Phyllosphere of Organically Grown Strawberries

Interactions between the Resident Microbiota, Pathogens and Introduced Microbial Agents

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Phyllosphere of Organically Grown Strawberries. Interactions between the Resident Microbiota, Pathogens and Introduced Microbial Agents

Abstract

The use of biological control agents (BCAs) is regarded as a promising measure to control important foliar strawberry diseases such as grey mould (*Botrytis cinerea*) and powdery mildew (*Podosphaera aphanis*) in the organic strawberry cultivation. However, the use of biological control agents (BCAs) in the phyllosphere is still challenging as this environment is very harsh and dynamic, in particular under field conditions. In this thesis, the simultaneous use of BCAs was studied for its potential to overcome the challenges biological control in the phyllosphere imposes and, thereby, to achieve more consistent efficacies against *B. cinerea* and *P. aphanis*.

In vitro tests revealed that inhibitory interactions can basically occur between two BCAs and that these are affected by nutritional factors. Leaf disc assays demonstrated that the simultaneous use of BCAs can result in improved suppression of *P. aphanis*, depending on the BCA constituents. Furthermore, several BCAs were applied as single or multiple strain treatments against *B. cinerea* in three years of field experiment and microbial interactions in the phyllosphere were investigated within these experiments. The simultaneous use of BCAs did not result in consistent *B. cinerea* control under field conditions. In the field experiment 2010, none of the tested single or multiple BCA treatments reduced *B. cinerea*. In the field experiments 2011 and 2012 *B. cinerea* incidence was significantly suppressed by simultaneously applied BCAs as opposed to single BCA treatments. Efficient BCA treatments, however, differed in 2011 and 2012. Microbial analyses on leaves from field grown strawberries by means of plate counts and 454 pyrosequencing revealed that the culturable and the non-culturable resident leaf microbiota considerably varied between different years of experiment but also in dependence on the strawberry's development stage. Likewise, the interactions between the resident microbial communities and introduced BCAs varied as well, which have shown to be associated with inconsistent efficacies of the tested BCAs in 2011 and 2012. Also, microbial investigations revealed shifts in fungal communities after introducing fungal BCAs, which however can be regarded as neglible due to the overall considerable dynamics of the phyllosphere microbiota.

Keywords: BCA compatibility, biological control, grey mould, microbial communities, microbial interactions, organic farming, phyllosphere, powdery mildew, strawberry

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Dedication

To my parents

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Justine Sylla, Beatrix W. Alsanius, Erika Krüger, Dorit Becker and Walter Wohanka (2013). *In vitro* compatibility of microbial agents for simultaneous application to control strawberry powdery mildew (*Podosphaera aphanis*). *Crop Protection* 51, 40-47.

II Justine Sylla, Beatrix W. Alsanius, Erika Krüger, Annette Reineke, Stephan Strohmeier and Walter Wohanka (2013). Leaf microbiota of strawberries as affected by biological control agents. *Phytopathology* 103, 1001-1011.

III Justine Sylla, Beatrix W. Alsanius, Erika Krüger, Annette Reineke, Monika Bischoff-Schaefer and Walter Wohanka (2013). Introduction of *Aureobasidium pullulans* to the phyllosphere of organically grown strawberries with focus on its establishment and interactions with the resident microbiome. *Agronomy* 3(4), 704-731.

IV Justine Sylla, Beatrix W. Alsanius, Erika Krüger and Walter Wohanka. Control of *Botrytis cinerea* in organically grown strawberries by biological control agents applied as single or multiple strain treatments (manuscript).

The contribution of Justine Sylla to the papers included in this thesis was as follows:

I Planned the experiments together with the co-authors. Performed most of the experimental work and supervised the students in performing some parts of the experimental work. Evaluated the data and wrote the manuscript together with the co-authors.

II Planned the field experiment together with the co-authors. Performed major parts of the experimental work in the field as well as in the laboratory. Evaluated the data and wrote the manuscript together with the co-authors.

III Planned the field experiments together with the co-authors. Performed major parts of the experimental work in the field. Evaluated the data and wrote the manuscript together with the co-authors.

IV Planned the field experiments together with the co-authors. Performed major parts of the experimental work in the field. Evaluated the data and wrote the manuscript together with the co-authors.

Abbreviations

ANOSIM	analysis of similarity
BCA	biological control agent
BSM	*Beauveria* selective medium
CFU	colony-forming units
CWDE	cell wall degrading enzymes
DAH	day after harvest
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DW	dry weight
ITS	internal transcribed spacer
MEGAN	Metagenome Analyzer
NCBI	National Center for Biotechnology Information
OTU	operational taxonomic unit
PAST	paleontological statistics software package
PCA	principal component analysis
PCR	polymerase chain reaction
PDA	potato dextrose agar
PLFA	phospholipid fatty acid
rRNA	ribosomal ribonucleic acid
SA	Sabouraud agar
t-RFLP	terminal restriction fragment length polymorphism
TSA	Tryptic soy agar
TSM	*Trichoderma* selective medium

1 Introduction

Severe strawberry diseases are caused by fungal pathogens such as *Botrytis cinerea* (grey mould), *Podosphaera aphanis* (powdery mildew), *Colletotrichum acutatum* (anthracnose), *Phytophthora cactorum* (leather rot, crown rot), *Phytophthora fragariae* var. *fragariae* (red stele root rot), and *Verticillium dahliae*, *Verticillium albo-atrum* (Verticillum wilt) (Jung, 2012; Agrios, 2005; Maas, 1984). Of these, *B. cinerea* and *P. aphanis* are regarded as the most economically important fungal pathogens in the phyllosphere of strawberries, i.e. on fruit and leaves, respectively (Jung, 2012). In conventional strawberry cultivation, these pathogens can be controlled by fungicide applications (Maas, 1984). In organically grown strawberry, however, fungicide use is strongly restricted (Mäder *et al.*, 2013) and, therefore, disease control of *B. cinerea* and *P. aphanis* is limited to the choice of less susceptible cultivars or cultural measures (Schmid, 2001).

The use of microbial biological control agents (BCAs) is regarded as a highly attractive disease control measure in organically grown strawberries. However, due to the hostile conditions for microorganisms in the phyllosphere control of foliar pathogens by introduced BCAs can generally be regarded as a great challenge (Andrews, 1992). In this thesis, the simultaneous application of BCAs with respect to its potential to overcome the challenges of biological control in the phyllosphere was investigated in laboratory and field experiments, which brought the microbial interactions in the phyllosphere into focus.

The present thesis was embedded in the project "Application of biocontrol agents to regulate diseases on strawberries – part: grey mould (*Botrytis cinerea*) and powdery mildew (*Podosphaera aphanis*)", which was conducted at Geisenheim University, Germany.

2 Background

2.1 Strawberry cultivation

Strawberries are cultivated on all continents. In 2011, the strawberry cultivated area made up 345,900 ha world-wide, with the largest cultivation area in Europe (approx. 162,000 ha) followed by Asia (approx. 125,000 ha), the USA (approx. 42,000 ha), Africa (approx. 16,000 ha) and Oceania (approx. 1,400 ha) (Dierend, 2012b). In 2009, highest yields in Europe were produced in Spain (264,000 t on approx. 7,100 ha), Poland (199,000 t on approx. 53,300 ha) and Germany (150,000 t on approx. 12 800 ha) (Dierend, 2012b). In Sweden, total yield of strawberries and the cultivation area corresponded to approx. 13,000 t and 1,800 ha in 2011, respectively (FAOSTAT, 2011).

A large number of strawberry cultivars is available on the market world-wide (StrawberryPlants.org). The cultivars might differ in terms of yield, fruit quality (e.g. colour, taste, firmness), harvest time or disease and pest resistance (Krüger, 2012). In addition, they vary in their environmental demands. Thus, one cultivar might perform differentially in distinct regions or environments (Maas, 1984). In Europe, the cultivar ‘Elsanta’ is predominant (Lieten, 2006), but other cultivars such as ‘Honeoye’, ‘Korona’, ‘Clery’ and ‘Darselect’ are often grown as well. The choice of cultivar is depending on the grower’s requirements and the environmental conditions (Krüger, 2012; Maas, 1984).

In Germany, strawberries are usually grown as annuals or perennials in open fields, whereas the cultivation period only in rare cases takes longer than two years. Frigo plants, i.e. bare-rooted and cold-stored plants, are planted from April to June or, alternatively, green plants are planted in August for harvest in the subsequent year. In perennial systems, plants can be mulched after the first harvest to initiate new leaf growth for a second harvest in the following year. When June-bearing strawberry cultivars, i.e. short-day cultivars (e.g. ‘Elsanta’,

'Korona'), are used in open fields, the harvest usually starts in the end of May / beginning of June and takes approx. four weeks in Germany (Dierend, 2012a). As an alternative to June-bearing cultivars, everbearing cultivars (e.g. 'Everest') can be used, i.e. long-day or day-neutral cultivars with extended period of flowering, fruit formation and harvest. The importance of everbearing cultivars, however, is generally considered to be negligible (Dierend, 2012a; Hancock, 1999).

There is a growing demand for off-season strawberries in Europe (Lieten, 2006). In open fields, the harvesting period can be prolonged by cultivation of early cultivars (e.g. 'Honeoye', 'Clery') or late cultivars (e.g. 'Yamaska', 'Florence') (Krüger *et al.*, 2012). Furthermore, application of cultural measures (e.g. plastic mulch covers) (Dierend, 2012a; Hancock, 1999) or cultivation of cold-stored plants (waiting bed plants) after the main growing season can prolong the period of harvest (Lieten, 2006). Strawberries outside the traditional and main season can as well be produced under plastic tunnels and in greenhouses (Lieten, 2002). Furthermore, protected strawberry cultivation offers different advantages such as improved fruit quality as strawberries are protected from rain, hail and wind. Also, applications of fungicides and herbicides may be reduced (Lieten, 2002). The latter one can, for instance, be linked to reduced *B. cinerea* incidence (Evenhuis & Wanten, 2006; Xiao *et al.*, 2001). For the above mentioned reasons, protected strawberry production has increased in Europe (Lieten, 2002).

2.2 Phyllosphere microbiology

The habitat on aerial plant surfaces (e.g. on leaves, flowers, fruit) is termed phyllosphere. It harbours a variety of epiphytic microorganisms (Whipps *et al.*, 2008; Lindow & Brandl, 2003; Lindow & Leveau, 2002). So far, most investigations on phyllosphere microbiology brought leaves into focus (Lindow & Brandl, 2003), on which bacteria are considered to be predominant and most diverse, followed by yeasts and filamentous fungi (Whipps *et al.*, 2008). It was suggested that leaves can be colonized by 10^2 to 10^{12} culturable bacterial cells g^{-1} leaf. Yeast and filamentous fungi can make up 10 to 10^{10} CFU and 10^2 to 10^8 CFU g^{-1} leaf, respectively (Whipps *et al.*, 2008).

The phyllosphere is regarded as a hostile habitat for microorganisms, which is predominantly characterized by inconstant environmental conditions (e.g. with regard to temperature, light, precipitation) and low water and nutrient availability (Lindow & Brandl, 2003). The ability of microorganisms to immigrate and to colonize this environment mainly determines the composition

of microbial phyllosphere communities. Immigration and colonization by the microorganisms in turn is linked to their ability to adapt to or to tolerate the hostile conditions in the phyllosphere (e.g. through production of pigments that provide protection from UV radiation, aggregate formation or release of surfactants) as well as to their ability to compete with the resident microbiota (Delmotte *et al.*, 2009; Whipps *et al.*, 2008; Lindow & Brandl, 2003; Lindow & Leveau, 2002; Beattie & Lindow, 1999; Kinkel, 1997). Additional factors such as plant species (Yang *et al.*, 2001) or leaf age (Redford & Fierer, 2009; de Jager *et al.*, 2001; Thompson *et al.*, 1993) can affect the composition of microbial communities in the phyllosphere as well. Furthermore, infections of plants by plant pathogens (Suda *et al.*, 2009) and even the cropping system (Schmid *et al.*, 2011; Ottesen *et al.*, 2009) have shown to affect microbial phyllosphere communities.

