by microorganisms in a higher abundance in organic treatments, as shown for apples from organic management (Granado *et al*. 2008). One reason for a higher ability of manure amended soils to build up microbial biomass probably is the increased water holding capacity and porosity (Haynes & Naidu 1998).

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Several studies have compared species richness or diversity indices of microorganisms between organic and conventional treatments. A higher richness among arbuscular mycorrhizal fungi was found by tRFLP analysis of organically managed maize and potato rhizosphere (Verbruggen *et al*. 2010). Together with this finding also higher beta diversity in organically managed fields was detected. Richness of fungal species and, in one case of bacterial species, was higher in organically managed grassland of different soil types (Yeates *et al*. 1997). In the long-term trial DOK the highest biodiversity based on Biolog analysis was found in the biodynamic system, followed by the bioorganic and a conventional system with farmyard manure amendment (Mäder *et al*. 2002). The conventional system without amendment of manure showed the lowest biodiversity (calculated as Shannon index of diversity). In a comparison of 13 pairs of farms in the Netherlands bacterial species richness, but not diversity (both calculated from DGGE analysis) was statistically significantly higher in organically managed fields (van Diepeningen *et al*. 2006). Leaves and apples of organically managed apple trees harboured a higher number of culturable microorganism species than conventionally managed trees or trees from integrated management (Waipara *et al*. 2002; Granado *et al*. 2008). Some studies investigated the influence of farming practices on single groups of microorganisms. For example in the long term trial NFSC in England a higher abundance and a higher Shannon index of diversity of *nif*H genes was found in a potato field with an organic crop rotation. In the conventional rotation barley and in the organic rotation bean were the pre-crops of potato. The abundance was assessed with RT qPCR and the index was calculated based on RT DGGE (Orr *et al*. 2011). In the endorhiza of organically managed maize a higher richness of type I Methanotrophs was found based on DGGE, showing that also the community of the endosphere can be influenced by farming practices. This effect is contributed to the differences in the fertilizing strategy used in both fields (in the organic system compost was used for fertilization) (Seghers *et al*. 2003).

Recently it was established that at least in faunal predator-pathogen interactions evenness of both, predators and pathogens, and not species richness is important for maintenance of a sustainable ecosystem (Crowder *et al*. 2010). Influence of agronomic practices on microbial evenness in general or on evenness in microbial interaction networks has not yet been subject of many studies. Most studies support presence of differences in community composition rather than evenness between organic and conventional systems. Application of high amounts of cattle manure over a long period of time led to an increase in

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abundance of archaea, but to a decrease of diversity and evenness (Gattinger *et al*. 2007). Whereas bacterial species diversity increased with time under organic management, evenness only increased right after change to organic farming and then decreased (van Diepeningen *et al*. 2006). Soil community of continuously cropped and rotationally cropped soybean was different in composition, but not in species richness and evenness, assessed by sequencing of clone libraries (Tang *et al*. 2009). Using an extended PLFA analysis no statistically significant difference in microbial evenness was found between fertilization and crop protection regimes (Esperschütz *et al*. 2007). In the contrary, differences in PLFA patterns were present in this study, suggesting influence of the farming regimes on the microbial community in a qualitative manner. Similar results were obtained using the DNA based techniques tRFLP and RISA within the same long term trial (Hartmann *et al*. 2006). In a long term trial in California, the SAFS project, different PLFA patterns were present between a cover cropped organic, a cover cropped low input, and a conventional tomato field (Bossio *et al*. 1998). Differences in the community structure also were present in the phylloplane of organically and conventionally managed grapevine (Schmid *et al*. 2011). Whereas significant differences in fungal community structure were revealed using SSCP analysis, differences in bacterial communities were not evident (Grube *et al*. 2011).

Plant health and microbial diversity

Organic agronomic measures lead to increased soil organic carbon content and to an elevated microbial biomass in comparison to soils under conventional management. Especially incorporation of organic manure or compost contributes to this difference (Fraser *et al*. 1988; Bossio *et al*. 1998; Marschner *et al*. 2003; Esperschütz *et al*. 2007; Fließbach *et al*. 2007; Gattinger *et al*. 2007). But also zero tillage or soft tillage operations develop soils with higher microbial mass and activity than heavily tilled systems (Angers *et al*. 1993; Curci *et al*. 1997). The higher microbial biomass in organic systems is traced back to the community´s ability to convert organic matter to biomass more readily and with lower costs of energy than communities present in conventional soils (Mäder *et al*. 2002). The presence of a functionally diverse microbial community therefore ensures more efficient resource utilization in organic systems with advantages for plant health. This is also reflected in lower nitrogen losses found in organic management systems (Drinkwater *et al*. 1998; Kramer *et al*. 2006).

