Dzenana Sarajlic, BSc

Interactions between beneficial microorganisms and soilborne plant pathogens

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Univ.-Prof. Dipl.-Biol. Dr.rer.nat. Gabriele Berg

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Abstract

Soil-borne fungal pathogens are responsible for major food losses and negatively affect plant health as well as human nutrition worldwide. Biological control agents that counteract these persistent fungi provide sustainable strategies to solve these increasing problems. In order to develop successful biocontrol strategies, it is important to understand the interactions between plant rhizosphere-associated bacteria and phytopathogenic fungi. Thereby, the focus of this study was to analyse the influence of microbial volatile organic compounds (mVOCs) on the inhibition of mycelial growth of fungi which are currently understudied. Different strains of the Gram-positive bacteria, *Bacillus amlyoliquefaciens* and *Bacillus cereus* as well as of the Gram-negative *Pseudomonas helmanticensis,* were tested in antagonistic volatile assays against the fungal pathogens *Rhizoctonia solani* and *Sclerotinia sclerotiorum.* Furthermore, various bacterial isolates and *Fusarium oxysporum* were subjected to a dual-culture assay in order to assess mVOCs-driven antagonism. BOX-PCR was used to select for unique isolates within the same species for more efficient construction of bacterial consortia. In a complementary approach, headspace solid phase micro extraction (HS SPME) GC/MS analysis of volatiles produced from bacteria grown on different growth media was performed to investigate the effect of growth media on the bacterial volatilome. In addition, this analytical method was also applied to deepening insights into fungal-bacterial interaction and mVOCs-based communication. Survival structures (sclerotia) from *S. sclerotiorum* were exposed to antagonistic bacteria and the liquid produced as a response was analysed using UHPLC-MS. The results indicated that specific strains of *B. amlyoliquefaciens*, *B. cereus* and *P. helmanticensis* were strong inhibitors of mycelial growth of *R. solani* and *S. sclerotiorum*. This effect was enhanced in a concurrent application of *P. helmanticensis* and *B. amlyoliquefaciens*, where the growth of *R. solani* was inhibited completely. *F. oxysporum* growth was most efficiently inhibited by strains belonging to *B. amyloliquefaciens* and *B. aerius*. Utilization of BOX-PCR as a genotype fingerprinting method had shown high similarity among the highly antagonistic *B. amyloliquefaciens* strains. HS-SPME GC-MS analysis revealed that different growth media have a substantial effect on the volatilome of bacterial isolates. Moreover, it was found that fungal volatiles are important communication molecules in fungal-bacterial interaction. They were shown to modify the bacterial volatilome and induce the production of novel compounds in the exposed bacterial

strains. LC-MS analysis of the liquid produced by sclerotia of *S. sclerotiorum* displayed a wide variety of protective compounds and additional compounds that are of importance for fungal metabolism. The findings of this study highlight that mVOCs are promising tools for the treatment of persistent crop pathogens and will likely be included in future agricultural applications.

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1 Introduction

1.1 Fungal diseases of plants

Fungi are a highly diverse group of eukaryotic, carbon heterotrophic organisms that occupy most natural habitats (Knogge, 1996). The vast majority of them are strict saprophytes (Schetfer, 1991). The significance of fungi as decomposers, plant disease factors and producers of pharmacological and industrial products has encouraged scientists worldwide to research their biology. The influence that fungi have on the health of the plant, food losses and human nutrition is remarkable. Some of the greatest world food shortages and human suffering can be assigned to the plant pathogenic fungi and fungi-like organisms (Knogge, 1996).

In order to colonize plants, fungi have developed strategies to occupy plant tissue, to optimize plant growth and to spread. Most of them, as well as bacteria and viruses, often depend on wounds or natural openings for invasion. Others can actively pass over the plant's outer structural barriers and the epidermal cell wall (Knogge, 1996). To enter the plant, fungi secrete a cocktail of hydrolytic enzymes like proteases, pectinases and cellulases (Kolattukudy, 1985).

After the fungi enter the plant, they colonize plants with the secretion of toxins or hormone-like compounds to manipulate plant's physiology to the benefit of the pathogen. This interference can simply kill plant cells or more subtly, redirect the cellular machinery (Keen, 1986). This is often achieved by production of phytotoxins with various specificity degrees toward various plants (Walton, 1996).

Phytopathogenic fungi, as causal agents of infectious diseases of crop plants, provoke epidemics and are the significant cause of crop yield losses that made fungi be the main economic factor. Approximately 300,000 species of flowering plants are attacked by pathogenic fungi. Single plants can be host to only a few fungal species, resulting in a limited host range. They appear in several levels of specialization inside a plant (Schetfer, 1991).

Opportunistic parasites which enter plants through wounds and cause mild disease symptoms are regarded as first level. The next level of fungi includes true pathogens that are

highly virulent on only few number of host species. Finally, the highest level of complexity is achieved by obligate pathogens for which the main condition of their complete life cycle is to have a living host plant (Keen, 1986).

Some fungi cause foliar diseases. Downy mildews, powdery mildews and white blister are some of the highly prevalent foliar diseases. Other fungi like *Plasmodiophora brassicae*, *Pythium spp., Fusarium spp., Rhizoctonia spp., Sclerotinia and Sclerotium spp.* – are soilborne pathogens (Abawi *et al*., 2000).

1.2 Soilborne pathogens

Soilborne pathogens reduce yield and crop quality. They are a big challenge because they can survive in soil for many years and each crop is susceptible to a wide range of species (Koike *et al*., 2003). As an example, lettuce anthracnose (*Microdochium panattonianum*) pathogen survives in the soil because of small resting structures (microsclerotia).

Important soilborne pathogens include fungi, fungi-like organisms, bacteria as well as viruses and plant parasitic nematodes (Baysal-Gurel F. *et al*., 2012). Simultaneous infections from few soilborne pathogens can result in a complex disease that can lead to a destruction of the crop. Many diseases are hard to predict, detect and diagnose. A highly complex environment makes it challenging to understand all aspects of diseases caused by soilborne pathogens (Koike *et al*., 2003).

Fusarium solani and *Rhizoctonia solani* (teleomorph *Thanatephorus cucumeris*) first described by Kühn J.G in 1858, are the most important soilborne fungal pathogens, which develop in both cultured and non-cultured soils. They cause damping-off and root rot diseases in a wide range of vegetable and crop plants including tomato (Nutter *et al*., 1993). The frequency of damping-off was increased from 19 to 90% with increasing *R. solani* inoculum levels, while the occurrence of root rots caused 10 to 80% vegetable losses (Patel *et al*., 2001). Host plants of *R. solani* include alfalfa, peanut, soybean, lima bean, cucumber, papaya, eggplant, corn and many more. (Patel *et al.*, 2001).

Some soilborne pathogens can cause foliar diseases with symptoms and damage appearing

on the phyllosphere. When raindrops splash pathogen-laden soil particles on the leaves of lettuce, fungi move to the plant and induce a leaf spot disease. *Sclerotinia sclerotiorum* (Lib.) de Bary (1884), which survives in soil as sclerotia, has a similar effect. Under certain conditions, the sclerotia produce small mushroom-like structure (apothecia) that release spores. The ability of soilborne pathogens in soil depends on the biological group to which it belongs. Few of them are long-term soil inhabitants, some survive for limited period on roots or plant debris or direct in the soil and some of them survive by secreting slimy material which makes protective layers around cells and allow them to survive unfavourable conditions (Koike *et al*., 2003).

There are many ways to control diseases caused by soilborne pathogens. The control must be economical; the value of the crop saved must exceed the cost of the control. Essential steps would be regular monitoring of fields and deep examination of symptomatic plants. Controls must be safe, simple and inexpensive to apply. Options for disease control would be to check on host resistance, cultural controls as well as to apply chemicals. For example, Pre-plant fumigants are highly successful in reducing soilborne inoculum. Fungicides are also used and applied to soil, plants or seed. Classical biological controls are about to become a prospering business. Biological control is a product which contains viable antagonistic organisms and is applied to work against target pathogens (Koike *et al*., 2003).

1.3 Target pathogens in this study

1.3.1 *Rhizoctonia solani*

Rhizoctonia solani (teleomorph *Thanatephorus cucumeris*) (Kühn, 1858) is one of the most recognized species of *Rhizoctonia*. It is a basidiomycete fungus which does not produce any asexual spores (conidia) and produces sexual spores (basidiospores) under certain conditions. Naturally, *R. solani* exists as vegetative mycelium and/or sclerotia. *R. solani* appears in a wide range of host plants and it is a ubiquitous soilborne pathogen. *R. solani* attacks primarily the parts of plants below ground like seeds, roots or hypocotyls, but it is also able to attack fruits, stems, leaves or pods. “Damping-off” is the most common

Rhizoctonia disease symptom which is characterized by non-germination of highly contaminated seeds after which infected seedlings can be destroyed before or after they emerge from the soil. *R. solani* attacking shoots often occur because the mycelium and/or sclerotia of the fungus are close to or splashed on the tissue of the plant. *Rhizoctonia* diseases can also be initiated by basidiosporal infection in beans, sugar beet and tobacco. Basidiospores germinate to produce hyphae which propagate into leaves during high humidity periods or extended wet weather (Ceresini *et al*., 2011).

1.3.2 *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is a plant pathogen infecting over 400 species of plants worldwide including important crops and numerous weeds (Bolton *et al*., 2006). It is a threat to dicotyledonous crops like soybean, chickpea, sunflower, peanut, oilseed rape and various vegetables as well as monocotyledonous species like onion and tulip (Boland *et al*., 1994). It is a member of the family *Sclerotiniaceae* which have gone through a lot of modification and redistribution of genera (Boland *et al*., 1994). This family includes species producing inoperculate asci from brownish apothecia which appear from sclerotial stroma within or associated with a host plant (Holst-Jensen *et al*., 1997). The development of sclerotial stroma, a melanized hyphal aggregate, is a common character of all members of the *Sclerotiniaceae* family. Symptoms of *S. sclerotiorum* vary among its wide variety of hosts. Leaves of plants usually have water-soaked lesions that expand fast. Those lesions develop into necrotic tissues that develop fluffy white mycelium which is the most evident sign of a *S. sclerotiorum* infected plant. Sclerotia appear inside but also may form on the surface of infected plant during high humidity conditions (Boland *et al*., 1994). A sclerotium is an aggregate of hyphae with outer black cells containing melanin, a compound that is believed to have important role in protection from microbial degradation in fungi as well as from adverse conditions (Bell, 1986). Sclerotia can produce inoculum and are able to survive up to eight years in soil (Adams *et al*., 1979). *S. sclerotiorum* sclerotia vary in size depending on host. They may be 1 cm thick and exceed 35 cm in diameter while some may be 2-10 mm in diameter (Latha et.al., 2009).

1.3.3 *Fusarium oxysporum*

Fusarium oxysporum is an asexual fungus which can produce three types of spores: micro-, macroconidia and chlamydospores. Microconidia are one or two celled and produced by *Fusarium* mostly within the infected plants. Macroconidia are three to five celled and commonly found on the plants surfaces which were killed by *Fusarium* wilt. The third type of the spores is chlamydospores which are one or two celled. They can remain latent in soil and infect other hosts for the next 30 years. These spores can spread through machinery, farm implements or running water (Agrios *et al*., 1998).

Fusarium oxysporum affects hundreds of crops and appears worldwide. The ability of *F. oxysporum* to induce vascular wilt on a certain host is the most researched characteristic of this pathogen. However, a lot of environmental studies of broad habitats often find *F. oxysporum* in the absence of plant disease (Stoner *et al*., 1981). Populations like these, mostly called "non-pathogenic", even if pathogenicity lack is not usually confirmed, are experienced component of soil communities. *F. oxysporum* can also invade plants without symptoms, like an endophyte (Kuldau *et al*., 2000).

The interaction of plants and *F. oxysporum,* which are endophytic, can give us information about the functioning between pathogen recognition and plant resistance. In vulnerable plants, pathogenic *F. oxysporum* invades the root, colonizes the cortex of the root and then disseminates through the xylem to the other parts of the plant. In this way, it causes the xylem occlusion and eventually wilting and death (Olivain *et al*., 1999, 2003). The spread of the fungus in host cultivars that are resistant to this pathogen is blocked and only limited colonization of the host tissue can occur (Baayen, 1988; Beckman *et al*., 1982; Harling *et al* ., 1985).

F. oxysporum strains that are non-pathogenic and inoculated onto plants can colonize the cortex of the root like the pathogens. Nevertheless, the response of host such as cell walls thickening and invasion into intercellular spaces can limit the endophytic growth, similarly to defences which limit the growth of pathogens in resistant plants (Olivain *et al.*, (1999); Olivain *et al*; 2003; Benhamou *et al* ., 2001). Most of the population genetics studies have focused on non-pathogenic *F.oxysporum* isolated from roots (Demers *et al* ., 2014).

Despite the ecological diversity of *F. oxysporum*, the diversity and the structure of the soil

and endophytic *F. oxysporum* are not understood sufficiently. O' Donnel *et al*., (2009) demonstrated that 256 sequence types out of 850 isolates of *F. oxysporum* are mainly plant pathogens (O' Donnell *et al*., 2009). Subsequent studies using the same typing scheme found 26 additional sequence types in isolates from Sardinia soil and 46 new ones from soil across Australia (Balmas *et al*., 2010; Laurence *et al*., 2012). These newly revealed sequence types suggest that focusing only on plant-pathogenic *F. oxysporum* species may underestimate the diversity of total species. Studies which compared non-pathogenic with pathogenic *F. oxysporum* populations in connection with the same host have found much higher diversity levels in the non-pathogenic populations than in the pathogenic ones (Demers *et al*., 2014).

Besides being very active as a plant pathogen, *F. oxysporum* is also known to be a serious, opportunistic human pathogen due to the high number of severe reported cases and to its broad resistance to available antifungal drugs (Boutati *et al*., 1997; Odds *et al*., 1998). *Fusarium* currently represents the second most frequent mould which causes fungal infections in immunocompromised patients, very often with lethal outcomes (Ortoneda *et al*., 2004). All fusariosis in humans are practically caused by *F. oxysporum* together with *F. solani* and *F. verticillioides* (Guarro *et al*., 1995).

1.4 Negative implifications of chemical pesticides in agriculture

Since the start of the development of agriculture about 10 000 years ago, farmers had to compete with harmful organisms like animal pests, plant pathogens and weeds; collectively referred to as pests. Biotic stressors, together with a lack of excess of water in the growth season and extreme temperatures, can reduce crop production in high levels. Organisms like these can be controlled by applying biological, physical and chemical measures (pesticides) (Russell, 2005).

Soil application of fungicides is expensive and deleterious to non-target microflora. Since plant pathogens are the main factor of crop reduction, the main goal is to protect crops from them. In the beginning, diseases caused by microscopic organisms were hardly perceived as pest-related and options for control were limited to the use of land races adapted to growth conditions (Russell, 2005).

The first usage of pesticides/fungicides started more than a century ago using copper, sulphur and organic mercury. The second generation of fungicides included organic chemicals which acted as surface protectants. Third generation fungicides are systemic in plants, they penetrate the tissue and are able to control infections. Pesticides are considered as quick and easy solution for controlling weeds and insect pests. However, the use of pesticides is also contaminating almost every part of our environment. Residues of pesticides are found in air, soil and water. The best way to reduce pesticide contamination is to use safer, non-chemical pest control methods (Russell, 2005).

Heavy treatment of soil with pesticides has deleterious effect on beneficial soil bacteria as well. Overuse of pesticides has the same effect as human overuse of antibiotics. Use of chemicals may work for the few years but after some time there are no more beneficial microorganisms able to hold onto the nutrients (Savonen, 1997). Resistance development to pesticides is generally considered to be one of the most serious barriers to effective control of the pest nowadays. Resistance is the development of an ability in a pest population to tolerate toxicant doses that would show to be lethal for the most of the individuals within one species (Stenersen, 2004). Plant pathogens can be protected from the chemicals by developing their physiological changes. One way of protection is the increase in the number of gene copies which allows the organism to produce more protective enzyme which will make pesticide less toxic. Another protection mechanism is to reduce the number of biochemical receptors that bind pesticides (Daly *et al.,* 1998).

There is evidence that pesticides pose a potential risk to humans and other forms of life (Forget, 1993; Igbedioh, 1991; Jeyaratnam, 1981). Certain pesticides elicit their effects by imitating natural hormones in the body. Their long-term exposure is reflected in the effects of human health such as cancer, immune suppression, reproductive abnormalities, etc (Brouwer *et al*., 1999; Crisp *et al* ., 1998; Hurley *et al* ., 1998)

1.5 Sustainable plant protection and growth promotion

In order to provide sufficient food for human population and to have renewable energy in higher amounts, enhanced production of crops is necessary. Current production methods in agriculture with usage of chemical pesticides and fertilizers cause a number of environmental and health problems (Russell, 2005).

Therefore, there is a demand to use ecological strategies in agriculture. New plant biotechnology approaches produce crop varieties with greater resistance and better nutritional value but unfortunately, plant-microbe interaction is usually ignored in those breeding strategies (Berg, 2009). Plant-associated microbes enhance stress tolerance, provide disease resistance and promote biodiversity (Berg, 2009). It is demonstrated that fungi and bacteria interact with their host plants and are able to promote plant growth as well as to act against plant pathogens (Berg *et al*., 2005).

The rhizosphere is the hot-spot of microbial colonization of plants. The possibility to affect antagonistic/plant growth-promoting potential is to apply microorganisms as biocontrol agents (Berg, 2009). Plant growth promotion can be successfully achieved by interaction between microbes and their host plants as well as indirectly due to their antagonistic activity against plant pathogens. In comparison with chemical pesticides, microbial inoculants are very safe, effective in small quantities, decompose quickly and have reduced risk on environment and human health (Berg, 2009).

In most of the research to date, biocontrol agents are applied alone to compete against the growth of pathogens. Although the potential benefits of a single biocontrol agent application are proved in many studies, in many cases they showed inconsistent performance because a single biocontrol agent is not likely to be active in all kinds of soil environments and all agricultural ecosystems (Raupach *et al*., 1998). These have resulted in inadequate colonization, limited tolerance to changes in environmental conditions and fluctuations in the production of antifungal metabolites (Weller *et al*., 2002). Combinations of biocontrol agents also have the advantage of utilizing a broad range of activities, enhancing the effectiveness and reliability of biological control and ensuring increased initiation of defence enzymes along individual strains (Latha et.al., 2009). A highly effective biocontrol strain

should be able both to compete and persist in the environment and to colonize and proliferate on plant parts. It should not be expensive and should maintain good viability without a specialized storage system (Latha et.al., 2009).