The composition of microbial communities can be studied by culture-dependent techniques that allow microorganisms from natural samples to be enriched on nutrient media (Madigan *et al.*, 2009). For this purpose, plant material (e.g. leaves, fruit) can be directly imprinted on nutrient media (Krimm *et al.*, 2005). Alternatively, phyllosphere microorganisms can be washed off from plant surfaces. The washing solution/suspension, containing the detached microorganisms, can be plated on nutrient media (Jensen *et al.*, 2013; Russell *et al.*, 1999). Single colonies can be isolated from the agar plates and identified, e.g. by morphological characteristics (Jensen *et al.*, 2013; Russell *et al.*, 1999) or gene sequencing (Krimm *et al.*, 2005). Furthermore, the use of washings allows the quantification of the microorganisms by counting the colonies on the agar plates (plate counts) and calculating the colony-forming units (CFU) ml^{-1}, g^{-1} or cm^{-2} (Madigan *et al.*, 2009). Although numerous nutrient media have been designed to recover as many microorganisms as possible, the majority of microorganisms still cannot be cultured (Madigan *et al.*, 2009; Hill *et al.*, 2000). For instance, only 0.1 - 3% of bacterial cells are considered to be culturable (Whipps *et al.*, 2008). Therefore, culture-dependent techniques are considered to be insufficient to reflect the leaf microbiota (Whipps *et al.*, 2008; Yang *et al.*, 2001). In contrast, culture-independent techniques allow the analysis of both culturable as well as non-culturable microorganisms of environmental samples (Madigan *et al.*, 2009; Hill *et al.*, 2000). DNA-based techniques make use of polymerase chain reaction (PCR) to amplify DNA from the environmental samples. Most often the 16S rRNA and the internal transcribed spacer (ITS) rRNA regions are amplified to identify bacterial and fungal species, respectively (Schoch *et al.*,

2012; Redford *et al.*, 2010; Buée *et al.*, 2009; Hamp *et al.*, 2009; Yang *et al.*, 2001).

Several DNA-based techniques were already applied to analyze phyllosphere microbial communities, e.g. denaturing gradient gel electrophoresis (DGGE) (Suda *et al.*, 2009; Zhang *et al.*, 2008a; Yang *et al.*, 2001), terminal restriction fragment length polymorphism (t-RFLP) (Kim *et al.*, 2010; Zhang *et al.*, 2008b), construction of gene clone libraries and sequencing (Ottesen *et al.*, 2009; Redford & Fierer, 2009) as well as 454 pyrosequencing (Leveau & Tech, 2011; Redford *et al.*, 2010).

Of the DNA-based techniques, next-generation sequencing (e.g. 454 pyrosequencing) enables an affordable and quick high-throughput sequencing of microbial communities of environmental samples without the construction of clone libraries (Jones, 2010; Harkins & Jarvie, 2007). Compared to Sanger sequencing, 454 pyrosequencing requires only 10% and 0.9% of the costs and time, respectively, for the same amount of sequences (Jones, 2010). For 454 pyrosequencing, the 454/Roche technology is used. With this technology, genomic DNA fragments, individually bound to microscopic beads in an oil and water mixture, are clonally amplified by emulsion PCR. Thereafter, the beads are transferred into wells of a picotiter plate (one bead per well) and sequenced by adding the four nucleotides sequentially into the wells. If a nucleotide is incorporated in a complementary template sequence, pyrophosphate is released and converted into adenosine triphosphate (ATP). ATP is used by luciferase for the oxidation of luciferin and, as a result, light is emitted from the respective well. Afterwards, the sequences can be determined in parallel by means of the light signals in each well (Thomas *et al.*, 2012; Jones, 2010; Clark, 2009; 454 LifeSciences).

In strawberries, phyllosphere microbial communities have been studied by culture-dependent techniques in several studies. Krimm *et al*. (2005) isolated epiphytic microorganisms by agar imprints of strawberry leaves, flowers and fruit and, thereafter, identified bacterial morphotypes by culture-independent techniques. In their study, most isolates were identified as bacteria and only approx. 4% of all isolates were identified as fungi. Among the bacterial isolates, *Pseudomonas* and *Bacillus* represented predominant genera in the strawberry phyllosphere (Krimm *et al.*, 2005). In another study, bacteria were dominant on strawberry fruit as well (approx. 10^4 CFU g^{-1} berry at maximum), with *Curtobacterium* spp., *Serratia* spp., *Pseudomonas* spp. and *Enterobacter* spp. being most commonly isolated (Jensen *et al.*, 2013). The filamentous fungi *Cladosporium* spp. and *Penicillium* spp. as well as the yeasts *Candida* spp., *Cryptococcus* spp. and *Rhodotorula* spp. were also commonly recovered from strawberry fruit (Jensen *et al.*, 2013). Furthermore, Parikka *et al*. (2009) investigated the microbial quality of marketable fruit from organically grown

strawberries and thereby identified *Mucor* spp., *Penicillium* spp., *Cladosporium* spp., *Alternaria* spp., *Acremonium* spp., *Fusarium* spp., *Trichoderma* spp. and *Botrytis cinerea* as abundant fungal epiphytes.

The phyllosphere is the playground for the interactions between phyllosphere applied BCAs and foliar pathogens, which is the subject in the present thesis. It is, therefore, important to investigate the resident microbiota in the phyllosphere as well as potential interactions with the introduced BCAs. Furthermore, biological control agents should not displace or harm non-target microorganisms (e.g. through pathogenicity or toxigenicity) (Cook *et al.*, 1996). However, only few investigations on the effects of introduced BCAs on non-target microorganisms in the phyllosphere were conducted (Kim *et al.*, 2010; Zhang *et al.*, 2008a; Okon Levy *et al.*, 2006; Russell *et al.*, 1999), which emphasizes the necessity to investigate the interactions between the resident microbiota and introduced microbial agents in this habitat.

2.3 Grey mould in strawberries

2.3.1 General aspects

In strawberries, *Botrytis cinerea* Pers.: Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Wetzel) is one of the most important pathogens worldwide and is causing grey mould (Crous *et al.*, 2009; Shtienberg, 2004). Infections of strawberries with *B. cinerea* typically start during flowering: the pathogen actively invades the flowers and grows towards the inflorescence and fruit, where it remains quiescent (i.e. fungal growth and symptoms are not visible) until the fruit ripen and, quite often, until cold storage of the fruit (Droby & Lichter, 2004; Kronstad, 2000; Jarvis, 1962b). Infected fruit tissue turns brown (Figure 1 B), before it is covered by the pathogen's mycelium, condiophores and grey conidia appearing as the characteristic grey mould symptoms (Figure 1 A and B) (Maas, 1984).

Figure 1. Characteristic grey mould symptoms in strawberries caused by *Botrytis cinerea*. **A:** Fruit covered with the mycelium of *B. cinerea* prior to harvest (photo courtesy of Winfried Schönbach). **B:** Stored fruit with brown tissues and mycelium of *B. cinerea* (photo: Justine Sylla).

Humidity and moderate temperatures favour the development of *B. cinerea*. Under such conditions, the pathogen can cause severe damage in strawberries in the field, but also during storage of ripe fruit because *Botrytis* is able to grow at temperatures close to 0 °C (Agrios, 2005; Elad *et al.*, 2004; Holz *et al.*, 2004).

2.3.2 Disease cycle of *Botrytis cinerea*

Outdoors, *Botrytis cinerea* survives winter time saprophytically on decaying plant material and in soils, or as survival structures termed sclerotia. The survival structures are involved in long-term survival of the pathogen (Agrios, 2005; Holz *et al.*, 2004). It is likely that primary inoculum of *B. cinerea* is generated in the crop. However, because of its wide host range, conidia of *B. cinerea* are ubiquitous. Therefore, other crops or weeds might also provide primary grey mould inoculum. The conidia of *B. cinerea* are spread by wind, rain or insects and can infect plant tissues (Holz *et al.*, 2004; Jarvis, 1962a; Jarvis, 1962b). Dispersed conidia of *B. cinerea* attach to plant tissues and germinate by producing germ tubes at high humidity and, preferably, in the presence of a water film (Holz *et al.*, 2004; Bulger *et al.*, 1987; Jarvis 1962b). Active penetration of plant tissues is accompanied by appressorium formation (Kars & Kan, 2004). Invasion of the plant tissue can also occur by passive entrance of the pathogen through natural openings (stomata) or wounds (e.g. insect wounds or lesions from other pathogens) (Droby & Lichter, 2004; Holz *et al.*, 2004). The fungus grows inside the host tissue inter- and intracellularly and kills its host cells (Tenberge, 2004). Infected tissue becomes soft and the fungus produces grey conidiophores and conidia, which serve as further inoculum (Maas, 1984). Dislodged conidia can be disseminated through the air, mainly by wind but also by precipitation (Jarvis, 1962a).

The sexual stage of *B. cinerea* and the formation of apothecia can be easily obtained in the laboratory but the occurrence of *B. fuckeliana* is not common in nature (Beever & Weeds, 2004).

2.3.3 Control of grey mould in strawberries

In the organic strawberry cultivation, the use of fungicides is highly restricted (Mäder *et al.*, 2013; Trapp, 2013). That is why organic farmers have to use cultivars which are less susceptible to *B. cinerea* to control grey mould in their fields. Legard *et al.* (2000) showed that the incidence of *B. cinerea* in strawberries was highly influenced by the choice of cultivar. Similarly, Daugaard and Lindhard (2000) tested 20 strawberry cultivars under conditions of organic strawberry production. In their study, the cultivar ‘Elsanta’, which is the most grown cultivar in Europe, showed approx. 11 % *Botrytis* incidence

and, thus, has to be regarded as a moderate resistant cultivar. For this reason, apart from the choice of cultivar there is still a need for further measures to reduce grey mould incidence in the organic cultivation of strawberries.

A moderate nitrogen fertilisation and straw mulch is recommended to prevent *B. cinerea* in strawberries (Schmid, 2001; Hancock, 1999). Moreover, leaf sanitation (Mertely *et al.*, 2000) and adequate spacing (Legard *et al.*, 2000; Maude, 1980) has shown to reduce the incidence of *Botrytis cinerea* in the field. The use of drip-irrigation in the field can also result in reduced leaf wetness and, therefore, reduce the risk for *B. cinerea* infections (Xiao *et al.*, 2001). Furthermore, protected strawberry cultivation offers great potential to prevent *B. cinerea* epidemics due to higher temperatures, shorter periods of leaf wetness, reduced light intensity, lack of precipitation for conidial dispersal and reduced influx of wind-dispersed conidia as opposed to outside tunnel conditions (Evenhuis & Wanten, 2006; Xiao *et al.*, 2001). Cultural measures alone, however, often do not lead to sufficient control of *B. cinerea* in strawberries (Legard *et al.*, 2000; Mertely *et al.*, 2000). For that reason, the use of microbial biological control agents (BCAs; see chapter 2.5) may offer a promising supplement to the cultural measures to suppress *B. cinerea* in organically grown strawberries.

The use of fungicides predominates in the suppression of *B. cinerea* in the conventional strawberry cultivation. There is, however, rising public concern about the use of fungicides due to the potential environmental and human health hazards of chemical pesticides (Hauschild, 2012). In recent years, the intensive use of fungicides in strawberries has also led to increasing reports on fungicide resistances towards *B. cinerea* (Leroch *et al.*, 2013; Weber, 2011). Therefore, fungicide sprays need to be reduced also in conventionally grown strawberries. The supplemental use of alternative measures (e.g. BCA treatments) might allow reduced fungicide applications in the conventional cultivation of strawberries.

2.4 Powdery mildew in strawberries

2.4.1 General aspects

Powdery mildews are obligate biotrophic plant pathogens (Yarwood, 1957). They feature epiphytic hyphal growth and produce excessive amounts of conidia on their host plants. Accordingly, the term powdery mildew has arisen from their powdery appearance (Glawe, 2008; Yarwood, 1978).

In strawberries, powdery mildew is caused by *Podosphaera aphanis* (Wallr.) U. Braun & S. Takam and is regarded as an important disease worldwide (Maas, 1984). *P. aphanis* typically infects strawberry leaves. The infections start with the emergence of mycelium patches on the upper or lower side of the leaves (Figure 2 A), which then grow and converge to cover the whole leaf surface (Figure 2 B). With disease progression, the leaves curl (Figure 2 C) and may show red spots on the lower leaf surface. Severe powdery mildew infections on the leaves can result in the occurrence of necrosis and subsequent defoliation (Maas, 1984). In addition, *P. aphanis* can infect leaf petioles, flowers (Figure 2 D) and fruit (Carisse & Bouchard, 2010; Maas, 1984).

