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Characterization of Bacillus amyloliquefaciens B47 for Biological Control

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Abstract

One of the major challenges in agriculture are phytopathogenic fungi which are the cause of various severe plant diseases and are therefore responsible for extensive yield losses in crop production every year. Today, the negative effects of chemical pesticide use on the whole ecosystem gets increasingly obvious and restriction of the most hazardous chemicals is under debate. Therefore, biological control which uses naturally occurring microbial enemies to combat plant diseases is considered to be one of the most important tools for sustainable plant protection. The present study is intended to conduct the research required for the application of *Bacillus amyloliquefaciens* B47 as a novel biological control agent.

The studies included whole genomic sequencing with a focus on detecting genes relevant for biocontrol activities and interactions with the plant host. The antagonism towards the five fungal phytopathogens *Verticillium longisporum, Alternaria alternata, Botritis cinerea, Phytophthora infestans* and *Sclerotinia sclerotiorum* was investigated by direct in vitro antagonism assays as well as by VOC (volatile organic compound) - assays. The elicitors of VOC mediated antagonism were identified by headspace solid phase micro extraction Gas chromatography coupled with mass spectrometry (SPME-GC-MS) measurements. The metabolic response of *B. amyloliquefaciens* B47 towards VOCs released by *Verticillium longisporum* and *Botrytis cinerea* was studied by high pressure liquid chromatography coupled with mass spectrometry (HPLC-MS) measurements of soluble metabolites. The interaction with the plant host was studied by plant growth promotion assays under sterile conditions as well as in soil, using tomato plants as a model system. Visualization of tomato root colonization was performed by FISH-CLSM (fluorescent in situ hybridisation - confocal laser scanning microscopy).

The *B. amyloliquefaciens* B47 strain showed a broad spectrum activity in biocontrol of all tested phytopathogenic fungi. The inhibitory activity was shown to be due to the production of antimicrobial metabolites like fengycin and surfactin on the one side and the release of a broad spectrum of antimicrobial volatile organic compounds on the other side. The results presented in this thesis suggest a complex mode of inter – species communication via exchange of VOCs. It was shown that *B. amyloliquefaciens* B47 can sense the presence of foreign VOCs emitted by phytopathogenic fungi and respond with changes in the spectrum and abundance of volatiles released. Detailed analysis of volatile compounds showed that VOC exchange with *Verticillium longisporum* as well as with *Botrytis cinerea* trigger the release of the two compounds acetoine and 2,3-butanediol well known to confer induced systemic resistance (ISR) in plants. Furthermore, results were obtained which lead to the suggestion that the bacterial biocontrol strain can discriminate between fungal pathogens by sensing the different VOCs transmitted via the air interface and adapt its metabolism in a pathogen-strain specific

manner. Regarding the aspect of plant growth promotion the *B. amyloliquefaciens* B47 strain had significantly positive effects on seed germination rate, plant development and plant health. Colonization of tomato roots was visualized but however did not result in reliable results concerning the colonization pattern of *B. amyloliquefaciens* B47. The studies conducted in this thesis are furthermore supported by in silico genomic analysis.

In conclusion, this thesis describes the high potential of *B. amyloliquefaciens* B47 for use as a biological control agent and emphasises the importance of VOCs not only for inter-microbial but also for BCA-plant communication.

Zusammenfassung

Eines der größten Probleme im landwirtschaftlichen Bereich sind pflanzenpathogene Pilze, welche diverse ernsthafte Pflanzenerkrankungen hervorrufen und daher für weitreichende Ernteausfälle verantwortlich sind. Heutzutage häufen sich die Hinweise auf den negativen Einfluss chemischer Pestizide auf das Ökosystem weshalb Verbote des Einsatzes der schädlichsten Stoffe werden diskutiert. Daher wird die biologische Schädlingsbekämpfung, bei der natürlich vorkommende Feinde zur Prävention und Behandlung von Pflanzenkrankheiten eingesetzt werden, als eines der wichtigsten Hilfsmittel in einer nachhaltigen Landwirtschaft angesehen. Das Ziel der vorliegenden Arbeit ist es, die notwendige Forschung für die Entwicklung eines Produktes zur biologischen Schädlingsbekämpfung durchzuführen. Beim untersuchten Organismus handelt es sich um einen bakteriellen Stamm, der als *Bacillus amyloliquefaciens* B47 bezeichnet wird.

Die Forschungsarbeit beinhaltet die Analyse der genomischen Information, wobei der Fokus auf der Identifizierung von Genen lag die für die Interaktion mit Pathogenen und der Wirtspflanze relevant sind. Der inhibierende Effekt auf die fünf Pflanzenpathogene *Verticillium longisporum, Alternaria alternata, Botritis cinerea, Phytophthora infestans* und *Sclerotinia sclerotiorum* wurde sowohl in direkten in vitro assays als auch in s.g. VOC (volatile organic compound) – assays bei denen die Mikroorganismen nur über den Luftraum in Kontakt treten können untersucht. Die volatilen Substanzen, die für den inhibierenden Effekt verantwortlich sind, wurden mittels Festphasenmikroextraktion und anschließender Analyse durch Gas Chromatographie gekoppelt mit Massenspektrometrie identifiziert. Die metabolische Reaktion von *B. amyloliquefaciens* B47 auf das Vorhandensein von volatilen Substanzen, die von *Verticillium longisporum* und *Botrytis cinerea* ausgestoßen werden, wurde durch Analyse der löslichen Metabolite mittels Hochleistungsflüssigkeitschromatographie gekoppelt mit Massenspektrometrie erforscht. Der Einfluss auf das Wachstum von Tomatenpflanzen wurde sowohl unter sterilen Laborbedingungen als auch unter natürlichen Bedingungen in herkömmlicher Erde getestet. Die Visualisierung der Kolonisierung von Tomatenwurzeln durch *B. amyloliquefaciens* B47 wurde mittels „fluorescent in situ hybridization“ (FISH) und anschließender Konfokalmikroskopie umgesetzt.

Der *B. amyloliquefaciens* B47 Stamm zeigte eine Breitbandwirkung in der Inhibierung aller getesteten Pflanzenpathogene. Es wurde gezeigt, dass der inhibitorische Effekt auf der Produktion von antimikrobiellen Sekundärmetaboliten wie Fengycin und Sufactin sowie der Freisetzung eines breiten Spektrums an antimikrobiellen flüchtigen Substanzen beruht. Die Studie kommt zu dem Ergebnis, dass eine komplexe Interaktion zwischen Mikroorganismen aufgrund des Austausches von flüchtigen Substanzen untereinander stattfindet. Die Ergebnisse zeigen, dass *B. amyloliquefaciens* B47 die Anwesenheit von pathogenen Pilzen

aufgrund der von diesen ausgesandten flüchtigen Stoffe erkennen kann und mit einer Anpassung des eigenen Spektrums an volatilen Substanzen reagiert. Detaillierte Analysen der Komponenten zeigten, dass der Austausch von flüchtigen Substanzen sowohl mit *Verticillium longisporum* als auch mit *Botrytis cinerea* die Produktion der zwei Substanzen Acetoin und 2,3-Butandiol aktiviert, welche hinreichend dafür bekannt sind, induzierte systemische Resistenz (ISR) in Pflanzen auszulösen. Im Weiteren wurden Ergebnisse erzielt, welche vermuten lassen, dass der bakterielle biokontroll-Stamm durch die Wahrnehmung der von den Pilzen ausgesandten unterschiedlichen volatilen Substanzen zwischen verschiedenen pathogenen Pilzen unterscheiden kann und auf spezifische Art und Weise metabolische Vorgänge adaptiert. Im Hinblick auf die Interaktion mit der Wirtspflanze wurden positive Effekte des *B. amyloliquefaciens* B47 Stamms auf die Keimungsrate von Tomatensamen, die Entwicklung der Keimlinge und auf die generelle Pflanzengesundheit festgestellt. Die Kolonisierung der Tomatenwurzeln wurde durch Konfokalmikroskopie visualisiert, wobei aufgrund experimenteller Schwierigkeiten keine Aussage über konkrete Kolonisierungsmuster gemacht werden kann. Die Untersuchungen dieser Studie wurden durch genomische *in silico* Analysen gestützt.

Zusammenfassend zeigt diese Studie das enorme Potential des untersuchten *B. amyloliquefaciens* B47 Stamms für die Produktformulierung als potentielles biologisches Pflanzenschutzmittel und betont die Wichtigkeit volatiler Substanzen sowohl für die inter-mikrobielle Kommunikation als auch für die Interaktion mit der Wirtspflanze.

1. Introduction

1.1. Biocontrol of plant pathogens

Plants live in close relationship with a huge number of microorganisms which coexist in the rhizosphere (the interface between soil and plant roots) as well as in the phyllosphere (above ground parts of plants). Among the microorganisms living in the rhizosphere bacteria are the most abundant ones, however fungi, algae and protozoa are also found in large amounts. Bacteria associated with plants can have beneficial influence on plant growth as well as health by different mechanisms. These bacteria are often described as plant growth promoting rhizobacteria (PGPR). Plant growth promoting rhizobacteria are soil-borne bacteria, enhancing plant growth by mechanisms such as nitrogen fixation, phosphorus solubilisation and production of phytohormones. Additionally PGPR often protect plant health by showing antagonistic activities towards phytopathogens or by inducing host resistance towards plant pathogens (Sivasakthi et al., 2014).

Microbial plant pathogens which are often fungi that naturally occur in the soil habitat can exhibit significant negative impacts on plant productivity. Management of microbial pests is accomplished primarily by use of pesticides which often results in pesticide resistance. Also pesticides often do not reach the target pests but instead negatively influence diversity and abundance of non-target species. (Matson et al., 1997). Therefore one of the major concerns our society is faced with nowadays, is the excessive use of pesticides in agriculture which brings along global negative impacts on ecosystems and in last instance, on human health. Finding not harmful alternatives for pest control becomes increasingly essential. A promising approach in this field is the use of bioresources as it is done in biological control. This method, also called biocontrol in short, is defined by the Centre for Agriculture and Biosciences International (CABI) as the control of pest populations by natural enemies in a way that the invasive species is not eradicated but becomes manageable.

Not only bacterial but also fungal strains often have the ability to act as biocontrol agents. An example is the *Trichoderma* genus which comprises a high number of strains with great potential for use in biocontrol of fungal phytopathogens due to their antagonistic activities. (Zachow et al., 2016)

1.2. The role of Bacillus species in biocontrol of plant pathogens

One of the most commonly used bacterial species in biocontrol is the *Bacillus* genus. *Bacillus* species are gram positive, rod shaped spore forming bacteria. The ability of forming spores facilitates survival in extreme environmental conditions and also contributes to the attractiveness of *Bacillus* for use as a biocontrol agent (BCA) as it provides advantages in production and storage of products (Emmert and Handelsman, 1999). As *Bacillus* species have a broad spectrum of biocontrol activities and are declared as GRAS (generally recognized as safe) organisms by the US Food and Drug Administration (FDA) the number of Bacillus species used in the field of biocontrol is rapidly increasing (Shafi et al., 2017).

A very powerful strain used commercially as biocontrol agent and biofertilizer is *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 for which beneficial effects on tomato, cucumber, cotton, tobacco, and lettuce were shown. The modes of action of this bacterium are very diverse (Chowdhury et al., 2015). A key aspect is the production of secondary metabolites aiming to suppress competitive bacteria and fungi in the rhizosphere habitat. It was shown that *B. amyloliquefaciens* FZB42 is capable of non-ribosomal synthesis of the cyclic lipopeptides surfactin, bacillomycin, fengycin - which all show antifungal activity - and the iron siderophore bacillibactin. The production of siderophores acts plant protecting by solubilizing and scavenging iron from the environment which makes it unavailable for other microorganisms like phytophathogens. Also synthesis of the antibacterial acting polyketides macrolactin, bacillaene, and difficidin was assigned (Chen et al., 2009). Another way by which *B. amyloliquefaciens* FZB42 protects plants from deleterious microorganisms is the induction of plant defence strategies called induced systemic resistance (ISR). ISR is defined as “enhanced defensive capacity of the entire plant against a broad spectrum of pathogens; acquired upon local induction, e.g., at roots, by beneficial microbes” (Pieterse et al., 2014). Research showed that *B. amyloliquefaciens* and several other Bacillus strains can elicit ISR in various vegetable plants including tomato and sugar beet. Mediators of ISR are i.e. jasmonic acid, ethylene, salicylic acid (Kloepper et al., 2004) but also microbial volatile organic compounds (Ryu et al., 2003) and the lipopeptides surfactin and fengycine (Ongena et al., 2007).

In the last years the attention was more and more drawn to the role of volatile organic compounds (VOCs) in biocontrol of pathogens. Studies showed that VOCs have a comprehensive influence on microbial communication and defence strategies (Rybakova et al., 2017).

By using this broad set of strategies *Bacillus* species are promising candidates for successful biocontrol which can be applied for a more sustainable agriculture.

1.3. Previous screening of potential biocontrol strains

In previous studies (Gassner, 2007) a *Bacillus amyloliquefaciens* B47 strain isolated from sclerotia of Rhizoctonia in Germany was shown to have an exceptionally strong β-glucanase activity which is an extracellular enzyme thought to be involved in biocontrol activities by antagonising phytopathogens such as Phytophthora (Sakdapetsiri, Fukuta, Aramsirirujiwet, Shirasaka, & Kitpreechavanich, 2016). Furthermore extracellular protease activity of *B. amyloliquefaciens* B47 was revealed. Proteases are often involved in biocontrol of pathogens like *Fusarium oxysporum*. (Guleria, Walia, Chauhan, & Shirkot, 2016). Also a first hint regarding the plant growth promotion potential was obtained from this previous study in which the ability of *B. amyloliquefaciens* B47 to produce Indole-3-acetic-acid was shown. IAA is a well-known phytohormone inducing phyto-stimulation and hence improving plant growth. (Spaepen, Vanderleyden, & Remans, 2007). Therefore *B. amyloliqeufaciens* B47 was considered as a promising candidate for development of a biocontrol agent.

1.4. Investigated phytopathogenic fungi

In this study the antagonism of a *B. amyloliquefaciens* B47 biocontrol strain against the three soil borne diseases *Verticillium longisporum*, *Alternaria alternata and Sclerotinia sclerotiorum* and the two foliar diseases *Botrytis cinerea* and *Phytophthora infestans* was investigated in detail.

Verticillium longisporum is a soil-borne fungal phytopathogen causing vascular disease called Verticillium wilt mostly in Bassicaceae and has become a global problem in oilseed rape production. (Depotter et al., 2016) Fungal hyphae develop from microsclerotia and enter the plant host by penetrating the root epidermis. During its infection cycle the fungus enters the xylem and spreads throughout the plant (Eynck et al., 2007). Disease symptoms can vary between host plants but often include wilting, stunting, chlorosis, vascular discoloration and early senescence (Fradin and Thomma, 2006). To date there is no effective fungicide treatment available for disease control (Depotter et al., 2016), therefore finding new strategies for *V. longisporum* pest control is essential.

Alternaria alternata is one of the most important fungal pathogens inducing postharvest decay within a broad host range. It is present in most of the world's natural environments, is able to infect both fruit and vegetative tissues and causes black spot. A dangerous characteristic of *A. alternata* is its ability to produce mycotoxins which are related to food poisoning. Most commonly *A. alternata* infects fruits by entering through wounds and natural openings. The fungus can sporulate and hence survive in extreme environmental conditions and survive cold periods in soil, seeds and perennial host tissues. (Troncoso-Rojas and Tiznado-Hernández, 2014)

Another devastating soil-borne pathogen is the fungus *S. sclerotiorum* which builds long-term survival structures called sclerotia which enables it to survive for up to 8 years in soil. It can infect over 400 plant species worldwide which are mostly dicotyledonous. The disease symptoms usually include water-soaked lesions on leaves and stems from which necrotic tissues develop. Further patches of white mycelium develop and wilting occurs. The infection can spread over fields by plant-to-plant contact. Host plants in most cases could not develop resistance, therefore often fungicides are applied to combat this disease (Bolton et al., 2006).

One of the most destructive phytopathogens worldwide with a very broad host spectrum of over 200 crop hosts is the fungus *Botrytis cinerea.* It infects mostly dicotyledonous hosts but also several monocotyledonous host plants. Most affected are vegetables (i.e. cabbage, lettuce, broccoli, beans) and small fruit crops (grape, strawberry, raspberry, blackberry) but also legumes and cut flowers like rose and gerbera. The symptoms of infected plants are soft rotting of all aerial plant parts, and rotting of vegetables, fruits and flowers and the appearance of grey conidia. In the dying host tissues *B. cinerea* can form sclerotia which facilitates survival in unfavourable conditions (Williamson et al.).

A serious potato and tomato disease known as late blight is caused by the oomycete *Phytophthora infestans*. This pathogen is a serious challenge in the production of potato which is the world's third largest staple crop and is considered as the most important disease in potato. Breeding programmes did not succeed in increasing the level of resistance of current potato varieties. (Haverkort et al., 2008) The disease destroys potato leaves as well as potato tubers and is similarly destructive on tomato plants. Repeated asexual cycles of reproduction cause extremely rapid destruction of foliage. (Fry et al., 2015)

1.5.Objectives of the present study

Biocontrol agents are thought to become one of the most important tools for sustainable crop production in future agriculture. Therefore there is a high demand for new products in this field. The aim of this study is to conduct the research required for the development of a new biocontrol agent.