1.6 Specific modes of action of antagonistic bacteria

There are a lot of reports on the production of metabolites against fungi by bacteria *in vitro* that can also be active *in vivo.* Some of them are: ammonia, hydrogen cyanide (HCN), kanosamine, butyrolactones, xanthobaccin, zwittermycin A, 2,4-diacetylphloroglucinol (Ph1), phenazine-1-carboxylic acid (PCA) (Whipps, 2001). In order to show the role of antibiotics in biocontrol, mutants which lack antibiotics production or mutants which over-produce them have been used. Both PCA and Ph1 were isolated from the rhizosphere of wheat following biocontrol strains of *Pseudomonas* introduction, which confirmed that such antibiotics are produced in vivo. Furthermore, production of Ph1 in the wheat rhizosphere proved the existence of high density of bacterial population and their ability of root colonization (Raaijmakers *et al*., 1999). The production of antibiotics by bacteria, especially pseudomonads, seems to be highly regulated by a two-component system including an environmental sensor (membrane protein) and a cytoplasmic response factor. (Keel and Defago, 1997). Mutation in each gene leads to multiple effects on the production of antibiotics (Whipps, 2001).

Bacteria, especially actinomycetes, are able to parasitize and degrade fungal plant pathogens spores. This ability is well established (El-Tarabily *et al*., 1997). Nutrients pass from the plant pathogen to bacterium and fungal growth is inhibited. Assuming this, the parasitism spectrum can range from simple attachment of cells to hyphae to complete lysis and hyphae degradation (Whipps, 2001). It is generally thought that if fungal cells are lysed and cell walls degraded, enzymes produced by bacteria are responsible, even if antibiotics can be produced in parallel. Even though there is little evidence obtained for the presence of cell wall-degrading enzymes in bacteria and their activity in the rhizosphere, a lot of effort has gone into their identification (Whipps, 2001). Important enzymes in biocontrol are chitinolytic enzymes, cellulases and ß-glucanases (Anand S. *et al*., 2009). Chitinases and glucanases as antifungal proteins are of important biotechnological interest because they

can be used as food and seed preservative agents as well as for plant engineering for the purpose of resistance to phytopathogenic fungi (Dempsey *et al*., 1998). These enzymes produced by *Bacillus cereus* and *Pantoea agglomerans* also seem to be included in the biocontrol of *R. solani* Kühn (Chernin *et al*., 1995; Pleban *et al* ., 1997).

The greatest growth area in biocontrol research has been concerned with induced resistance defined as "the active resistance process dependent on the host plant's chemical and physical barriers induced by biotic or abiotic agents (inducing agents)" (Kloepper *et al*., 1992). Most of the work was focused on the systemic resistance caused by non-pathogenic *Bacillus* and *Pseudomonas* species which colonize the rhizosphere. This resistance occurs in systems where the inducing bacteria and the specific pathogen remain separated spatially during the experiment and there was no interaction between pathogen and bacteria (Whipps, 2001). Bacteria induce resistance differently with some being active on specific plant species and not on the others. All inducing compounds from bacteria are probably not discovered yet but siderophores and lipopolysaccharides are known and already researched (Métraux *et al*., 1990; Leeman *et al*., 1996b). The earlier mentioned definition of induced resistance by Kloepper *et al*. (1992) explained both biotic and abiotic inducers. Even if the phenotypic effects of root inoculation with bacteria can be similar to treatment with microbes or agents that affect damage, the changes like biochemical and mechanical can be slightly different. This led to the introduction of the term "induced systemic resistance (ISR)" for resistance induced by bacteria (Pieterse *et al*., 1996). Some of the changes observed in roots of the plants which exhibit ISR are: epidermal and cortical cell walls strengthening, increased enzyme levels, higher production of phytoalexin and enhanced stress-related genes expression (Whipps, 2001).

1.7 Volatile organic compounds as interaction molecules between microbes and fungi

Secreted metabolites and proteins play different and important roles in organism-environment interactions. Volatile organic compounds (VOC) can travel long distances from the production point through soil, atmosphere and liquid. This makes them ideal information chemicals for mediating short as well as long both intercellular and organismal interactions (Bitas *et al*., 2013). Biogenic VOCs have certain physical and chemical properties in common: they belong to chemical classes like alcohols, aldehydes, thiols, terpenoids, esters and fatty

acid derivatives, they are usually lipophilic, they have high vapour pressure and low molecular weight (Schulz and Dickschat, 2007).

Plant-associated microbes play important roles in plant ecology at individual and community level. Besides some microbes like nitrogen-fixing bacteria, mycorrhizal fungi and major pathogens, the question of influence of other microbial communities on plants remains to be clarified. One of the mechanism by which microbes influence the growth and fitness of plants is the release of VOCs (Bitas *et al*., 2013). Bacteria produce a broad spectrum of bioactive VOCs which have multiple functions and are not restricted to one species. It has also been shown that bacterial volatiles are able to supress growth of soil-borne pathogenic fungi (Cernava *et al*., 2015). By producing VOCs, bacteria inhibit spore germination and mycelial growth or they cause abnormal changes in the morphology of fungi (Effmert *et al*., 2012). From 197 bacterial strains which were cultured from soybean and canola plants, 14 of them belonging to four *Pseudomonas spp.,* produced VOCs that caused inhibition of sclerotia germination and mycelial growth of *S. sclerotiorum* both in vitro and in soil tests (Fernando *et al*., 2005). Current researches about volatile-mediated antagonism are mostly dealing with cultivable fungi. Among the fungi that are responsible for many diseases in crops, those that were tested against bacterial volatiles include species from the genera *Alternaria*, *Botrytis, Fusarium*, *Rhizoctonia*, *Sclerotinia* and Vertilicium. So far, mostly *Bacillus, Streptomyces* and *Pseudomonas* have been reported to inhibit growth of phytopathogenic fungi while others are still in investigation (Kai *et al*., 2009; Moore-Landecker *et al*., 1972). Not all bacterial volatiles have a positive influence on plants. Members of some bacterial genera, including *Chromobacterium, Serratia, Pseudomonas, Burkholderia and Stenotrophomonas*, produce a number of volatiles that inhibit plant growth or induce phytotoxicity (Bailly and Weisskopf, 2012). Compounds mediating negative effects on plants include: ammonia, hydrogen cyanide, dimethyl sulfide and 3-phenylpropionic acid (Blom *et al*., 2011b). The search for the active compounds that are responsible for the VOCs-mediated antifungal activity of bacterial strains is rather difficult and challenging. Besides difficult adjustment of the concentration required for the testing, other challenges also appear. One of them is a relatively high amount of unresolved peaks in GC-MS profiles; another one could be found in application technology, where volatiles are applied just once on the target while emission of the bacteria results in slowly release, as the fungus is growing. The last challenge would be that

pure compounds are rarely bioactive enough, while a mixture of different compounds can increase the activity (Fierer, 2006).

2 Materials and Methods

2.1 Materials

2.1.1 Growth media

- 10% TSA (Tryptic Soy Agar)
- **Waksman agar** – prepared according to **„**Handbook of microbiological media“ (Atlas, 2004).

Typtone Peptone	4 g
Glucose	8 g
Yeast extract	2,4 g
NaCl	4 g
Agar-Agar	16 g
Distilled water	800 g

- **Kings'B medium (modified)**

Protease peptone	12,8 g
Glucose	6,4 g
K2HPO4	0,96 g
Gylcerine	9,6 g
MgSO4 X 7H20	0,96 g
Agar-Agar	12 g
Distilled water	800 g

- prepared according to: „Kings medium B Base“- HiMedia Labs.
- **DNA extraction buffer**

  200 mM Tris/HCl: → 20 ml of 1 M stock solution

  250 mM NaCl: → 25 ml of 1 M stock solution

  0.5 % SDS → 5 ml of a 20% stock solution

  → fill up to 100 ml with distilled water and autoclave

- **3 M Sodium acetate**

  20 ml: 4.92 g Sodium-acetate

15.5 ml distilled water

4.5 ml 1N HCl

The pH was adjusted to 5.2.

- **Artificial root exudate medium (ARE)** was prepared according to Schulz-Bohm *et al*., (2015). The medium was filter sterilized and stored at -20°C.

Instant media like NA and PDA were prepared according to the instructions of the manufacturer (Carl Roth, Germany).

Chemicals, growth media and hardware used were obtained from following companies: Carl Roth (Karlsruhe, Germany), Sifin Diagnostics (Berlin, Germany), Merck KGaA (Darmstadt, Germany), Sarstedt (Nümbrecht, Germany), Chromtech (Idstain, Germany).

If not specified, all used solutions and growth media were autoclaved at 121°C for 15 min to ensure sterility.

2.1.2 Fungal strains

One of the plant pathogens utilized in experiments, *Sclerotinia sclerotiorum* Goa11, was obtained from an infected pumpkin field in 2011 by Eveline Adam (Institute of Environmental Biotechnology, TU Graz) (Figure 1).

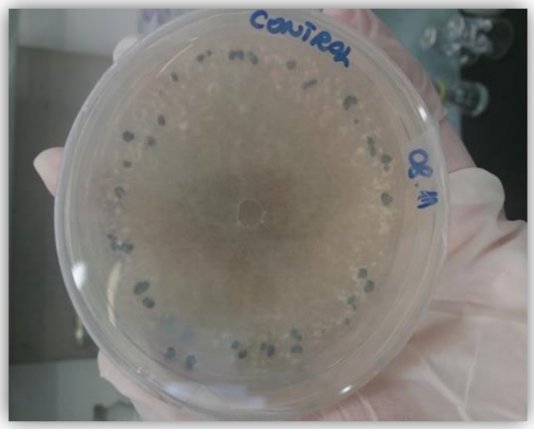


Figure 1. Mycelial growth and sclerotia buildings at the outer range of *Sclerotinia sclerotiorum* Goa 11.

Fungal dried sclerotia were stored for the period of 4.5 years at room temperature and afterwards recultivated on PDA plates. This pathogen strain is available in the collection of strains of the Institute of Environmental Biotechnology (Graz University of Technology).

Another plant pathogen, *Rhizoctonia solani* AG-8, which infected a potato field in Sanitz (Germany) was provided by Kristin Dietel (ABiTEP, Germany) (Wagner P., 2017) (Figure 2).

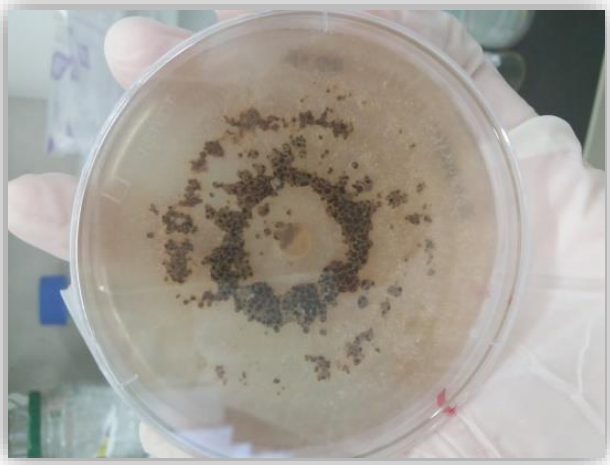


Figure 2. Mycelial growth and brown sclerotia of *Rhizoctonia solani* AG-8.

Fusarium oxysporum is an additional plant pathogen used in experiments. It infected tomato plants and was provided by Manuela Rändler (TU Graz) (Figure 3).

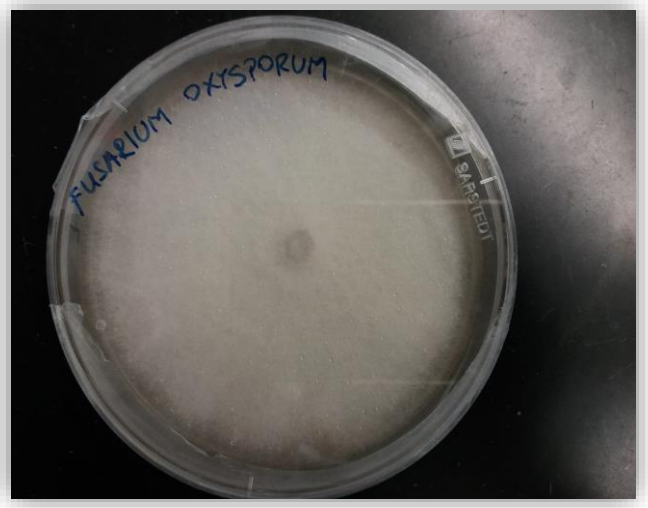


Figure 3. Fluffy white mycelial growth of phytopathogenic fungi *Fusarium oxysporum* on PDA plate.

2.1.3 Bacterial strains

Antagonistic bacterial strains used in this study can be found in the strain collection of the Institute of Environmental Biotechnology (Graz University of Technology) (Table 1) (Wagner, 2017).

Table 1. Antagonistic bacterial strains used in assays. Isolates were identified by 16S RNA Sequencing and BLAST.

Isolates from Potato Tubers infected with *Rhizoctonia solani*	
Rs-MS14	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Rs-MS28	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Rs-MS53	*Bacillus sp.* (potential hit: *Bacillus cereus*)
Rs-MS87	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Rs-mS115	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Rs-Ts222	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Rs-Ts269	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Rs-Ts276	*Bacillus sp.* (potential hit: *Bacillus aerius*)
Rs-So360	*Bacillus sp.* (potential hit: *Bacillus aerius*)
Rs-So365	*Bacillus sp.* (potential hit: *Bacillus aerius*)
Rs-So386	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Isolates from Sclerotia of *Sclerotinia sclerotiorum*	
Sc-S1	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-S7	*Bacillus sp.* (*Bacillus subtilis* group)
Sc-S8	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-S11	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-S14	*Bacillus sp.* (potential hit: *Bacillus cereus*)
Sc-K19	*Buttiauxella noackiae*
Sc-K20	*Enterobacter aerogenes*
Sc-K29	*Bacillus sp.* (potential hit: *Bacillus aerius*)
Sc-K36	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-K38	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-K55	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-T59	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-T63	*Bacillus sp.* (potential hit: *Bacillus cereus*)
Sc-T64	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-T67	*Bacillus sp.* (potential hit: *Bacillus cereus*)
Sc-T71	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-T74	*Buttiauxella warmboldiae*
Sc-B83	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-B84	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-B89	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)

Sc-B94	*Pseudomonas sp. (*potential hit*: Pseudomonas helmanticensis)*
Sc-B98	*Pseudomonas sp. (*potential hit: *Pseudomonas helmanticensis)*
Sc-B99	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-T107C	*Enterobacter soli*
Sc-S112C	*Bacillus sp.* (potential hit: *Bacillus aerius*)
Sc-S114C	*Bacillus sp.* (*Bacillus subtilis* group)
Sc-K121C	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-B137C	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-B142C	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-K143	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-K146	*Bacillus sp.* (potential hit: *Bacillus aerius*)
Sc-K155C	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)
Sc-K159	*Bacillus sp.* (potential hit: *Bacillus aerius*)
Sc-K161	*Bacillus sp.* (potential hit: *Bacillus amyloliquefaciens*)

The other group of bacterial strains were obtained from Manuela Rändler (TU Graz) (Table 2). They were isolated from tomato (*Solanum lycopersicum* L.). Tomato plants were subjected to ionic stress which was generated by a higher value of EC (electrical conductivity) = 5 of the Hoagland solution and osmotic stress which was induced by lowering the amount of irrigation (40% of the water holding capacity).

Table 2. The second group of bacterial isolates used in experiments. C=control, CF=pathogen control for *Fusarium oxysporum*, CV=pathogen control for *Verticillium dahliae*, I=ionic stress, O=osmotic stress.

C-B4	*Microbacterium flavescens*
CV(CF)-C7	*Microbacterium flavescens*
I-B5	*Microbacterium maritypicum*
IOF-G4	*Microbacterium flavescens*
IF-C6	*Sporosarcina koreensis*
IO-C6B	*Bacillus aryabhattai*
OV-C9	*Stenotrophomonas maltophilia*
C-E1	*Ralstonia pickettii*
CF-B11	*Ralstonia insidiosa*
IO-A9	*Agrobacterium tumefaciens*
C-E6	*Agrobacterium tumefaciens*

2.2 Methods

2.2.1 Characterization of antagonistic bacteria

2.2.1.1 Characterization of isolates using BOX-PCR

Before executing BOX-PCR to characterize 47 antagonistic bacterial strains, DNA extraction of bacterial isolates was performed. A small portion of bacteria was placed into 2 ml tube with glass beads and 450 µl of DNA extraction buffer was added. The solution was ribolyzed for 15 seconds and 225 µl of Na-acetate was added to the tube. Tubes were stored at-20°C for 1 hour and afterwards centrifuged for 10 minutes at 13000 rpm (4°C). The supernatant was transferred to a new tube and after the addition of phenol/chloroform, the centrifugation was repeated. The upper phase of the tube content was moved to a new tube and an equal amount of isopropanol was added. The process of incubation at room temperature lasted for 1 hour. The isopropanol was decanted and equal amounts of 70% ethanol were added onto the pellet. Following the last cycle of centrifugation, the ethanol was decanted, pellet was dried and resuspension was done in 50 µl of nuclease-free water. After the completion of the protocol, the extracted DNA material could be used to perform BOX-PCR.

Each BOX-PCR tube contained 25 µl of reaction mixture which consisted of: template, nuclease-free water, Primer Box A1 and Taq 840 Polymerase. Antagonistic bacterial strains were used as templates for this purpose. Table 3 represents the BOX-PCR reaction mixture and amounts of ingredients used.

Table 3. BOX-PCR reaction mixture and ingredient volumes.

Reagent	Volume (µl)
Template	1,6
Nuclease-free water	15,9
Primer Box A1 (10 mM)	2,5
Taq 840 Polymerase	5
Reaction volume	25

The temperature program for the BOX-PCR reaction mixture can be found in Table 4. In order to determine the correct fragment length of the BOX-PCR samples, an electrophoresis gel was performed. The gel consisted of 1.5% of agarose and 0.5x TBE buffer. GeneRuler^{TM} 1 kb DNA Ladder was used for approximate quantification of DNA in samples.