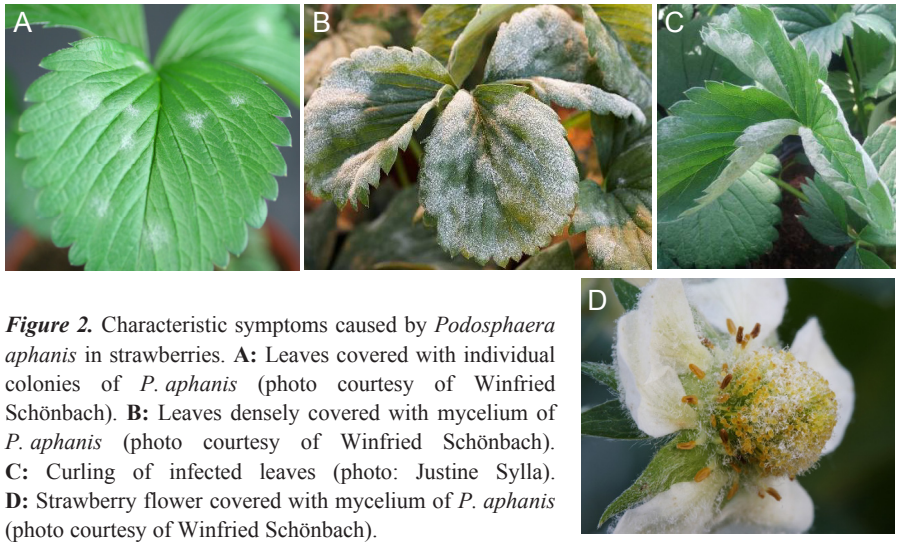

Figure 2. Characteristic symptoms caused by *Podosphaera aphanis* in strawberries. **A:** Leaves covered with individual colonies of *P. aphanis* (photo courtesy of Winfried Schönbach). **B:** Leaves densely covered with mycelium of *P. aphanis* (photo courtesy of Winfried Schönbach). **C:** Curling of infected leaves (photo: Justine Sylla). **D:** Strawberry flower covered with mycelium of *P. aphanis* (photo courtesy of Winfried Schönbach).

Strawberry leaves and fruit exhibit age-related (ontogenic) resistance to *P. aphanis* (Carisse & Bouchard, 2010; Amsalem *et al.*, 2006). Recently, Carisse and Bouchard (2010) reported that fully expanded strawberry leaves and ripe strawberry fruit were significantly less susceptible to *P. aphanis* than young leaves, flowers and green fruit. Thus, powdery mildew does commonly not occur before harvested and mulched plants start to re-grow in field grown strawberries. Infections with *P. aphanis* at this period might eventually have an impact on winter survival but do not have severe effects on the strawberry yield in the current or in the following year (Carisse & Bouchard, 2010; Maas,

1984). Therefore, the economic losses caused by *P. aphanis* can be regarded as insignificant in field grown strawberries (Xiao *et al.*, 2001; Maas, 1984).

In protected strawberry cultivation, however, severe epidemics of *P. aphanis* can occur. Here, powdery mildew infections of fruit are favoured and, therefore, yield and fruit quality can be considerably affected (Willocquet *et al.*, 2008; Xiao *et al.*, 2001). Rain has a negative effect on the development of most powdery mildew species. It is suggested that not germinated conidia are easily washed off from plant tissues and mycelium and conidiophores become easily damaged by rain drops (Blanco *et al.*, 2004; Sivapalan, 1993a). Furthermore, conidial germination is considered to be inhibited in the presence of water (Sivapalan, 1993b). Therefore, the increased occurrence of strawberry powdery mildew in protected cultivation can mainly be explained by the absence of precipitation (Amsalem *et al.*, 2006). In addition, further microclimatic conditions conducive to *P. aphanis* are prevailed in protected cultivation as well: long periods of temperatures higher than 20°C, high humidity and reduced light intensity (Amsalem *et al.*, 2006; Xiao *et al.*, 2001).

2.4.2 Disease cycle of powdery mildews

Most powdery mildew species can develop as both anamorphs (asexual reproduction) and teleomorphs (sexual reproduction).

After landing on a susceptible host plant, the conidia (in case of asexual reproduction) or ascospores (in case of asexual reproduction) of powdery mildews germinate and form germ tubes, appressoria as well as penetration hyphae. The penetration hyphae actively enter the epidermal host cells through enzyme production and turgor pressure and, thereafter, form haustoria within the plant cells. After successful infection, conidiophores arise from the hyphae of powdery mildews, followed by production of conidia for further asexual reproduction (Glawe, 2008; Braun *et al.*, 2002; Green *et al.*, 2002). The hyaline and usually rather large conidia (Braun *et al.*, 2002) can easily be dispersed by wind or insects but also by shaking of leaves (Glawe, 2008; Yarwood, 1957).

The sexual reproduction comprises the plasmogamy of antheridia and ascogonia and the production of cleistothecia (also called chasmothecia), which contain asci and ascospores (Glawe, 2008). Under unfavourable conditions (e.g. cold periods, hot periods, absence of green host plants), cleistothecia can serve as survival structures (Gadoury *et al.*, 2010; Jarvis *et al.*, 2002). For strawberry powdery mildew, for instance, cleistothecia are embedded in a thick mycelium that makes them less sensitive to rain and subsequent removal from strawberry leaves during winter (Gadoury *et al.*, 2010).

2.4.3 Control of powdery mildew in strawberries

The use of fungicides against *P. aphanis* is highly limited in the organic cultivation of strawberries as well. For instance, sulfur is the only fungicidal agent that is allowed for the regulation of *P. aphanis* in greenhouse grown strawberries in Germany (Trapp, 2013). For this reason, there is an urgent need for biological measures such as the use of resistant cultivars to control powdery mildew in the organic cultivation of strawberries. Only few cultivars are available being "more or less resistant" against *P. aphanis*. These are, for instance 'Senga Sengana', 'Cesena' and 'Dania' (Daugaard & Lindhard, 2000). None of these cultivars, however, is coevally satisfactory with respect to resistance against other economically important strawberry diseases, fruit quality and shelf-life. Therefore, these cultivars are not suited for the organic strawberry cultivation (Pertot *et al.*, 2008; Daugaard & Lindhard, 2000). Furthermore, it was reported that UV-B radiation might suppress *P. aphanis* in strawberries due to induced plant resistance (Kanto *et al.*, 2009). But still, it is unclear whether the use of UV-B radiation can be realized in the field. Therefore, the application of microbial BCAs displays an important measure to suppress *P. aphanis* in organically grown strawberries.

In conventionally grown strawberries, powdery mildew is commonly controlled with fungicides. In the perennial strawberry cultivation in open fields, fungicides are mainly applied after harvest, i.e. when plants start to re-grow after mulching (Jung, 2012). In contrast, frequent fungicide use is required in the protected strawberry cultivation (in tunnels or greenhouses) (Jung, 2012; Sombardier *et al.*, 2010), which poses an increased risk for fungicide resistances (Sombardier *et al.*, 2010; Maas, 1984) Again, the integration of BCA treatments in conventionally grown strawberries might be a promising approach to reduce fungicide treatments, in particular in the protected strawberry cultivation.

2.5 Microbial biological control agents

2.5.1 Modes of action

Microbial biological control agents are characterized by their modes of action causing direct or indirect antagonism towards the plant pathogens. Direct antagonism requires occupation of the same niches by both the BCAs and the plant pathogens. The most important modes of action, which might be involved in the control of plant pathogens, are antibiosis, parasitism,

competition and induced resistance (Pal & McSpadden Gardener, 2006; Elad & Stewart, 2004; Bélanger & Labbé, 2002; Blakeman & Fokkema, 1982).

Antibiosis is a form of direct antagonism between a BCA and a pathogen. It relies on the BCAs' production of secondary metabolites (preferably *in planta*) that inhibit plant pathogens (Alabouvette *et al.*, 2006; Elad & Stewart, 2004). Recently, the whole genome of *Bacillus amyloliquefaciens* FZB42 was sequenced. It was thereby revealed that 8.5% of this agent's genetic capacity is devoted to secondary metabolite synthesis (Chen *et al.*, 2009a). Accordingly, control of foliar plant pathogens by secondary metabolites has repeatedly been reported for *B. amyloliquefaciens* (Li *et al.*, 2012; Zhang *et al.*, 2012; Chen *et al.*, 2009b), but also for other *Bacillus* species (Romero *et al.*, 2007a; Touré *et al.*, 2004; Guetsky *et al.*, 2002b). Further important representatives of BCAs causing antagonism by antibiosis are, for instance, *Pseudomonas* spp. and *Trichoderma* spp. (Alabouvette *et al.*, 2006; Elad & Stewart, 2004; Tronsmo & Dennis, 1977).

Parasitism of plant pathogenic fungi, also termed mycoparasitism, represents another mechanism of direct antagonism. The penetration of the pathogen's mycelium, however, is dependent on the BCAs' ability to produce cell wall degrading enzymes (CWDE) such as chitinases, mannases and proteinases (Elad & Stewart, 2004). Members of the genus *Trichoderma* (Verma *et al.*, 2007; Elad & Stewart, 2004; Tronsmo & Dennis, 1977) as well as *Ampelomyces quisqualis* (Angeli *et al.*, 2012; Romero *et al.*, 2007b; Romero *et al.*, 2003) are prominent representatives of mycoparasites.

Antagonism can also arise from microbial BCAs that compete with plant pathogens for nutrients and space. A more rapid nutrient uptake and colonization of plant tissues by microbial agents will reduce the amount of available nutrients as well as the available space for fungal pathogens, which in turn will reduce spore germination and further growth of fungal pathogens (Alabouvette *et al.*, 2006; Elad & Stewart, 2004; Blakeman & Fokkema, 1982). Competition for nutrients is, for instance, the main biocontrol mechanism of *Aureobasidium pullulans* (Castoria *et al.*, 2001; Lima *et al.*, 1997).

BCAs can also induce plant resistance locally or systemically by activating the plant's defence mechanisms (e.g. hypersensitive reaction, formation of papilla, production of pathogenesis-related proteins) and thereby prevent subsequent infections by plant pathogens (Alabouvette *et al.*, 2006; Elad & Stewart, 2004; Bélanger & Labbé, 2002). This mode of action was, for instance, reported for *Bacillus* spp. (Li *et al.*, 2012; Zhang *et al.*, 2012; Guetsky *et al.*, 2002b) and *T. harzianum* (Elad, 2000; Elad *et al.*, 1998).

Many biological control agents do not exclusively feature one single mode of action to suppress plant pathogens (e.g. *Trichoderma* spp.). It is, instead, assumed that several mechanisms are involved in the biological control of plant pathogens (Elad & Stewart, 2004; Elad, 2000; Tronsmo & Dennis, 1977).

2.5.2 Biological control of grey mould in strawberries

It is essential to protect flowers from infections with *B. cinerea*. Accordingly, BCA applications during flowering are considered to be most efficient to suppress preharvest as well as postharvest *B. cinerea* incidence (Droby & Lichter, 2004; Ippolito & Nigro, 2000; Lima *et al.*, 1997). In contrast, postharvest BCA applications are considered to be less effective in suppressing previously established infections of postharvest pathogens such as *B. cinerea.* It was suggested that postharvest BCA applications may suppress weak infections in wounded fruit only (Ippolito & Nigro, 2000).

Competition for nutrients is considered to be the most effective mode of action to control *B. cinerea* (Sharma *et al.*, 2009), which - as a necrotrophic pathogen - requires high levels of nutrients to germinate and to grow (Elad & Stewart, 2004; Blakeman & Fokkema, 1982). Accordingly, *A. pullulans* can be regarded as a promising microbial agent because it efficiently competes for exogenous nutrients as its main mode of action (Lima *et al.*, 1997). Furthermore, *A. pullulans* is well adapted to the phyllosphere (Chi *et al.*, 2009), which is another prerequisite for efficient suppression of *B. cinerea* (Ippolito & Nigro, 2000). Lima *et al.* (1997) reported that preharvest applications of *A. pullulans* significantly reduced postharvest *B. cinerea* infections on strawberries. Furthermore, treatments with *A. pullulans* significantly suppressed the growth of *B. cinerea* on wounded, green strawberry fruit under controlled conditions and delayed the incidence of *B. cinerea* on stored, ripe fruit after preharvest treatments with *A. pullulans* (Adikaram *et al.*, 2002).