As *B. amyloliquefaciens* B47 is thought to have a very strong potential for serving as a biocontrol agent, in the present study it was focused on the molecular and functional characterization of this particular strain. The investigations conducted within this study included analysis of genomic information of *B. amyloliquefaciens* B47 with a focus on genes relevant for control of phytopathogens, survival in soil and plant growth promotion. To get an impression of the range of biocontrol activity, the direct and volatile mediated antagonism against different plant pathogens was examined. By applying gas chromatography coupled with mass spectrometry the elicitors of volatile mediated antagonistic effects were investigated. The volatile interaction of *B. amyloliquefaciens* B47 with one soil borne (*Verticillium longisporum*) pathogen and one foliar pathogen (*Botrytis cinerea*) was further studied on the level of emitted VOCs as well as on the level of changes in bacterial metabolome due to VOC exchange with pathogens. To characterize the plant growth promoting effects, the impact of *B. amyloliquefaciens* B47 on the growth of tomato plants was tested under sterile laboratory conditions as well as under non-sterile conditions in soil. Additionally, the plant-growth-promotion trials were performed also with the fungal biocontrol strain *Trichoderma harzianum* 3TSMI. The plant competence was investigated by visualizing the colonization of tomato roots by *B. amyloliquefaciens* B47 using confocal laser scanning microscopy.

Taken together this work is intended to give an overview of the biocontrol potential of *B. amyloliquefaciens* B47 and describe the modes of action more in detail, providing essential information for BCA product development.

2. Materials and Methods

2.1. Growth Media and Buffers

Potato Dextrose Agar (PDA) (Roth. Karlsruhe. Germany)

Potato infusion	6.5 g/l
Glucose	20 g/l
Agar-Agar	15 g/l

Nutrient Agar II (NA) (Sifin, Berlin, Germany)

Nährbouillon II	15 g /l
Agar-Agar	15 g /l

Waksman-Agar

Trypton	5,0 g/l
NaCl	5,0 g/l
Meat extract	3,0 g/l
Glucose	10,0 g/l
Agar-Agar	20,0 g/l

Pea Agar

Frozen peas	150 g cooked in 1L of distilled H_2O for 50 minutes, remove peas and bring volume of broth back up to 1l
Glucose	5 g/l
Agar-Agar	10 g/l

V8 Juice Agar

Vegetable Juice	200 ml/l
$CaCO_3$	3 g/l
Agar-Agar	15 g/l
pH	7.2

Gamborg B5 medium (Duchefa Biochemie)

Basal salt mixture	3.04 g /l

All media were sterilized at 121°C for 20 min.

Phosphate Buffer

20 : 80 (v/v) mixture of 200 mM NaH_2PO_4 and 200 mM Na_2HPO_4

1x PBS Buffer

NaCl	130mM
Phosphate buffer	5%
pH	7.2

Hybridization Buffer

Formamide concentration	*15%*	*35%*
5 M NaCl	36 µl	36 µl
1 M Tris/HCl	24 µl	24 µl
2% SDS	6 µl	6 µl
FA	180 µl	429 µl
ddH_2O	768 µl	528 µl

Washing Buffer

FA conc. during hybridization	*15%*	*35%*
5 M NaCl	636 µl	140 µl
1M Tris/HCl	200 µl	200 µl
0.5 M EDTA		100 µl
ddH_2O	9164 µl	9560 µl

2.2. Bacterial and fungal strains

Bacterial and fungal strains used in this study are listed in **Table 1**. All fungal strains except *Phytophthora infestans* were cultivated for reproduction on PDA agar plates at 20°C and stored at 4°C. *Phytophthora infestans* was cultivated either on V8 agar or pea agar at 20°C. The bacterial strain *B. amyloliquefaciens* B47 was cultivated either on NA agar plates or in NA liquid medium at 30°C.

Table 1 Bacterial and fungal strains used in this study

Strain description	Resource
Bacterial biocontrol strain	
Bacillus amyloliquefaciens B47	strain collection of TU Graz, Institute of Environmental Biotechnology
Fungal biocontrol strain	
Trichoderma harzianum 3TSMI	strain collection of TU Graz, Institute of Environmental Biotechnology
Fungal phytopathogens	
Verticillium longisporum	strain collection of TU Graz, Institute of Environmental Biotechnology
Botrytis cinerea	strain collection of TU Graz, Institute of Environmental Biotechnology
Alternaria alternata	strain collection of TU Graz, Institute of Environmental Biotechnology
Phytophthora infestans	Wageningen University & Research, Business Unit Biointeractions and Plant Health
Sclerotinia sclerotiorum	strain collection of TU Graz, Institute of Environmental Biotechnology
Rhizoctonia solani	strain collection of TU Graz, Institute of Environmental Biotechnology

Preparation of *V. longisporum* mycelial suspension

For inoculation of *V. longisporum* to agar plates, some mycelium grown for 3 days on a PDA agar plate was resuspended in sterile H_2O by adding 1 ml of H_2O to a piece of agar overgrown with mycelium and scraping off some fungal material with a drigalski spatula until a turbid solution was obtained.

2.3. Genomic analysis of *B. amyloliquefaciens* B47

The genomic DNA was extracted by CNR-IPSP (National Research Council of Italy - Institute for Sustainable Plant Protection) and sequenced at IGA Technology Services. A total of 2,852,486 raw reads of 300 bp were sequenced using MiSeq2500 (Illumina). The raw reads were assembled into scaffolds using ABySS de novo sequence assembler (Galaxy Version 2.0.1.0) with default parameters (Simpson et al., 2009). Scaffolds generated by ABySS were further extended using SSPACE-Standard ver. 3.0 (Boetzer et al., 2011) with default parameters (-k 5 -a 0.7 -x 1 -m 30 -o 20), and gaps were closed using GapFiller ver. 1.10 with default parameters (-m 30 -o 2 -r 0.7 -n 10 -d 50 -t 10 -T 1 -i 1). The number of de novo assembled scaffolds was 249 which formed the draft genome of *B. amyloliquefaciens* B47. The draft genome was annotated using Prokka prokaryotic genome annotation (Galaxy Version 1.12.0) (Cuccuru et al., 2014) with default parameters. The NCBI BLAST Alignment tool available at http://blast.ncbi.nlm.nih.gov/Blast was used for the alignment of *B. amyloliquefaciens* B47 genomic sequence with genes found in the genomes of *B. amyloliquefaciens* subsp. plantarum UCMB5113 (Niazi et al., 2014) and genes from *B. amyloliquefaciens* FZB42 (Chen et al., 2007). Secondary metabolite biosynthesis gene clusters were predicted using antiSMASH 3.0 (Weber et al., 2015).

2.4. Direct in vitro antagonism test

The *B. amyloliquefaciens* B47 strain was tested for its direct in vitro antagonistic activity against the plant pathogens *Sclerotinia sclerotiorum, Botrytis cinerea, Verticillium longisporum, Rhizoctonia solani* and *Alternaria alternata* by dual culture assay on Waksman agar plates as described in (Zachow et al., 2008). Additionally the antagonism towards the plant pathogen *Phytophthora infestans* was tested on pea agar and V8 juice agar due to the inability of *Phytophthora infestans* to grow on Waksman agar. Depending on the speed of mycelium development either one or 4 agar plugs (5-mm-diameter) were cut from each plate

with the pathogens and were placed on the Waksman agar plate. *B. amyloliquefaciens* B47 was streaked on the Waksman agar plates in four lines starting from the centre of the plate to the edge. *V. longisporum* was inoculated to the plate by plating 100 µl of the above described mycelial suspension on a Waksman agar plate. After drying the plate for 10 minutes, *B. amyloliquefaciens* B47 was streaked on the plate. To assess growth inhibition of *V. longisporum* by quantitative measurements of fungal diameter, 10 µl of the before mentioned turbid mycelia solution were dropped on a Waksman agar plate on four locations. As a control the fungal strains were inoculated to the agar plates in the same manner, but no bacterial strain was streaked. After 6-10 days of incubation at 20°C the fungal diameters were determined using a ruler.

2.5. Volatile mediated antagonism against plant pathogens

To test for possible antagonistic effects mediated by volatile organic compounds of *B. amyloliquefaciens* B47 towards different plant pathogens, a two clamp VOC-assay as described in (Cernava et al., 2015) was applied. The VOC assay was performed in six replicates, using 6-well-TC plates. The cavities of the 6-well-plates were filled with 4 ml of PDA or NA respectively. A single colony of *B. amyloliquefaciens* B47 grown over night at 30°C was streaked out very densely onto the NA agar surfaces of the six wells on one plate. Agar plugs (0.6 cm) overgrown with fungal mycelium of the plant pathogens *A. alternata*, *S. sclerotiorum*, *P. infestans* and *B. cinerea* were cut out and placed on the outer corner of the wells of a second plate filled with PDA or pea agar. The plant pathogen *V. longisporum* was inoculated by dropping 20 µl from a mycelial suspension in the middle of the PDA agar. The bacterial and the fungal plates were put together, separated by a perforated silicone foil (2 mm). As a control the fungal pathogens were clamped together with a sterile 6-well-plate filled with NA and a bacterial plate was clamped together with a sterile PDA plate. The setup was fixed by four clamps, one at each side. The plates were co-incubated at 20°C in the dark for 5 to 12 days depending on the fungal growth rate. The diameters of fungal mycelia were determined in regular time intervals.

2.6. Identification of volatile organic compounds by SPME-GC-MS measurements

Sample preparation

For growing the bacterial and fungal strains, 20 ml open head space glass vials (75.5 × 22.5 mm; Chromtech, Idstein, Germany) were filled with 6.5 ml NA or PDA slope agar respectively. A single colony of *B. amyloliquefaciens* B47 grown overnight at 30°C was streaked very densely onto the surface of the NA slope agar. One fungal plug (0.5 cm) of *B. cinerea* per vial was cut out and transferred to PDA slope agar. The pathogen *V. longisporum* was inoculated to the PDA slope agar by distributing 20 µl of a before described mycelial solution of *V. longisporum* over the agar surface. To allow exchange of volatile compounds, the openings of two vials, one inoculated with *B. amyloliquefaciens* B47 and the other containing either *B. cinerea* or *V. longisporum* were sealed together with parafilm (**Figure *1***). In control experiments, to eliminate agar-derived background a vial inoculated with *B. amyloliquefaciens* B47 was sealed together with not inoculated PDA agar and the vials containing fungal strains were sealed together with not inoculated NA agar. To detect compounds solely derived from the growth medium, vials containing sterile NA were connected and coincubated with vials filled with PDA. All connected vials were coincubated for 3 days at 20°C in the dark to exchange volatile organic compounds. To achieve high reproducibility the measurements were performed two times in three biological replicates each. After the incubation period, vials were separated and sealed with crimp seals two hours before measurements were started.

Figure 1 Coincubation setup for GC-MS measurements allowing exchange of volatile organic compounds of two strains without direct contact to each other.

GC-MS measurement

Solid phase micro extraction (SPME) was performed as described in (Cernava et al., 2015) with an automated sampler and 50/30 µm Divinylbenzen/ Carboxen TM / Polydimethylsiloxane (PDMS) 2 cm Stableflex/ SS fiber (Supelco, Bellefonte, PA, USA). Volatile compounds were enriched for 30 min at 30°C. Compound separation and detection was performed on a system

combining a GC7890A with a quadrupole MS5975C (Agilent Technologies, Waldbronn, Germany). Samples were run through a (5%-phenyl) methylpolysiloxane column, 30 m × 0.25 mmi.d., 0.25 µm film thickness (HP-5MS; Agilent Technologies, Waldbronn, Germany), followed by electron ionization (EI;70eV) and detection (mass range 25–350). The inlet temperature was adjusted to 270°C. For the temperature gradient the GC column was kept at 40°C for two minutes, raised to 110°C at a rate of 5°C/min, then to 280°C at 10°C/min and finally maintained at 280°C for 3 minutes. The helium flow rate was set to 1.2 mL/min.

Compound identification

The identification of unknown compounds was done by comparing the mass spectra obtained from MS measurements to NIST mass spectral database and comparison of calculated retention index to the theoretical retention index specified in the NIST database. The retention index is used to convert system-dependent retention times into system-independent constants and thus allows identification of substances by comparing retention indices to theoretical values for specific compounds found in databases. The retention index was calculated from the retention times obtained from a series of straight alkanes C_5-C_{20} (*Table 2*). The retention times of alkane series were measured with the same method as described above.

Table 2 Retention times of alkane series measured by GC-MS.

Alkane	**RT**	**Alkane**	**RT**
Pentane	1.607	**Tridecane**	18.876
Hexane	2.023	**Tetradecane**	20.605
Heptane	3.02	**Pentadecane**	22.069
Octane	5.115	**Hexadecane**	23.371
Nonane	7.725	**Heptadecane**	24.563
Decane	10.722	**Octadecane**	25.675
Undecane	13.761	**Nonadecane**	26.721
Dodecane	16.664	**Eicosane**	27.713

Calculation: Ix=100n+(100(tx-tn))/((tn+1-tn))

n: retention time of adjacently eluting n-alkane beneath

x: Retention time of inquired substance

n+1: retention time of adjacently eluting n-alkane above

Statistical analysis

Statistical analysis was based on average peak area values over all replicates. The peak areas of detected compounds released by the fungal and bacterial strains after coincubation were compared to the peak areas detected in control experiments without exchange of volatiles. To test for significance of differences between these two groups the p-value was calculated by applying a two sided, paired t-test. For compounds with a p-value <0.05 the log2 fold change was calculated.

2.7. Bacterial metabolome analysis by HPLC-MS measurements

Sample preparation

B. amyloliquefaciens B47 was coincubated with *V. longisporum* and *B. cinerea* in order to exchange their VOCs without direct contact to each other. For that three single colonies of *B. amyloliquefaciens* B47 grown over night at 30°C were streaked very densely onto a NA plate. The bacterial plate was placed on top of a PDA plate inoculated with 5 agar plugs (0.6 cm) of *B. cinerea* mycelium or 100 µl of a before described suspension of *V. longisporum* mycelium respectively. As a control the bacterial plate was placed on top of a sterile PDA plate (*Figure 2*). The assays were performed in three independent biological replicates. To allow accumulation of VOCs the plates were sealed with parafilm and incubated for 3 days in the dark at 20°C.

Figure 2 Incubation setup enabling exchange of volatile organic compounds between microorganisms

After 3 days of incubation the whole cell material was harvested by adding 3 ml of 0.85% NaCl to the plate overgrown with bacteria and resuspending the cell material with a drigalski spatula. The biomass obtained from 3 plates was merged to achieve high metabolite concentration.

The cells were pelleted by centrifugation (2000 rpm, 20min.) and washed 3 times with 9 ml of 0.85% NaCl. After washing the pellet was transferred to a 2 ml tube and pelleted by centrifugation (15 min, 13500 rpm, 4°C.) 1.3 g of cell material were transferred to a screw cap micro tube containing Methanol/H_2O (90/10) cooled to -70°C and glas beads (250 mg ∅ 0,25-0,5 mm and 3 beads ∅ 3 mm). Cell disruption was done by using Ribolyser FastPrep-24 (MP Biomedicals, Santa Ana, California, USA) for 2 times at 30 seconds 6 m/s. After centrifugation (15 min, 13500 rpm, 4°C) the supernatant was taken off and stored at -70°C until samples were measured by HPLC-MS.

HPLC-MS measurement

The bacterial and fungal metabolite extracts were measured with a combined HPLC-hybrid quadrupole-orbitrap mass spectrometer (Q Exactive; Thermo Scientific, Bremen, Germany). This analytical method combines separation capabilities of liquid chromatography (HPLC) with mass analysis capabilities of mass spectrometry (MS). Bacterial metabolite extracts were separated by using a Atlantis dC18 (3µm, 2,1x100mm) column (Waters Corporation). Aqueous formic acid (0.1%, v/v) was used as solvent A and formic acid (0.1 %, v/v) in acetonitrile was used as solvent B. Starting concentrations for the gradient elution were 10 % of solvent B and 90 % of solvent A. The conditions were gradually changed to 80 % B and 20 % A within the first 35 min. This step was followed by five minutes of 10 % of solvent B and 90 % of solvent A for readjustment to initial conditions. The eluent flow was maintained at 0.3 mL/min and the column temperature was kept at 25 °C. Sample analysis was carried out separately with positive and negative ion ESI detection. ESI conditions were set to 3.5 kV spray voltage and a capillary temperature of 250°C for positive ESI ionisation and 2.9 kV spray voltage and a capillary temperature of 256.25°C for negative ESI ionisation. Scans were recorded in the range 100.0-1500.0m/z with the AGC target set to 1×10^6 and maximal accumulation time of 200 ms. The resolution was adjusted to 70,000. Altering full MS-SIM and data-dependent MS^2 cycles were carried out.

Data analysis

An initial automated compound identification was conducted by Compound discoverer 2.1 software. As the software is not always capable of correct compound identification, substances of special interest were identified manually by comparing mass spectral data and retention times to databases (mzCloud and ChemSpider). To analyse differential abundance of metabolites due to volatile exchange with pathogens the peak areas of detected compounds

after coincubation were compared to the peak areas detected in control experiments without exchange of volatiles. To test for significance of differences between these two groups the p-value was calculated by applying a two sided, paired t-test. The differential abundance of metabolites was expressed as log2 fold change values.