Table 4. BOX-PCR amplification program. n = number of cycles

Steps	Temperature	Time
1. Initial DNA denaturation	95 °C	5 min
2. DNA denaturation	95 °C	1 min
3. Primer annealing	53 °C	1 min
4. Elongation	65 °C	8 min
5. Final extension	65 °C	16 min
6. Rest	15 °C	Endless

n=35

2.2.2 Screening of the antagonistic activity via microbial VOCs towards *Sclerotinia sclerotiorum* and *Rhizoctonia solani*

This screening assay was performed in order to determine the effect of another media (KING'S B) on the antagonistic effect via VOCs of the isolated bacterial strains. Two plant pathogens were used for testing, *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, separately. The bacterial strains used in this experiment were: *Pseudomonas helmanticensis* Sc-B94, *Bacillus cereus* MS53, *Bacillus amyloliquefaciens* Sc-K143, one consortia consisted of *Bacillus cereus* Rs-MS53 and *Bacillus amyloliquefaciens* Sc-K143 and another consortia consisted of *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis Sc*-B94. The assay was carried out at room temperature. PDA and KING'S B plates were used for fungi and bacteria, respectively. Bacterial strains were previously freshly grown whereas fungal mycelium was a few days old. The test was done by placing the fungus on a PDA plate and the bacterial strain or consortia above, sealed together and afterwards left to grow at room temperature (Figure 4). Assays for each bacteria and consortia were performed in triplicates in order to get

consistent and reliable results. Control plates were prepared without bacteria. Before the fungal mycelium on control plate reached the edge, the radius of mycelia on other plates was measured and compared to controls to determine the intensity of growth inhibition.

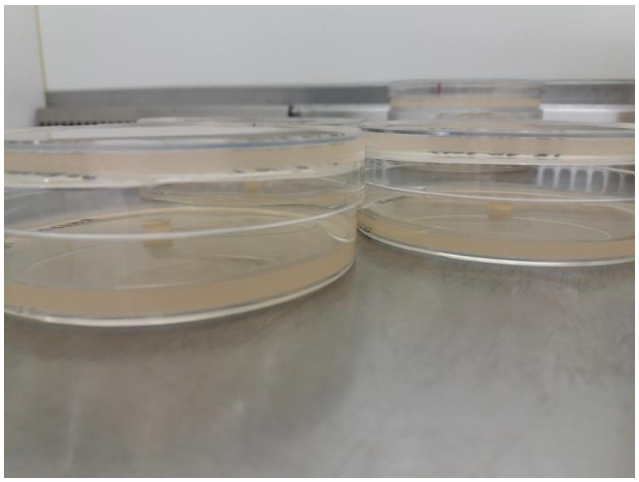


Figure 4. Bacterial volatile antagonistic screening assay against fungal mycelium.

2.2.3 In vitro dual-culture assay with *Fusarium oxysporum*

The aim of this dual-culture assay was to detect the antagonistic behaviour of 40 isolated bacterial strains against the plant pathogen *Fusarium oxysporum.* Referring to the BOX-PCR results from the experiment done previously, the number of 47 bacteria was reduced to 40 because of the fact that few of them are belonging to the same species and revealed very similar characteristics. Therefore, it was assumed that these isolates are identical. In contrast to previously described antagonistic screening assay, bacterial strains and fungal mycelium were supposed to grow on the same plate to screen for both volatile and non-volatile antifungal activities. The growth media used for this purpose was Waksman agar. Two different bacteria were placed on opposite sides of the plate and the fungus was placed in the middle (Figure 5). Controls without bacteria were made in order to compare the results and determine the difference. Inhibition of fungal growth could be surveyed as a zone of no growth around the bacterial isolates. The bacteria which showed antagonistic potential were subjected to volatile assay which will be further explained below.

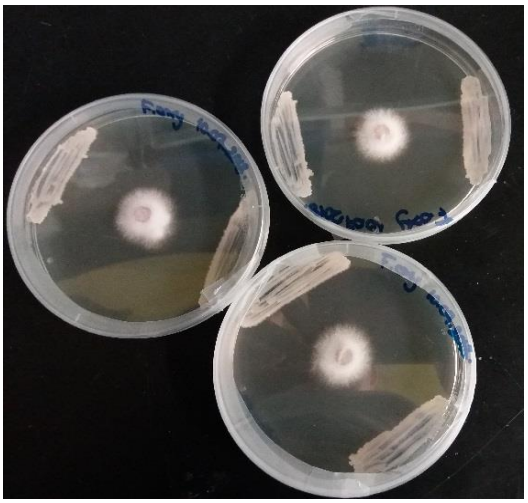


Figure 5. Dual-culture assay with *Fusarium oxysporum* on Waksman agar.

2.2.3.1 Volatile antagonistic screening assay against *Fusarium oxysporum*

After performing the dual-culture assay in which we tested which of the 40 bacteria had antagonistic behaviour, the volatile antagonistic screening assay was done for each of the bacteria that revealed antifungal activity in the previous experiment. In the case of screening assay against plant pathogen *Fusarium oxysporum* the initial number of 40 bacteria was reduced to 23 for further analysis. Each bacterium was placed on an individual NA plate while on the opposite side of the set up a plug of fungal mycelium was positioned on a PDA plate. These were left to grow until the fungal control reached the edge. The experiment was performed at room temperature. The assay was done in triplicates.

Table 5. Antagonistic bacterial strains used in volatile assay against *F. oxysporum*.

Rs-MS-S14	*Bacillus amyloliquefaciens*
Rs-MS28	*Bacillus amyloliquefaciens*
Sc-B84	*Bacillus amyloliquefaciens*
Rs-MS115	*Bacillus amyloliquefaciens*
Rs-Ts222	*Bacillus amyloliquefaciens*
Rs-Ts259	*Bacillus amyloliquefaciens*
Rs-Ts269	*Bacillus methylotrophicus*
Rs-So386	*Bacillus amyloliquefaciens*
Sc-K29	*Bacillus aerius*
Sc-S1	*Bacillus amyloliquefaciens*
Sc-S11	*Bacillus amyloliquefaciens*

Sc-K36	*Bacillus amyloliquefaciens*
Sc-K38	*Bacillus amyloliquefaciens*
Sc-K55	*Bacillus amyloliquefaciens*
Sc-T71	*Bacillus amyloliquefaciens*
Sc-B84	*Bacillus amyloliquefaciens*
Sc-K121C	*Bacillus amyloliquefaciens*
Sc-B142C	*Bacillus amyloliquefaciens*
Sc-K143	*Bacillus amyloliquefaciens*
Sc-K161	*Bacillus amyloliquefaciens*
Sc-T59	*Bacillus amyloliquefaciens*
Sc-T64	*Bacillus amyloliquefaciens*
Sc-B89	*Bacillus amyloliquefaciens*

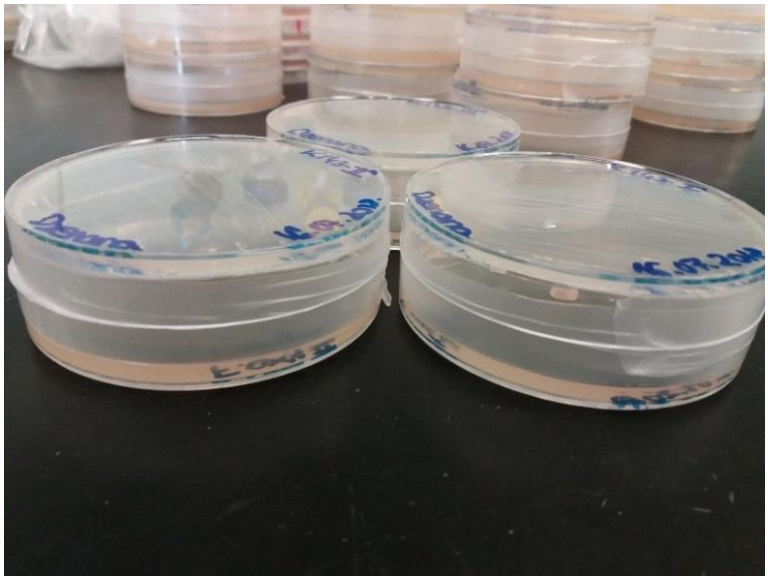


Figure 6. Antagonistic screening assay against *Fusarium oxysporum.*

2.2.4 Bacterial VOCs production as a response to different nutrients

In this approach bacteria were grown on four different media to test the effect of different nutrients on the production of microbial volatile organic compounds (mVOCs) using Headspace - Solid Phase Microextraction Gas chromatography – Mass spectrometry (HS-SPME GC-MS). Media used were: Nutrient agar, Water agar, Tryptic Soy Agar and Artificial Root Exudate medium. For this purpose, 20 mL headspace vials (75.5 × 22.5 mm; Chromtech, Idstein, Germany) were filled with 8 mL of each of the four media. Bacteria were cultivated in vials and left to grow on 30°C for 24 hours. The next day HS-SPME GC-MS was performed.

Eleven bacteria from six different genera were tested (Table 6). All of the measurements were done in triplicates for result consistency.

Table 6. Bacterial isolates tested for mVOCs on four different nutrient media.

C-B4	*Microbacterium flavescens*
CV(CF)-C7	*Microbacterium flavescens*
I-B5	*Microbacterium maritypicum*
IOF-G4	*Microbacterium flavescens*
IF-C6	*Sporosarcina koreensis*
IO-C6B	*Bacillus aryabhattai*
OV-C9	*Stenotrophomonas maltophilia*
C-E1	*Ralstonia pickettii*
CF-B11	*Ralstonia insidiosa*
IO-A9	*Agrobacterium tumefaciens*
C-E6	*Agrobacterium tumefaciens*

2.2.5 Bacterial response to signal molecules produced by fungi

In order to examine the influence of mVOCS of bacteria on fungi and vice versa, the following experiment was performed. At first volatile metabolites of the plant pathogens *Sclerotinia sclerotiorum* and *Rhizoctonia solani* were detected using HS-SPME GC-MS. 20 mL headspace vials (75.5 × 22.5 mm; Chromtech, Idstein, Germany) were filled with 8 mL of PDA and fungal mycelium was placed on it. Vials were left to grow at room temperature for four days. Then, HS-SPME GC/MS was performed. Triplicates were measured for each sample to get consistent and reliable results. Afterwards, common substances of both fungi were identified and ordered from the manufacturer "Merck KGaA" (Darmstadt, Germany). To check if the measured substances match the ordered ones, we prepared standard solution of 100 ppb concentration using hexane. Subsequently, bacteria were exposed to the fungal metabolites to examine if they are able to induce the production of new volatiles in bacteria. All stimulation experiments were done on three bacteria: *Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94. These three were stimulated with five substances in total. Before stimulation, substances were diluted with ultra-pure water by Carl Roth (Karlsruhe, Germany). For all tests we used 1:100 dilutions. Before the stimulation was done, bacteria were cultivated in the vial on NA medium, left to grow on 30°C and after 8 hours subjected to stimulation. The first stimulation trial was

performed with mixture of five substances with the volume of 25 µl in total (5 µl of each substance). Vials were returned to 30°C and on the next day bacteria were ready to be measured and analysed. This approach was also executed with solely 20 µl of each substance.

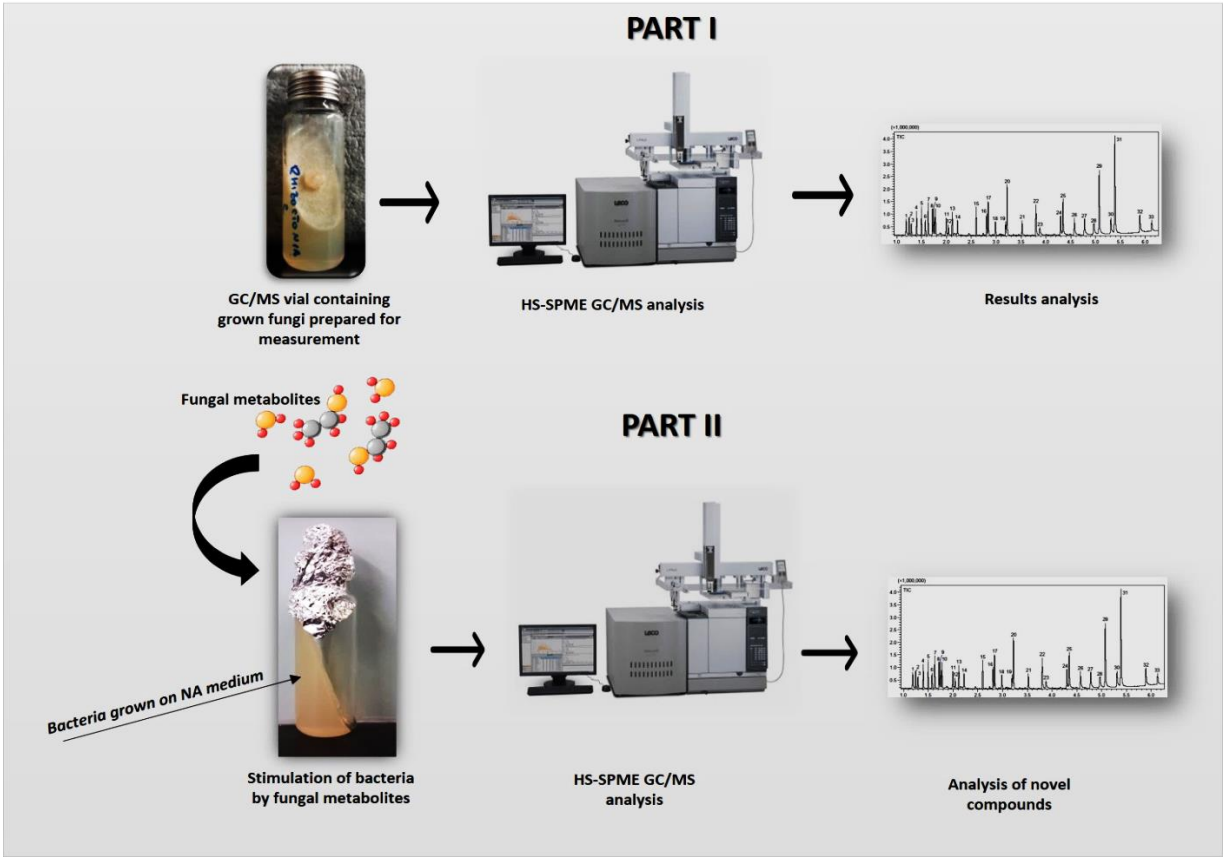


Figure 7. Illustration of the bacteria stimulation experiment. Part I: GC-MS measurement of fungal metabolites as well as result analysis. PART II: Fungal metabolites were used to stimulate bacteria on NA medium.

2.2.6 Stress response by *Sclerotinia sclerotiorum* sclerotia

This experiment was performed with the intention to analyse the liquid produced by sclerotia of *Sclerotinia sclerotiorum* when exposed to biotic stress in form of mVOCs released by different antagonistic bacteria. The following bacterial strains were used: Pseudomonas helmanticensis Sc-B94, *Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143, one consortia consisting of *Bacillus cereus* Rs-MS53 and *Bacillus amyloliquefaciens* Sc-K143 and another consortia of *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94. There were 25 sclerotia on the inner side of each petri dish (Figure 8). All of the set

ups were done in five replicates in order to have enough reliable information about the results. Petri dish lid served as a base for sclerotia and connected with parafilm to bacteria streaked on the bottom of the same dish. The incubation lasted for 27 days and was performed at room temperature. Bacteria were replaced every 84 hours and the liquid from sclerotia was collected after 9, 18 and 27 days. Afterwards, liquid chromatography was executed to investigate the composition of the liquid produced by sclerotia under stress. Liquid chromatography - mass spectrometry (LC-MS) was performed in positive ion mode.

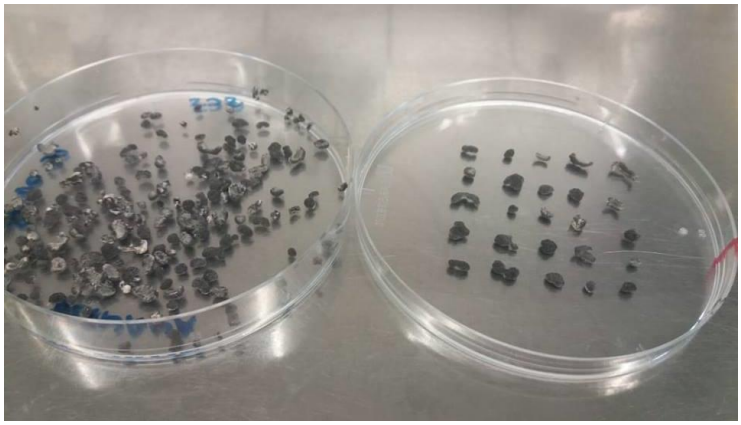


Figure 8. Dried sclerotia of *Sclerotinia sclerotiorum*.

3 Results

3.1 Characterization of antagonistic bacteria

3.1.1 Characterization of isolates using BOX-PCR

BOX-PCR of bacterial strains belonging to the same species provided information on scattered DNA regions of the bacterial genome. Based on its strong differentiation power, we could form sub-groups of isolates within the species of *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus aerius, Bacillus subtilis* and *Pseudomonas helmanticensis*. Agarose gel electrophoresis was used to visualize fingerprints of the bacterial isolates (Figure 9). By comparing gel bands between the isolates within a species, they were distinguishable from each other. Out of 28 *B. amyloliquefaciens* isolates 4 sub-groups were identified from which the largest group consisted of 9 isolates. It was also shown that all four isolates of the *Bacillus cereus* isolates share the same fingerprint. Two sub-groups of isolates in the *Bacillus aerius* group were discovered to share the same BOX pattern while all *B. subtilis* and *P. helmanticensis* isolates were clearly distinguishable.