Also, several *Trichoderma* species are considered as efficient BCAs against grey mould in strawberries, presumably due to the involvement of more than one mode of action (Elad, 2000). The first investigation on the use of BCAs against *B. cinerea* in strawberries was performed with several *Trichoderma* species (Tronsmo & Dennis, 1977). In this investigation, applications of *Trichoderma* to field grown strawberries during flowering reduced the incidence of *B. cinerea* in the field and on stored fruit. Freeman *et al.* (2004) tested several strains of *Trichoderma harzianum* (T-39, T-166, T-161) against *B. cinerea* in strawberries under greenhouse conditions as well. It was shown that *T. harzianum* T-39 significantly reduced *B. cinerea* in flowers when applied at concentrations of 0.04% (approx. 4×10^7 conidia plant^{-1}) at 2 d, 7 d

and 10 d intervals, whereas the same strain was not effective when applied at 0.8% concentrations or at 0.4% concentration when applied simultaneously with another *T. harzianum* strain (T-166) (Freeman *et al.* 2004). Kovach *et al.* (2000) reported on effective reductions in *Botrytis* damaged strawberries under field conditions and in mist chambers, particularly when the microbial agent was delivered by bumble bees and honey bees. Likewise, honey bees delivered *T. harzianum* conidia reduced the development of strawberry grey mould in two seasons (Shafir *et al.*, 2006).

Furthermore, the fungus *Clonostachys rosea* can be regarded as a promising agent against *B. cinerea*. Applications of *C. rosea* reduced the incidence of *B. cinerea* in flowers and fruit under field conditions (Cota *et al.*, 2009; Cota *et al.*, 2008). Mamarabadi *et al.* (2008) showed that inhibition of *B. cinerea* in strawberry leaves is linked to the expression of chitinase genes by *C. rosea*.

Apart from fungal BCAs, bacteria and yeasts can be important microbial agents towards *B. cinerea* as well (Elad & Stewart, 2004). The density of conidiophores of *B. cinerea* in strawberry leaves was effectively reduced by *B. subtilis* (Helbig & Bochow, 2001) as well as by the yeast *Cryptococcus albidus* (Helbig, 2002). Under field conditions, the incidence of *B. cinerea* was reduced by *B. subtilis* by 40% in one field experiment as well, whereas insignificant reductions of *B. cinerea* incidence (21% and 17%) were observed in the two other trials (Helbig & Bochow, 2001).

2.5.3 Biological control of powdery mildews

Due to their biotrophic life style, powdery mildews are not depending on exogenous nutrients. Therefore, powdery mildews cannot be controlled by BCAs that deplete nutrients in the phyllosphere (Kiss, 2003). Instead, powdery mildews can directly be controlled by BCAs through antibiosis or mycoparasitism or indirectly through inducing resistance in host plants (Kiss, 2003; Bélanger & Labbé, 2002).

The most prominent biological control agent of powdery mildews is the hyperparasitic fungus *A. quisqualis*, which already provided good efficacies against powdery mildews in different crops (Romero *et al.*, 2007b; Kiss *et al.*, 2004; Kiss, 2003; Romero *et al.*, 2003; Elad *et al.*, 1998; Elad *et al.*, 1996). *A. quisqualis* parasitizes and kills the cells of powdery mildew (e.g. hyphae and conidiophores) and thereby restrains the conidiation of the pathogen (Kiss, 2003). Recently, Angeli *et al.* (2012) tested 24 strains of *A. quisqualis* with respect to their mycoparasitic activity against powdery mildew in strawberry, grapevine and cucumber plants under controlled conditions. In their study, all tested strains were effective against the powdery mildews. However, the grapevine as well as the cucumber powdery mildew was generally more

susceptible to the tested *A. quisqualis* strains than *P. aphanis* in strawberries. It, was furthermore, shown that mycoparasitic activity of the different *A. quisqualis* strains positively correlated with the *in vitro* activity of two cell wall degrading enzymes, namely chitobiase and protease (Angeli *et al.*, 2012).

But also other BCAs than *A. quisqualis* have shown biocontrol efficacies against powdery mildew. For instance, *T. harzianum* effectively suppressed *Sphaerotheca fusca* in greenhouse grown cucumber plants and was almost as efficient as *A. quisqualis* in young leaves (Elad *et al.*, 1998). In strawberries, the effects of *A. quisqualis*, *T. harzianum* and *B. subtilis* on the suppression of *P. aphanis* alone and in alternation with fungicides were studied in the greenhouse and in tunnels (Pertot *et al.*, 2008). It was shown that the tested BCAs alone were able to reduce powdery mildew incidence in the greenhouse and in tunnel-protected strawberries as compared to the untreated control samples. Further investigations of Pertot *et al.* (2007) revealed efficient suppression of *P. aphanis* by *T. harzianum* and two strains of *B. subtilis* (*B. subtilis* QST 713 and *B. subtilis* F77) in leaf bioassays as opposed to *A. quisqualis*. Several strains of *B. subtilis* also significantly controlled cucurbit powdery mildew in melon seedlings (Romero *et al.*, 2007b). It was demonstrated that the efficacy of *B. subtilis* against cucurbit powdery mildew was linked to the production of secondary metabolites (Romero *et al.*, 2007a). In strawberries, also applications of *Penicillium oxalicum* suppressed powdery mildew on runners of different cultivars and strawberry lines under controlled (growth chambers) as well as under field conditions (De Cal *et al.*, 2008).

2.6 Challenges of biological control in the phyllosphere

Despite the promising results from various studies on the use of microbial agents against foliar diseases, only comparably few antagonistic micro-organisms were registered as biological control agents (Ehlers, 2006; Fravel, 2005). The most limiting factor is the inconsistent treatment success of BCAs, which was particularly observed under field conditions (Alabouvette *et al.*, 2006; Fravel, 2005; Magan, 2004; Butt & Copping, 2000; Andrews, 1992). For instance, sprays with the commercially available products Binab®TF-WP (*T. polysporum* and *T. harzianum*) and Prestop (*Gliocladium catenulatum*) did not effectively reduce *B. cinerea* in field grown strawberries (Prokkola & Kivijärvi, 2007; Prokkola *et al.*, 2003). Similarly, three commercially available *Trichoderma* products (Binab®TF-WP, Trichodex WP, Rootshield®) and one experimental *Trichoderma* strain (*T. harzianum* P1) failed to control *B. cinerea* in greenhouse grown strawberries (Hjeljord *et al.*, 2000).

Indeed, the use of BCAs in the phyllosphere is considered to be more challenging than in the rhizosphere, which represents a more stable habitat for introduced BCAs (Andrews, 1992).

The phyllosphere is a very dynamic environment and is, therefore, strongly affected by fluctuating environmental factors (e.g. rain, temperature, radiation, water availability, relative humidity, dew) (Lindow & Brandl, 2003; Kinkel, 1997). Already in this context, the introduced BCAs, like other immigrating microorganisms, have to tolerate a very hostile environment (Jacobsen, 2006; Magan, 2006). Furthermore, the introduced microbial agents have to cope with nutrient paucity in the phyllosphere and have to compete with the resident phyllosphere microbiota for nutrients and space (Jacobsen, 2006; Hjeljord *et al.*, 2000). Hjeljord *et al.* (2000), for instance, reported on better colonization of different *Trichoderma* strains on disinfected, senescent strawberry leaf discs, i.e. in the absence of an indigenous microflora and in the absence of competition for nutrients, as compared to non-disinfected leaf discs. Nutrient availability and the composition of resident microbial communities, however, can in turn considerably vary in dependence of multiple factors such as environmental conditions and plant age (Jacobsen, 2006; Kinkel, 1997). The problem is, accordingly, that inconsistent conditions in the phyllosphere result in inconsistent establishment of the introduced BCAs and, therefore, in inconsistent efficacies against the target pathogens.

There have been several investigations on the establishment and survival of BCAs in the phyllosphere under controlled conditions, which in most cases revealed a quick decline of the BCA populations in the phyllosphere. Guetsky *et al.* (2002a) studied the survival of the bacterium *B. mycoides* and the yeast *Pichia guilermondii* on greenhouse grown strawberry leaves and fruit using plate counts. They revealed a rapid decline of the BCA populations to 1/50 and 1/500 after 5 and 19 days in single strain treatments, respectively. In another study, it was shown that the survival of several *T. harzianum* strains on strawberry leaves considerably declined within 3 days under greenhouse conditions (Freeman *et al.*, 2004). Likewise, populations of *T. atroviride* SC1, which were applied to greenhouse grown strawberries, rapidly declined from approx. 3×10^5 to 1×10^1 cfu mm^{-2} leaf area within 7 days (Longa *et al.*, 2008). Elad *et al.* (1998) reported that the population size of *T. harzianium* T39 was reduced from 7×10^3 to 5×10^3 cfu mm^{-2} within six days on cucumber leaves under greenhouse conditions. Accordingly, BCA applications usually have to be repeated to compensate for the BCAs' rapid decline in the phyllosphere (Jacobsen, 2006).

There are several approaches to improve the BCAs' efficacies in the phyllosphere. Biological control might be improved, for instance, by using BCA strains with increased tolerance to the hostile conditions in the phyllosphere (Ippolito & Nigro, 2000) or by improved formulations (e.g. containing components that protect the BCAs from UV radiation or that facilitate their adherence to plant surface) (Fravel, 2005; Butt & Copping, 2000). There is also increasing evidence that the manipulation of the growth conditions during the fermentation process already affects the accumulation of endogenous compounds (e.g. sugars) in the propagules, which might result in increased tolerance of the BCAs to environmental stress in the phyllosphere (e.g. protection from desiccation) (Magan, 2006). Furthermore, pre-activation of the BCAs' conidia, i.e. the preliminary initiation of conidial germination in nutrient solution prior to application, might reduce the conidia's germination time at the target site under suboptimal temperature conditions and, thereby, improve their efficacy against *B. cinerea* as it was shown for *T. harzianum* under field conditions (Hjeljord *et al.*, 2001).

The combined use of BCAs represents another promising approach to overcome inconsistent BCA efficacies in the phyllosphere. In strawberries, for instance, mixtures of the BCAs *P. guilermondii* and *B. pumilis* enhanced the suppression of *B. cinerea* on leaf discs as well as on leaves and fruit from greenhouse grown plants as compared to single BCA applications. It was suggested that improved disease suppression arose from distinct ecological requirements (Guetsky *et al.*, 2001) and different modes of action of the two BCAs (Guetsky *et al.*, 2002a; Guetsky *et al.*, 2002b). According to a theoretical model, increased disease suppression can as well result from the use of two BCAs which are differentially adapted to distinct habitats (Xu & Jeger, 2013). However, most of the investigations on simultaneous use of BCAs were performed under controlled conditions. Therefore, there is still little available knowledge if disease suppression can as well be improved under field conditions. Furthermore, it is worth to note that also antagonistic effects between BCAs might occur. Studies with mixtures of the biocontrol agents *B. subtilis* (Serenade^{TM}), *T. harzianum* Rifai T22 (Trianum^{TM}) and *T. atroviride* (Sentinel^{TM}) resulted in reduced suppression of *B. cinerea* in strawberries as compared to some single BCA treatments (Xu *et al.*, 2010; Robinson-Boyer *et al.*, 2009). Therefore, potential antagonistic interactions between simultaneously applied BCAs should be considered as well.

2.7 Objectives

The objective of the present thesis was to investigate whether the simultaneous use of BCAs represents a solution for a more consistent biological control of foliar strawberry diseases under controlled and field conditions. The investigations focused on the interactions between the BCAs as well as their interactions with the resident phyllosphere microbiota and the target pathogens (Figure 3) to gain more knowledge of the microbial interactions in the phyllosphere and, thereby, of potential limitations for phyllosphere applied BCAs with regard to biological control of foliar strawberry diseases.