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It is shown by Crowder *et al*. that biodiversity indices and species richness need not necessarily be a crucial factor for the health status of an ecosystem. Experiments in potato field plots revealed that, with a larger evenness of predators and (insect) pests, biomass production and mortality of pest species increased (Crowder *et al*. 2010). Evenness of microbial communities plays a crucial role for stress tolerance of physiological functionality, which has been shown in microcosm experiments (Wittebolle *et al*. 2009). Resource availability and concentration of toxic compounds are important factors influencing functional diversity and microbial evenness (Moffett *et al*. 2003; Langehnheder & Prosser 2008). To our knowledge there has not been any study that showed direct associations between increased plant health or plant growth and increased microbial diversity or evenness.

Little is known about the influence of agronomic practices on the communities of plant beneficial microorganisms or their functional activities. Many plant-associated microorganisms, and especially those associated with the rhizosphere of plants, are known to exhibit traits and functions beneficial to plant health (Berg 2009; Raaijmakers *et al*. 2009). These functions include production of plant growth hormones, provision of nutrients (e.g. sulphate oxidation, phosphate solubilisation), induction of plant´s systemic resistance, and antagonism to plant pathogens. Microorganisms present in the endosphere of plants can exhibit plant beneficial functions such as biosynthesis of plant growth stimulating substances and antagonism to phytopathogens (Sessitsch *et al*. 2004; Ryan *et al*. 2008). Saprophytic microorganisms residing on aerial parts of plants are known to inhibit germination of fungal conidia due to nutrient competition (Blakeman & Fraser 1971).

Results from several studies suggest that a higher microbial biodiversity also harbours a higher potential of plant beneficial traits. A higher suppressiveness towards growth of the plant pathogenic fungus *Rhizoctonia solani* was found in soil from a pristine grassland than in soil from arable land under oat/maize/potato rotation as well as under continuous maize cultivation (van Elsas *et al*. 2002). Microbial diversity was higher in the grassland soil, whereas no significant differences in diversity and suppressiveness were present between the two arable soils. In general the phenomenon of suppressiveness of soils to certain plant diseases is more frequently found and more pronounced in organically managed soils (van Bruggen 1995; van Bruggen & Termorshuizen 2003). In barley and wheat the higher suppressiveness to take all decline in organically managed fields was contributed to a lower

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native abundance of fluorescent *Pseudomonas*, key factors of take all decline (Hiddink *et al*. 2005). Grapevine phyllosphere from organic viticulture harboured *Aureobasidium pullulans* in a higher abundance than phyllosphere from conventional viticulture (Schmid *et al*. 2011). Antagonistic activity towards the plant pathogenic fungus *Botrytis cinerea* and the high abundance of *A. pullulans* in the organic treatment led to the conclusion that organic farming measures increased the overall antiphytopathogenic potential of the plant associated community. Physiological properties of *A. pullulans* isolates from this study suggested a causative involvement of agronomic measures in its enrichment. Whether enrichment of *A. pullulans* also was caused by the overall higher abundance of fungi in the organically treated grape plants remains to be investigated. Organic measures have less adverse impact on arbuscular mycorrhizal fungi, which have positive effects on plant nutrition and plant pathogen defence, than conventional farming methods (Gosling *et al*. 2006)

Conclusions

As though yields and labour input are higher in most organically grown crops there are several advantages of organic farming. The higher microbial biodiversity, which can develop under organic farming leads to more stable physiological networks and thus ensures more effective resource utilization. Furthermore a higher microbial biodiversity increases the potential of plant beneficial microbial effects, like for example plant disease suppressiveness. Therefore more focus should be addressed to integration of elements from organic agriculture into existing production systems and their impact on microbial biodiversity and microbial community functions. Most cultivars are bred for conditions present in conventional agriculture. Therefore these cultivars often are not adapted properly to conditions provided by organic farming. Plant breeding with the aim to develop cultivars better adapted to organic farming conditions would improve performance in organic agriculture and thus may reduce the gap between organic and conventional yields.

Decrease in biodiversity caused by agronomic practices is a dangerous threat to sustainability, soil and plant health, and productivity of agricultural systems. In order to face lowered microbial diversity in agricultural ecosystems it is important to understand the impacts of human interventions on microbial communities and to identify measures that

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increase biodiversity and favour emergence of microorganisms with beneficial traits like antagonism towards plant pathogens, nitrification or phosphate solubilisation.

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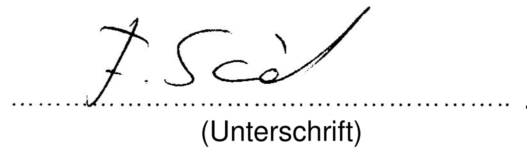

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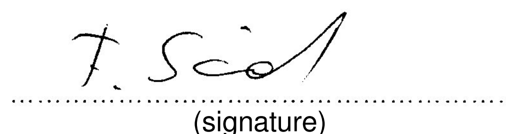

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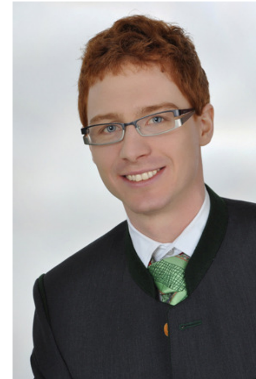

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Publications and public contributions

Publications in scientific Journals and conference proceedings

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Posters and Abstracts

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