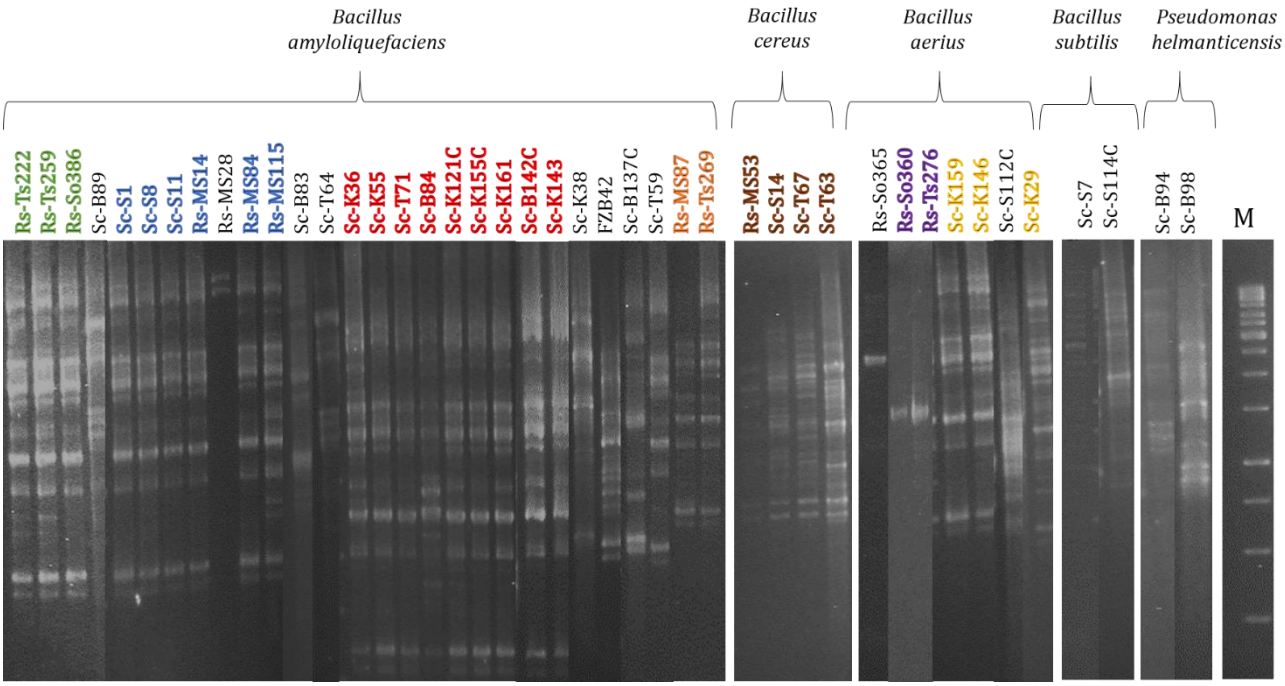


Figure 9. Agarose electrophoresis gel of BOX-PCR and similarity of isolates within *B. amyloliquefaciens, B. cereus, B. aerius, B. subtilis* and *Pseudomonas helmanticensis* species previously detected using 16S rRNA sequencing. The molecular size marker is 1 kb ladder.

3.1.2 Screening of the antagonistic activity via microbial VOCs towards *Sclerotinia sclerotiorum* and *Rhizoctonia solani*

After being treated with three bacteria (*Pseudomonas helmanticensis* Sc-B94, *Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143) and two consortia grown on KING's B agar plates, we were able to measure the mycelial growth inhibition of *S. sclerotiorum* (Figure 10) and *R. solani* (Figure 11). The mycelial growth of the *Sclerotinia sclerotiorum* was inhibited for almost 90% in all cases. Likewise, in a case of *R. solani*, consortia Sc-K143/Sc-B94 and Sc-B94 inhibited mycelial growth completely.

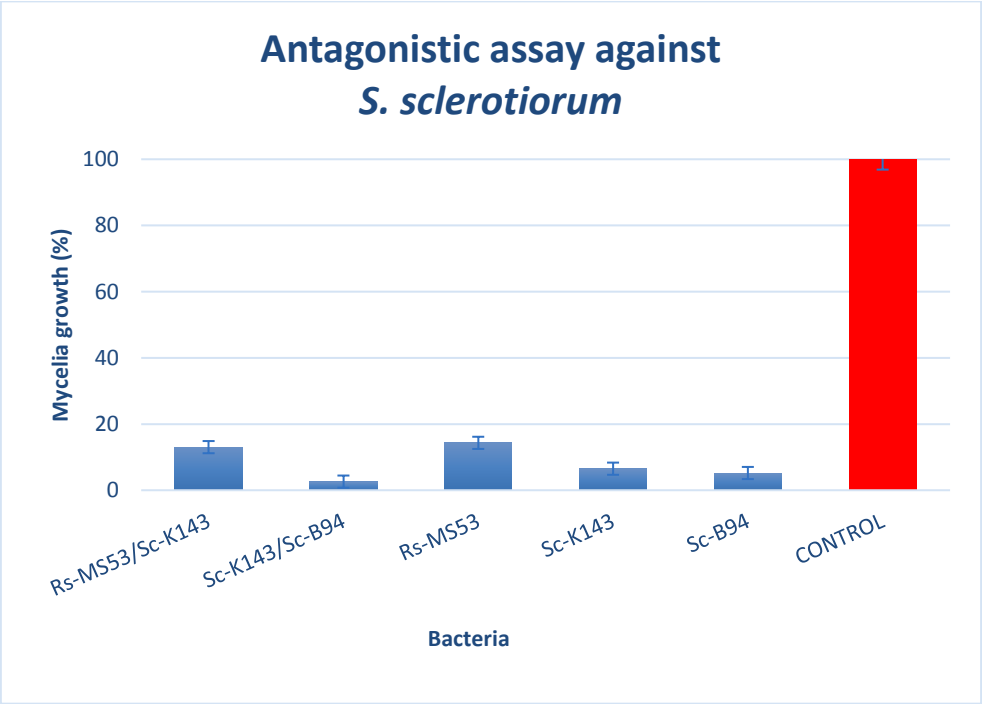


Figure 10. Growth inhibition of *S. sclerotiorum*. Fungal growth inhibited for 94% in the best case of consortia Sc-K143/Sc-B94 (*Bacillus amyloliquefaciens* and *Pseudomonas helmanticensis*).

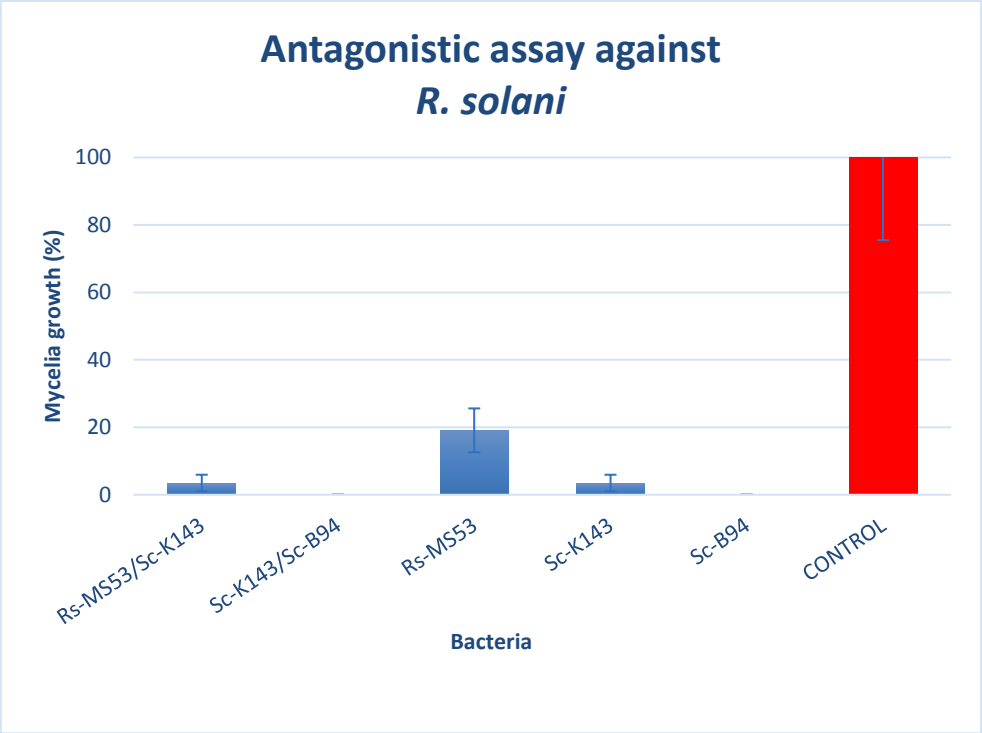


Figure 11. Growth inhibition of *R. solani*. Fungal growth completely inhibited in the case of Sc-B94 (*Pseudomonas helmanticensis*) and consortia Sc-K143/Sc-B94 (*Bacillus amyloliquefaciens and Pseudomonas helmanticensis*).

3.1.3 In vitro dual-culture assay with *Fusarium oxysporum*

Dual-culture assay with *Fusarium oxysporum* indicated that 25 bacterial strains out of 40 tested were able to inhibit the growth of this plant pathogen. The rest of the isolates showed no inhibitory effect. The radius of the mycelial growth of *F.oxysporum* of the controls was 3.74 cm in average. Based on this we could classify isolates which caused the inhibition zone of 20 mm or more or isolates whose zone of inhibition was 10 mm or higher (Table 7). Bacterial isolates which showed inhibitory effect were later subjected to the volatile assay.

Table 7. Inhibitory effect of bacterial isolates in *F. oxysporum* in dual-culture assays. The size of the inhibition zone for 17 bacteria was 20 mm or higher while 6 isolates inhibited the mycelium for 10 mm or more.

Inhibition of *F.oxysporum*	
>20 mm	>10 mm
Sc-K143	Sc-K29
Sc-T59	Sc-K121C
Sc-S1	Sc-B84
Sc-K161	Sc-T71
Sc-S11	Sc-B142C
Sc-B89	Sc-T64
Rs-Ts222	
Rs-mS115	
Sc-K36	
Sc-K38	
Rs-MS14	
Rs-So386	
Sc-K55	
Rs-Ts269	
Rs-MS28	
Rs-Ts259	
Rs-MS84	

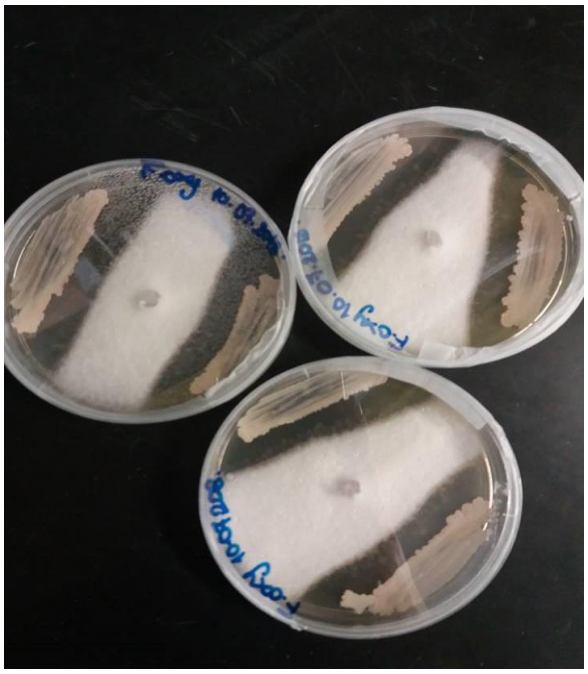


Figure 12. Antagonistic isolates which had inhibitory effect on *F. oxysporum.*

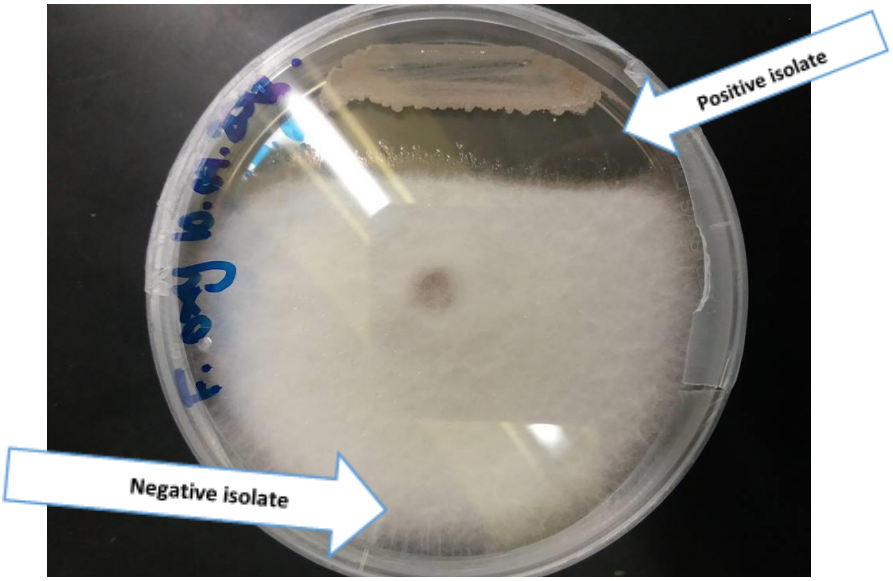


Figure 13. An example of inhibition where only one of the isolates showed the positive effect.

3.1.4 Antagonistic screening assay against *Fusarium oxysporum*

Analysis of results demonstrated that 10 of 25 bacteria did not inhibit mycelial growth of this plant pathogen at all. More precisely, all of them were overgrown by *F. oxysporum*. However, there were also strains that had an inhibition effect of up to 10%. Some strains were most successful with an inhibition of more than 15%. The best strains in this trial were mostly *Bacillus amyloliquefaciens spp.* (Sc-S1, Sc-B89, Rs-MS84, Rs-Ts259, Sc-K36).

Table 8. Inhibition of mycelial growth in percentage.

Isolate	Inhibition of mycelial growth in %
Sc-K38 Sc-T59 Sc-K29 Sc-B142C Sc-T71 Rs-So386 Rs-Ts269 Sc-T64 Sc-K146 Sc-K159	No inhibition or overgrowth
Rs-MS14 Sc-S11 Rs-MS28 Rs-mS115 Sc-K121C Sc-K143 Rs-Ts222 Sc-K161 Sc-B84 Sc-K55	0-10%
Sc-S1 Sc-B89 Rs-MS84 Sc-K36 Rs-Ts259	15% and higher

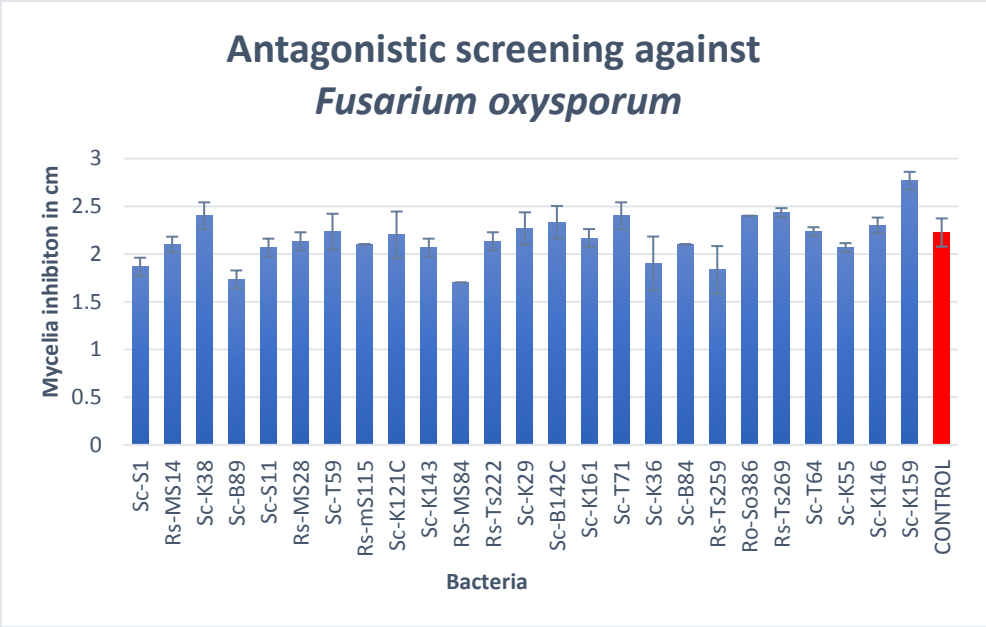


Figure 14. Antagonistic screening assay inhibition results in cm.

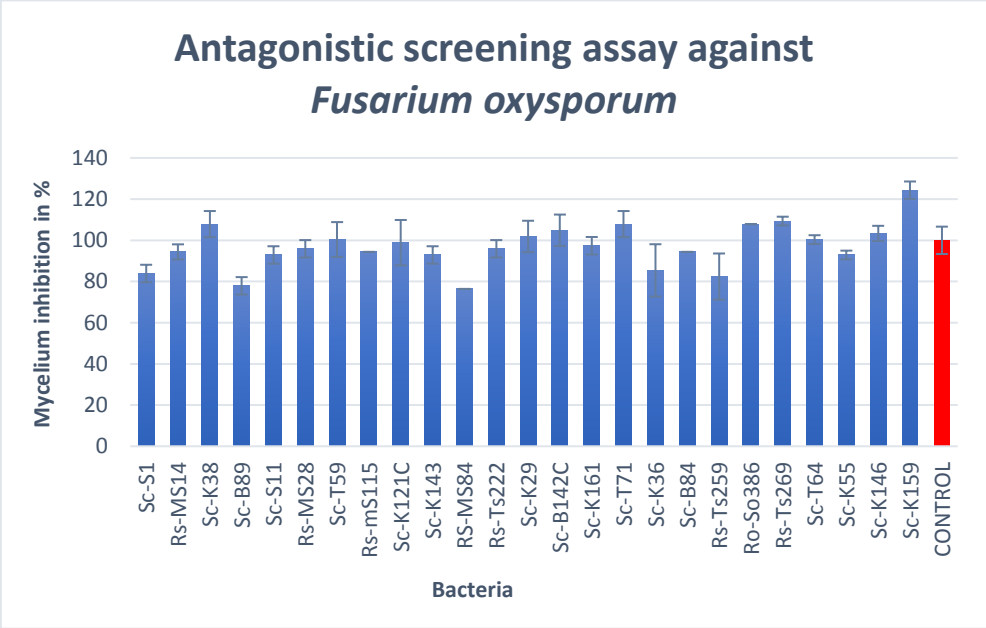


Figure 15. Antagonistic screening assay inhibition results in percentage. 10 strains of the total 25 made no inhibitory effect, 10 of them made the inhibition of up to 10% and 5 of them inhibited the mycelial growth more than 15% inhibition.

3.2 Bacterial mVOCs production as a response to different nutrients

The aim of this experiment was to find out if bacteria produce different volatiles on different media. It was expected that various number of compounds will be revealed because of the fact that each of the media has different composition of carbon and nitrogen sources which are important for bacteria and represent the basis for bacterial growth and metabolism. First of all, raw data from the GC-MS were processed and with the help of the chromatogram and mass spectrum peaks, we were able to determine the volatilome of each bacterium. One of the parameters for confirmation of volatile existence was the retention index (RI).