2.8. Plant growth promotion trials

Surface sterilization of tomato seeds

Tomato seeds were surface sterilized by treatment with 2% NaOCl for five minutes. Following, seeds were washed five times with 0.85% NaCl and dried in a petri dish.

Priming of tomato seeds with biocontrol strains

The *B. amyloliquefaciens* B47 strain was grown over night on a NA plate at 30 °C. The following day, cell material was harvested by adding 10 ml of 0.85% NaCl to the agar plate and resuspending the cell material with a drigalski spatula. From this cell solution (approximately $4*10^7$ cfu/ml) 3 ml were transferred into a TC 60 dish and incubated together with the tomato seeds at the shaking incubator for 3 h at room temperature. Seeds incubated with sterile 0.85% NaCl for 3 h served as a control. After the incubation time, seeds were washed three times with sterile H_2O and dried in a sterile environment.

For inoculation of the seeds with the *Trichoderma harzianum* 3TSMI biocontrol strain, a piece of agar overgrown with spores was cut out, placed into a tube containing 0.85% NaCl and shaken until a light green spore-solution was obtained. The spore solution was mixed with a covercoat solution (20% covercoat end-concentration). For inoculation 250 µl of this mixture were used to cover 24 seeds. The seeds were treated for one minute and put into a fresh petri dish for drying. As a control, seeds were treated the same way with sterile 20% covercoat solution.

Plant growth in sterile germination pouches

Sterile germination pouches were filled with 20 ml of sterile, deionized H_2O. Into each of the soaked pouches, 6 seeds were placed aseptically. Six pouches containing 6 seeds each were prepared for both groups, bioprimed with *B. amyloliquefaciens* B47 and sterile control. The pouches were placed in sterile, closed plastic boxes. Plants were grown under artificial 12/12

h day/night in the greenhouse for 13 days. The phyllosphere was separated from the roots by cutting with a sterilized scalpel and root lengths were determined. The phyllospheres and roots of five plants were each collected in sterile whirlpacks (VWR, Avantor) and weighed.

Plant growth in soil

The dried seeds were planted into pots containing approximately 30 ml of unsterile soil. In each pot there were placed 4 seeds which were covered with soil. Six pots were prepared for each of the experimental groups, primed only with *Trichoderma harzianum* 3TSMI, primed only with *B. amyloliquefaciens* B47, primed with both biocontrol strains and the sterile control group. Plants were grown under artificial 12/12 h day/night in the greenhouse for 13 days. Plants were harvested by separating the phyllosphere from the roots. The fresh weight of the phyllosphere was determined immediately after harvesting by weighing the individual plants. For determination of the dry weight the plants were stored in small paper bags and incubated at 103°C for 5 days. To prevent the plants from rehydrating after removing them from the incubator, they were stored in an exsiccator containing silica gel until weighing.

2.9. Visualization of root colonization by FISH-CLSM

Plant growth and inoculation with *B. amyoliquefaciens* B47

As a model system for plant colonization the tomato plant was chosen. The initial surface sterilization of tomato seeds was performed as described in section 2.8. Two different approaches of applying the bacterial biocontrol strain were tested.

- Biopriming

  Priming of tomato seeds with *B. amyloliquefaciens* B47 and plant growth in sterile germination pouches was performed as described in section 2.8.

- Root dipping

  Surface sterilized tomato seeds were placed aseptically onto germination paper soaked with sterile distilled water in a sterile closed plastic box. Two boxes containing each 20 seeds were prepared. Plants were grown under artificial 12/12 h day/night in the greenhouse. After seven days, the roots of the seedlings were inoculated with *B. amyloliquefaciens* B47. For that, a bacterial suspension of *B. amyloliquefaciens* B47 was prepared by centrifugation of 10 ml of a culture of the *Bacillus* strain grown in NA medium

overnight at 30°C and resuspending the pellet in 10 ml of sterile 0.85 % NaCl. The roots of seven days old seedlings were incubated in 1 ml of the bacterial suspension for 20 min. After incubation time the plants were carefully planted into sterile vermiculite soaked until saturation with sterile Gamborg B5 medium in a sterile plastic box. As a control, roots of seven days old seedlings were treated the same way with sterile 0.85% NaCl. Plants of both, the experimental and the control group were grown for additional 7 days in vermiculite before harvested for fixation.

As an additional control, root dipping of tomato plants was done the same way as described above, roots covered with bacterial suspension were dried for 40 minutes and directly preserved with paraformaldehyde fixation.

Fixation of plant material

Preservation of plant material was achieved by applying paraformaldehyde fixation. Roots were washed with PBS after harvesting. The roots were then incubated in a mixture of 1 vol. PBS (1x) + 3 vol. PFA (4%) for 20 hours at 4°C. After incubation time, PFA was removed and the samples were washed 3 times with PBS (1*-rinse, 2* -5 min, 3* -10 min.). Samples were stored in 1 vol. PBS(1x) /1 vol. EtOH at -20°C until fluorescent in situ hybridization (FISH) was performed.

FISH probes

Bacteria of all species were stained using the oligonucleotide probe EUB338 which is specific for the domain Bacteria, labelled with the fluorophore Cy3 (extinction wavelength, 555 nm; emission wavelength, 570 nm). The labelled probe was obtained from MWG Biotech. To exclude unspecific binding of EUB338 a nonsense NONEUB probe labelled with the Cy5 fluorophore (extinction wavelength, 650 nm; emission wavelength, 670 nm) was used. To specifically stain *Firmicutes* species the probe LGC354A/B/C labelled with Cy5 was used. Plant cell walls were additionally stained with Calcofluor white.

Hybridization protocol

The formaldehyde fixed plant material was cut into small pieces and placed in a 1.5 ml tube and rinsed with 200 µl of 1x PBS. To increase cell wall permeability the plant material was incubated in 200 µl Lysozyme (1mg/ml) for 10 min. After removal of Lysozyme it was washed twice in ice-cold 1x PBS. To destroy the bacterial membranes, a series of ethanol solutions (50%-70%-96%, 3 min each) was applied by exchanging the ethanolic solutions in the same tube. Following, samples were first rinsed and then washed for 3 minutes with ice-cold 1x

PBS. After washing, 200 µl of the hybridisation buffer (35% formamide, FISH probe LGC354A/B/C – Cy5) were added and samples were incubated at 43°C for 2h in the dark. After removing the hybridization buffer, samples were rinsed twice with 500 µl of pre-warmed (44°C) washing buffer. Next, 1 ml of washing buffer was added and incubated in a water bath (44°C) for 15 min. The washing buffer was removed and samples were rinsed with 500 µl of ice-cold double distilled water. In a second hybridization step, again 200 µl of the hybridization buffer were added (15% formamide, FISH probe EUB338 – Cy3) and samples were incubated at 43°C for 2h in the dark. The washing procedure was repeated as in the first hybridization step, but with the respective washing buffer. For Calcofluor white (CFW) staining the sections were incubated in 350 µl 0.17% CFW staining solution at room temperature for 20 min in the dark. Samples were first washed with and then incubated with 1 ml ice-cold double distilled water for 10 min in the dark. The sections were placed onto a microscope glass slide and dried quickly with soft compressed air. The dried sections were covered with ProLong Gold antifadent (Thermo Fisher scientific), sealed with nail polish and incubated in the dark for at least 24 hours.

Imaging and image analysis

For visualization a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) was used. The fluorophores Cy3, and Cy5 were excited with 532 and 635 nm laser beams. Calcofluor staining was excited with a 405 nm laser beam. The confocal stacks were acquired with a Leica ACS APO 63x oil CS objective lens (NA, 1.30). For image-analysis the software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used.

3. Results

3.1. Analysis of *B. amyloliquefaciens* B47 genome

Genome sequencing of *B. amyloliquefaciens* B47 resulted in a draft genome sequence consisting of 4,002,528 bp and 249 scaffolds, predicted to contain 3,955 genes. The GC content of the genomic DNA was 46.47%.

The annotated genome was analysed with a focus on the identification of genes relevant for survival in the soil habitat, interaction with plants and biocontrol mechanisms (*Table 3*). Genes were compared to the annotated genomes of *Bacillus amyloliquefaciens* FZB42 (Chen et al., 2007) and *Bacillus amyloliquefaciens* subsp. plantarum strain UCMB5113 (Niazi et al., 2014). Several genes and gene clusters enabling root colonization such as genes involved in biofilm formation (eps-operon, tasA, sinR, sigW, resE, abrB, sigH, spo0A) and swarming motility (swrC, efp, hag, flg-, flh-, fli-, che-operon) were detected. Also genes for breakdown, utilization and transport of plant-derived substances were predicted. These included genes for xylan degradation, lactose metabolism, galactose metabolism, cellulose hydrolysis, pectin lyase, pectate lyase and chitosanases. Further, 3-phytase and other genes (alsDS, trpP, trpE) which products are thought to act as plant growth promoting agents or elicitors of induced system resistance were identified. A great amount of genes which facilitate persistence in soil by protecting from environmental stresses were present in the *B. amyloliquefaciens* B47 genome. Examples are thiol peroxidase, cold shock protein, heat shock protein, superoxide dismutase, catalase, glutathione peroxidase, organic hydroperoxide resistance proteins and flavohemoprotein. Another useful trait for the persistence of *B. amyloliquefaciens* B47 in soil is genetic competence, for which several operons conferring to the ability of DNA uptake were identified (comK, comAP, comeAC, comfAB, comgACG, coi). To compete with other microorganisms such as phytopathogens, *Bacillus* species developed defence strategies like antibiotics and siderophores. Several of these substances involved in biocontrol mechanisms were found in the investigated *B. amyloliquefaciens* B47 genome. Biosynthesis cluster for the antibiotic substances bacilysin, bacillaene and plipastatin were found to be present in the genome. Additionally genes for production of siderophores like bacillibactin, Ferri-bacillibactin, enterobactin and the feuABC operon coding for iron-uptake system binding proteins were annotated.

Table 3 Genes relevant for biocontrol activities, predicted to be present in the *B. amyloliquefaciens* B47 genome

Annotation ID	gene	gene product	Gene function
Siderophores			
BVE_00319	ymfD	bacillibactin exporter	secretion of bacillibactin
BVE_01826 - BVE_01830	dhbABCEF	Bacillibactin biosynthesis cluster	bacillibactin biosynthesis
BVE_01825	BesA	Ferri-bacillibactin esterase	Cytosolic iron release
BVE_01831	YbdZ	Enterobactin biosynthesis protein	Enterobactin biosynthesis
BVE_03927	EntB	Enterobactin synthase component B	Enterobactin biosynthesis
BVE_01691	yusV	siderophore transport system	import of bacillibactin and enterobactin
BVE_00642	yfiY	siderophore-binding lipoprotein	Siderophore import
BVE_03888 - BVE_03890	feuABC	Iron-uptake system binding protein	Siderophore import
Antibiotics			
BVE_00733 - BVE_00739	BacABCDEFG	Bacilysin biosynthesis cluster	Bacilysin - antibiotic synthesis
BVE_00343- BVE_00346	BaeBCDE	Bacillaene biosynthesis cluster	Bacillaene – antibiotic synthesis
BVE_03877 - BVE_03879; BVE_00492 - BVE_00493	ppsABCDE	Plipastatin biosynthesis cluster	Plipastatin – antibiotic synthesis
DNA uptake			
BVE_02071	comK	competence transcription factor	Regulator of late competence genes
BVE_01855 - BVE_01856	comAP	Two component system ComP/ComA	Regulation of comK transcription
BVE_01390; BVE_01392	comeAC	comE operon protein 1/ 3	DNA uptake
BVE_00966; BVE_00967	comfAB	ComF operon protein 1/ 2	DNA uptake
BVE_01475; BVE_01477; BVE_01481	comgACG	ComG operon protein 1 / 3/ 7	DNA uptake
BVE_01975	coiA	Competence protein CoiA	For optimal transformation
Root colonization, swarming motility and biofilm formation			
BVE_02827	sfp	Phosphopantetheinyl transferase	surfactin and plipastatin B1 production
BVE_03942; BVE_02829- BVE_02831	srfaABCD	Surfactin biosynthesis cluster	Surfactin synthesis
BVE_03100	swrC	swarming motility protein	For swarming motility and surfactin self-resistance
BVE_01503	efp	elongation factor P	Essential for swarming motility

Scaffold 3-manually identified	Spo0A	Stage 0 sporulation protein A	Master regulator, essential for biofilm formation
BVE_03485	sigH	RNA polymerase sigma H factor	Biofilm formation
BVE_03664	abrB	Transition state regulator	Transcription control of biofilm formation
BVE_02690	sigW	ECF sigma factor W	Transcription control of biofilm formation
BVE_00462	resE	sensor histidine kinase	Transcription control of biofilm formation
BVE_01060	sinR	transcription regulator	Master regulator of biofilm formation
BVE_01063; BVE_01065-BVE_01076	epsC-O	exopolysaccharide operon	Synthesis of biofilm extracellular matrix
BVE_01486	tasA	spore coat associated protein	Development of complex colony structures
BVE_02103	ecsA	ABC multidrug transport	Protein secretion to extracellular matrix
BVE_00254-BVE_00255; BVE_00264-BVE_00265; BVE_00869; BVE_00972	flgBCDFGK	flagellar body proteins	For swarming motility
BVE_00274-BVE_00276	flhABF	flagellar biosynthetic proteins	For swarming motility
BVE_00256-BVE_00258; BVE_00261; BVE_00267-BVE_00268; BVE_00271; BVE_00975; BVE_00978-BVE_00979	fliEFGJMNPWDS	flagellum proteins	For swarming motility
BVE_00029; BVE_00269; BVE_00278-BVE_00282; BVE_02357	cheVYBAWCDR	chemotaxis proteins	For chemotaxis
BVE_00977	hag	flagellin	Flagella subunit
Breakdown, transport and utilization of plant derived substances			
BVE_00850 BVE_00464; BVE_00465 BVE_00385	xynABCD	endo-1-4-beta xylanase A Glucuronoxylanase Arabinoxylan-arabinofuranohydrolase Beta Xylosidase	Xylan degradation Extracellular degradation of plant cell walls
BVE_03603	lacG	6-phospho-beta-galactosidase	Lactose metabolism

BVE_03605	lacE	Phosphotransferase system	Lactose metabolism
BVE_03023	lacF	Lactose transport system permease	Lactose metabolism
BVE_00682	galK_1	Galactokinase	Galactose metabolism
BVE_03607	galE	UPD-glucose 4-epimerase	Galactose metabolism
BVE_03608	galT_1	Galactose-1-phosphate uridyltransferase	Galactose metabolism
BVE_03609	ganB	Arabinogalactan endo-beta-1,4-galactanase	extracellular degradation of plant cell wall
BVE_00591	pelB	pectin lyase	extracellular degradation of plant cell wall
BVE_03833	pel	pectate lyase	extracellular degradation of plant cell wall
BVE_00460	eglS	Endoglucanase	Cellulose hydrolysis
BVE_00603	bglS	Beta-glucanase	Beta-D-Glucan hydrolysis
BVE_01755	csn	Chitosanase	Degradation of fungal cell wall
PGP and ISR			
BVE_02470	phy	3-phytase	Increase of inorganic phosphorus
BVE_02106	trpP	putative tryptophan transport protein	Tryptophan uptake
BVE_00911-BVE_00912	alsDS	Alpha-acetolactate decarboxylase/ Acetolactate synthase	Acetoin synthesis
Response to environmental stress			
BVE_01105	tpx	thiol peroxidase	protection against oxidative stress
BVE_02195-BVE_02196; BVE_03319; BVE_02446	cspABCD	cold shock protein	adaptation to atypical conditions
BVE_02849	sodA	superoxide dismutase	Destroys superoxide anion radicals
BVE_00632	katA	catalse	protects from hydrogen peroxide
BVE_00605	katE	Catalase HPII	protects from hydrogen peroxide
BVE_00535	ahpC	Alkyl hydroperoxide reductase C	detoxifying peroxides
BVE_02449	bsaA	Glutathione peroxidase homolog BsaA	response to oxidative stress
BVE_03727-BVE_03729	ohrARB	Organic hydroperoxide resistance proteins	organic hydroperoxide resistance
BVE_01914	hmp	Flavohemoprotein	NO detoxification
BVE_03642	hsIR	Heat shock protein 15	Response to heat shock
BVE_03105	OpuE	Osmoregulated proline transporter	Response to high osmolarity growth conditions

To detect additional secondary metabolite biosynthesis gene clusters the genome was analysed by using antiSMASH 3.0. A total of 32 secondary metabolite gene clusters were predicted. Among them were gene clusters for synthesis of previously described bacilysin, surfactin, bacillibactin and bacillaene but also gene clusters for synthesis of the antifungal substance fengycin, the antibiotics bacitracin and difficidin and several other putative clusters for unknown substances.

3.2. Direct in vitro antagonistic activity against plant pathogens

The in vitro antagonism of *B. amyloliquefaciens* B47 towards six plant pathogens (*V. longisporum, B. cinerea, A. alternata, S. sclerotiorum, P. infestans and R. solani*) was determined by dual culture assay on agar plates. The *B. amyloliquefaciens* B47 strain showed a high antagonistic activity against all six tested pathogens.