The specific objectives of the present thesis were:

- to study the *in vitro* compatibility of microbial agents (paper I)
- to characterize the resident leaf microbiota of strawberries (paper II and III)
- to describe the interactions between resident microbiota and introduced BCAs in the strawberry phyllosphere (paper II and III)
- to study the effects of introducing the BCAs as single and multiple strain treatments on strawberry grey mould under field conditions (paper II and IV)

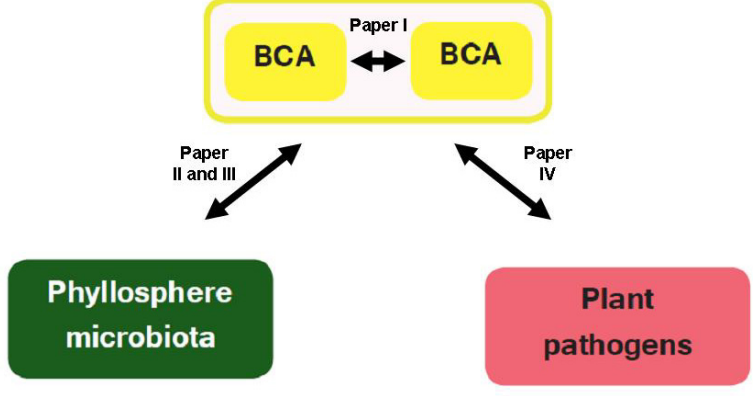

Figure 3. Overview of microbial interactions as brought into focus in the present thesis.

The hypotheses included in the present thesis were determined as follows:

(i) constituents in multiple strain treatments do not counteract each other *in vitro* (paper I)

(ii) multiple BCA treatments with known compatible biological control agents increase their effectiveness with respect to powdery mildew control on strawberry leaf discs (paper I)

(iii) resident microbiota of strawberry leaves changes in dependence of the crop's phenological stage (paper II and III)

(iv) introduced BCAs do not have a significant impact on the resident leaf microbiota of strawberry, irrespective of their application as single or multiple strain treatments (paper II and III)

(v) multiple strain treatments improve the biological control of grey mould in field grown strawberries (paper II, IV)

3 Materials and methods

3.1 Biological control agents

The BCAs used in the present study are displayed in Table 1. These microbial agents (including the two entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*) were selected from approx. 90 isolates because of their good efficacies towards both *B. cinerea* and *P. aphanis* in previously performed laboratory assays (dual culture tests, assays on detached flowers and leaves; unpublished data, not included in the present thesis). A further selection from these BCAs was made for the field experiments. This selection was based additionally on the availability of registered BCA products in Germany in 2009 in order to enable the performance of large-scale applications.

All isolates were stored in cryoculture (-80 °C) and re-cultured when required for laboratory experiments. For the field experiments, the respective commercially available products were used according to the manufacturers' instructions.

Table 1: *List of biological control agents included in the present study*

Organism	Preparation	Kindly provided by
Ampelomyces quisqualis AQ10	AQ10® WG	Intrachem Bio Deutschland GmbH&Co.KG
Trichoderma harzianum T58	Trichostar®	Gerlach Natürliche Düngemittel GmbH&Co.KG
Trichoderma harzianum T22	Trianum-P	Koppert Biological Systems
Penicillium oxalicum DSM 898	-	German Collection of Microorganisms and Cell Cultures (DSMZ)
Aureobasidium pullulans DSM 62074 [1]	-	German Collection of Microorganisms and Cell Cultures (DSMZ)
Aureobasidium pullulans DSM 14940 & DSM 14941 [2]	BoniProtect®forte	bio-ferm GmbH
Metarhizium anisopliae 43	-	Julius Kühn-Institute, Institute for Biological Control, Germany
Beauveria bassiana ATCC 74040	Naturalis®	Intrachem Bio Deutschland GmbH&Co.KG
Bacillus subtilis FZB24	FZB24®fl.	ABiTEP GmbH
Bacillus amyloliquefaciens FZB42	RhizoVital®42 fl.	ABiTEP GmbH
Enterobacter radicincitans	Experimental strain	ABiTEP GmbH

[1] This strain of *Aureobasidium pullulans* was used in the laboratory experiments.

[2] These strains of *Aureobasidium pullulans* were used in the field experiments.

3.2 Experimental set-up

3.2.1 Laboratory experiments (paper I)

Inhibition assays

The *in vitro* compatibility of the tested BCAs was investigated by means of different inhibition assays on nutrient media.

For the inhibition assays, conidial and bacterial suspensions and the culture filtrates were produced as described in paper I. Not all BCAs could be included in all inhibition assays as *A. quisqualis* did not grow radially on any of the two tested nutrient media and *A. pullulans* did not grow on one of the two selected nutrient media.

Antagonistic effects of bacterial BCAs on the mycelial growth of fungal BCAs were tested by inoculating PDA (potato dextrose agar, Merck, Germany) and 0.1 TSA (tryptic soy agar, Difco, BD, France) plates with two mycelial plugs (Ø = 5 mm) from the extension zone of the mycelium of a fungal BCA (6-8 d old culture). Bacterial strains were streaked between the mycelial plugs according to the fungal expansion. The plates were incubated until the mycelial fonts in the respective control samples merged.

Inhibitory effects of fungal BCAs on bacterial growth were determined by mixing diluted (1:100) bacterial suspensions with nutrient media (PDA and 0.1 TSA) as described in paper I. The solidified agar was inoculated with two mycelial plugs from a fungal BCA and the plates were incubated until a uniform bacterial lawn was visible on the nutrient media.

Inhibitory interactions between two bacterial BCAs were investigated by mixing diluted (1:100) bacterial suspensions with PDA and 0.1 TSA as well. Two heated stainless steel tubes (Ø = 8 mm) were placed onto the solidified plates and filled with aliquots of another, undiluted bacterial suspension as described in paper I. In control plates, no bacterial suspensions were transferred into the steel tube. The plates were incubated until a uniform bacterial lawn developed in the media.

Antagonistic effects between two fungal BCAs were tested by mixing PDA with aliquots of selected fungal culture filtrates as described in paper I. In control plates, PDA was not augmented with fungal culture filtrates. Aliquots of diluted (1:100) conidial suspensions were plated onto the solidified plates using a spiral plater (Whitley Automatic Spiral Plater, Don Whitley Scientific, England). The plates were incubated until fungal colonies were visible.

Each inhibition assay was carried out twice.

Leaf disc assays

Leaf discs assays were performed to investigate if biological control of strawberry powdery mildew is improved by compatible BCA combinations. These assays were divided into two experiments, each comprising five BCA (see paper I for more details).

For the leaf disc assays, plant material was provided by growing strawberry plants (cv. Elsanta) in the greenhouse. Detailed information about the growth conditions are found in paper I. In addition, a constant inoculum of the obligate biotrophic pathogen *P. aphanis* was needed for the leaf disc assays. The pathogen was collected from naturally infected strawberry plants in Hesse (Germany) and conserved on strawberry plants (cv. Elsanta) by regular inoculation of young and healthy plants with *P. aphanis* (see paper I for more details).

The leaf disc assays were conducted as follows. For single BCA treatments, 10 ml of BCA suspensions were used for the assays, whereas each 5 ml of two different BCA suspensions were mixed carefully and used for multiple BCA treatments. Sterile 1/8 strength Ringer solution (Ringer tablets, Merck, Germany) was used for the control treatments. Five detached leaf discs (Ø = 1 cm), which were obtained from young leaves of macroscopically healthy strawberry plants, were dipped into the respective BCA suspension and positioned onto water agar in a Petri dish (Figure 4 A). When the leaf discs appeared dry, the Petri dishes were covered with the lid. Six plates per treatment were incubated for 24 h (at approx. 22.5 °C and 65 % RH in average, photoperiod: 12 h (light) and 12 h (dark)). After incubation, leaf discs were inoculated with *P. aphanis* conidia using a paint-brush and, thereafter, were incubated for nine more days to allow *P. aphanis* to grow and to conidiate (Figure 4 B). Each experiment of the leaf disc assays was carried out twice.

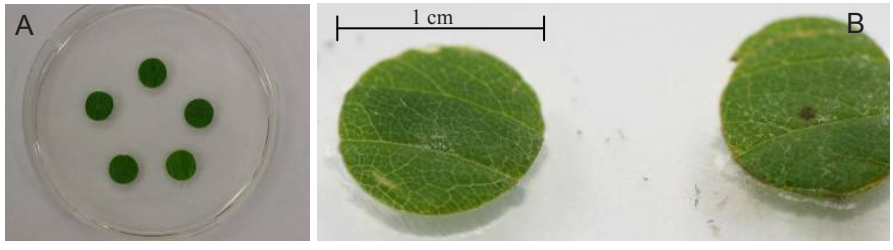

Figure 4. Leaf disc assays. **A:** Leaf discs on water agar (Photo: Justine Sylla). **B:** Powdery mildew infections on leaf discs (Photo: Justine Sylla).

3.2.2 Field experiments (paper II-IV)

All field experiments were performed at the same experimental site (1300 m²) at Geisenheim, Germany. At the experimental site, soil was characterized as follows: sandy silty loam (haugh), pH 7.2 and 4% carbonate content.

Strawberry plants cv. Elsanta were used for all field experiments. Green plants were planted on August 4th, 2009 for the field experiment in 2010 (paper II). In the following year, new green plants were planted on August 12th, 2010 at the same site. These plants were used for the field experiments in 2011 and 2012 (paper III and IV). After the first cropping season in 2011, plants were mulched immediately after harvest to initiate the re-growth of leaves.

The experimental field consisted of 36 plots with 80 strawberry plants each. Nine plots were arranged in four rows, respectively. Each plot was randomly assigned to one of the nine treatments within each of the four rows. Individual plots consisted of four single rows with 20 plants each.

Strawberry plants were mulched with straw at the end of flowering and drip irrigated in each year. Furthermore, weeds were removed mechanically when needed, whereas no other plant protection measures than the treatments described below were applied for pest and disease control.

From flowering through harvest, strawberry plants were treated with the BCAs in weekly intervals. The different treatments were applied with a compression sprayer (Mesto GmbH, Germany), which was connected to a three-nozzle spray system (Christian Schachtner Gerätetechnik, Germany) to allow an even delivery of the BCAs on aerial plant surfaces (Figure 5).

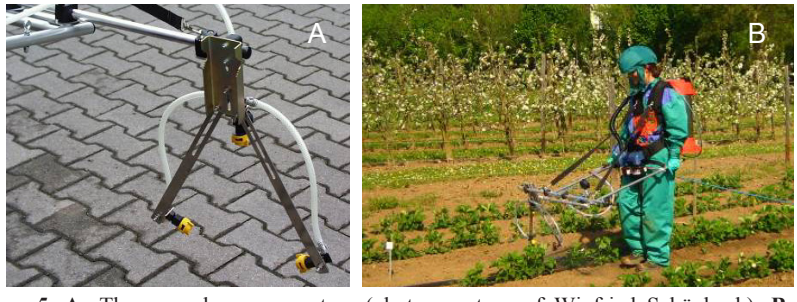

Figure 5. **A:** Three-nozzle spray system (photo courtesy of Winfried Schönbach). **B:** BCA applications in the field using a compression sprayer connected to the three-nozzle spray system (photo courtesy of Winfried Schönbach).

In the field experiment 2010, the BCA preparations RhizoVital®42 fl. (2.5×10^{10} endospores ml^{-1} of *Bacillus amyloliquefaciens* FZB 42), Trianum-P (1.0×10^{9} conidia g^{-1} of *Trichoderma harzianum* T22) and Naturalis® (2.3×10^{7} conidia ml^{-1} of *Beauveria bassiana* ATCC 74040) were applied to the strawberry plants as single strain treatments as well as multiple strain treatments (paper II). In the field experiments 2011 and 2012 (paper III and IV), the *Trichoderma*-preparation was replaced by the BCA preparation BoniProtect®forte (7.5×10^{9} blastospores g^{-1} of *Aureobasidium pullulans* DSM 14940 and DSM 14941) as *Trichoderma*-treated fruit have shown to be covered by *Trichoderma* mycelium during storage in 2010. Control plots were treated with surface water or with fungicides (see paper II and IV for further details) in all field experiments.

In all field experiments, a sprinkler was installed in the center of each plot to simulate nightly precipitation for creating conducive conditions for *B. cinerea* infections (see paper IV for more details). Furthermore, 70 of 80 plants per plot were not harvested in order to facilitate *B. cinerea* development in the field.