The most comprehensive volatile profile was found out on NA (Table 9). There was a high similarity of the volatilome of all bacteria. In the case of TSA there was a smaller number of compounds detected but still they were similar for all bacteria (Table 10). Bacteria that grew on water agar produced significantly less volatiles than in two previous cases (Table 11). Moreover, there were three bacteria (IO-C6B, I-B5, C-E6A) for which we could not confirm any of the volatiles produced. It could be also noticed that volatiles differ among the bacteria. The last media tested was ARE. In this case there were also no volatiles produced for the following bacteria: IO-C6B, IF-C6, IO-A9 (Table 12).

Table 9. Bacterial VOCs detected on NA medium.

Bacterial strain	Nutrient Agar (NA)
CF-B11	2,5-Dimethylpyrimidine; anti-2-Acetoxyacetaldoxime; Methylenecyclobutane; Dimethyl trisulfide; Methyl glyoxal; Methyl thiolacetate; S-Methyl 2-methylpropanethioate; S-Methyl propanethioate
IO-C6B	3-methyl-1-butanol,; 2,3-Butanedione; 3-methoxy-3-methyl-2-butanone,; Benzene; Dimethyl disulfide,; 1,1-dimethyl-hydrazine,; Isoprene; Methyl thiolacetate; 2,3,4-trimethyl (2.alpha.,3.alpha.,4.beta.)-oxetane,; S-Methyl 2-methylpropanethioate
C-E1	2,5-Dimethylpyrimidine; 1-chlorobutane-; S-methyl ester-butanethioic acid; Methylenecyclobutane; Propylclopropane; Dimethyl trisulfide; Dimethyl disulfide; Methyl thiolacetate; S-Methyl 3-methylbutanethioate; S-Methyl propanethioate
I-B5	1,4-Pentadiene; 3-methylbutanol; 2-Ethyl-1-hexanol; anti-2-Acetoxyacetaldoxime; 3-methyl-S-ethyl ester-butanethioic acid; S-methyl ester-butanethioic acid; Methylenecyclobutane; Dimethyl trisulfide; Dimethyldisulfide; 1,1-dimethyl-hydrazine; Methyl glyoxal; Methyl thiolacetate; Oxetane, 2,3,4-trimethyl-, (2.alpha.,3.alpha.,4.beta.)-; Pyrrole; S-Methyl 2-methylpropanethioate; S-Methyl propanethioate; Thiopivalic acid; Toluene
C-E6A	3-methyl-butanol-; 2,5-Dimethylpyrimidine; 2-Ethyl-1-hexanol;, 3-methyl-S-ethyl ester-butanethioic acid; S-methyl ester-butanethioic acid; Methylenecyclobutane; Dimethyl trisulfide; Dimethyldisulfide, Methyl thiolacetate; Oxetane, 2,3,4-trimethyl-(2.alpha.,3.alpha.,4.beta.)-; 2-methyl-propanethioic acid; S-Methyl propanethioate; Thiopivalic acid
IF-C6	2-methyl-1-butanole-; 2,5-dimethyl-tetrahydro-2H-Pyranmethanol; 1-chlorobutane; S-methyl ester-butanethioic acid; Methylenecyclobutane; Propylcyclopropane-; Dimethyldiazene; Dimethyl trisulfide; Dimethyl disulfide; 2,5-dipropyl-tetrahydro-furan; Methyl thiolacetate; vinyl ester n-Caproic acid; , 2,5-dimethyl-pyrazine; S-Methyl propanethioate; Thiopivalic acid
CV(CF)-C7	1,4-Pentadiene; 3-methyl-Butanol; 2-Ethyl-1-hexanol; Benzaldehyde; Methylenecyclobutane; Dimethyl trisulfide; Dimethyl disulfide; Methyl thiolacetate; S-Methyl 2-methylpropanethioate; S-Methyl 3 methylbutanethioate; S-Methyl propanethioate; isobutyl pentyl ester-sulfurous acid; Thiopivalic acid
OV-C9	1,4-Pentadiene; 2-methyl-1-butanol; 3-methyl-1-butanol; 2,5 Dimethylpyrimidine; Methylenecyclobutane; Dimethyl sulfone; Dimethyl trisulfide; Disulfide, dimethyl
IOF-G4	2-methyl-1-butanol; 3-methyl-1-butanol; 2-Ethyl-1-hexanol; Benzyl alcohol; Dimethyl trisulfide; Methyl thiolacetate; S-Methyl 2-methylpropanethioate; S-Methyl 3-methylbutanethioate; S-Methyl propanethioate; Thiopivalic acid
IO-A9	3-methyl-1-butanol; Methylenecyclobutane; Dimethyl sulfone; Dimethyl trisulfide; Methyl thiolacetate; S-Methyl 2-methylpropanethioate; S-Methyl 3-methylbutanethioate; S-Methyl propanethioate
C-B4	3-methyl-1-butanol; 2-Ethyl-1-hexanol; S-methyl ester-butanethioic acid; Dimethyl-diazene; Dimethyl trisulfide; Methyl thiolacetate; S-Methyl 2-methylpropanethioate; S-Methyl propanethioate; Thiopivalic acid

Table 10. Bacterial VOCs detected on TSA medium.

Bacterial strain	Tryptic Soy Agar (TSA)
CF-B11	Methylenecyclobutane; Dimethyl trisulfide; Methyl thiolacetate; S-Methyl propanethioate
IO-C6B	
C-E1	Isoprene; 2,3-diethyl-5-methyl-pyrazine
I-B5	1,4-Pentadiene; Acetone; Dimethyl trisulfide; Dimethyl disulfide; Methyl thiolacetate; S-Methyl 2-methylpropanethioate; S-Methyl propanethioate
C-E6A	
IF-C6	Methylenecyclobutane; Dimethyl trisulfide; Dimethyl disulfide; Methyl thiolacetate; S-Methyl propanethioate; Vinyl butyrate
CV(CF)-C7	
OV-C9	1-chlorobutane; Dimethyl trisulfide; Methyl thiolacetate; S-Methyl propanethioate
IOF-G4	Acetic acid; Dimethyl trisulfide; Methyl thiolacetate; Octanal; 2-methyl-1-nitro-Propane; S-Methyl 2-methylpropanethioate; S-Methyl 3-methylbutanethioate; Tridecane
IO-A9	
C-B4	2,3-Butanedione; Acetoin; Methylenecyclobutane; Dimethyl trisulfide; 1-chloropentane

Table 11. Bacterial VOCs detected on WA medium.

Bacterial strain	Water agar (WA)
CF-B11	Dimethyl disulfide
IO-C6B	
C-E1	2,2,4,4,7,7-Hexamethyloctahydro-1H-indene; 2-Ethyl-1-hexanol; 2-methyl-2-propanol; Tridecane
I-B5	
C-E6A	
IF-C6	2-propyl-1-heptanol; 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate; 2,2,5-trimethyl-3,4-Hexanedione; 1-methyl-3-(1-methylethenyl)-benzene; Dimethyl disulfide; Isobutyl pentyl ester-sulfurous acid
CV(CF)-C7	2,3-Butanedione
OV-C9	Isobutyl pentyl ester-sulfurous acid
IOF-G4	2,3-Butanedione; Dimethyl sulfone
IO-A9	Isobutyl pentyl ester-sulfurous acid
C-B4	2,3-Butanedione; Acetic acid

Table 12. Bacterial VOCs detected on ARE medium.

Bacterial strain	Artificial Root Exudate (ARE) medium
CF-B11	Pentyl ester-propanoic acid; Isobutyl pentyl ester-sulfurous acid
IO-C6B	
C-E1	2-Ethyl-1-hexanol
I-B5	2,3-Butanedione
C-E6A	2,3-Butanedione; 2-methyl-propanal
IF-C6	
CV(CF)-C7	2-methyl-1-butanol; 2,3-Butanedione; Acetoin
OV-C9	9-hydroxy-2-Nonanone; Acetone; Dimethyl disulfide
IOF-G4	2-methyl-1-butanol; Acetoin
IO-A9	
C-B4	2-methyl-1-butanol; Acetoin

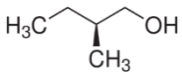
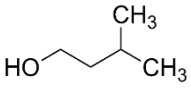
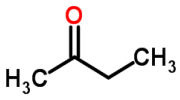
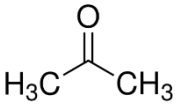
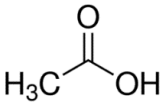
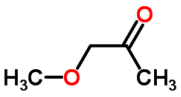
3.3 Bacterial response to signal molecules produced by fungi

The first measurements with GC-MS were done in order to identify the fungal volatilomes of *Sclerotinia sclerotiorum* and *Rhizoctonia solani.* From the list of all detected compounds (Table 13) 6 of them were chosen and ordered from the "Merck KGaA" (Darmstadt, Germany). We were choosing substances that are simple and easier to work with. We also wanted to confirm if they were certainly present in the samples. Substances that we ordered were subjected to measurement and represented the reference (Table 14). The confirmation was done as with all the other compounds.

Table 13. The list of all detected fungal metabolites.

Detected fungal metabolites	
Scerotinia sclerotiorum	***Rhizoctonia solani***
2-methyl-1-butanol	2-methyl-1-butanol
3-methyl-1-butanol	2-n-propylaziridine
1H-Tetrazole-1,5-diamine	Acetic acid
2-Butanone	Acetone
2-Propenal	Dimethyl ether
3,4-Dimethyldihydrofuran-2,5-dione	Ethylenimine
Acetone	Isobutane
Methoxyacetone	

Table 14. Compounds tested for the analysis accuracy.

2-methyl-1-butanol	
3-methyl-1-butanol	
2-butanone	
Acetone	
Acetic acid	
Methoxyacetone	

The end results showed that from these compounds, five of them could be confirmed and used in stimulation of bacteria (Table 15). The compounds could be confirmed by comparing the peak in single measurements of the substance to the fungal samples. The only one which was not confirmed is Methoxyacetone whose chromatogram did not match with the peak of reference/standard compounds.

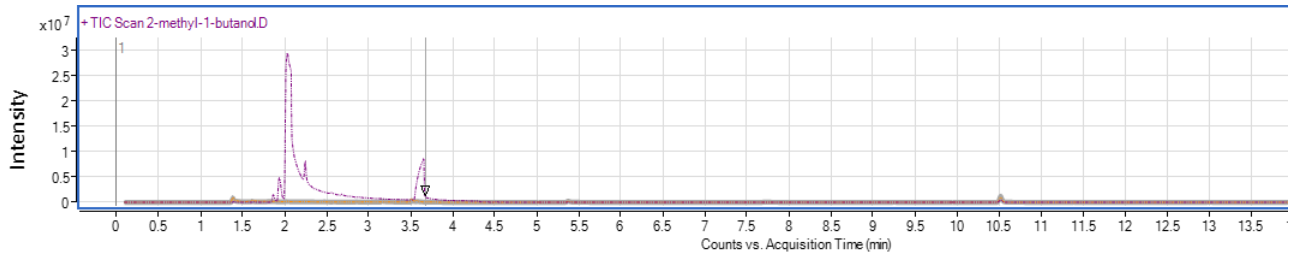


Figure 16. Chromatogram of reference compound; 2-methyl-1-butanol.

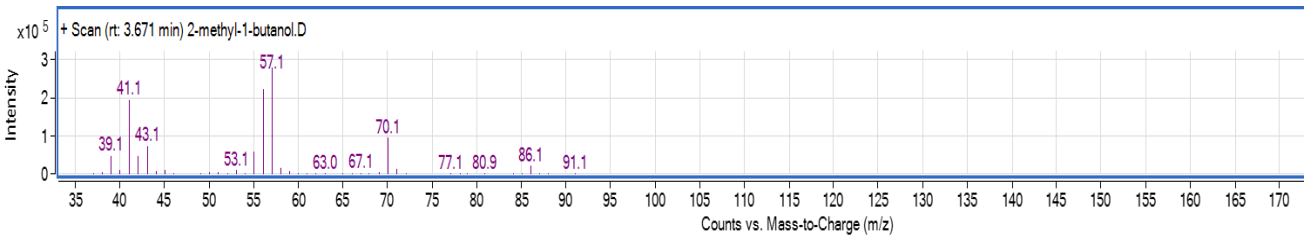


Figure 17. Mass spectrum of reference compound; 2-methyl-1-butanol

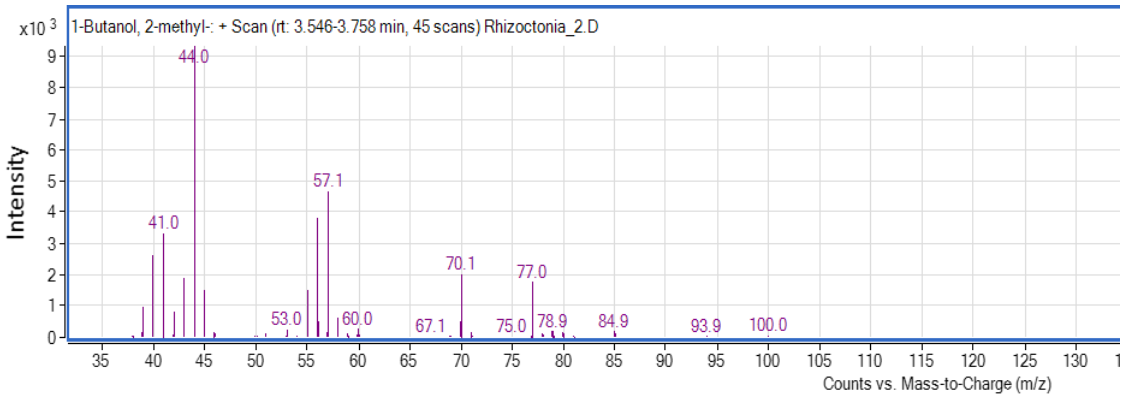


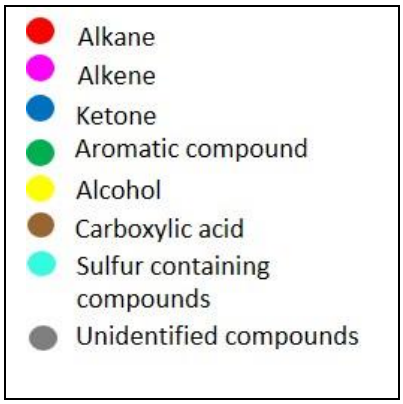
Figure 18. Mass spectrum of detected 2-methyl-1-butanol in *Rhizoctonia solani* sample. Peak 44 appears as a background.

Table 15. Compounds used for the stimulation of selected bacterial strains.

2-methyl-1-butanol
3-methyl-1-butanol
Acetic acid
2-Butanone
Acetone

The stimulation of bacteria (*Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94) with fungal volatiles resulted in an altered volatile profile. Volatiles were confirmed on the same way as in the previous measurements. The ones which were positive were sorted by their main chemical groups. All of them which could be certified with mass spectrum but not with the RI were labeled as „Unidentified“ (Table 16).

Table 16. Color code for main compound groups detected with GC/MS after stimulation.



It can be noticed that the smallest number of new compounds was produced after the bacteria were stimulated with the mixture of five fungal volatiles (Figures 19, 20, 21). Most of them were unidentified while the positive ones were mostly alkanes and alkenes. In the case where bacteria were stimulated with 2-methyl-1-butanol we could find ketones and alcohols (Figure 22). Stimulation with 3-methyl-1-butanol revealed novel compounds from the groups of alkenes, ketones, alkanes, aromatic compounds and carboxylic acids (Figure 23). Bacteria stimulated with 2-butanone produced novel compounds containing sulfur, alcohols, alkanes and ketones (Figure 24). After the stimulation of bacteria with acetic acid we found new compounds from the groups of alcohols, alkanes, alkenes, ketones, carboxylic acids and sulfur – containing compounds (Figure 25). The last case where strains were stimulated with acetone, mostly resulted in a production of alcohols, ketones, alkanes, aromatic and sulfur – containing compounds (Figure 26). Figures 19 – 26 represent novel compounds in „circles“ whose size correspond to the area of the chromatogram peak. Therefore, small circles represent compounds with lower presence in the sample and accordingly bigger circles correlates with compounds which were present in higher amounts in the sample.

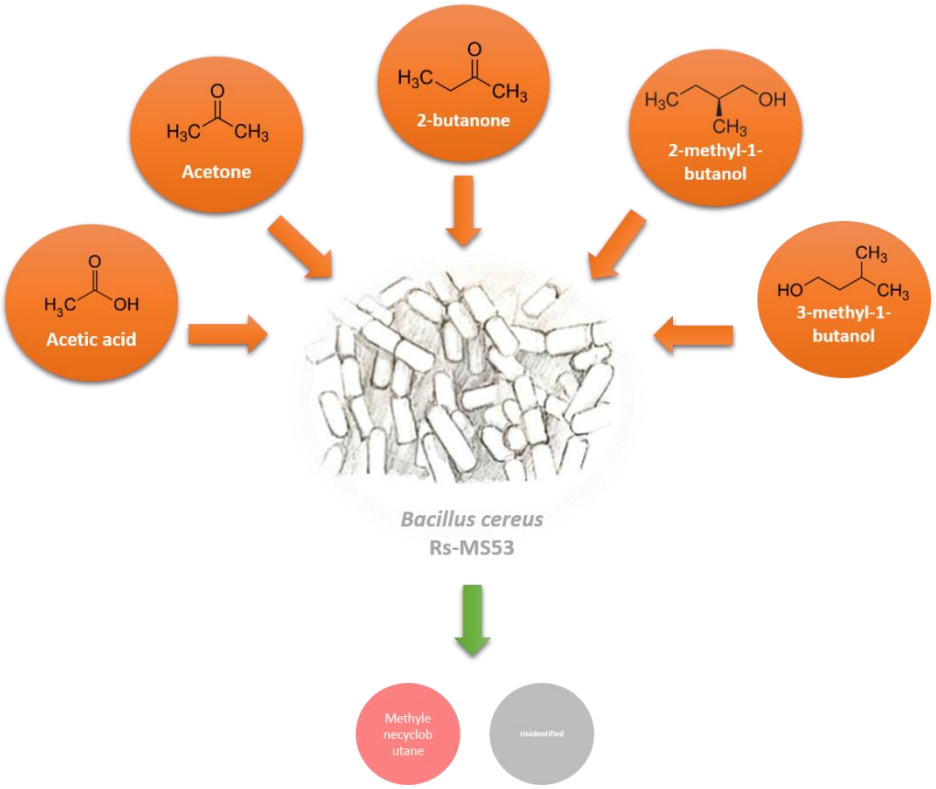


Figure 19. Novel compounds detected after *Bacillus cereus* Rs-MS53 was stimulated with the mix of five substances.