The fungal diameters of plant pathogens were significantly decreased due to the presence of the *Bacillus* biocontrol strain (**Figure *3***and **Figure *4***). The strongest inhibitory effect was observed against *S. sclerotiorum* and *B. cinerea* which were almost completely defective in developing fungal mycelia in presence of the bacterial strain. After 6 days of growth the fungal diameters were reduced by 88 % (*S. sclerotiorum*) and 74% (*B. cinerea*) relative to the control. *R. solani* was still able to grow although there was a clear zone of inhibition in close proximity to the bacterial strain. The plant pathogens *A. alternata* and *V. longisporum* were also affected in their growth by *B. amyloliquefaciens* B47 and showed a reduction of fungal diameters of 54% (*A. alternata*) and 44% (*V. longisporum*). As *P. infestans* was not able to grow on Waksman agar the antagonism test was performed on pea agar. On pea agar, growth of *P. infestans* was completely inhibited, however this was most likely due to direct contact of *B. amyloliquefaciens* B47 with *P. infestans* which resulted from the induction of swarming motility of *B. amyloliquefaciens* B47 on pea agar. Therefore the antagonism towards *P. infestans* was additionally tested on V8 agar. On V8 agar a decrease in fungal diameter of 69 % relative to the control was measured.

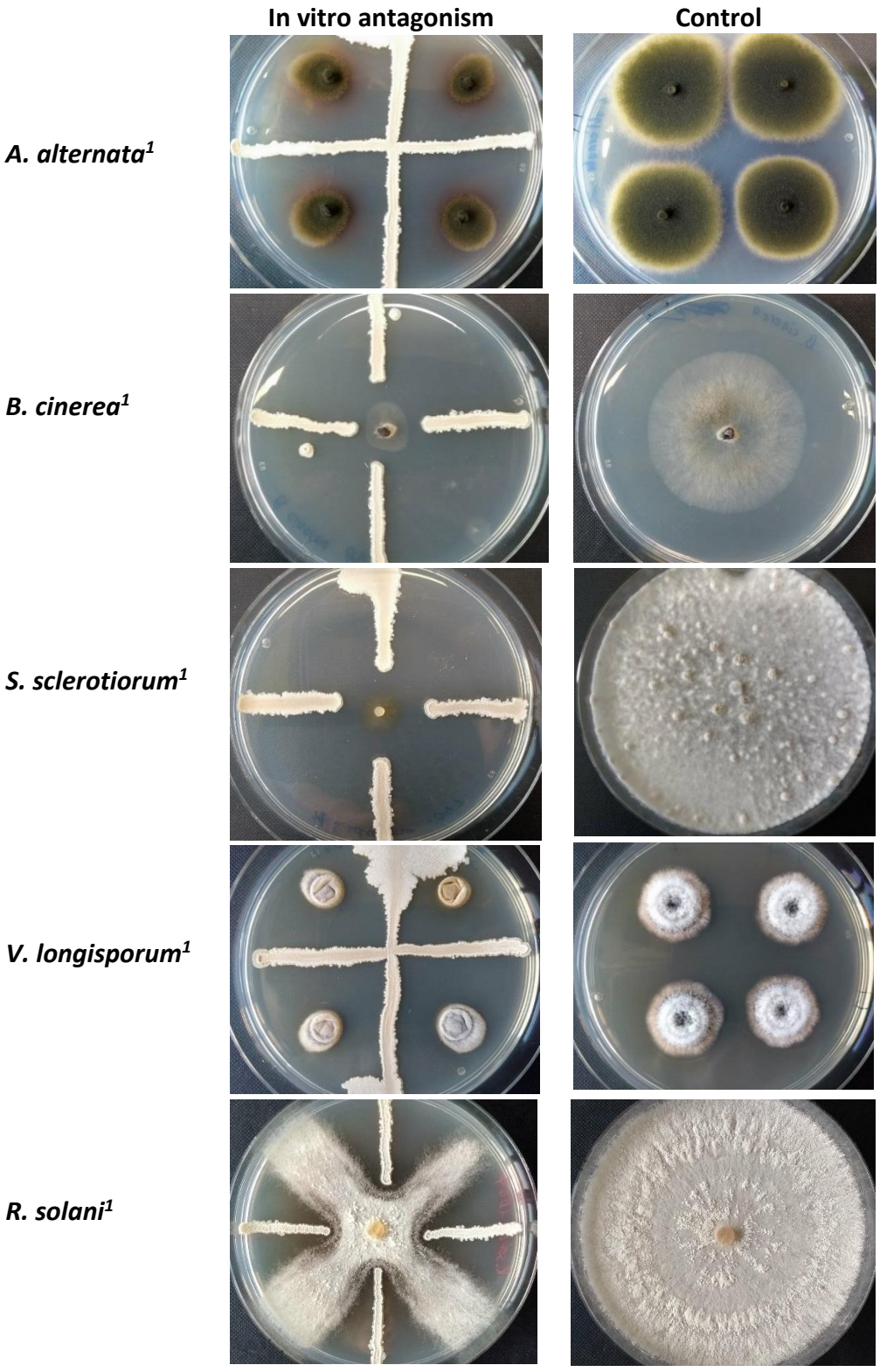

Figure 3 Direct in vitro antagonism tests by dual culture assay. The potential biocontrol strain *B. amyloliquefaciens* B47 was tested for antagonistic activities towards different phytopathogens by observing zones of inhibition due to cocultivation on agar plates. [1] growth medium = Waksman agar

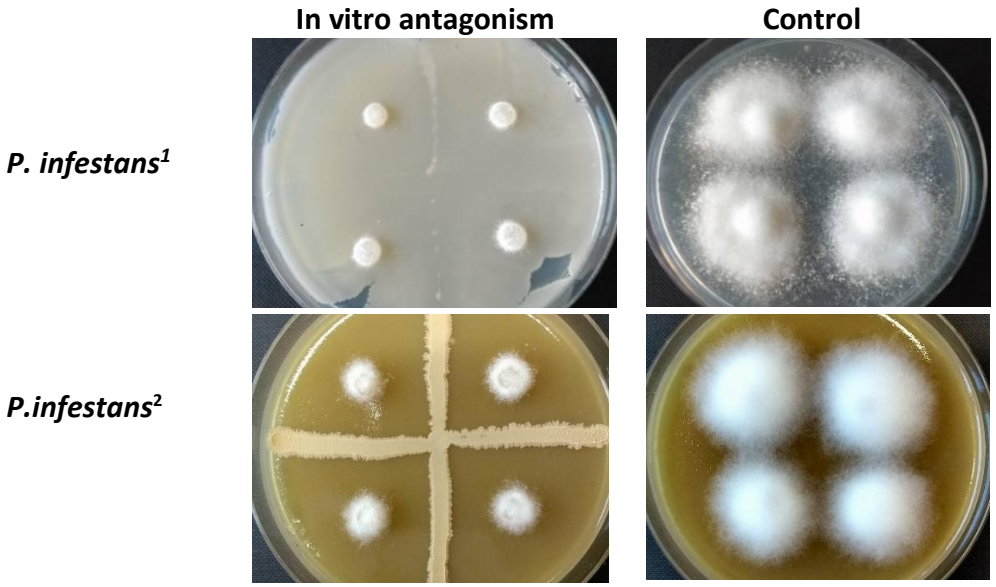

Figure 4 Direct in vitro antagonism tests by dual culture assay. The potential biocontrol strain *B. amyloliquefaciens* B47 was tested for antagonistic activities towards *P. infestans* on two different media by observing zones of inhibition due to cocultivation on agar plates.; [1] growth medium = pea agar; [2] growth medium = V8 agar

A graphical representation of the observed inhibitory effects of *B. amyloliquefaciens* B47 towards the fungal plant pathogens *V. longisporum, B. cinerea, A. alternata, S. sclerotiorum and P. infestans* is shown in *Figure 5*.

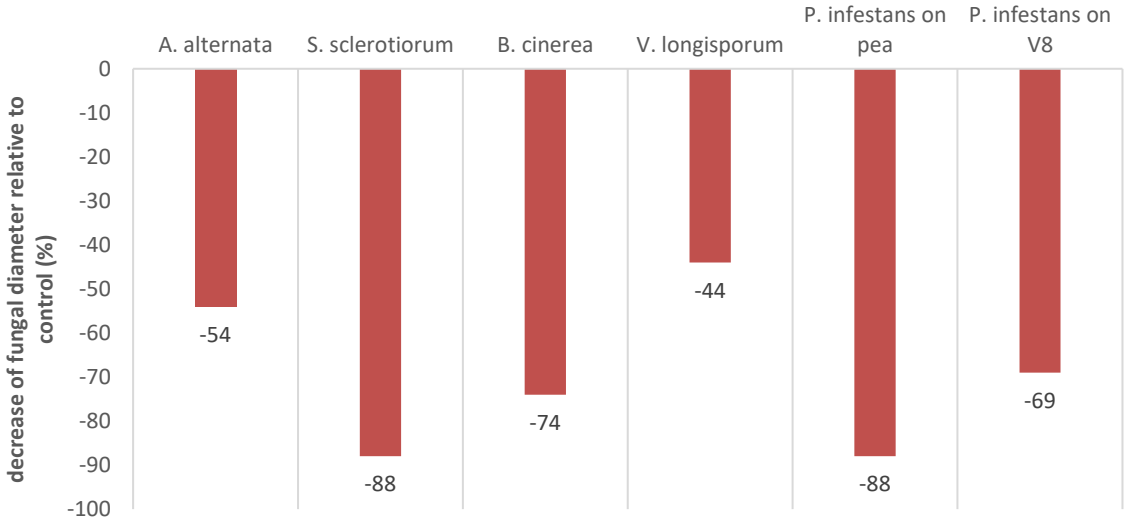

Figure 5 Quantification of in vitro antagonism of *B. amyloliquefaciens* B47 towards selected phytopathogens. The values indicate the decrease in mycelium diameter due to coincubation with *B. amyloliquefaciens* on the same plate and are expressed as percentage of fungal diameter relative to control. The antagonistic activity was tested towards *A. alternata, S. sclerotiorum, B. cinerea, V. longisporum* and *P. infestans*.

3.3. Volatile mediated antagonism against fungal pathogens

3.3.1. Inhibition of phytopathogens by volatile organic compounds

In order to investigate the role of VOCs in the observed antagonism of *B. amyloliquefaciens* B47 towards fungal pathogens, a two clamp VOC assay was applied. The VOC mediated inhibition of five plant pathogens (*A. alternata, V. longisporum, B.cinerea, P. infestans* and *S. sclerotiorum*) was tested. VOCs emitted by *B. amyloliquefaciens* B47 clearly showed antagonistic effects by repressing growth of fungal pathogens to different extents (*Figure 6*).

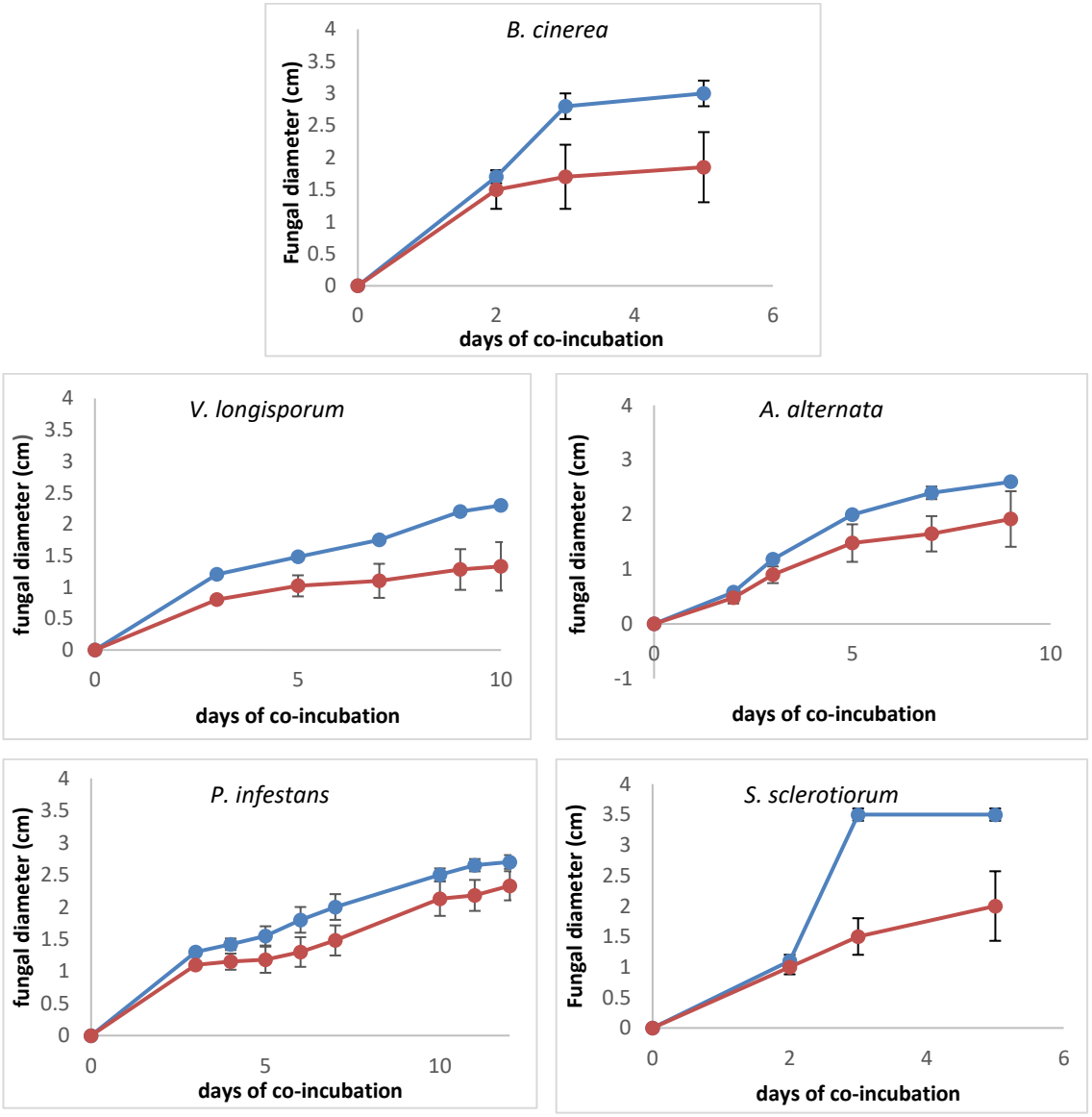

Figure 6 Volatile mediated growth inhibition of different phytopathogens. Development of fungal mycelia was measured over time in presence (red) and absence (blue) of volatile compounds emitted by *B. amyloliquefaciens* B47.

Fungal mycelium development of *B. cinerea* was strongly inhibited by *Bacillus* VOCs and reached a plateau after 2 days of growth, whereas the fungal mycelium in the control experiment continued to grow until it reached maximal well diameter after 4 days. The phytopathogen *V. longisporum* was also strongly inhibited in growth due to VOC exchange with *B. amyloliquefaciens* B47 and after 10 days of growth only reached half of the fungal diameter measured in control experiments. The growth of *A. alternata* was, although not completely repressed, slowed down in comparison to the control. Only a weak inhibition of *P. infestans* mycelium development was observed.

The volatile compounds released by *B. amyloliquefaciens* B47 had a big impact on mycelium development of the plant pathogen *S. sclerotiorum* and the ability of sclerotia formation of this particular fungal strain (*Figure 7*). A very clear growth inhibition was observed in presence of VOCs released by the *Bacillus* strain, as development of fungal mycelium was very sparse due to exposure of VOCs emitted by the biocontrol strain. Fungal mycelium did only develop very slowly, whereas in the control group the *S. sclerotiorum* mycelium continued to rapidly grow until the whole well was covered with dense mycelium after only 3 days of growth. To observe the sclerotia formation ability of *S. sclerotiorum,* the plates were further coincubated for a total timespan of 7 days. After one week, formation of sclerotia was observed in the control experiments whereas sclerotia formation was completely inhibited due to VOC exchange with *B. amyloliquefaciens* B47.

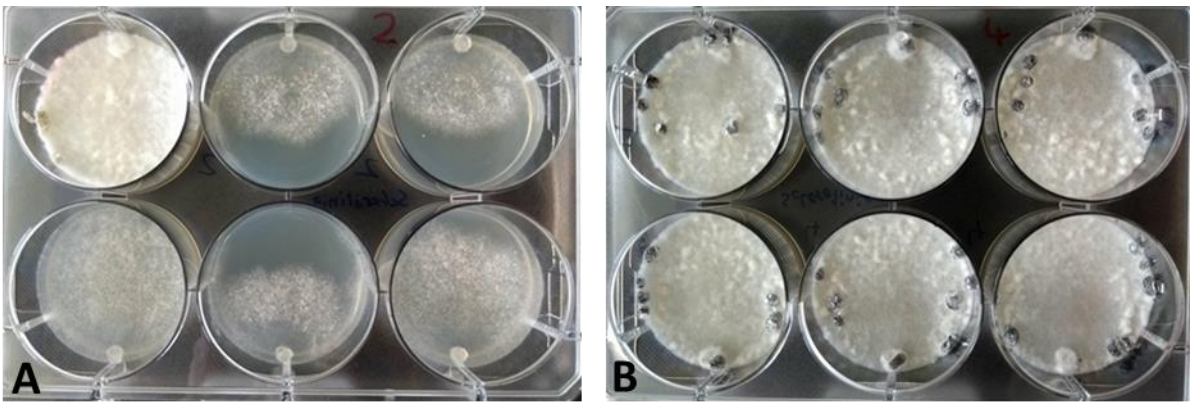

Figure 7 VOC mediated inhibition of *S. sclerotiorum* sclerotia formation. **A** *S. sclerotiorum* mycelium grown in presence of *B. amyloliquefaciens* B47 VOCs in six replicates. **B** Control experiment - *S. sclerotiorum* mycelium grown in absence of VOCs released by the biocontrol strain.