3.3 Analyses

3.3.1 Laboratory experiments (paper I)

Inhibition assays

In the inhibition assays, zones of inhibition (cm) were measured on PDA and 0.1 TSA plates. For the inhibition assay using fungal culture filtrates, fungal colonies were counted on PDA plates and the amount of colony-forming units (CFU) ml^{-1} was calculated for each tested conidial suspension.

Leaf disc assays

In the leaf disc assays, the leaf discs of each Petri dish were suspended in Ringer solution to detach powdery mildew conidia from the leaf discs into the solution. The number of powdery mildew conidia was counted microscopically three times per sample using a Thoma counting chamber and, thereafter, the number of conidia cm^{-2} leaf area was calculated.

3.3.2 Field experiments (paper II-IV)

Quality of commercially available BCA products

The viability of BCAs in the BCA preparations was examined for the field experiments 2011 and 2012 (paper IV). For this purpose, each of the tested BCA preparations (RhizoVital®42 fl., BoniProtect®forte and Naturalis®) was serially diluted in sterile 1/8 strength Ringer solution on each day of BCA treatment and, thereafter, spiral-plated on nutrient media. The CFU ml^{-1} of the microbial agents was calculated for the respective (undiluted) BCA preparation.

Sample collection and microbe extraction for microbiological analyses

In all field experiments, leaf samples were collected once prior to BCA applications and twice after BCA applications in each experimental plot of selected treatments (see paper II and III for further information). According to the identification key for phenological stages (BBCH) of strawberries (Meier *et al.*, 1994), leaf samples were collected at the phenological stages BBCH 59, BBCH 65 and BBCH 78 in 2010 (paper II), whereas in 2011 and 2012 leaf samples were taken at BBCH 60, BBCH 73 and BBCH 93, respectively (paper III). The leaf samples were washed as described in paper II and III and used for plate counts as well as for 454 pyrosequencing. In the field experiments 2011 and 2012, fruit samples were collected as well (paper IV). In both years, fruit samplings took place at BBCH 91. The fruit were washed as described in paper IV.

Plate counts

Aliquots of the wash solutions obtained from leaf and fruit samples were used for plate counts of different microbial groups (Table 2) on different nutrient media. The nutrient media were augmented with antibiotics or fungicides (see paper II - IV for details on nutrient media).

Undiluted wash solutions were spiral-plated on R2A (R2A agar, Difco, BD, France), diluted PDA, *Beauveria* selective medium (BSM) and *Trichoderma* selective medium (TSM) to determine the CFU of total bacteria, total fungi, *Beauveria* spp. and *Trichoderma* spp., respectively (paper II-IV). Furthermore, wash solutions were spiral-plated on SA (Sabouraud dextrose agar, BD, France) and morphologically identified colonies of *Aureobasidium* spp. were enumerated to determine the CFU of these yeast-like fungi. Heat-treated (80 °C for 15 min) wash solutions were spiral-plated on 0.1 TSA to determine the CFU of endospore-forming bacteria. Plate count results were expressed as CFU g dry weight $(DW)^{-1}$.

Table 2: *Overview of plate count analyses of different microbial groups in papers II-IV*

Plate counts of	**Paper II** (leaf samples)	**Paper III** (leaf samples)	**Paper IV** (fruit samples)
Total bacteria	x	x	x
Total fungi	x	x	x
Endospore-forming bacteria	x	x	x
Beauveria spp.	x	x	x
Trichoderma spp.	x		
Aureobasidium spp.		x	x

454 pyrosequencing

To analyze the fungal and bacterial communities by 454 pyrosequencing only leaf samples were used. For 454 pyrosequencing, the wash solutions were processed as described in papers II and III.

The obtained pellets were used for extraction of genomic DNA (Power Soil® DNA Isolation Kit, Sued-Laborbedarf GmbH, Gauting, Germany). Fragments of the fungal internal transcribed spacer ribosomal RNA (ITS rRNA) gene were amplified using the primer pairs ITS1 and ITS2 (Buée *et al.*, 2009), whereas fragments of the bacterial 16S rRNA gene were amplified using the primer pairs 27F and 337R (Hamp *et al.*, 2009). Prior to amplification, primers have been modified for 454 pyrosequencing as described in paper II and III. After amplification, fungal and bacterial PCR products were purified (HiYield PCR Clean-up/Gel Extraction Kit, Sued-Laborbedarf GmbH, Gauting, Germany) and pooled at equal molar

concentrations, respectively. Afterwards, the fungal and bacterial amplicon pools were sent to LGC Genomics GmbH (Berlin, Germany) for 454 pyrosequencing.

Harvest assessments

In the field experiments 2011 and 2012, ten plants per plot (from the two inner rows) were used for harvest assessments (paper IV). Both healthy berries as well as *Botrytis* infected fruit were harvested twice a week. The fresh weights of healthy and infected fruit per plant (g plant^{-1}) for the entire fruiting season were calculated and expressed as percentage values as described in paper IV.

Assessment of Botrytis cinerea incidence

In the field experiments 2010, 2011 and 2012, ten plants per plot (from the two inner rows) were randomly selected and labelled for disease assessments. Disease assessments were done once maturity of fruit began, i.e. at BBCH 85, BBCH 87 and BBCH 89 in 2010 (paper II) as well as at BBCH 89, BBCH 91 and BBCH 92 in 2011 and 2012 (paper IV). The number of healthy and *Botrytis* infected fruit was counted for each plant. The disease incidence of *B. cinerea* and disease reduction was calculated as described in paper II and IV.

Development of Botrytis cinerea during storage

A set of thirty fruit was harvested (or all fruit if less than thirty fruit were harvested) and used for the assessment of *B. cinerea* development during storage two times per fruiting season (2011 and 2012). The visually healthy strawberry fruit were placed in plastic trays, stored at 20°C for 7 days and examined for *B. cinerea* infections after two, four, six and seven days after harvest (DAH). Infected fruit were enumerated and removed from the trays. Healthy fruit remained in the trays until they became infected or until 7 DAH. The incidence of *Botrytis cinerea* and disease reduction was calculated as described in paper IV.

3.3.3 Statistics

Basic statistic analyses (paper I-IV) were performed with Statistica software package, version 7.1 (StatSoft, 2005). Taxonomic assignment of 454 pyro-sequencing data was done using blast sequence analysis (BLASTn 2.2.25+) of individual sequence reads against NCBI (National Center for Biotechnology Information) NT database and using Metagenome Analyzer (MEGAN), version 4.62.3 (Huson *et al.*, 2007) in paper II.

Using QIIME Virtual Box version 1.6.0 (Caporaso *et al.*, 2010), 454 pyrosequencing data were analyzed in paper III, including picking of operational taxonomic units (OTUs) and alignment of representative sequences of OTUs with reference sequences from databases (Greengenes database and UNITE database) using RPD classifier (Wang *et al.*, 2007). Diversity indices were calculated using BioToolKit 320 (Chang Bioscience, 2005) in paper II and using the paleontological statistics software package (PAST), version 2.17b (Hammer *et al.*, 2001) in paper III. PAST was also used for analyses of similarity (ANOSIM) in paper III. Calculation of correlations, regressions as well as principal component analysis (PCA) in paper III was performed using Minitab statistical software, version 16.1.0.0 (Minitab, 2010). A detail description of statistical analyses is available in the individual papers.

4 Results and discussion

4.1 *In vitro* compatibility of microbial agents (paper I)

The simultaneous use of BCAs is regarded as a promising approach to achieve more consistent BCA efficacies against foliar pathogens in the phyllosphere (Xu & Jeger, 2013; Guetsky *et al.*, 2002a; Guetsky *et al.*, 2002b; Guetsky *et al.*, 2001). Therefore, the concept of the present thesis was to investigate the microbial interactions in the strawberry phyllosphere with regard to the potential of combined use of BCAs to meet the challenges of biological control in this habitat. Apart from the interactions between the introduced BCAs with the resident phyllosphere microbiota (chapter 4.3) and with the plant pathogens (chapter 4.4), potential interactions between the BCAs might have an impact on the outcome of biological control and, therefore, were considered as well.

Loss in consistency of a multiple strain treatment may be provoked if the ingredient strains counteract one another. This is a basic phenomenon to be foreseen when designing a multiple strain treatment. One precondition for a reliable prediction of the interactions between BCA strains in a multiple strain cocktail is that the assay mimics the conditions at the future sites of action.

In this thesis (paper I), different dual culture tests were employed. Inhibitory interactions between BCAs were observed in dual culture tests, in particular between bacterial and fungal BCA strains (Figure 6), whereas no or only few inhibitory effects were observed between the tested bacterial BCAs and fungal BCAs, respectively.

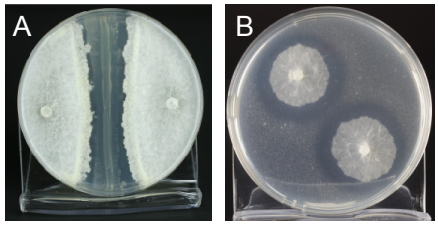

Figure 6. Inhibition assays **A:** Inhibition of mycelial growth (fungal BCA) in dual culture with a bacterial BCA (photo courtesy of Winfried Schönbach). **B:** Inhibition of bacterial growth (bacterial BCA) in the medium in dual culture with a fungal BCA (photo courtesy of Winfried Schönbach).

Inhibitory interactions were significantly different on the two tested nutrient media (PDA, 0.1 TSA) in some BCA combinations. This indicates that nutritional factors affect the interactions between the chosen BCAs. As none of the tested nutrient media reflected the nutritional conditions prevailing in the phyllosphere, it is however difficult to conclude from the results of the inhibition assays to probable BCA interactions in the phyllosphere (Knudsen *et al.*, 1997). The applied inhibitory assays, however, provide evidence that biological control agents can counteract each other under laboratory conditions.

Leaf disc assays allow to study microbial interactions when a biotrophic organism is involved, in the present case to study if biological control of strawberry powdery mildew is improved in multiple strain treatments with compatible BCAs, i.e. which have shown no or few inhibitory interactions in the dual culture tests. Some of the tested multiple strain treatments significantly enhanced biological control of *P. aphanis* on leaf discs as compared to single strain treatments. Surprisingly, these included also BCA treatments whose constituents have shown inhibitory interactions in dual culture tests (e.g. *B. subtilis* + *M. anisopliae*). This finding demonstrates that biological control of *P. aphanis* can be improved by multiple strain treatments under controlled conditions but that improved efficacies do not necessarily arise from compatible BCAs as determined on nutrient media only and emphasized the role of the plant as a matrix and of the associated resident microbiota. This finding from the leaf disc assays also underlines that results from the dual culture tests must be interpreted with care. On the contrary, impaired efficacies of the multiple strain treatments *B. subtilis* + *T. harzianum* T58 with respect to powdery mildew control were in line with the results from the inhibition assays.

So far, there is only little knowledge on the simultaneous use of BCAs against *P. aphanis*. However, both effects, i.e. improved as well as reduced disease suppression under controlled conditions were previously observed for

simultaneously applied BCAs, albeit with regard to *B. cinerea* suppression (Xu *et al.*, 2010; Robinson-Boyer *et al.*, 2009; Guetsky *et al.*, 2002a; Guetsky *et al.*, 2002b; Guetsky *et al.*, 2001). Together with the findings from this study, this implies that the outcome of simultaneous BCA use is dependent on the BCA constituents and their interactions in the multiple strain treatments.

One needs, though, to keep in mind that the leaf discs were kept under controlled conditions. It is suggested that distinct environmental and nutritional conditions as well as the resident microbiota in the phyllosphere will affect the development of BCAs and, most probably, also their interactions (e.g. formation of secondary metabolites) when applied in multiple strain treatments under greenhouse or field conditions (Alsanius *et al.*, 2009; Jacobsen, 2006; Magan, 2006; Lindow & Brandl, 2003; Yoshida, 2001; Andrews, 1992). This is supported by investigations of Hjeljord *et al.* (2000), where several *Trichoderma* strains have shown to be strongly affected by environmental factors, nutrient availability and the resident microflora. Therefore, it is difficult to draw final conclusions from the results of the leaf disc assays for the outcome of simultaneously applied BCAs with respect to *P. aphanis* control under greenhouse or field conditions. However, we suggest that BCAs combinations, which have shown to improve powdery mildew control on leaf discs, can be considered as promising candidates with respect to improved powdery mildew control in further studies *ad planta*. In this context, it should be also considered that screening for candidates for BCA combinations is hampered under field conditions due to different reasons, e.g. inconsistent environmental conditions, time consuming, space consuming (Knudsen *et al.*, 1997).