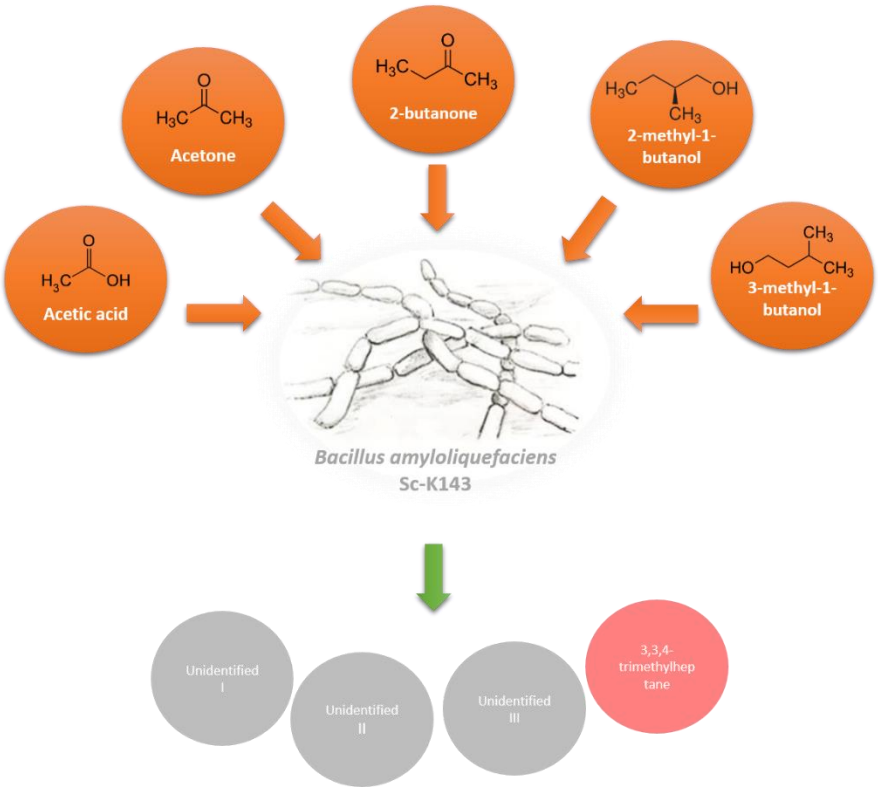


Figure 20. Novel compounds detected after *Bacillus amyloliquefaciens* Sc-K143 was stimulated with the mix of five substances.

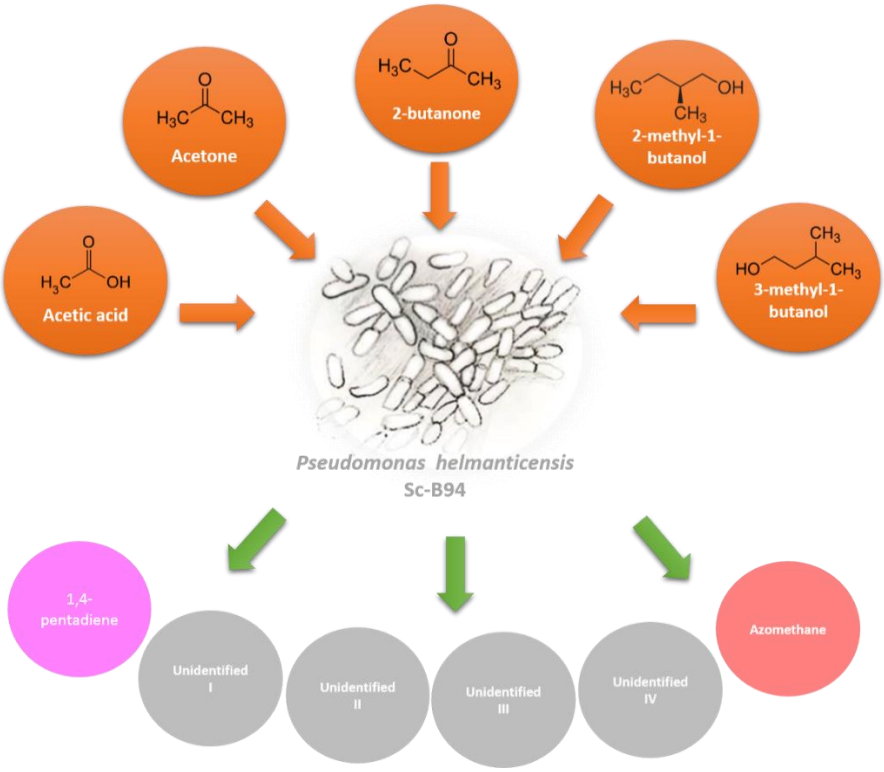


Figure 21. Novel compounds detected after *Pseudomonas helmanticensis* Sc-B94 was stimulated with the mix of five substances.

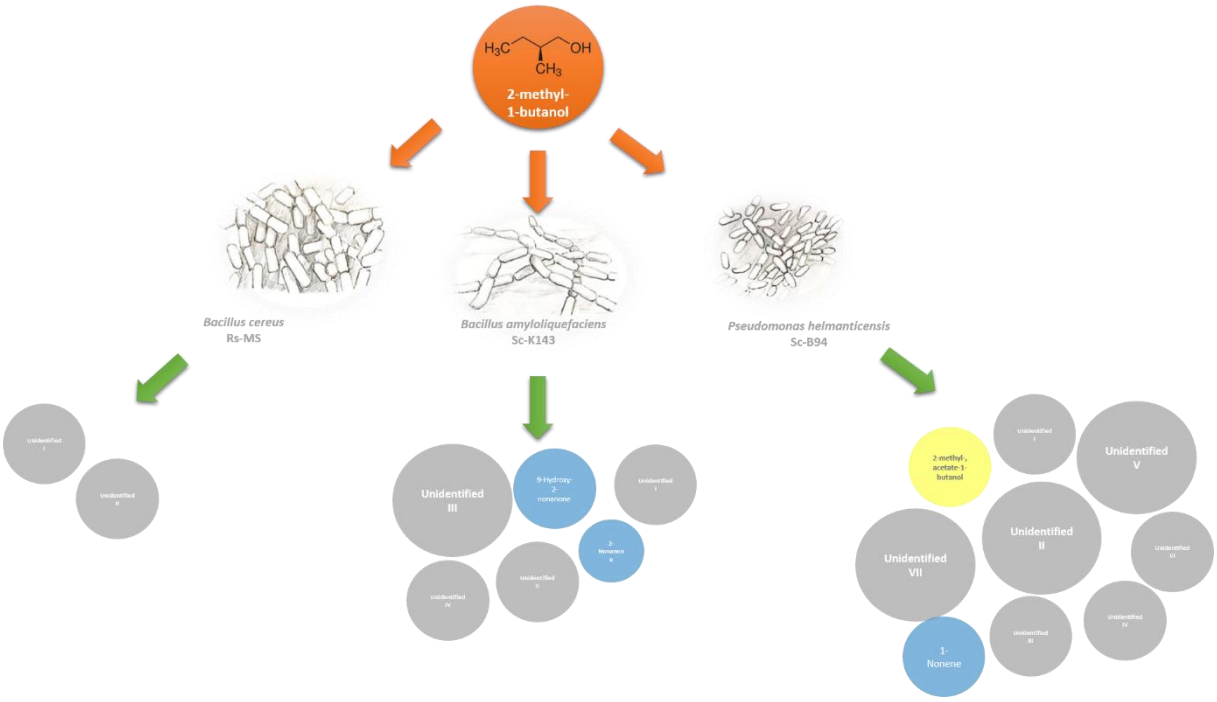


Figure 22. Novel compounds detected after *Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94 were stimulated with 2-methyl-1-butanol.

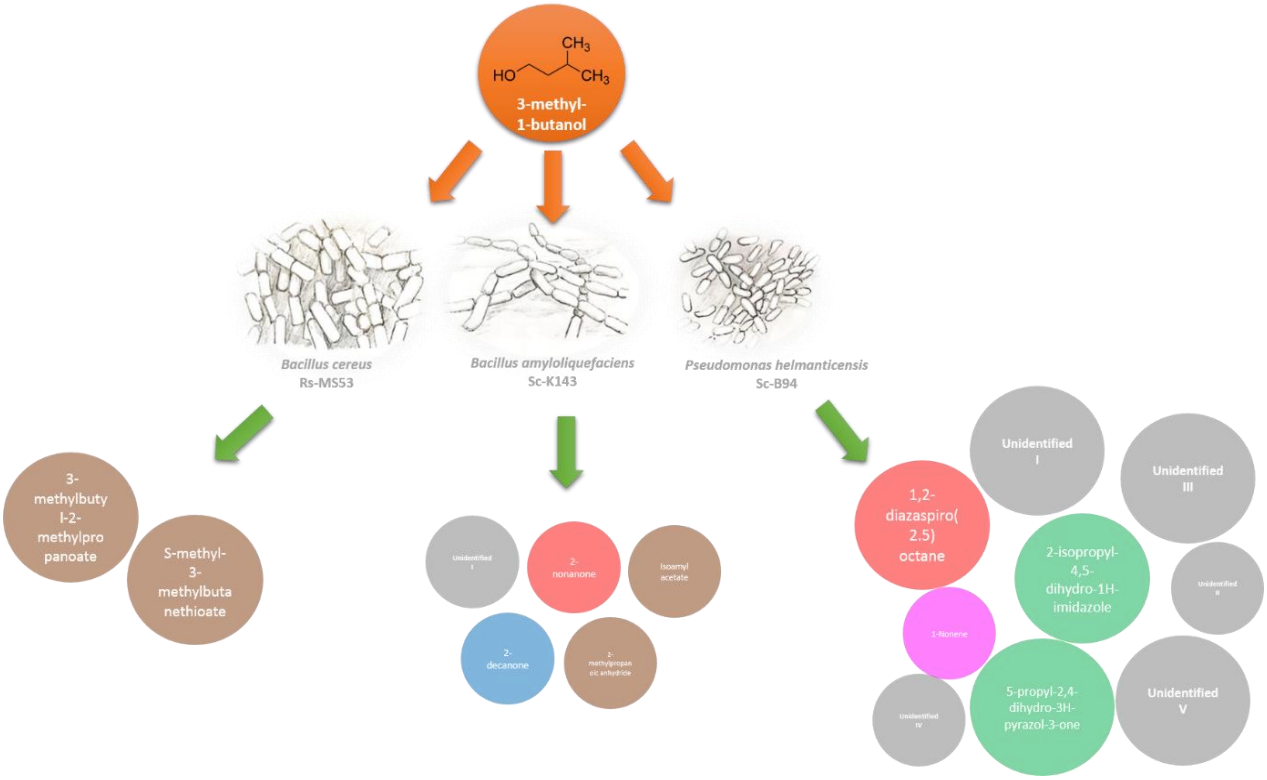


Figure 23. Novel compounds detected after *Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94 were stimulated with 3-methyl-1-butanol.

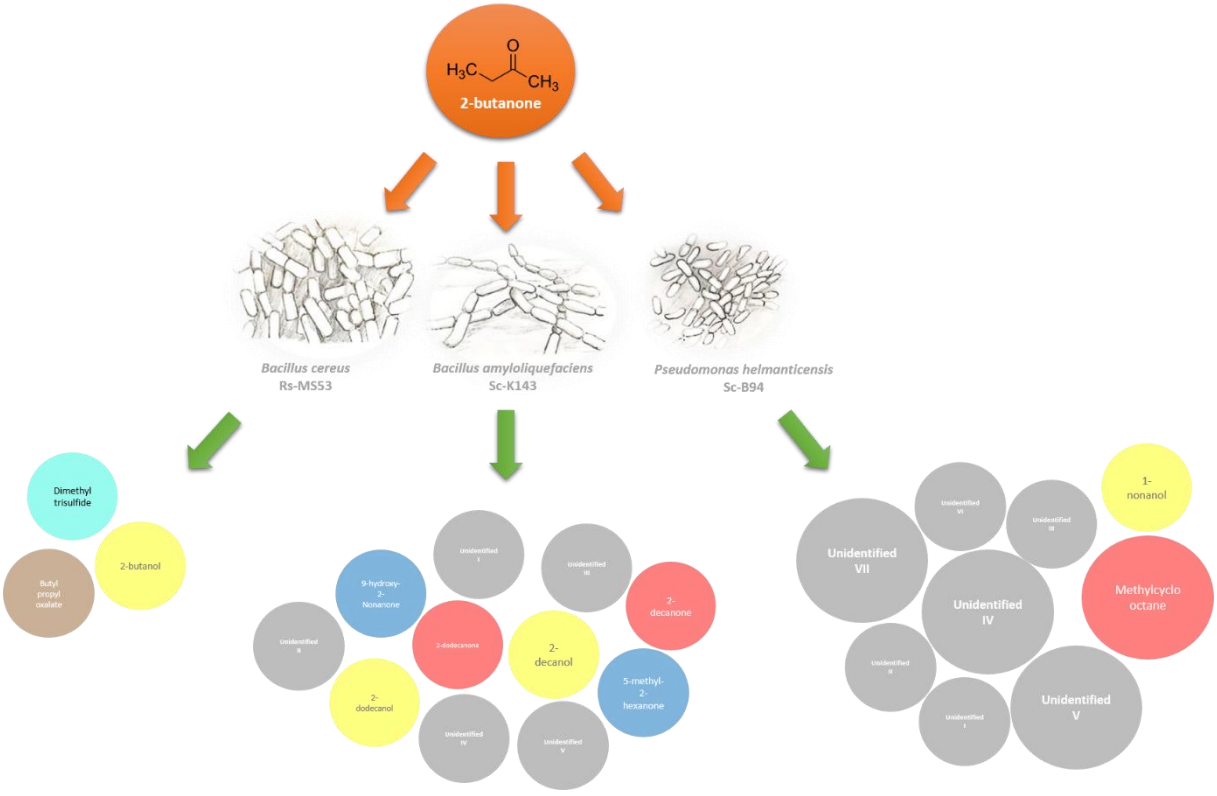


Figure 24. Novel compounds detected after *Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94 were stimulated with 2-butanone.

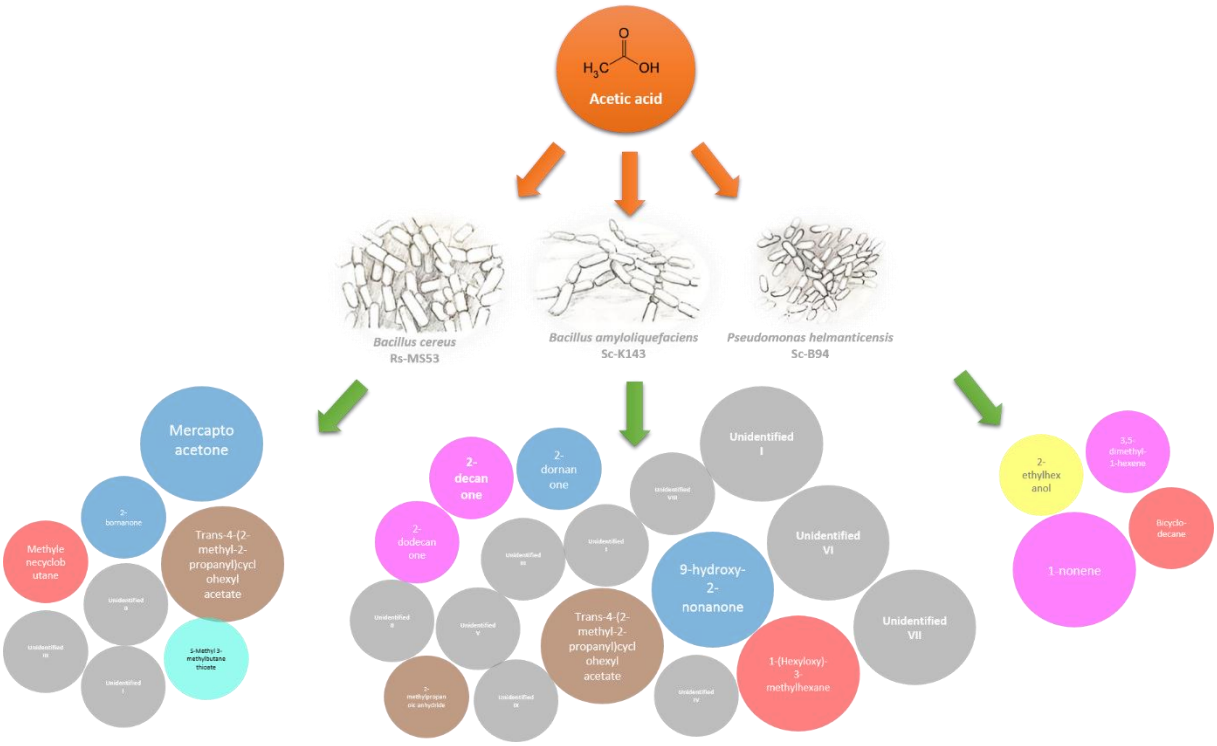


Figure 25. Novel compounds detected after *Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94 were stimulated with Acetic acid.