3.3.2. Identification of released volatile organic compounds by GC-MS analysis

To identify the elicitors of the observed antagonistic effects mediated by VOC exchange, a gas chromatography coupled with mass spectrometry analysis was carried out. In order to assess differences in compounds released by *B. amyloliquefaciens* B47 in response to VOC exchange with the plant pathogens *V. longisporum* and *B. cinerea,* the spectrum of volatiles emitted by *B. amyloliquefaciens* B47 was measured in absence and presence of pathogenic volatiles. To exclude compounds derived from the medium, substances emitted by sterile PDA and NA were identified in GC-MS analysis.

Spectrum of volatiles released by *B. amyloliquefaciens* B47 in absence of pathogens

After three days of growth a total of 16 volatile substances released by *B. amyloliquefaciens* B47 were identified (*Table 4*). Among these substances seven compounds are described in literature to have antimicrobial effects. One of these compounds, 1,3-pentadiene is known to show specific antifungal activity against *B. cinerea* when applied as pure substance. (Gotor-Vila et al., 2017) The substance 1-butanol-3-methyl has antimicrobial activity against several phytopathogens. (Dalilla et al., 2015). Isobutyric acid was detected, which is also a potential antifungal substance (Mercier and Jiménez, 2004). Further, 2-heptanone, 2-nonanone and 2-undecanone all have a strong inhibitory effect towards *Agrobacterium tumefaciens* (Plyuta et al., 2016). The detected compounds 2-nonanone and 2-undecanone are thought to act as plant growth promoting agents by increasing root lengths (Fincheira et al., 2017). A compound indetified as 2-tridecanone is described in literature to inhibit the growth of the fungal plant pathogen *Fusarium oxysporum* (Raza et al., 2015) and to protect plants from bacterial infections (López-Lara et al., 2018).

B. amyloliquefaciens B47 VOC profile in response to volatile exchange with *B. cinerea*

The response of *B. amyloliquefaciens* B47 to the presence of volatiles emitted by the phytopathogenic fungus *B. cinerea* was investigated by the identification of volatile compounds and analysis of their abundances after VOC exchange of both strains (*Table 4*). From the 16 compounds measured in absence of pathogens, 15 compounds were also detected after coincubation and VOC exchange with *B. cinerea*. The compound 2-heptanone,5-methyl was not produced by the *Bacillus* strain after coincubation with *B. cinerea*. Interestingly, two new compounds, acetoine and 2,3-butanediol, were released into the headspace by the bacterial strain in response to volatile exchange with the phytopathogen.

These two compounds are very well described in literature as elicitors of induced systemic resistance and plant growth promoting agents (Farag et al., 2006). To quantify compound abundances, in a first step p-values were calculated to test for significance of the observed effects. A probability value of $p<0.05$ was set to confirm differences between groups (presence/absence of *B. cinerea* VOCs). For compounds with statistically significant changes in abundance the log2 fold change was calculated. A major downregulation of compounds (log2 fold change < -1.5) due to VOC exchange with *B. cinerea* was detected for 2-heptanone, 2-heptanone-6 methyl and 2-nonanol. A major upregulation (log2 fold change > 1.5) was detected for only one compound, 1-butanol, 3-methyl. For all other compounds no statistically significant changes in abundance were observed.

Table 4 Compounds released by *B. amyloliquefaciens* B47 identified by GC-MS headspace SPME measurements

RT(min)	Predicted substance	log2FC - *B. cinerea*[1]	log2FC - *V. longisporum*[1]	Predicted function
1.64	1,3-Pentadiene	n.a.	n.a.	Antifungal specifically against Botrytis cinerea
2.82	2-Pentanone	-0.97*	-0.98*	n.a.
3.2	Acetoine	UNIQUE	UNIQUE	ISR and plant growth promotion
3.52	1-Butanol 3-methyl	1.62*	n.a.	Antimicrobial
4.2	Isobutyric acid	n.a.	n.a.	Potentially antifungal
4.46	2-Hexanone	n.a.	n.a.	n.a.
4.5	2,3-Butanediol, [S-(R*,R*)]-	UNIQUE	UNIQUE	ISR and plant growth promotion
6.26	Butanoic acid, 3-methyl-	0.87*	1.24*	n.a.
6.67	Butanoic acid, 2-methyl-	n.a.	n.a.	n.a.
7.16	2-Heptanone	-2.59*	-1.97*	Antibacterial
9.08	2-Heptanone, 6-methyl-	-3.72*	-1.84*	n.a.
9.37	2-Heptanone, 5-methyl-	N.d.	N.d.	n.a.
10.17	2-Octanone	n.a.	n.a.	n.a.
13.28	2-Nonanone	-1.43*	-2.57*	Antibacterial, PGP
13.52	2-Nonanol	-2.30*	N.d.	n.a.
18.56	2-Undecanone	n.a.	-2.44*	Antibacterial, PGP
18.7	2-Undecanol	n.a.	n.a.	n.a.
21.86	2-Tridecanone	n.a.	n.a.	Plant protection, antifungal
21.94	2-Tridecanol	n.a.	n.a.	n.a

* p-value <0.05

[1] The regulation of *Bacillus* VOCs due to VOC exchange with the pathogens *B. cinerea* and *V. longisporum* is expressed as log2 fold change. N.a. means compounds were not found to be regulated with statistical significance. "UNIQUE" denotes compounds which were only detected after VOC exchange with pathogens. Compounds labelled with N.d. (not detected) were only detected in absence of pathogens but not after VOC exchange with pathogenic strains.

B. amyloliquefaciens B47 VOC profile in response to volatile exchange with *V. longisporum*

The reaction of *B. amyloliquefaciens* B47 towards *V. longisporum* volatiles was additionally tested to detect possible pathogen-strain specific differences in volatile communication (*Table 4*). Two compounds, 2-heptanone, 5-methyl and 2-nonanol released in absence of pathogens were not detected after coincubation with *V. longisporum*. The two compounds acetoine and 2,3-butanediol, produced in response to coincubation with *B. cinerea* were also detected to be released after VOC exchange with *V. longisporum*. Figure 8 shows the overlay of chromatograms derived from headspace measurements of blank controls (sterile medium), *V. longisporum* and *B. amyloliquefaciens* B47 strains after three days of growth alone and after VOC exchange with each other. At 4.5 minutes the peaks representing 2,3-butanediol appear in two of the three biological replicates of *B. amyloliquefaciens* headspace measurements after VOC exchange with *V. longisporum*, but in none of the other measured samples. A downregulation of log2 fold change < -1.5 was detected for 2-heptanone, 2-heptanone-6 methyl, 2-nonanone and 2-undecanone. No major upregulation of compounds was detected due to coincubation with *V. longisporum.*

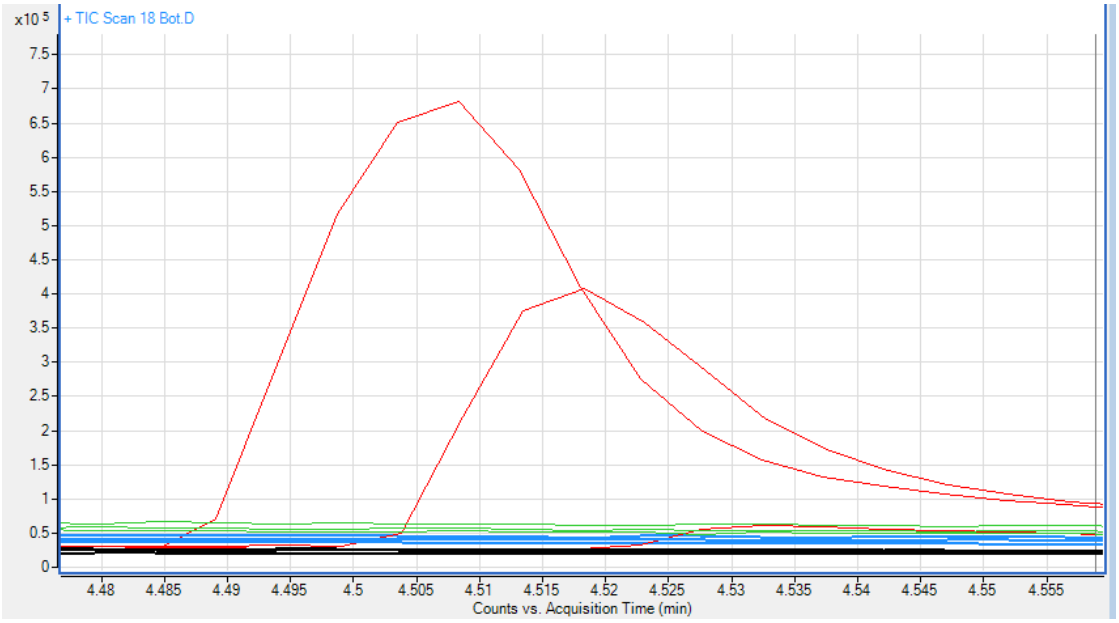

Figure 8 Gas chromatography peaks representing 2,3-butanediol released by *B. amyloliquefaciens* B47 in response to *V. longisporum* VOCs. The overlay of chromatograms shows compound peaks eluting at 4.5 minutes. Chromatograms of SPME headspace measurements of the two strains without VOC exchange with each other are shown in green (*V. longisporum*) and black (*B. amyloliquefaciens*). Chromatograms after coincubation and VOC exchange of both strains are shown in blue (*V. longisporum*) and red (*B. amyloliquefaciens*). All samples were measured in 3 biological replicates.

B. cinerea VOC profile

Furthermore the spectrum of volatiles emitted by *B. cinerea* was evaluated (*Table 5*). When *B. cinerea* was cultivated alone, 5 compounds were detected to be released into the headspace by the fungal strain. These compounds were identified as acetic acid, 1-butanol, 3-methyl, 1-butanol, 2-methyl, 2-methyl-2-bornene and 2-methylisoborneol. Analysis of compounds released after 3 days of coincubation with *B. amyloliquefaciens* B47 showed that the production of 2 compounds was prevented due to VOC exchange with the biocontrol strain. *B. cinerea* still produced acetic acid, 1-butanol, 3-methyl and 1-butanol 2-methyl but did not emit 2-methyl-2-bornene and 2-methylisoborneol. Changes in the abundance of compounds could not be evaluated as the probability value was $p > 0.05$ and therefore statistical significance was not given.

Table 5 Compounds released by *B. cinerea*, identified by GC-MS SPME headspace measurements

RT(min)	Predicted substance	Fold change[1]	Predicted function
2.3	Acetic acid	n.a.	n.a.
3.51	1-Butanol, 3-methyl-	n.a.	n.a.
3.57	1-Butanol, 2-methyl-	n.a.	n.a.
10.91	2-Methyl-2-bornene	Not detected	n.a
15.88	2-Methylisoborneol	Not detected	n.a.

1 Regulation of *B. cinerea* VOCs due to VOC exchange with *B. amyloliquefaciens*. N.a. means compounds were not found to be regulated with statistical significance. Compounds denoted with "not detected" were not identified in the spectrum of volatiles released by *B. cinerea* after VOC exchange with *B. amyloliquefaciens B47.*

V. longisporum VOC profile

Additionally, the VOC profile produced by the fungus *V. longisporum* was analysed (*Table 6*). When *V. longisporum* was grown in absence of the bacterial strain it released six compounds into the headspace which were identified as 1-propanol, 2-methyl; acetic acid; 1-butanol, 2-methyl; 1-butanol, 3-methyl; 1-pentanol and phenylethyl alcohol. The substance 1-butanol, 3-methyl, as mentioned before is known to show antifungal effects (Dalilla et al., 2015). Phenylethyl alcohol is a known antibacterial and antifungal substance (Pubchem). The spectrum of emitted compounds did not change due to coincubation and VOC exchange with *B. amyloliquefaciens* B47. A statistically significant downregulation (log2 fold change < -1.5) was detected for 1-butanol, 2-methyl.

Table 6 Compounds released by *V. longisporum*, identified by GC-MS SPME headspace measurements

RT(min)	Predicted substance	Log2FC	Predicted function
2.21	1-Propanol 2-methyl	n.a.	n.a.
2.3	Acetic acid	n.a.	n.a.
3.5	1-Butanol, 3-methyl-	n.a.	Antimicrobial
3.59	1-Butanol, 2-methyl-	-1.58*	n.a.
4.1	1-Pentanol	n.a.	n.a.
13.9	Phenylethyl Alcohol	n.a.	antimicrobial, antiseptic, and disinfectant

* p-value <0.05

[1] The regulation of VOCs emitted by *V. longisporum* due to VOC exchange with *B. amyloliquefaciens* is expressed as log2 fold change. N.a. means compounds were not found to be regulated with statistical significance.

3.4. Bacterial metabolome studies in reaction to phytopathogens

As it was shown before that the interchange of VOCs between microorganisms can influence metabolism of both interaction partners (Rybakova et al., 2017), metabolomics studies were performed aiming to detect changes in bacterial metabolite levels due to VOC exchange with fungal pathogens. The response of *B. amyloliquefaciens* B47 to VOC exchange with *B. cinerea* and *V. longisporum* was investigated on metabolic level.

Since correct identification of metabolites derived from whole cell extracts is extremely time consuming and requires a lot of expertise, in this study it was focused on quantitative analysis of metabolite abundances. A total of 2845 compounds were found to be present in each of the samples analysed by HPLC-MS. *Figure 9* and *Figure 10* show the changes in abundance of *B. amyloliquefaciens* B47 metabolites due to VOC exchange with the pathogens, expressed as log2 fold change on the x-axis and the value of statistical significance on the y-axis. Dots lying in the areas coloured red or green are significantly up or downregulated with a threshold of log2 fold change >1.5 or <1.5 respectively and a p-value <0.05. Comparing shifts in metabolome due to VOC exchange with the two different pathogens *V. longisporum* and *B. cinerea* shows a much stronger reaction to the VOCs of the fungal pathogen *V. longisporum*. Whereas in presence of VOCs emitted by *B. cinerea* only few *Bacillus* metabolites significantly changed in their abundances, an exceeding amount of metabolites were up or down regulated in response to VOC exchange with *V. longisporum*.

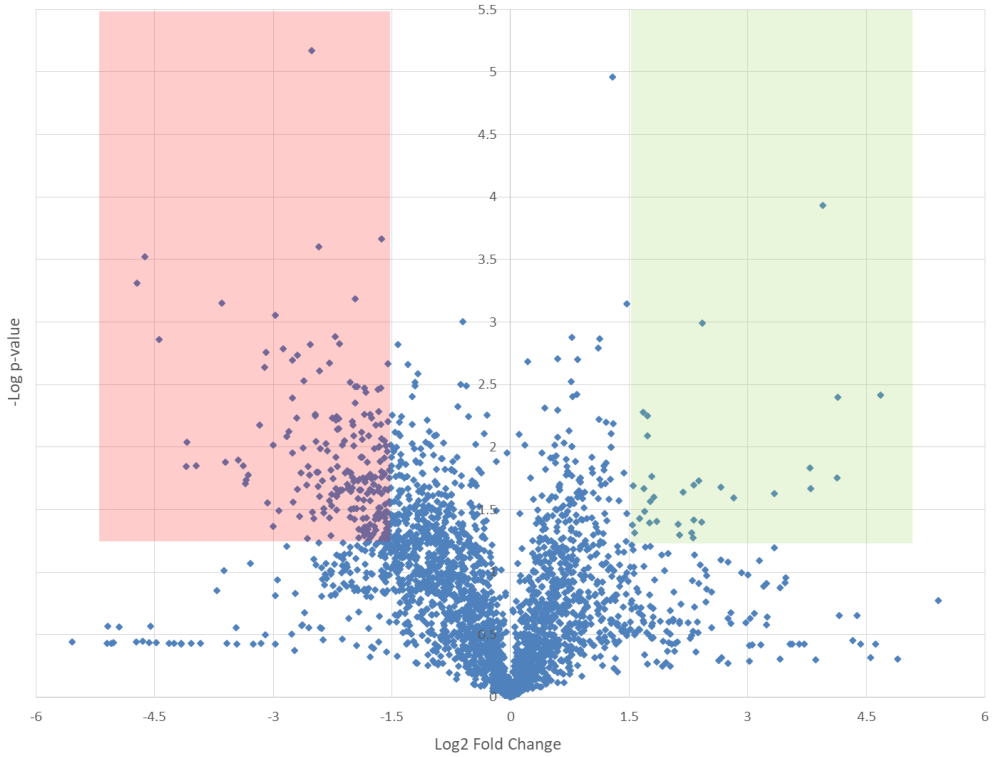

Figure 9 Differential analysis of changes in *B. amyloliquefaciens* B47 metabolite abundances in response to *B. cinerea* VOCs. Each data point stands for one specific metabolite. Data points lying in coloured areas are considered to be significantly upregulated (green) or downregulated (red) with thresholds of log2 fold change < -1.5 or > 1.5 and a p-value < 0.05.