4.2 Resident leaf microbiota of strawberries (paper II-III)

Introduced BCAs may be viewed as immigrants to already existent microbial assemblies or biofilms on the target plant organs. Therefore, there is the need for the basic understanding of the composition of resident microbial communities in the strawberry phyllosphere. In the phyllosphere, resident microbial communities considerably vary depending on a variety of factors, e.g. environmental factors, nutrient and water availability and leaf properties (Hunter *et al.*, 2010; Whipps *et al.*, 2008; Lindow & Brandl, 2003; Kinkel, 1997). For this reason, we investigated the resident leaf microbiota (to exemplify the phyllosphere microbiota) in strawberries in three years of field experiment, i.e. in 2010 (paper II) as well as in 2011 and 2012 (paper III), by plate counts and 454 pyrosequencing.

It is generally suggested that bacteria are more successful colonizers of the phyllosphere than filamentous fungi (Whipps *et al.*, 2008; Krimm *et al.*, 2005). In the present thesis, culturable bacteria made up the most abundant group of microbial residents in the phyllosphere when strawberry plants began to flower (BBCH 59 - 60) in all years, whereas fungi were less abundant in two of three years (paper II and III). Investigations on the natural abundance of *Trichoderma* spp. and *Aureobasidium* spp. in the phyllosphere were included in 2010 (paper II) as well as in 2011 and 2012 (paper III), respectively. *Trichoderma* spp. were detected at BBCH 59 in 2010, albeit at low level. This finding is in line with results from other investigations, where this soil-borne fungus was isolated from leaves (Inácio *et al.*, 2002; de Jager *et al.*, 2001) and strawberry fruit (Jensen *et al.*, 2013). *A. pullulans* is regarded as a common phyllosphere resident (Chi *et al.*, 2009; Blakeman & Fokkema, 1982). It was, therefore, surprising that *Aureobasidium* spp. were not detected or only detected at a low level on strawberry leaves of flowering plants in 2011 and 2012, respectively (paper III). Interestingly, *Aureobasidium* was also not found on strawberry fruit in other investigations (Jensen *et al.*, 2013; Parikka *et al.*, 2009).

Plate count results displayed considerable differences in the culturable resident leaf microbiota at early flowering of strawberry plants between the three years of experiment. Fungal counts, for instance, were comparably low at this stage in 2010 and 2011 but surprisingly high in 2012 (Figure 2; paper II and Figure 1; paper III). Indeed, statistical analyses performed in paper III revealed that plate counts of total bacteria, total fungi and endospore-forming bacteria significantly differed on leaves from flowering plants between 2011 and 2012, respectively, although leaf samples were taken at exactly the same phenological stage of the strawberry plants. By comparing the plate counts of different samplings within one growing season, considerable changes in the culturable leaf microbiota in the course of the plant's development in the three years were evident as well (paper II and III).

Although plate counts already provided interesting results, one needs to keep in mind that this technique generally does not sufficiently reflect the leaf microbiota (Rappe & Giovannoni, 2003; Yang *et al.*, 2001) as only a small fraction of viable microorganism, e.g. only 0.1 - 3% of viable bacterial cells, are culturable (Whipps *et al.*, 2008). For this reason, 454 pyrosequencing was employed as complementary technique to obtain more detailed information on both the culturable as well as the non-culturable residents of the phyllosphere. But one should also keep in mind that 454 pyrosequencing results reflect viable and dead microorganisms. Furthermore, the information value of 454

pyrosequencing results is depending on the number of reads. The average number of bacterial 16S rRNA sequences was comparably low in most samples as compared to fungal ITS rRNA sequences in 2010 (Table 1; paper II, p. 1005) as well as in 2011 and 2012 (Table 1; paper III, p. 707). It is therefore questionable if the entire bacterial phyllosphere community was described by 454 pyrosequencing in the present thesis.

In 2010, the resident leaf microbiota was predominantly composed of members of the fungal classes Tremellomycetes and Dothideomycetes and the bacterial classes Alphaproteobacteria, Actinobacteria and Cytophagia as determined by 454 pyrosequencing shortly before flowering (Table 2 and 5; paper II). From these classes, the fungal orders Cystofilobasidiales, Filobasidiales and Capnodiales and the bacterial orders Actinomycetals, Sphingomonadales and Cytophagales were most abundant in the phyllosphere (Table 3).

Table 3: *Relative abundance of the most abundant fungal and bacterial orders in the strawberry phyllosphere at phenological stage BBCH 59[1] as determined by 454 pyrosequencing in 2010*

	Order	Mean[2]	SEM[3]
Fungi	Cystofilobasidiales (class: Tremellomycetes)	0.217	0.024
	Filobasidiales (class: Tremellomycetes)	0.188	0.018
	Capnodiales (class: Dothideomycetes)	0.126	0.014
	Tremellales (class: Tremellomycetes)	0.094	0.007
	Pleosporales (class: Dothideomycetes)	0.087	0.006
Bacteria	Actinomycetales (class: Actinobacteria)	0.286	0.026
	Sphingomonadales (class: Alphaproteobacteria)	0.187	0.054
	Cytophagales (class: Cytophagia)	0.134	0.020
	Rhodospirillales (class: Alphaproteobacteria)	0.066	0.013
	Rhizobiales (class: Alphaproteobacteria)	0.062	0.001

[1] At this stage most flowers with petals are forming a hollow ball; i.e. shortly before flowering

[2] Mean values refer to relative abundances (%) of the respective orders in nine different leaf samples (untreated).

[3] Standard error of the mean.

Members of the Filobasidiales, Cystofilobasidiales, Capnodiales and Pleosporales were also among the most abundant fungal orders in the strawberry phyllosphere at early flowering in 2011 and 2012 (Table 2 and 3; paper III, pp.710-711). The composition of the most abundant bacterial orders on leaves of flowering plants as determined in 2011 and 2012 (Table 4 and 5; paper III, pp. 715-716), however, seemed to differ from that in 2010. Members of the order Cytophagales, for instance, were highly abundant in 2010 but not

in 2011 and 2012. At this point, however, it is worthwhile to note that different bioinformatic analyses were adopted in 2010 (paper II) and 2011/2012 (paper III). In this context, Hirsch *et al.* (2013) recently demonstrated that different bioinformatic approaches for the analysis of 454 pyrosequencing data, namely taxonomy-dependent analysis (MEGAN) and taxonomy-independent analysis (OTU clustering), provided different results with respect to fungal species composition in the same samples. Likewise, in this thesis taxonomy-dependent analysis was used for 2010 (paper II), whereas taxonomy-independent analysis was used for 2011 and 2012 (paper III). Furthermore, different databases were consulted in this thesis as well, namely NCBI NT database (version of 12 May 2011) in 2010 (paper II) and Greengenes database (version 12_10) as well as UNITE database (version 12_11; alpha release) in 2011 and 2012 (paper III). Moreover, replicates of each sample were pooled, albeit after individual DNA amplification, for 454 pyrosequencing in 2010 (paper II), whereas they were not pooled in 2011 and 2012 (paper III). The data from 2011 and in particular from 2012 (paper III) display also considerable variations between replicates at BBCH 60 (prior to BCA introduction). For all these reasons, we suggest that microbial communities from 2010 should be compared with microbial communities from the following years (2011, 2012) only with care. In contrast, the comparison of microbial communities between 2011 and 2012 as determined by 454 pyrosequencing is valid.

Resident microbial communities differed between flowering plants in 2011 and 2012, which was most pronounced for bacteria at order (Table 4 and 5; paper III, pp. 715-716) but also at genus level (Figure 5; paper III; p. 717). This finding was in line with plate count results from the three years of experiments that also indicated variations in microbial communities between the years. It has previously been demonstrated that the composition of microbial communities is dependent on environmental factors such as UV-light (Kadivar & Stapleton, 2003) and temperature (Finkel *et al.*, 2011). In the present thesis, environmental conditions varied between 2011 and 2012, in particular with respect to precipitation during flowering (paper III and IV). We, therefore, suggested that changes in microbial communities most probably arose from differences in weather conditions. In this context, however, it cannot be ruled out if other than environmental factors, e.g. nutrient availability, water availability, plant habitus might have been involved, too.

Furthermore, the composition of fungal and bacterial phyllosphere residents considerably changed in the course of the strawberry season in 2010 (paper II) as well as in 2011 and 2012 (paper III). This finding is in line with the plate count results and with results from further studies (e.g. Thompson *et al.*, 1993). Leaf ageing is associated with changes in e.g. leaf morphology (Hunter *et al.*,

2010) and nutrient exudation (Kinkel, 1997). We, therefore, suggested that leaf ageing (Redford & Fierer, 2009; de Jager *et al.*, 2001; Thompson *et al.*, 1993) but also changes in environmental conditions (increased temperatures, radiation and day length) (Whipps *et al.*, 2008; Kinkel, 1997) in the course of the growing season contributed to a large extent to the observed seasonal changes in the microbial communities of strawberry leaves.

The most important findings from the microbial investigations on the resident microbiota were that the microbiota of the strawberry phyllosphere was not consistent with (i) respect to the growing season and (ii) with respect to the phenological stage of plants. This finding leads to the question if consistent interactions between the phyllosphere microbiota and the introduced BCAs as well as between the BCAs and the target pathogens can be anticipated in the phyllosphere.

4.3 Interactions between resident microbiota and introduced BCAs in the strawberry phyllosphere (paper II-III)

It is assumed that the resident leaf microbiota is one important determinant of the establishment of BCAs in the phyllosphere and, thereby, also of the BCAs' interactions with foliar plant pathogens. Also, there is currently little knowledge with regard to detrimental effects (e.g. displacement, toxigenicity) of phyllosphere applied BCAs on the resident leaf microbiota (Kim *et al.*, 2010; Zhang *et al.*, 2008a; Okon Levy *et al.*, 2006; Russell *et al.*, 1999), although this issue is important in terms of safety (Cook *et al.*, 1996). We, therefore, investigated microbial interactions in the phyllosphere after BCA applications in three years of field experiments by means of plate counts and 454 pyrosequencing.

The introduced BCAs *Trichoderma* and *Aureobasidium* established on leaves from field grown strawberry plants in 2010 and 2011, respectively, as determined by plate counts and indicated by 454 pyrosequencing (paper II and III). Surprisingly, *A. pullulans* poorly established on strawberry leaves in 2012 as compared to 2011 (paper III), although this agent is generally regarded to be well adapted to the phyllosphere (Chi *et al.*, 2009). As for other microorganisms, immigration of introduced BCAs is highly depending on environmental factors and plant physiological factors (Whipps *et al.*, 2008; Kinkel, 1997). Based on the varying weather conditions in 2011 and 2012, (Table 6; paper III, page 722 and Figure 7; paper IV, page 18) and the negative correlation between high precipitation and establishment of *Aureobasidium* as determined by plate counts (paper III), we concluded that environmental

factors were involved in the establishment of *A. pullulans*. Furthermore, introduced BCAs must compete with microbial residents for nutrients and space (Jacobsen, 2006; Elad & Kirshner, 1993). We, therefore, concluded that the resident leaf microbiota might have been also involved in the establishment of *A. pullulans*, especially as the resident microbial communities differed between 2011 and 2012 (see chapter 4.2.). In this context, it should be also kept in mind that the composition of microbial phyllosphere residents displayed more variation in 2012 as compared to 2011 (paper III). Another aspect that has to be considered is that the experiments in paper III (2011 and 2012) were performed within a perennial strawberry cultivation system, i.e. plants were treated with BCAs in both years, albeit the plants were mulched after harvest in the first year (2011). This leads to the question if perennial long-term effects due to foliar BCA applications in the previous year may be also involved in the composition of resident phyllosphere microbiota and the establishment of BCAs. In further studies, this question should be elucidated.

The entomopathogenic fungus *B. bassiana* did not establish epiphytically in any of the three years (paper II and III).