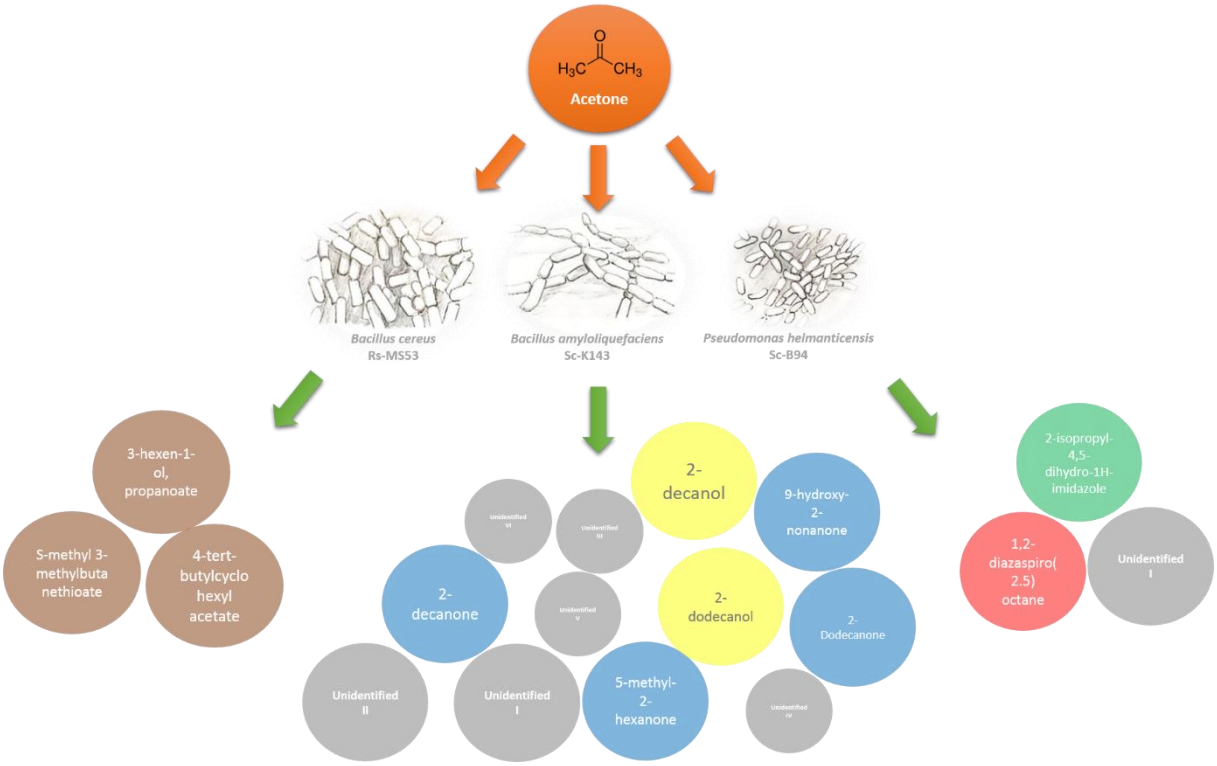


Figure 26. Novel compounds detected after *Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94 were stimulated with Acetone.

3.4 Stress response by *Sclerotinia sclerotiorum* sclerotia

LC-MS analysis of the data showed that various of compounds were produced in the sclerotia liquid after they were subjected to stress by bacteria. During the data analysis it could be noticed that the area of mass spectrum of majority of compounds was decreasing with time. In some cases, the area of compounds was even increasing or stable (Figures 28-42). The compounds were present in the liquid exudates in the highest amount until the 9th day of experiment (Figure 27A). Accordingly, the liquid collected on the 27th day of the experiment contained the lowest number of compounds within (Figure 27C). During the second time of the liquid collection it could be already noticed that less liquid is present than it was on the first collection and the germination of the sclerotia could be observed as well.

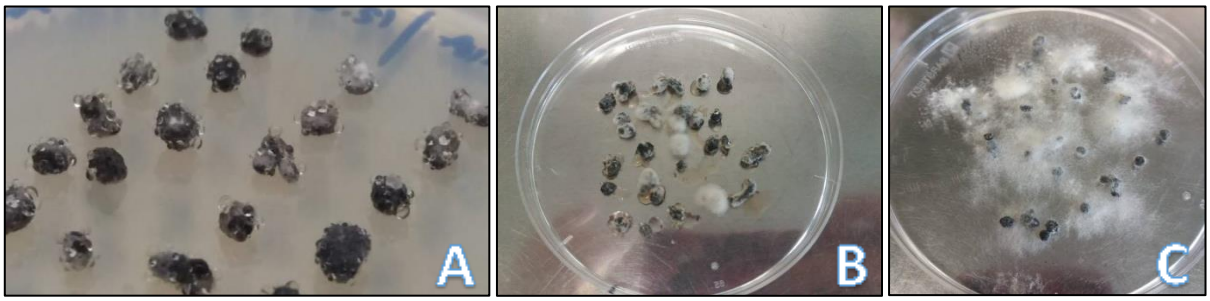


Figure 27. **A)** Sclerotia liquid production (9th day of experiment). **B)** Liquid exudate production (18th day of experiment). Germination of sclerotia can be noted. **C)** Sclerotia liquid production (27th day of experiment). The mycelium is spreading and the volume of the liquid decreased.

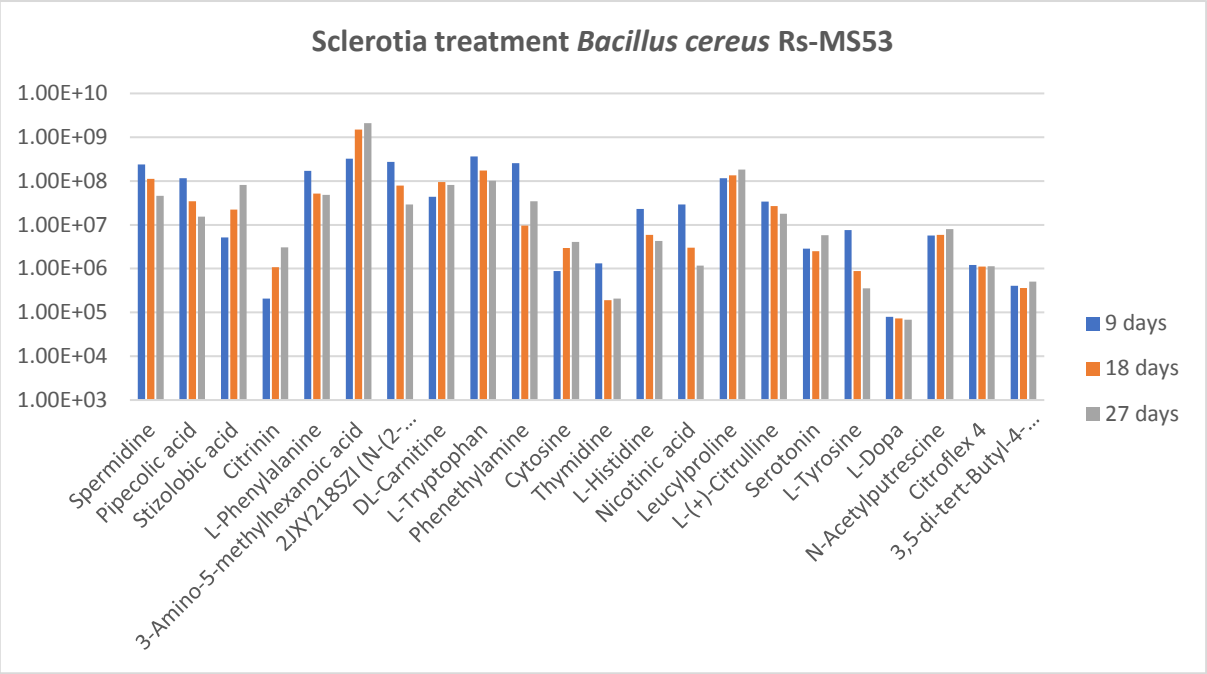


Figure 28. Area of compounds detected from sclerotia exudates stimulated with *Bacillus cereus* Rs-MS53. Presented area corresponds to the liquid collection time; 9, 18 and 27 days. Substances that were decreasing over time were: Spermidine, Pipecolic acid, L-Phenylalanine, L-Tryptophan, L-Histidine, Nicotinic acid, L-Citrulline and L-Tyrosine while the increase in area can be noticed in case of Stizolobic acid, Citrinin, 3-Amino-5-methylhexanoic acid and Cytosine. L-Dopa, Citroflex 4 and 3,5-di-tert-Butyl-4-hydroxybenzaldehyde were stable over time.

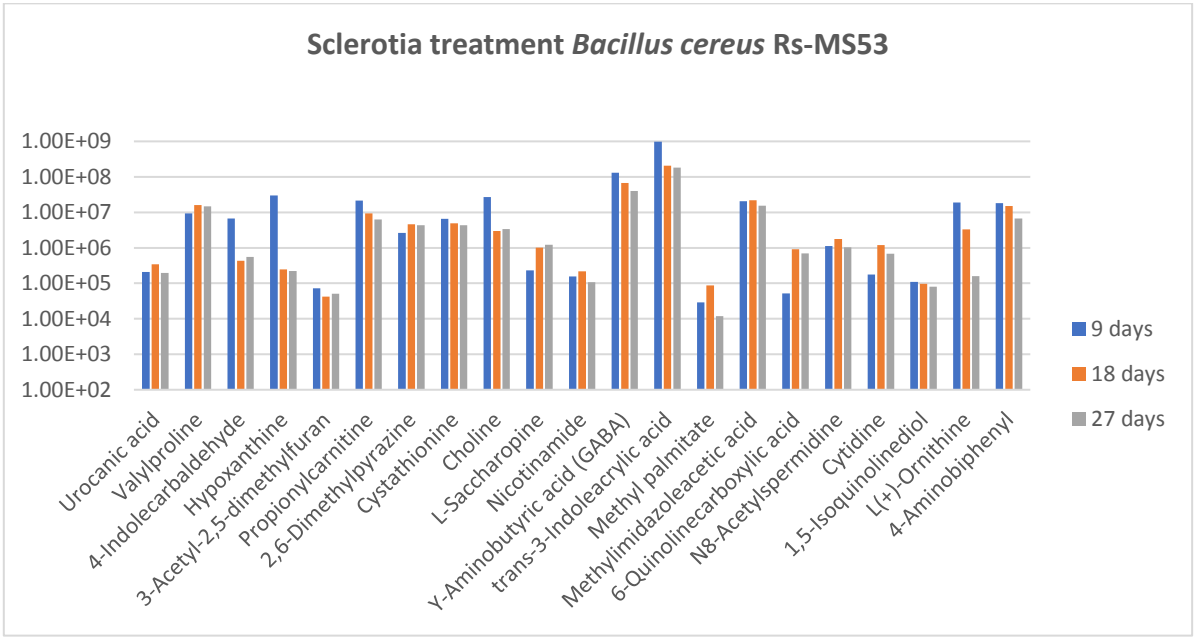


Figure 29. Area of compounds detected from sclerotia exudates stimulated with *Bacillus cereus* Rs-MS53. Significant decrease in area was noticed in case of Propionylcarnitine, GABA and L-Ornithine while 3-acetyl-2,5-dimethylfuran, Nicotinamide and 1,5-Isoquinolinediol were rather stable. The increase in area over time was recorded in L-Saccharopine.

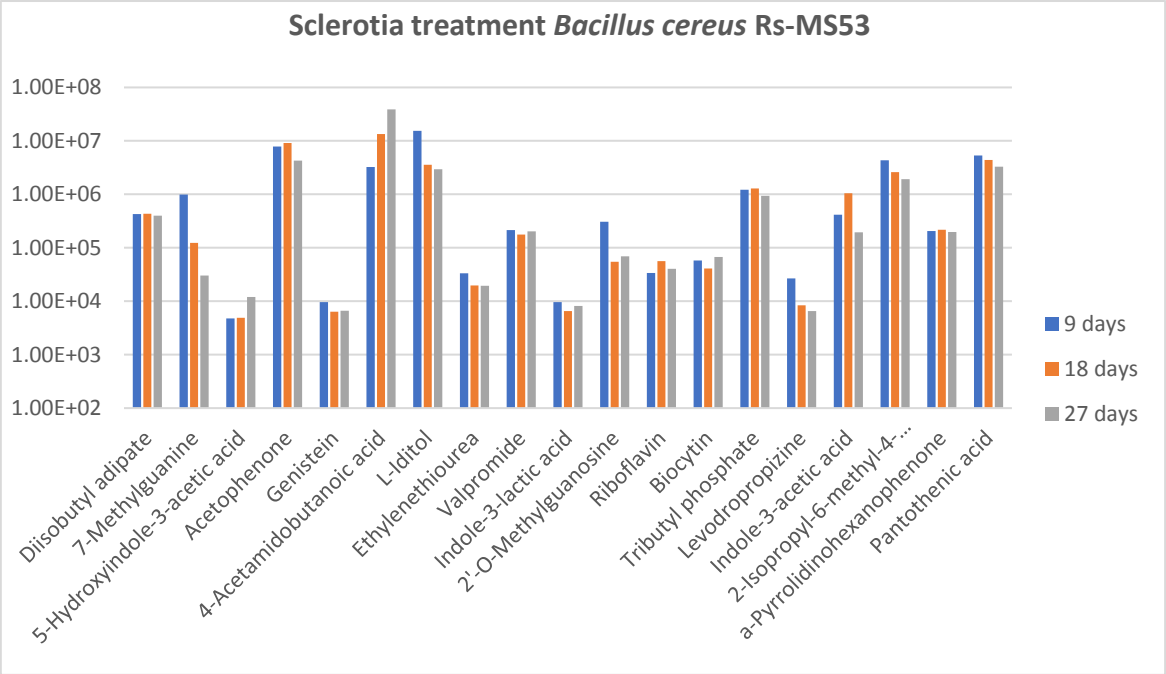


Figure 30. Area of compounds detected from sclerotia exudates stimulated with *Bacillus cereus* Rs-MS53. Diisobutyl adipate, Genistein, Indole-3-acetic acid, Riboflavin and Biocytin displayed stable area over the experiment time. Decrease in area can be noticed in case of 7-Methylguanine, L-Iditol, Levodropropizine and Pantothenic acid. 4-Acetamidobutanoic acid had significant rise in area.

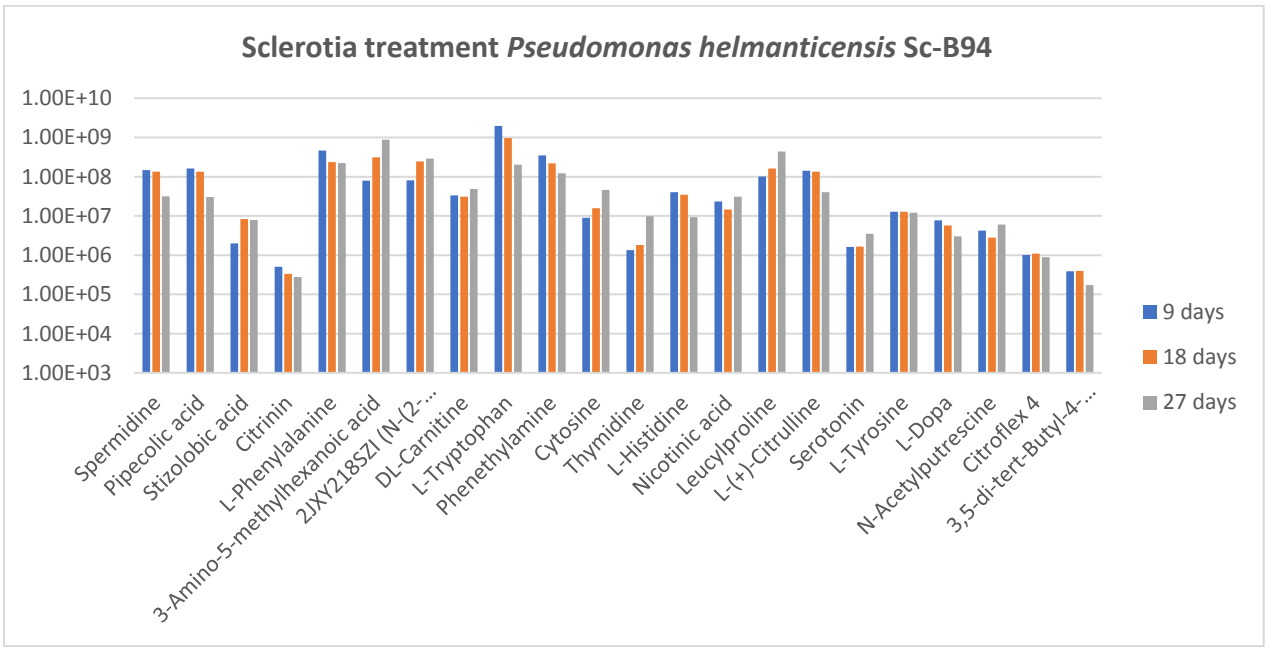


Figure 31. Area of compounds detected from sclerotia exudates stimulated *Pseudomonas helmanticensis* Sc-B94. Compounds area was mostly in decrease as in the case of Pipecolic acid, Citrinin, L-Tryptophan, Phenethylamine, L-Citrulline and L-Dopa. Slight increase in area was noticed in Stizolobic acid, Cytosine and Leucylproline.

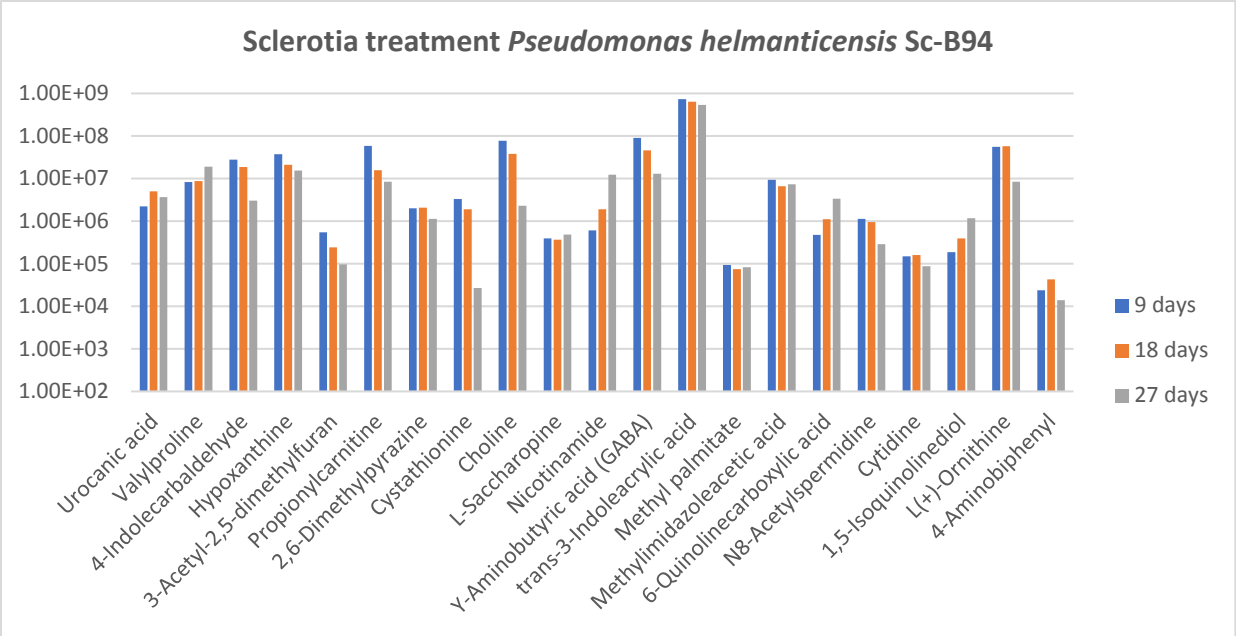


Figure 32. Area of compounds detected from sclerotia exudates stimulated *with Pseudomonas helmanticensis* Sc-B94. Compounds like 2,6-Dimethylpyrazine, L-Saccharopine, Methyl palmitate, Cytidine and 4-Aminobiphenyl had stable area over the experiment duration. Significant decrease in area was notified in 3-Acetly-2,5-dimethylfuran, Propionylcarnitine, Choline and GABA.