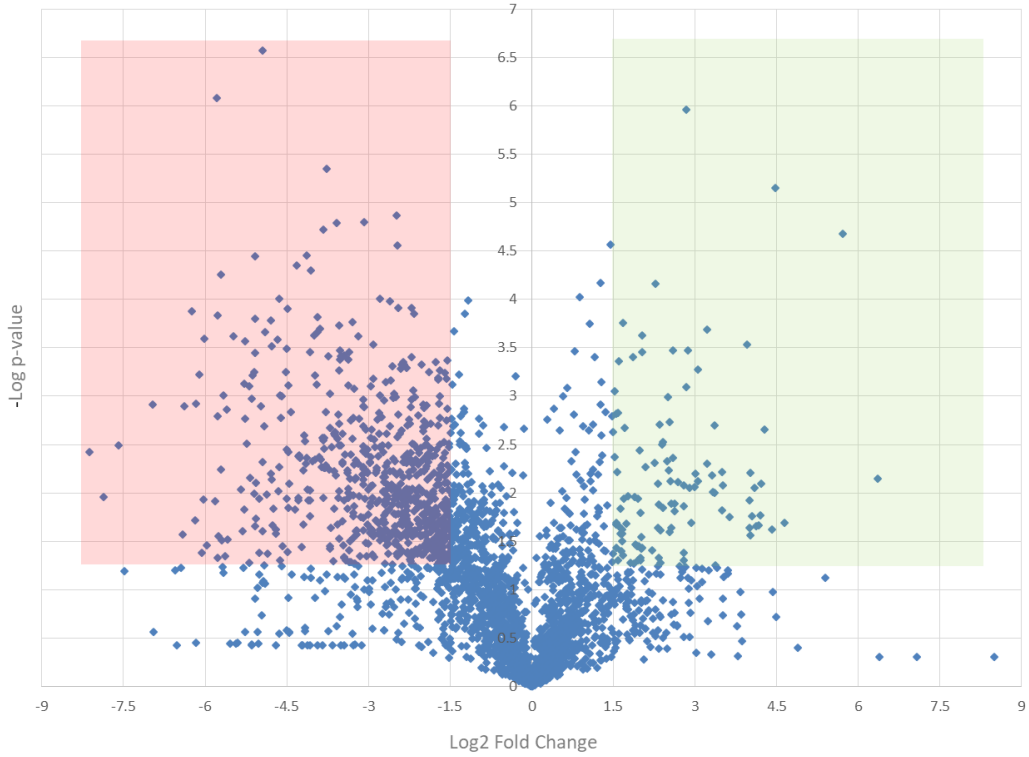

Figure 10 Differential analysis of changes in *B. amyloliquefaciens* B47 metabolite abundances in response to *V. longisporum* VOCs. Data points lying in coloured areas are considered to be significantly upregulated (green) or downregulated (red) with thresholds of log2 fold change < -1.5 or > 1.5 and a p-value < 0.05.

Further analysis showed, that VOC exchange with *V. longisporum* induced upregulation of 106 compounds and downregulation of 675 compounds whereas contact to *B. cinerea* VOCs only led to upregulation of 32 compounds and downregulation of 210 compounds. A common effect observed was, that approximately 6 times more compounds were downregulated than upregulated in reaction to both pathogens. A total of 781 compounds were found to be differentially produced by *B. amyloliquefaciens* B47 due to *V. longisporum* VOCs. Considering the 2845 compounds detected in total, this makes up a percentage of 27.5% of all metabolites which were differentially expressed due to VOC exposure. The presence of *B. cinerea* VOCs induced changes in abundance of 242 metabolites which represents 8.5 % of total detected metabolites.

The distribution of shared and unique up and down regulated metabolites in response to both pathogens is visualized in a Venn diagram (*Figure 11*). A total of 89 compounds were found to be upregulated only in response to *V. longisporum* volatiles but not in response to *B. cinerea* volatile compounds. Unique upregulation in response to *B. cinerea* was detected for 15 compounds. Only a portion of 14% (17 compounds) of all upregulated compounds was shared between *B. amyloliquefaciens* B47 metabolomes reacting to the two different pathogens. In the spectrum of all downregulated compounds, a percentage of 30.5% (207 compounds) were found to be downregulated in response to both pathogens and 468 compounds were specifically downregulated in presence of *V. longisporum* VOCs whereas only 3 compounds were uniquely downregulated in response to VOC exchange with *B. cinerea*.

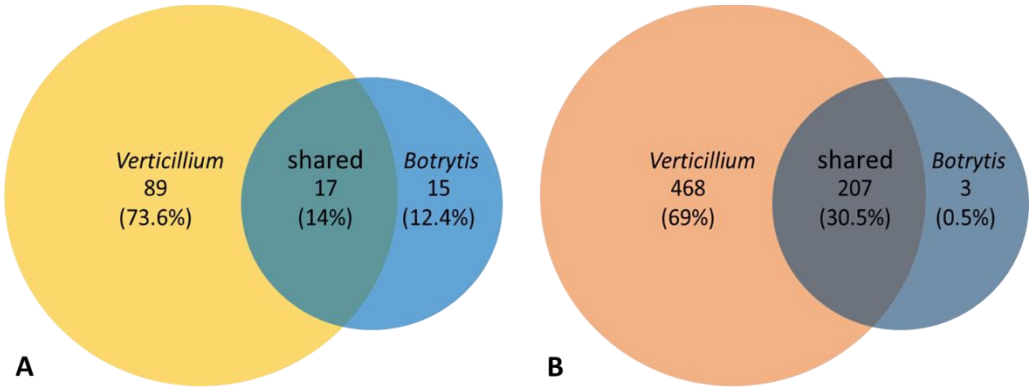

Figure 11 Graphical representation of common patterns in *B. amyloliquefaciens* B47 metabolite regulation in response to the VOCs of two different pathogens *V. longisporum* and *B. cinerea*. **A** Distribution of unique and shared significantly upregulated *B. amyloliquefaciens* B47 metabolites in reaction to VOC exchange with *B. cinerea* and V. *longisporum*. **B** Distribution of unique and shared significantly downregulated *B. amyloliquefaciens* metabolites in reaction to VOC exchange with *B. cinerea* and *V. longisporum.*

To gain information about relationships between groups and identify class differences in this multivariate dataset, a principal component analysis (PCA) was applied. PCA provides an unbiased approach of data analysis because separation is only observed between groups when within-group variation is significantly less than between-group variation. PCA analysis showed a clustering of the three groups each consisting of three biological replicates in the 3D plot (*Figure 12*). Therefore existing metabolic differences between groups due to the exchange of volatile compounds was confirmed.

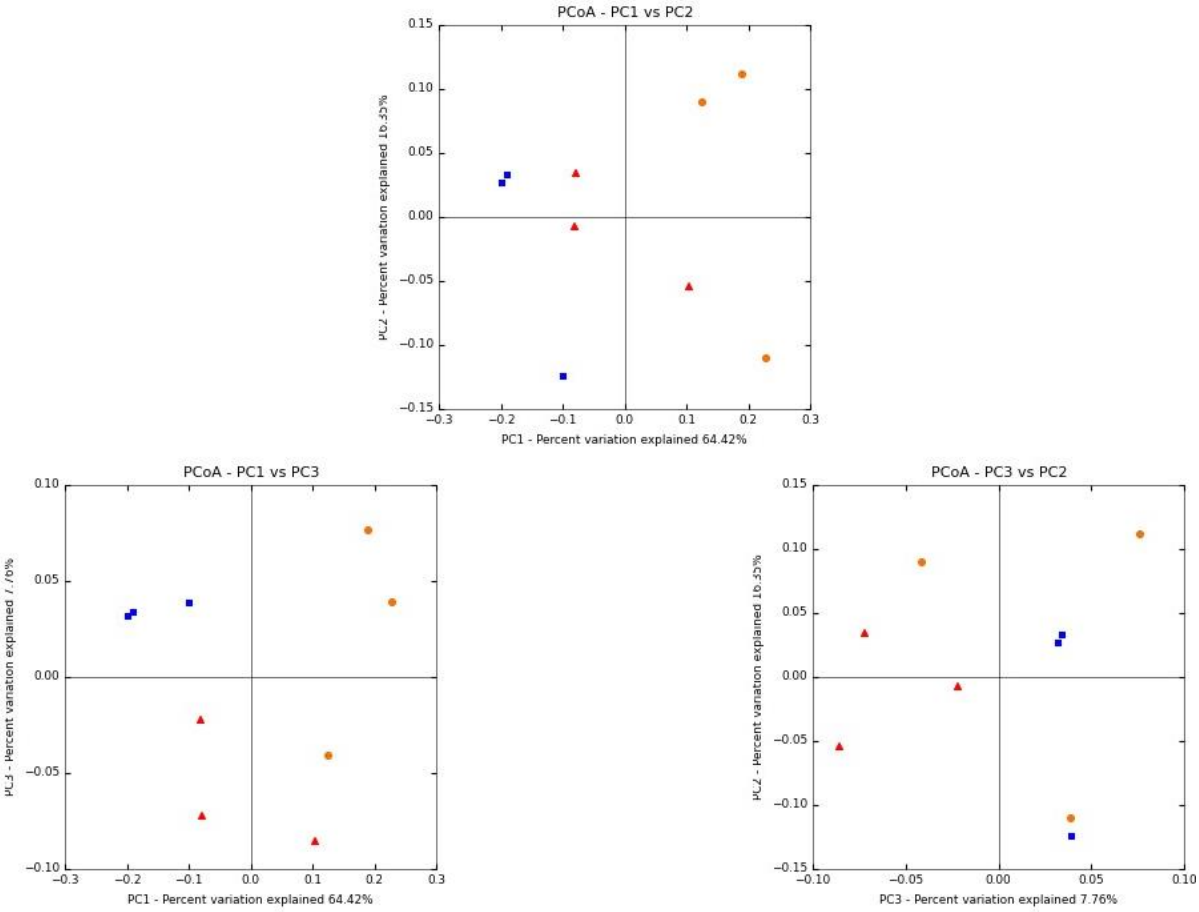

Figure 12 Principal component analysis of *B. amyloliquefaciens* B47 metabolomics data. Two dimensional PCA plots show separation of metabolite profiles due to VOC exchange with phytopathogens. Distinct colours are used to visualize experimental groups, each consisting of three biological replicates (blue= control; red= VOC exchange with *B. cinerea*; orange= VOC exchange with *V. longisporum*)

Identification of *Bacillus* secondary metabolites

With a targeted approach of looking for compound specific fragment masses described in literature, the secondary metabolites fengycin and surfactin were identified to be produced by *B. amyloliquefaciens* B47. Fengycin is a lipopeptide which effectively inhibits filamentous fungi. (Vanittanakom et al., 1986). Surfactin has a strong antimicrobial, antiviral and biofilm promoting activity (Raaijmakers et al.). Fengycin and surfactin may also be involved in elicitation of ISR in plants (Ongena et al., 2007). However these two substances were not found to be significantly up or down regulated neither in response to *B. cinerea* nor to *V. longisporum* volatiles.

3.5. Investigation of plant growth promotion abilities

The plant growth promoting effects of *B. amyloliquefaciens* B47 on tomato plants were assessed under sterile conditions in germination pouches as well as under non sterile conditions in soil.

3.5.1. Plant growth promotion under sterile conditions

After 13 days of growth, tomato plants were harvested and different parameters for assessing plant growth were compared between plants developed from sterile seeds (control) and plants developed from seeds primed with *B. amyloliquefaciens* B47 after initial surface sterilization (*Figure 13*). The first measured parameter was the total length of roots ranging from the germination site to the root tip, not taking into account any branches. Relative to the control plants, the average root length of bioprimed plants reached was increased by 36%. However the standard deviation for root lengths was exceedingly high, so that no confident conclusion can be drawn about the positive impact of *B. amyloliquefaciens* B47 on root length. After determination of root lengths, the phyllosphere was separated from the roots and weighed. The fresh weight of phyllospheres was increased by 16% and root weight was increased by 29% due to priming of seeds with *B. amyloliquefaciens* B47. Calculation of standard deviation was not possible for phyllosphere and root weight as weighing was done by pooling the roots/phyllospheres of 5 plants. Germination rate was determined by counting the sprouting

seeds of both groups. A 46% higher germination rate was observed due to priming of seeds with *B. amyloliquefaciens* B47.

Figure 13 Quantification of plant growth promotion abilities of *B. amyloliquefaciens* B47 under sterile conditions. Phyllosphere weight, root weight, root length and germination rate were assessed for plants developed from seeds primed with *B. amyloliquefaciens* B47 and compared to a sterile control group. Results are expressed as percentage of the measured values relative to the control group.

The overall health impression of plants was improved by application of the biocontrol strain, as plants treated with the *Bacillus* strain developed healthy green leaves whereas plants in the control group looked stunted and showed partly yellowish leaves (*Figure 14*).

Figure 14 Plant growth promoting effect of *B. amyloliquefaciens* B47 on tomato plants under sterile conditions. **A** 13 days old seedlings developed from seeds primed with *B. amyloliquefaciens* B47, grown under sterile conditions **B** negative control- 13 days old seedlings primed with sterile NaCl and grown under sterile environment.

3.5.2. Plant growth promotion under non-sterile conditions

In a recent study (Rybakova et al., 2016) it was suggested "that the natural non-sterile soil is the best medium for studying plant-bacterium interaction as it reflects the field conditions on the best way". Therefore to get a more realistic view of the plant growth promotion potential of *B. amyloliquefaciens* B47 under natural conditions, the effects of priming tomato seeds with the biocontrol strain were also investigated in the non-sterile soil habitat. Additionally in this trial a second biocontrol strain, the fungus *Trichoderma harzianum* 3TSMI was tested for its plant growth promoting effects towards tomato plants grown in soil. After 13 days of growth the phyllosphere was harvested and fresh weight was determined. The distribution of

measured fresh weights of individual plants in all four groups is plotted in a box-plot diagram (*Figure 15*). The box-plot diagram was chosen as this representation shows variation in samples without making assumptions of the underlying statistical distribution. The minimum and the maximum values are represented by whiskers, the box represents the first quartile, the median and the third quartile. Therefore a box-plot diagram displays the full range of variation. Although values for highest and lowest fresh weights were similar in all groups, a difference of weight was found considering the majority of data lying in the first to third quartile. The median values of phyllosphere fresh weight were 0.39 g in the control group, 0.43 g in the group primed only with *T. harzianum* 3TSMI, 0.51 g in the group primed only with *B. amyloliquefaciens* B47. These values show that a clear increase in fresh weight was obtained due to priming of tomato seeds with *B. amyloliquefaciens* B47. Treatment of seeds with *T. harzianum* 3TSMI led to a lesser plant growth promoting effect. Interestingly, when *T. harzianum* 3TSMI and *B. amyloliquefaciens* B47 were both applied the median value of phyllosphere fresh weight was 0.38 g and hence almost identical to the control group.

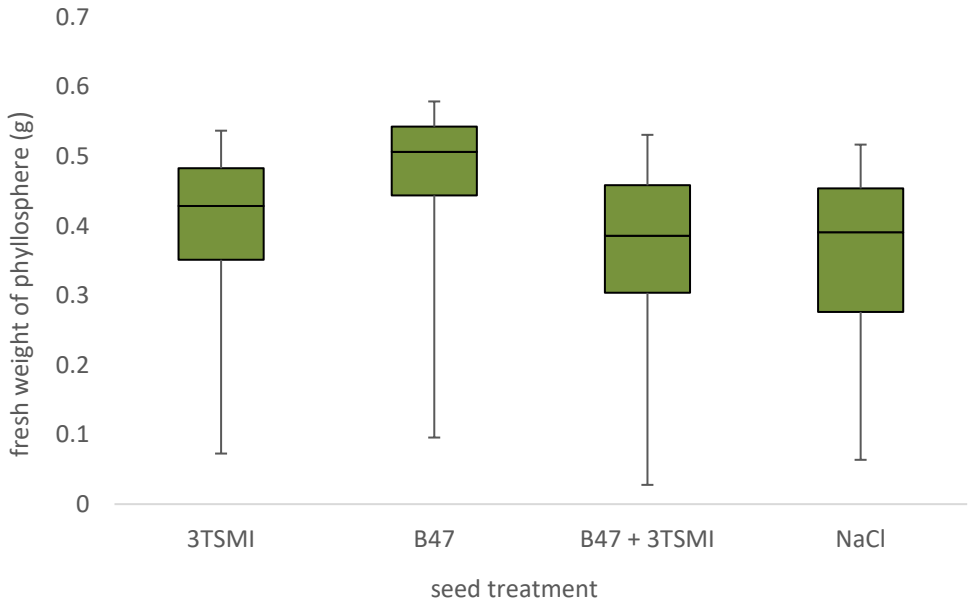

Figure 15 Plant growth promoting effects of *B. amyloliquefaciens* B47 and *T. harzianum* 3TSMI on tomato plants under non-sterile conditions. As a parameter for assessing plant growth, fresh weight of the plants green parts was determined directly after harvesting. The distribution of determined weights is shown in a box-pot with whiskers indicating the lowest and the highest values. The plant growth promotion potential of both biocontrol strains was tested by priming seeds with *either T. harzianum* 3TSMI (3TSMI) or *B. amyloliquefaciens* B47 (B47). Additionally the effect of combining both biocontrol strains on plant growth was tested (B47 + 3TSMI). As a negative control seeds were primed with sterile NaCl.