According to plate counts, the resident microbiota was not clearly affected by the introduced BCAs in three years of field experiments (paper II and III) which is supported by reports of Russel et al. (1999). In contrast, considerable shifts in the composition of fungal phyllosphere communities were observed shortly after the introduction and successful establishment of *T. harzianum* in 2010 (paper II) and *A. pullulans* in 2011 (paper III) by means of 454 pyrosequencing. These observed short-term effects occurred in single as well as in multiple strain treatments and were accompanied by a decrease in fungal diversity in most cases. In 2010, a decrease in the relative abundance of some classes, e.g. Dothideomycetes, was observed after the introduction of *T. harzianum* into the phyllosphere, while the relative abundance of Sordariomycetes increased (Table 3; paper II, p. 1005). This finding indicates that the introduced *Trichoderma* strain, belonging to the Sordariomycetes, displaced members of the other classes in the phyllosphere by means of antagonistic interactions. It was reported that *Trichoderma* spp. possesses several modes of action with respect to control of foliar pathogens, e.g. mycoparasitism, induced resistance, antibiosis and competition for nutrients (Elad, 2000; Elad & Stewart, 2004). These modes of action might have been involved in the displacement of the resident fungi as well.

Likewise, the Dothideales and the genus *Aureobasidium* were predominant in the phyllosphere after applications of *A. pullulans* in 2011 (Table 2 and Figure 3; paper III) and fungal diversity was significantly reduced (Figure 4;

paper III, p. 713), thus, indicating that resident fungi were also displaced by introducing *A. pullulans*. For single strain treatments with *A. pullulans* in 2011, the impact on the composition of the resident fungal microbiota persisted for approx. four weeks. This, however, was linked to long-term establishment of *A. pullulans* (paper III). *A. pullulans* is considered to be well adapted to the phyllosphere (Chi *et al.* 2009) and to efficiently compete for nutrients in this habitat (Lima *et al.*, 1997). In future studies, displacement mechanisms of *A. pullulans* as related to nutrient competition in the phyllosphere of strawberries need to be followed up.

Interestingly, diversity and composition of bacterial classes were not affected by phyllosphere applications of *T. harzianum* (paper II) and *A. pullulans* (paper III). These findings indicate that under field conditions these microbial agents either did not show any antagonistic effects against the bacterial residents (e.g. through production of antibacterial compounds) or that the bacterial communities were more stable than the fungal ones in the phyllosphere of strawberries. The latter speculation, however, is not in accordance with reports of Okon *et al.* (2006), who demonstrated that bacterial populations on leaves were indeed affected by applications of *T. harzianum* under controlled conditions. Furthermore, another aspect should be also kept in mind in this context, i.e. the comparably low numbers of 16S rRNA sequences and the general question if the entire bacterial microbiota was efficiently described in this thesis.

Another interesting finding of this thesis was that microbial communities were not affected shortly after *B. amyloliquefaciens* was introduced into the phyllosphere in 2010, although increased plate counts of endspore-forming bacteria were detected in the respective leaf samples. We suggested that the introduced *Bacillus* strain endured the conditions in the phyllosphere rather as endospores than as vegetative cells without disrupting the resident microbial communities (paper II). Likewise, two strains of *B. subtilis* did not affect microbial communities on leaves from field grown pepper as determined by culture-independent technique (Kim *et al.*, 2010). In contrast, Zhang *et al.* (2008a) observed considerable changes in bacterial communities after applying a *Bacillus* strain (*B. thuringiensis*) to the phyllosphere of greenhouse grown pepper plants. The authors hypothesized that *B. thuringiensis* competed with bacterial communities for nutrients or that the crystal protein, that is produced by *B. thuringiensis*, was either toxic to resident bacteria or was used as substrate (Zhang *et al.* 2008a).

The findings of this thesis in the light of Cook's postulates (Cook *et al.*, 1996) imply that short-term (paper II and III) but also long-term (paper III)

impacts on fungal communities may occur after applications with fungal BCAs, provided that the introduced agent established in the phyllosphere. To the best of our knowledge, these are the first reports about shifts in fungal communities after BCA introduction under field conditions.

With respect to safety issues, however, we suggest that the impact of the introduced BCAs on the resident microorganisms was less severe than the ones of weather related factors (chapter 4.2). It was also demonstrated that many more factors can have a significant impact on the phyllosphere microbiota, e.g. the use of pesticides (Zhang *et al.*, 2009; Zhang *et al.*, 2008b; Walter *et al.*, 2007), infections with pathogens (Suda *et al.*, 2009) or the cropping system (Schmid *et al.*, 2011; Ottesen *et al.*, 2009). Taking all these aspects into consideration, it seems that the displacement effects observed in this thesis can be seen as negligible. Furthermore, some of the undesired effects of introduced BCAs on nontarget microorganisms (e.g. displacement and toxigenicity) are definitely intended with regard to the control of target pathogens (Cook *et al.*, 1996). This leads to the inevitable question if BCA treatments can effectively control the pathogen without having a direct or indirect (as a result of the interactions with the pathogen) impact on microbial communities in the phyllosphere.

4.4 Effects of introducing BCAs as single and multiple strain treatments on grey mould in field grown strawberries (paper II and IV)

According to our concept, we have already demonstrated that microbial communities, but also the interactions between the resident and the introduced microorganisms in the phyllosphere varied in dependence on different factors (e.g. environmental conditions, leaf ageing). This leads to the question if the interactions between the introduced BCAs and pathogen may vary as well.

The effect of simultaneously applied BCAs was tested in three years of field experiment with regard to *B. cinerea* control in strawberries.

In 2010, *B. cinerea* incidence was not reduced by repeated applications of three BCAs (*B. amyloliquefaciens*, *T. harzianum* and *B. bassiana*) as single and multiple strain treatments (Figure 1; paper II, p. 1003). This finding implied that biological control of *B. cinerea* could not be improved by simultaneous use of BCAs as it was also shown in other experiments under controlled conditions (Xu *et al.*, 2010; Robinson-Boyer *et al.*, 2009). This finding, however, was not confirmed in the subsequent field experiments. Instead, biological control of *B. cinerea* was significantly improved by

simultaneously applied BCAs in 2011 and 2012 (Figure 1; paper IV, p. 9). The field experiments in 2011 and 2012, however, included other BCA treatments than that in 2010, namely *B. amyloliquefaciens*, *A. pullulans* and *B. bassiana* (applied as single and multiple strain treatments).

Based on these findings, it might be initially tempting to draw the conclusion that the outcome in simultaneously applied BCAs against strawberry grey mould is governed by the choice of BCA constituents, as we have also concluded from the leaf disc assays for powdery mildew control (paper I). This kind of conclusion would also support the contradictory reports from studies using different BCA combinations for the control of strawberry grey mould (Xu *et al.*, 2010; Robinson-Boyer *et al.*, 2009; Guetsky *et al.*, 2002a; Guetsky *et al.*, 2002b; Guetsky *et al.*, 2001). However, in 2010 none of the tested BCAs was efficient against *B. cinerea* as single strain treatment (Figure 1; paper II, p. 1003). This was in particular surprising for *T. harzianum* treatments because we have already demonstrated that this BCA successfully established on strawberry leaves and, furthermore, featured antagonistic effects against members of the fungal leaf microbiota (paper II). Therefore, we suggested that failed efficacy of *T. harzianum* against *B. cinerea* might have resulted from inefficient delivery of the BCAs to the flowers. As a consequence, disease suppression could not have been affected by multiple strain treatments and, therefore, no final conclusion can be drawn on BCA constituents in multiple strain treatments as determinants of the outcome with regard to grey mould control in strawberries.

The findings of paper IV initially suggest that the simultaneous use of the BCAs *B. amyloliquefaciens*, *A. pullulans* and *B. bassiana* can be considered as a promising approach to meet the challenges regarding the suppression of *B. cinerea* in strawberries even under field conditions. Different possible mechanisms were already suggested to be involved in improved disease control in combined BCA treatments (Xu & Jeger, 2013; Guetsky *et al.*, 2002a; Guetsky *et al.*, 2002b; Guetsky *et al.*, 2001). In the present thesis, however, investigations on modes of action were not included and, therefore, it was difficult to draw final conclusions on causal mechanisms for the observed effects *ad planta*.

Interestingly, the co-application of *B. bassiana* seemed to be involved in disease suppression as well (paper IV). This finding was in line with e.g. increased abundances of the genus *Aureobasidium* on leaves when *B. bassiana* was co-applied as determined by 454 pyrosequencing (Figure 7; paper III, p. 720). Further experiments are needed to verify if co-application of *B. bassiana* promote grey mould suppression. In this context, it is worthwhile to note that

endophytic growth of *B. bassiana* was already reported for other crops (Tefera & Vidal, 2009; Vega *et al.*, 2008). Furthermore, endophytically growing entomopathogenic fungi, including *B. bassiana*, have already shown to suppress plant pathogens (Ownley *et al.*, 2010). These reports together with the findings from the present thesis lead to the questions if endophytic growth of *B. bassiana* occurs in strawberries and if endophytic growth might be involved in disease suppression or in the promotion of BCAs' establishment.

The most important finding of paper IV, however, was that efficient multiple strain treatments in 2011 were not efficient in 2012 and vice versa (Figure 1; paper IV, p. 9). Furthermore, postharvest disease control was not observed at all in 2012 (Table 3; Paper IV) as opposed to 2011 (Table 2; paper IV), although *B. amyloliquefaciens* and *A. pullulans* established on strawberry fruit treated with the respective BCAs (Figure 2 and 3; paper IV). This finding was sobering and indicates still inconsistency of multiple strain treatments with regard to distinct growing seasons.

As already demonstrated in the present thesis (chapter 4.2), the resident leaf microbiota considerably varied in dependence of the year, most probably due to different environmental conditions in the three years of experiment (paper II and III). Furthermore, the interactions between the resident microbiota and the introduced BCAs varied as well and the introduced yeast-like fungus *A. pullulans* did not establish in the phyllosphere in 2012 (paper III).

With regard to disease suppression, the findings of paper IV indicate that *A. pullulans* played a key role in multiple strain treatments in 2011 as opposed to 2012. Based on these findings and the microbial investigations on leaves (paper III), we concluded that the inconsistent efficacies of different multiple strain treatments against *B. cinerea* between the two years of experiments were most probably linked to the poor establishment of *A. pullulans* in 2012. Microbial analyses on flowers might have shed light on the BCAs' establishment at the main sites of infections and might have allowed to draw final conclusions on the causes for the lacking effects of the BCAs against *B. cinerea* in 2010 (paper II) and 2012 (paper III). We, therefore, encourage microbial analyses on flowers for future studies.

Interestingly, the poor effects observed in treatments including *A. pullulans* in 2012 (paper IV) were in line with the poor establishment of *A. pullulans* in 2012 on strawberry leaves (Figure 6; paper III) as opposed to its good establishment on fruit (Figure 3; paper IV). This finding indicates that leaves, which were chosen to exemplify the microbial interactions in the phyllosphere, might be suitable as indicator for the potential interactions between the BCAs and *B. cinerea* on flowers, whereas fruit were not.

5 Conclusion and outlook

The present thesis demonstrates that the road to success of phyllosphere applied BCAs is very complex. While some multiple BCA treatments improved the control of powdery mildew under controlled conditions, the simultaneous use of BCAs only partially overcame the challenges with respect to consistent biological control of grey mould under field conditions.

Under field conditions, consistent efficacies of multiple BCA treatments do not only rely on efficient delivery of the BCAs to the target sites but also on consistent interactions between (i) the BCA constituents, (ii) between the resident microbiota and the introduced BCAs as well as (iii) between the BCAs and the target pathogen. In this thesis, however, these interactions have shown to considerably vary in the phyllosphere of field grown strawberries which has shown to be linked to multiple varying factors prevailing in the phyllosphere such as environmental conditions, leaf properties and nutritional factors. In future studies, more factors than precipitation and temperature should be monitored (e.g. wind, light intensities and spectrum, plant physiological parameters such as plant nutrition as well as abiotic factors in the boundary layer of leaves) to shed light on how these factors altogether are involved in the microbial interactions in the phyllosphere.

The complementary employment of plate counts and 454 pyrosequencing has shown to be suitable for investigating microbial communities in the phyllosphere. Furthermore, the 454 pyrosequencing technique turned out to be useful for detecting potential impacts of introduced BCAs on resident microbial communities in the phyllosphere. Due to the considerable dynamics of the resident leaf microbiota, however, we strongly recommend to interpret these effects with care. In future studies, the employment of metabolomic or metaproteomic approaches should be also taken into consideration to get further insights on the activities and functions of microbial communities.

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