Additionally, the dry weight of tomato plants was measured and compared between groups (*Figure 16*). Determination of dry weight can be considered to be less accurate as plants have a very low dry weight and rehydrate extremely quickly after removing them from the exsiccator which makes weighing error-prone. The median values for dry weight differed less between the experimental groups than the values did for fresh weight. The median dry weight in both, the control group and in the group treated with *T. harzianum* 3TSMI was 0.023 g, in the group treated with *B. amyloliquefaciens* B47 the median value was 0.027 g. When both biocontrol strains were applied a median value of 0.025 was achieved. In summary, this data showed the highest plant growth promoting effect when seeds were primed with *B. amyloliquefaciens* B47*.* However, this effect vanished when seeds were additionally treated with *T. harzianum 3TSMI.* The fungal biocontrol strain applied alone also stimulated plant growth although to a lesser extent.

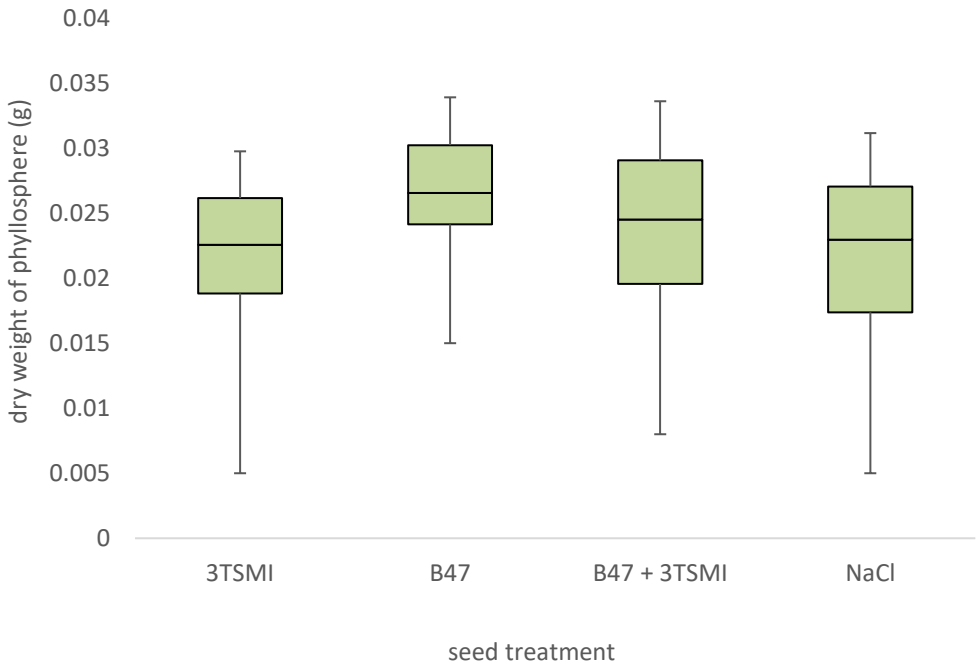

Figure 16 Plant growth promoting effects of *B. amyloliquefaciens* B47 and *T. harzianum* 3TSMI on tomato plants under non-sterile conditions. As a parameter for assessing plant growth, dry weight of the plants green parts was determined. The distribution of determined weights is shown in a box-pot with whiskers indicating the lowest and the highest values. The plant growth promotion potential of both biocontrol strains was tested by priming seeds with either *T. harzianum* 3TSMI (3TSMI) or *B. amyloliquefaciens* B47 (B47). Additionally the effect of combining both biocontrol strains on plant growth was tested (B47 + 3TSMI). As a negative control seeds were primed with sterile NaCl.

The priming of tomato seeds with *B. amyloliquefaciens* B47 had a great impact on the germination behaviour of the seeds planted in soil. *Figure 17* shows, that the addition of the bacterial biocontrol strain not only led to an increased germination rate but also speeded up the germination process. This beneficial effect on germination was observed when seeds were either primed with *B. amyloliquefaciens* B47 alone or in combination with *T. harzianum* 3TSMI but not for seeds solely primed with *T. harzianum* 3TSMI. After 4 days, 80% of seeds primed with *B. amyloliquefaciens* B47 and 75% of seeds primed with both biocontrol strains were germinated whereas only 55 % of both, control seeds primed with sterile NaCl and seeds primed with *T. harzianum* 3TSMI were germinated.

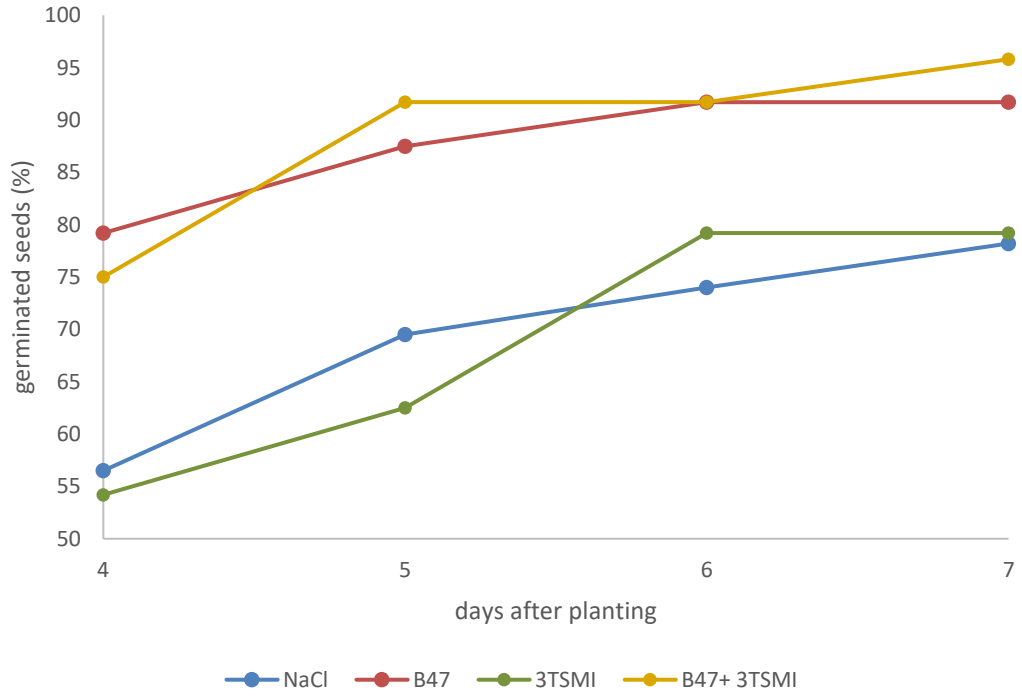

Figure 17 Germination behaviour of tomato seeds in non-sterile soil. Seeds were primed either with *B. amyloliquefaciens* B47 (red), *T. harzianum* 3TSMI (green) or with both biocontrol strains (yellow). As a control, seeds were primed with sterile NaCl (blue).

3.6. Visualization of tomato root colonization by *B. amyloliquefaciens* B47 using FISH – CLSM

Several bio-primed plants obtained from plant growth promotion experiments in sterile germination pouches were preserved by paraformaldehyde fixation until stained by fluorescent in situ hybridization (FISH). To discriminate between the *Bacillus* biocontrol strain and other bacteria possibly being present on the roots, originating from the inner of the seeds, two different FISH probes were used.

Figure 18 exemplarily shows a confocal laser scanning microscopy image generated in this study, of bacterial cells colonizing tomato roots of two weeks old seedlings. Fluorescence emitted by the Cy3 fluorophore is shown in red and indicates binding of the hybridization probe to sequences specific for all *Bacteria.* No strong signal was obtained from the Cy5 fluorophore specifically labelling all bacteria belonging to the phylum of *Firmicutes*. However, it has to be mentioned that while setting parameters for imaging a weak Cy5 signal was observed, but disappeared during the process of imaging due to photobleaching. Colonization patterns of *B. amyloliquefaciens* B47 did not show the expected density. In fact, only few clusters of bacterial cells were detected. No biofilm structures were shown to be present on the roots.

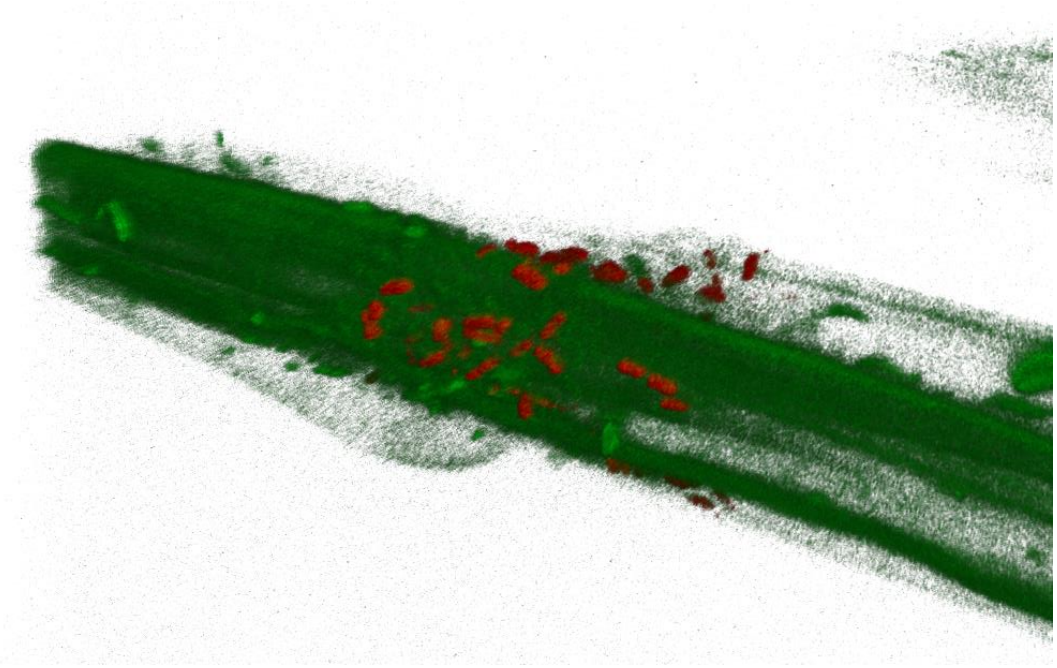

Figure 18 Visualization of 13 days old tomato seedlings grown in sterile germination pouches, primed with *B. amyloliquefaciens* B47. All bacterial strains were visualized using Eub338-Cy3 FISH probes binding to *Bacteria* (red). To specifically stain bacteria belonging to the class of *Firmicutes* the LGC354 Mix labelled with Cy5 was used (yellow).

Figure 19 shows a mixed model rendering of the colonization of tomato roots generated with Imaris 7.3. software.

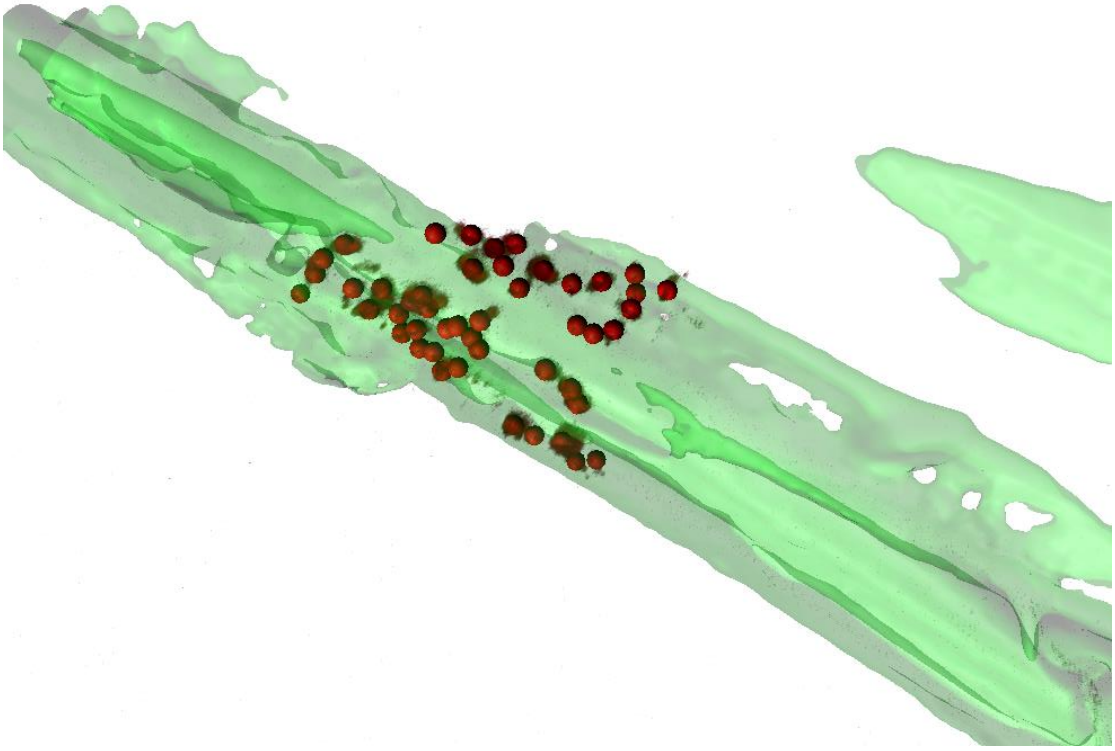

Figure 19 Mixed model rendering of a CLSM image of 13 days old tomato seedlings grown in sterile germination pouches, primed with *B. amyloliquefaciens* B47.

It was suggested that the low density of bacterial cells on roots developed from bio-primed seeds is due to inefficient colonization after bio-priming. Therefore, in further experiments the root-dipping approach was tested. Roots were grown in sterile vermiculite for one week after inoculating the roots with *B. amyloliquefaciens* B47 by root dipping. Visualization by FISH – CLSM was performed and again showed only few bacterial cells on the roots stained with only the *Bacteria* specific Cy3 labelled probe (not shown). The colonization density was comparable to the density of cells found on roots originating from bio-primed plants. No biofilm structures were identified.

To probe the causes of problems in visualization of *B. amyloliquefaciens* B47 cells, control experiments were performed and fixation of plant material was done directly after *B. amyloliquefaciens* B47 cells were applied to the roots. Following, fluorescent in situ hybridization was conducted and roots were observed using CLSM. These control experiments neither did result in successful visualization of *B. amyloliquefaciens* B47 by FISH using the *Firmicutes* specific Cy5 labelled probe. Several single cells were stained by the *Bacteria* specific Cy3 labelled probe, however by far not in the expected amounts.

4. Discussion

In this study it was shown that B*. amyloliquefaciens* B47 has a great potential for use as a biocontrol agent due to its broad spectrum of plant protection abilities. Living in the soil habitat, this bacterium is in constant interaction with the host-plant on the one side and the competing microflora including phytopathogens on the other side. The great effectiveness in plant protection by this bacterial strain is based on the combination of both of these interaction mechanisms.

4.1. Mechanisms utilized in biocontrol of pathogens by *B. amyloliquefaciens* B47

The role of extracellular soluble metabolites in biocontrol of pathogens

Analysis of the *B. amyloliquefaciens* B47 genome revealed the capability to produce several antibacterial and antifungal secondary metabolites like polyketides and nonribosomal peptides which are thought to act both as antibiotics and as signalling molecules for intercellular and interspecies communication. Gene clusters for the synthesis of four different antibiotics, bacilysin, bacillaene, surfactin and plipastatin (also referred to as fengycin (Honma et al., 2012)) were identified. The polyketide bacillaene acts as a primary defensive molecule that confers resistance to other soil dwelling bacteria like *Myxococcus xanthus* by providing sufficient time to develop spores and hence escaping the attack by *M. xanthus* (Müller et al., 2014). Bacilysin, plipastatin (fengycin) and surfactin all belong to the group of non-ribosomal peptide antibiotics and share a common mode of synthesis by non-ribosomal peptide synthetases (NRPS). Bacilysin acts against a wide range of bacteria and the yeast *Candida albicans*. Surfactin has haemolytic, antimicrobial and antiviral activities by altering membrane integrity and can therefore effectively inhibit competing organisms. The second role of surfactin, which has detergent properties, is promotion of root colonization and biofilm formation. This substance therefore provides advantages in root colonization by exhibiting antagonistic activities. The cyclic lipodecapeptide fengycin specifically antagonizes filamentous fungi (Chen et al., 2009).

The production of two of these antibiotic substances identified on the genomic level was also confirmed in metabolome studies. Surfactin and fengycin were produced by *B. amyloliquefaciens* B47during growth on NA agar plates even without contact to pathogens. These compounds are therefore a proposed mechanism by which the biocontrol strain conducts the observed direct antagonistic effects against several phytopathogens on agar

plates. The zones of inhibition of mycelial growth in the surroundings of the biocontrol strain lead to the conclusion that the bacterial strain supposedly secretes surfactin and fengycin which then diffuse through the agar and lead to inhibition of fungal mycelium growth. Another prominent mechanism of competition in soil is the production of iron chelators called siderophores which are secreted to the environment where they form complexes with iron and are again taken up by the cells by an active transport system through the cell membrane. Siderophores therefore can deprive other microorganisms of essential iron and hence act in an antagonistic way against them (Beneduzi, Ambrosini, & Passaglia, 2012). Several siderophores like bacillibactin and enterobactin were identified in genomic analysis of *B. amyloliquefaciens* B47.

Under natural conditions in soil it is most likely that *B. amyloliquefaciens* B47 synthesizes and secretes a whole spectrum of secondary metabolites for which coding regions were found on the genome but which however were not identified in metabolomics studies. The cultivation methods used in laboratory conditions, like growth on a solid substrate with defined composition can give a good hint about mechanisms of interaction but do not resemble the complete spectrum of interactions happening in the soil habitat. Especially production of secondary metabolites often is not induced under laboratory conditions as the triggering signals arising from soil, plants and interaction with other microorganisms are missing. A further investigation of the elicitors of the antagonistic effects could include identification of compounds released into the agar by *B. amyloliquefaciens* B47 when in direct contact with phytopathogens.

VOCs play a crucial role in pathogen antagonism directly and by strengthening the plant

Differences in the extent of inhibitory effects were observed towards different phytopathogens. Whereas *R. solani* growth was only inhibited in close proximity to the bacterial strain, some pathogens like *S. sclerotiorum* and *B. cinerea* were completely inhibited in fungal mycelium development and showed no growth in any direction. Based on these results an involvement of volatile organic compounds released by *B. amyloliquefaciens* B47 was suggested. The production of antimicrobial volatile compounds implies, that the biocontrol strain can elicit its antagonistic activities not only via secretion of soluble compounds for which direct contact in close proximity is needed but also through the air interface. The ability of antagonizing phytopathogenic fungi solely through production of antimicrobial volatile organic substances was assessed in VOC assays. The strain exhibited effective inhibition of a broad spectrum of phytopathogens mediated by volatile substances. However, strong differences in the magnitude of inhibitory effects towards different fungal pathogenic strains were observed. Also

in this trial the plant pathogens *B. cinerea* and *S. sclerotiorum* were most susceptible to inhibiting volatiles released by the biocontrol strain. The enormous power of *B. amyloliquefaciens* B47 VOCs was obvious when sclerotia formation of *S. sclerotiorum* was found to be completely inhibited by volatile compounds. The formation of sclerotia is an essential step in the life cycle of *S. sclerotiorum* and significantly facilitates its survival in soil.

To identify the specific compounds on which the observed antimicrobial effect is based, the volatile interaction of *B. amyloliquefaciens* B47 with one soil borne (*V. longisporum*) and one foliar (*B. cinerea*) phytopathogen was studied by SPME GC-MS analysis of emitted volatile compounds of both the bacterial and the fungal interaction partner in presence and absence of each other. The biocontrol strain was found to produce a broad spectrum of antibacterial and antifungal substances also without volatile contact to phytopathogens. Among the identified substances 1,3-pentadiene which is a compound known to be specifically and highly active against *B. cinerea* (Gotor-Vila et al., 2017) was present. This compound most likely contributes to the especially high antagonistic effect observed against *B. cinerea*. Interestingly, two antimicrobial compounds 2-heptanone and 2-nonanone were downregulated in response to the volatile exchange with both, *B. cinerea* and *V. longisporum*. Further, the antibacterial compound 2-undecanone was downregulated in response to *V. longisporum* volatiles. The finding of downregulation of antimicrobial compounds due to volatile exchange with phytopathogens was surprising as it was assumed that *B. amyloliquefaciens* B47 might respond to the presence of phytopathogenic volatiles with increased production of antimicrobial substances in order to protect itself by keeping the growth of the competing strain at bay.

Furthermore, changes in volatiles released by *B. cinerea* and *V. longisporum* due to contact to the biocontrol strain were examined in this study. *B. cinerea* completely stopped release of the substances 2-metylbornene and 2-methylisoborneol in presence of *Bacillus* VOCs, whereas *V. longisporum* still produced the whole spectrum of compounds but decreased production of 1-butanol, 2-methyl. These findings, in conclusion show that simplification of complex microbial interactions is often not possible and needs extensive studies. However, from these studies it became obvious that the exchange of volatiles is a very important factor in microbial communication and a powerful tool in biocontrol of pathogens.

Another interesting finding was the release of two novel substances by *B. amyloliquefaciens* B47 induced by exchange of VOCs with both pathogenic strains *B. cinerea* and *V. longisporum*. The compounds acetoine and 2,3-butanediol which are known for their plant growth promotion potential were only produced when *B. amyloliquefaciens* B47 was grown in presence of pathogenic volatiles. In literature, these two substances are also described as

elicitors of induced systemic resistance (ISR) in plants. The induction of ISR is a very powerful trait for a biocontrol agent as it enables the plant to show immunity against diverse microbial pathogens by activating innate defence mechanisms. These results suggest, that *B. amyloliquefaciens* B47 senses the presence of pathogenic fungi through volatile signals and responds by producing elicitors of induced systemic resistance to support the plant in fighting the invading pathogens and thus help maintaining plant health. These results show that VOCs are crucial for biocontrol activities not only in a direct way by production of antimicrobial substances but also by strengthening the plants immunity to resist pathogens present in the soil.

Taken together the production of secondary metabolites and the release of volatile organic substances are important mechanism by which the biocontrol strain can suppress phytopathogens and preserve plant health (Figure 20).

B. amyloliquefaciens B47 adapts its metabolome specifically in response to foreign VOCs

As the study showed interesting results regarding the communication of microbial strains through volatile organic compounds, this way of interaction was studied more in detail. The main question was, how the *Bacillus* biocontrol strain adapts its metabolism in response to exposure to VOCs released by phytopathogenic fungi. Further it was examined if *B. amyloliquefaciens* B47 is capable of changing its metabolism in a specific way depending on the fungal strain present.

In metabolome studies it was revealed that *B. amyloliquefaciens* B47 reacts with massive changes in metabolism to the presence of VOCs released by *V. longisporum* whereas only minor changes in metabolism were found in response to VOCs released by *B. cinerea*. More than one quarter (27%) of total metabolites were differentially regulated due to VOC exchange with *V. longisporum*. This remarkably high change in metabolite abundances leads to the suggestion that major changes in metabolism were induced by sensing the presence of V*. longisporum*. In contrast, when *B. cinerea* volatiles were present only 8.5 % of total cell metabolites changed in their abundance. It can therefore be reasoned that *B. amyloliquefaciens* B47 shows a lesser response on metabolic level to *B. cinerea* and does not change major metabolic pathways. This finding indicates pathogen-specific reactions of *B. amyloliquefaciens* B47 and implies the sensing of different VOCs and an adapted response on metabolic level. No conclusion could be drawn regarding the affected metabolic pathways as the analysis of individual compounds is extremely time consuming and requires a lot of expertise. A common effect observed in response to both pathogens was the predominant downregulation of compounds. In reaction to both pathogens an equal value of 15 % of

differentially abundant compounds were upregulated whereas 85 % of compounds were downregulated.

4.2. Plant – bacteria association and strategies for survival in soil

In genome studies a broad set of genes was identified supposedly being of great importance for interacting with the plant host. An extracellular enzyme increasing the plants phosphorus nutrition and thereby substantially increasing plant growth is 3 – phytase (Idriss et al., 2002) which was found to be present in the *B. amyloliquefaciens* B47 genome. A tryptophane transport protein was annotated which is thought to serve for tryptophane uptake from the environment. Tryptophane is the main precursor for synthesis of the auxin phytohormone indole-3-acetic-acid (IAA) (Spaepen et al., 2007). Spaepen et al. summarize that "bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms." Although not the whole genomic information for the biosynthetic pathway of IAA was found, the production of IAA was shown by an in situ assay in previous studies (Gassner, 2007). The missing genomic information is most likely due to incomplete sequencing of the *B. amyloliquefaciens* B47 genome which resulted in 249 scaffolds. The biosynthetic genes for the previously described volatile compound acetoine which confers plant growth promotion and ISR are also encoded in the genome. *B. amyloliquefaciens* B47 therefore is thought to produce soluble metabolites as well as volatile compounds to interact with the host plant and stimulate its growth.

The *Bacillus* strain naturally also draws benefits for its own survival from the close interaction with the plant. Several genes were identified which enable the bacterial strain to use plant derived substances as nutrients. Gene clusters coding for proteins involved in extracellular degradation of plant compounds like xylan, arabinogalactan, cellulose, pectin and pectate were annotated. By Beauregard et al. (Beauregard et al., 2013) it was shown that arabinogalactan, pectin and xylan induce biofilm formation and can further be digested, used as a carbon source and incorporated into the extracellular biofilm matrix in *B. subtilis*. The ability of biofilm formation is thought to be a prerequisite for successful root colonization which was shown exemplarily for *A. thaliana* and *B. subtilis* by Beauregard et al. The authors suggest, that the sensing of plant polysaccharides and their use as matrix components is conserved among *B. subtilis* and closely related species. This is therefore also thought to be a mechanism of root colonization for the *B. amyloliquefaciens* B47 strain investigated in this study. The biofilm component genes eps and tasA and the biofilm master regulator Spo0A, described to be essential for root colonization by biofilm formation, were identified in the

genome analysed in this study. This interaction is an interesting way by which the plant itself can stimulate its colonization by *Bacillus* biocontrol strains and benefit from its effects.

The plant growth promotion ability of *B. amyloliquefaciens* B47 on tomato plants was clearly shown in vivo under sterile laboratory conditions as well as under more natural conditions in soil. Fresh weights of the plants green parts were increased in both experiments due to priming the tomato seeds with *B. amyloliquefaciens* B47. Also plant health was improved, which became obvious especially in growth assays conducted in sterile environment where priming with *B. amyloliquefaciens* B47 had strongly beneficial effects. Further, a positive influence of the *Bacillus* strain on the germination rate and germination time of tomato plant seeds was observed.

To study the ability of plant colonization more in detail, visualization of root colonization was performed by fluorescent in situ hybridization (FISH) combined with confocal laser scanning microscopy (CLSM). Contradictory to the expected dense colonization of tomato roots by the formation of biofilms, the colonization of tomato roots by *B. amyloliquefaciens* B47 appeared to be very sparse and only few bacterial clusters were observed. However, considering the conducted control experiments, it is most likely that these results are due to methodological problems and do not reflect the actual colonization density of plant roots. A major problem was the bad signal obtained from Cy5 labelled *Firmicutes* specific probes. Therefore an identification of the observed bacteria as *B. amyloliquefaciens* B47 is not possible with high enough reliability. It is not entirely clear why the Cy5 signal was significantly weaker than the Cy3 signal emitted by Cy3 tagged *Bacteria* specific probes. Possible reasons are insufficient binding of the *Firmicutes* specific probe to complementary sequences or a not optimized protocol for hybridization of this specific probe. Also interference with the *Bacteria* specific probe applied in a second hybridization step might lead to the observed problems. In control experiments where roots were observed directly after dipping them into the bacterial suspension, the density of bacteria detectable on the roots was similar to the observed density in plant-competence experiments (bio-priming of tomato seeds and root-dipping). These results suggest problems in staining the bacterial cells by FISH. A crucial step in the hybridization protocol is the lysis of bacterial cell walls, enabling the probes to bind their target sequences. A thick cell wall and naturally also spore formation could negatively affect the efficiency of this lysis step. Another problem that might be considered is the extensive washing of samples during the FISH protocol. The numerous washing steps could lead to removal of not extremely tightly attached bacterial cells colonizing the root surfaces. When it comes to visualizing bacterial cells in biofilm structures by FISH a restricting factor might be the extracellular matrix, consisting of polysaccharides and proteins, surrounding the cells. Biofilm

components are specific for every bacterial strain und could prevent the FISH probes from reaching their targets in the inner of the cells.

In its natural environment the soil, *B. amyloliquefaciens* B47 is also exposed to a lot of abiotic stresses like heat, cold, osmotic and oxidative stresses. The bacterium is armed to resist those stresses by several detoxifying enzymes including catalases, peroxidases, osmoprotective enzymes, cold and heat shock proteins. To enable adaption to changing environmental conditions and assure persistence in the competitive soil habitat the acquisition of new genes is an essential trait for *B. amyloliquefaciens* B47. Several gene clusters for competence genes were identified. Therefore the *Bacillus* strain is most likely able to dynamically adjust its genomic repertoire to successfully survive diverse environmental conditions and adapt to different biotic and abiotic factors.

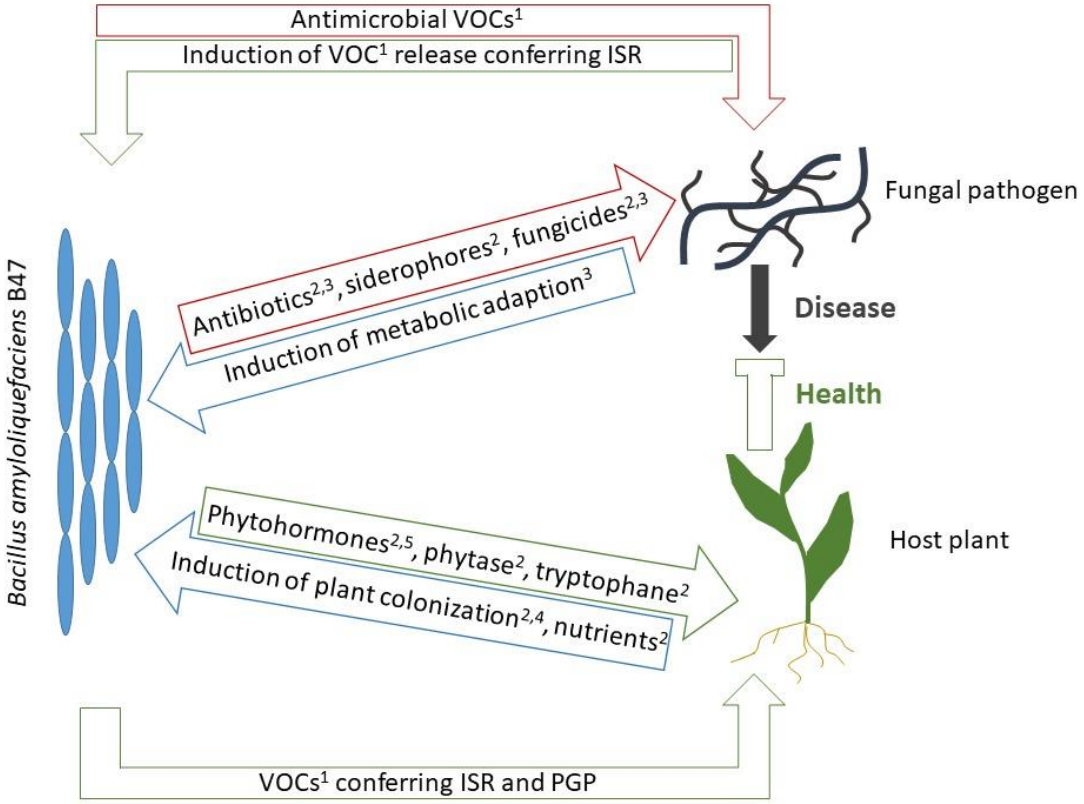

Figure 20 Interactions revealed in the present study between the biocontrol strain, the host plant and phytopathogenic fungi. [1] Identified by SPME headspace GC-MS measurements of microbial volatiles. [2] Based on genomic analysis of *B. amyloliquefaciens* B47. [3] Revealed in metabolome studies by HPLC-MS. [4] Visualized by FISH-CLSM. [5] production shown by physiological assays

Conclusion

This study revealed that the investigated bacterial strain *B. amyloliquefaciens* B47 is indeed a very promising candidate for BCA product development. Its outstandingly high antagonistic activity against a broad spectrum of devastating plant pathogens makes it a powerful agent in biocontrol of various diseases. Furthermore it has a great ability to promote plant growth and also strengthens the plant by induction of self-defence mechanisms. The mechanisms of action include the release of various soluble metabolites as well as a broad spectrum of volatile organic compounds interfering with both, the plant as well as other microbial strains.

Throughout the study it was shown that the biocontrol strain is strongly influenced by biotic and abiotic factors and is constantly interacting and responding to its environment. Especially the communication with microbial pathogens seems to be very complex and results were obtained suggesting the ability to not only sense microbial pathogens through the air interface but also to discriminate between different pathogenic strains and adapt its reaction depending on the present pathogen.

The ability to form endospores is a very desirable trait for product development as it improves stability of the product during production, storage and in the environment.

Further studies on this project could include improvement of visualization of plant colonization. Also the ability of colonizing not only tomato but also other host plants of importance in crop production could be studied. Concerning inter-microbial communication much remains to be investigated. A lot can be learned from better understanding the role of exchange of volatile organic compounds between microbial strains. To get a deeper insight into the reaction of *B. amyloliquefaciens* B47 to the VOCs of fungal pathogens on molecular level, a transcriptomics approach was conducted towards the end of this thesis. Unfortunately due to time constraints results cannot be shown here but will lead to a more comprehensive understanding of VOC mediated communication.

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