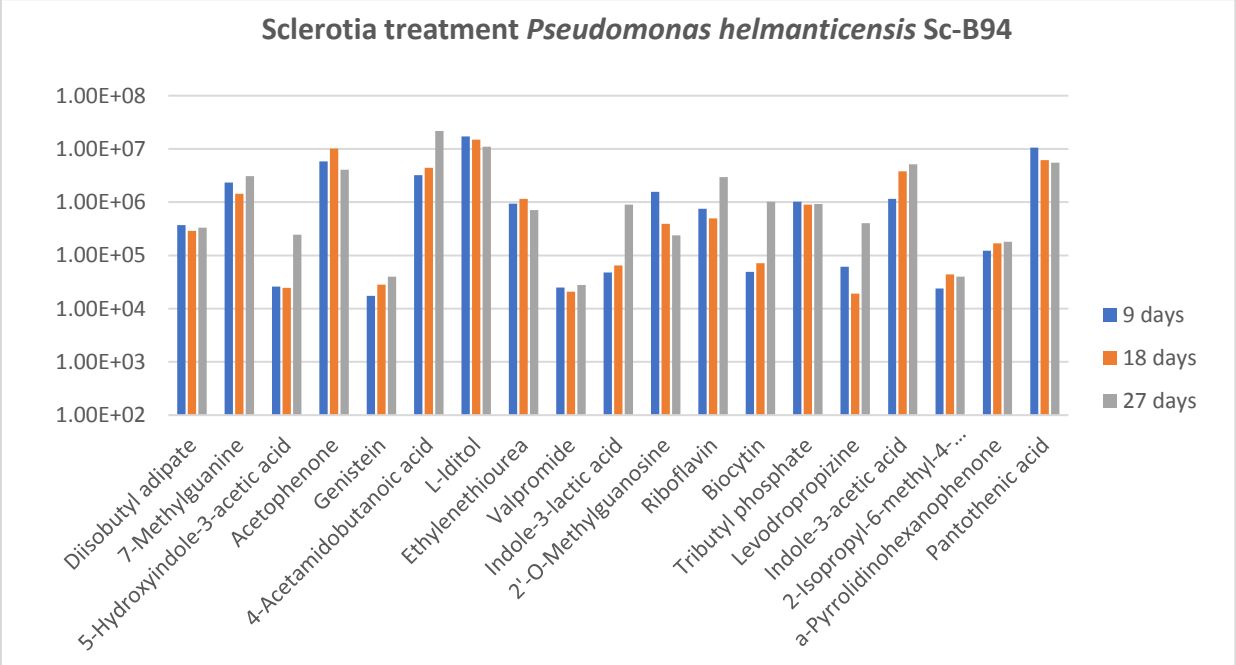


Figure 33. Area of compounds detected from sclerotia exudates stimulated with *Pseudomonas helmanticensis* Sc-B94. Stable compound area over time was measured in case of Diisobutyl adipate, Valpromide and Tributyl phosphate. In the case of 7-Mehtylguanine, Acetophenone, Ethylenethiourea, Riboflavin and Levodropropizine the area was fluctuating over time.

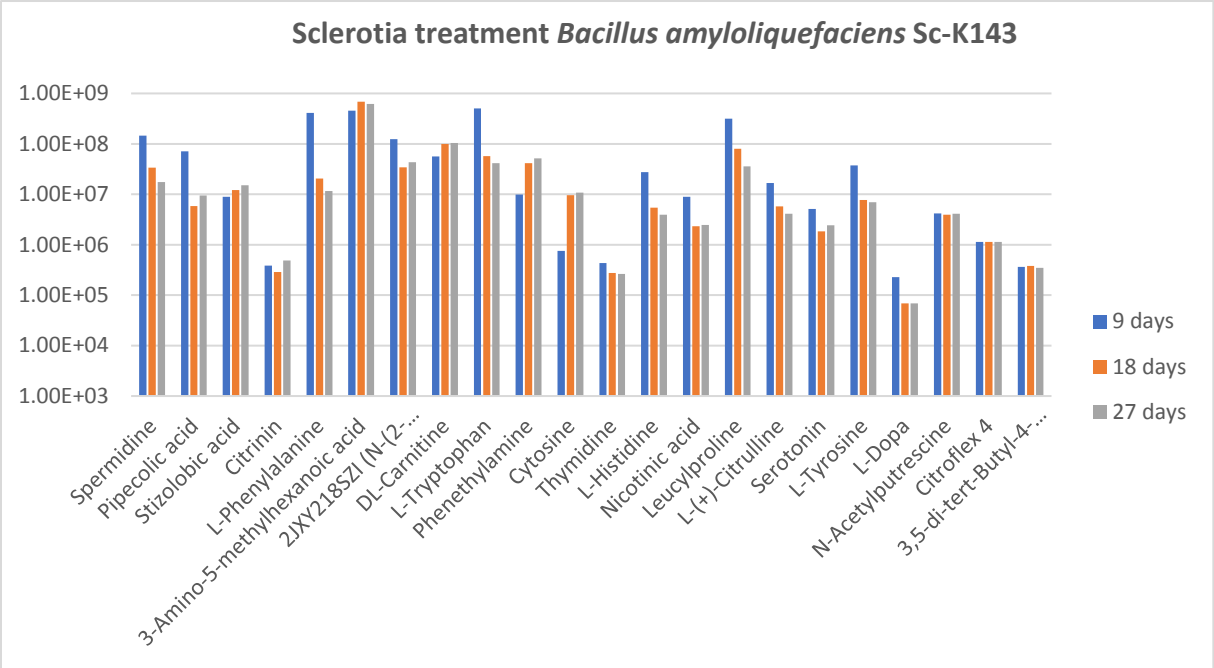


Figure 34. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143. Citrinin, Thymidine, Citroflex 4 and N-acetylputrescine did not have significant changes in area over time. Area decrease could be observed in the case of Spermidine, L-Phenylalanine, L-Histidine, Leucylproline and L-Citrulline.

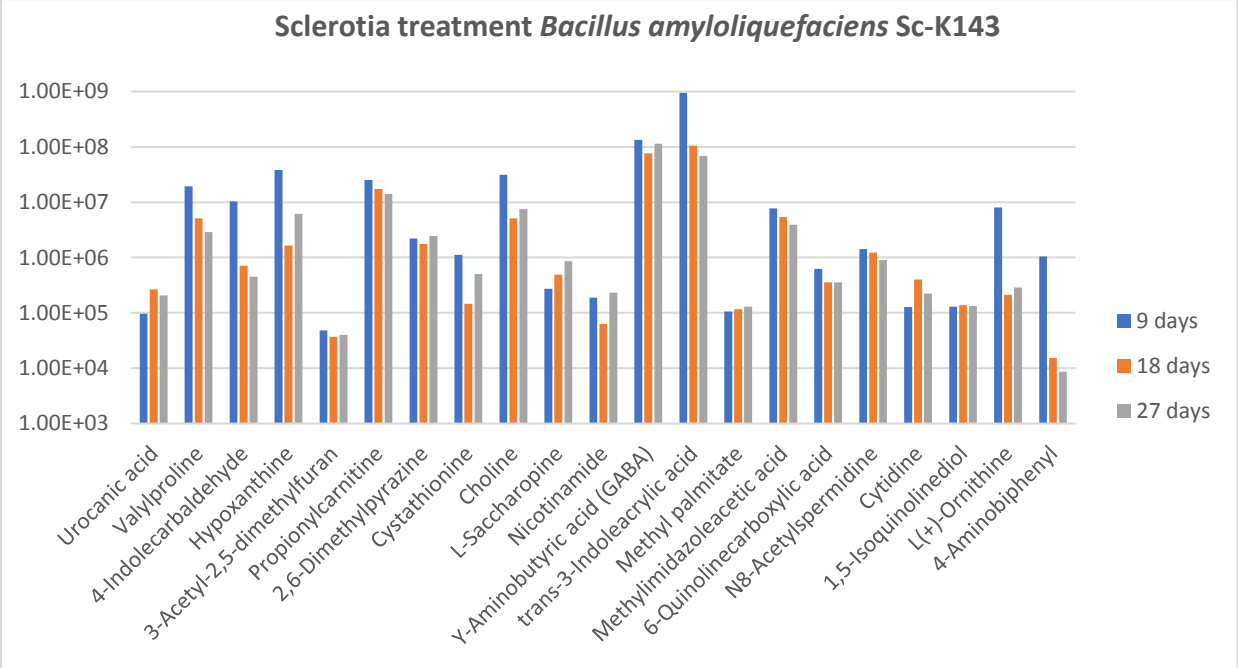


Figure 35. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143. Significant fall in area was observed in case of Valylproline, 4-Aminobiphenyl, 4-Indolecarbaldehyde and trans-3-Indoleacrylic acid. Compounds as N8-Acetylspermidine, 1,5-Isoquinolinediol and Methyl palmitate were notified to have stable area over time.

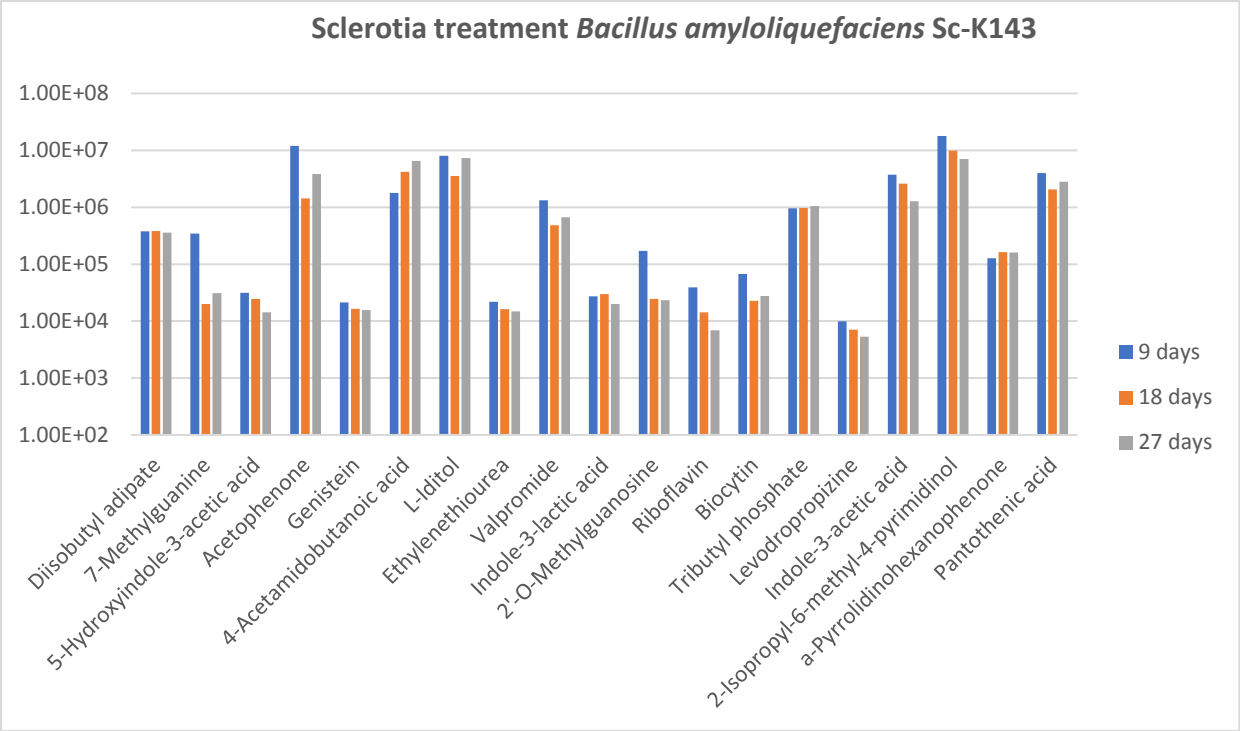


Figure 36. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143. Most of the compounds (Diisobutyl adipate, Genistein, Ethylenethiourea, Tributyl phosphate) were observed to have similar area over time. Levodropropizine and Riboflavin area was in fall during the experiment period.

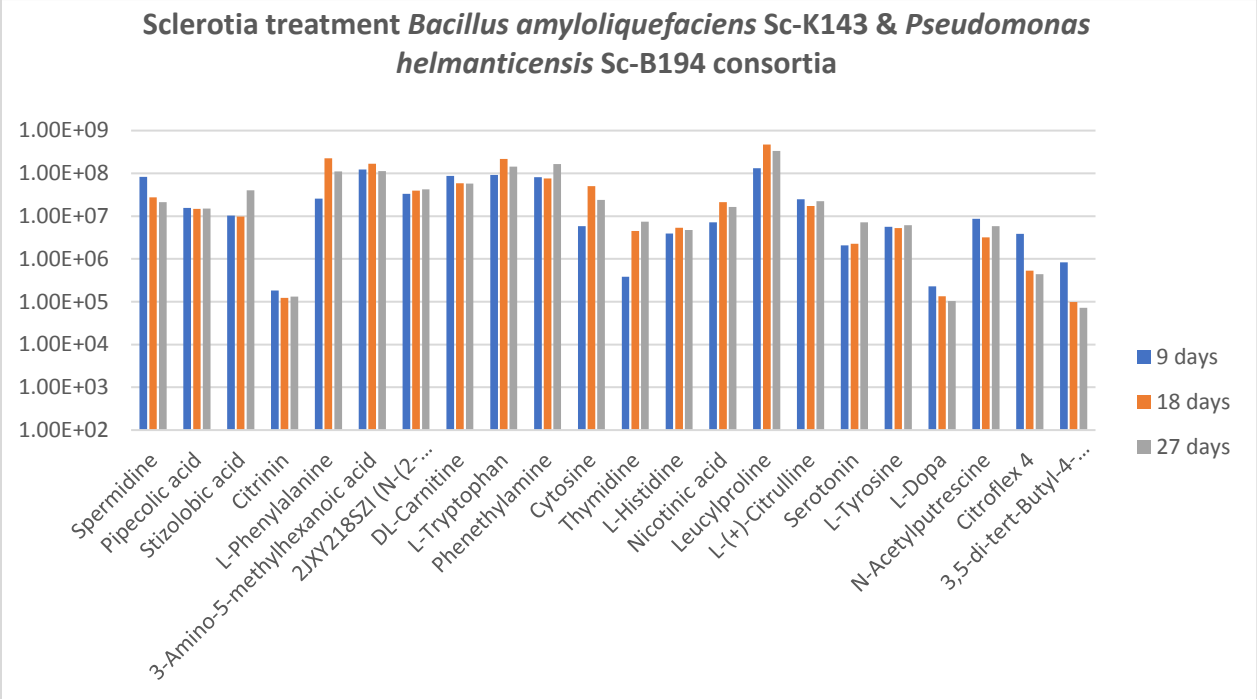


Figure 37. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B194 consortia. Spermidine and L-Dopa were observed to have area in decrease while some of them (Pipecolic acid, Citrinin, L-Histidine, L-Tyrosine) had stable area value through experiment duration.

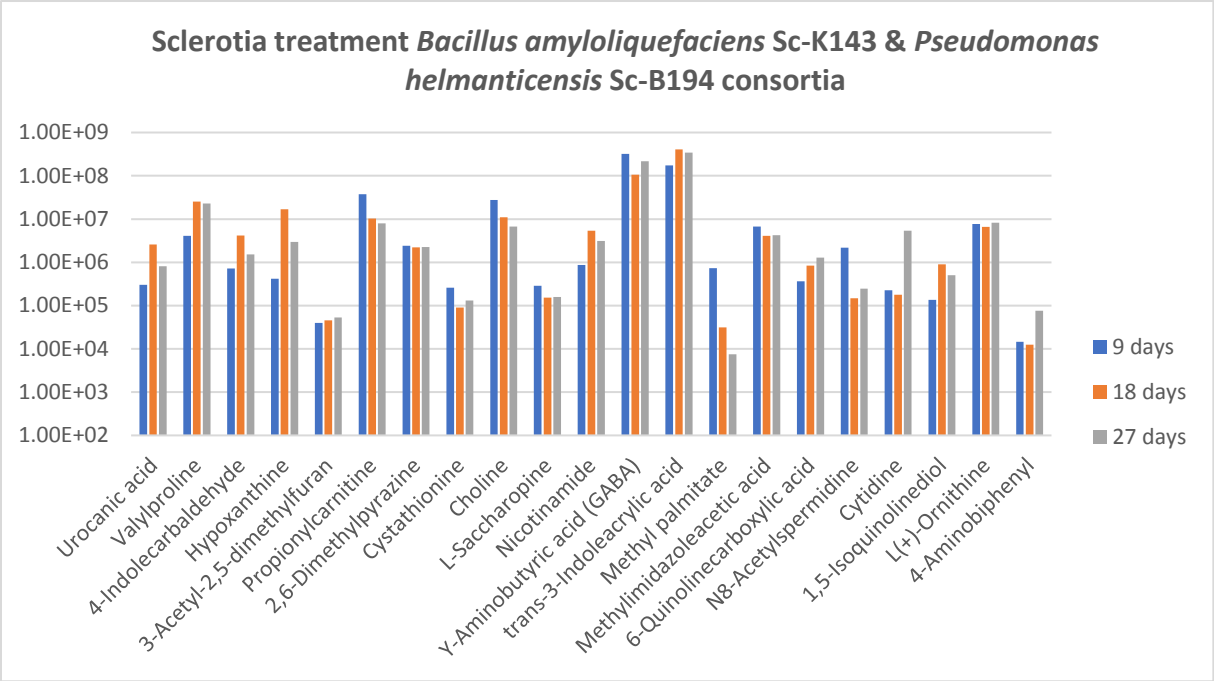


Figure 38. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B194 consortia. In the case of Urocanic acid, 4-Indolecarbaldehyde, Hypoxanthine, Nicotinamide and 1,5-Isoquinolinediol the area increase could be observed until 18th day while this value decreases along the time. Stable compound area was noticed with 3-Acetyl-2,5-dimethylfuran, L-Saccharopine and L-Ornithine.

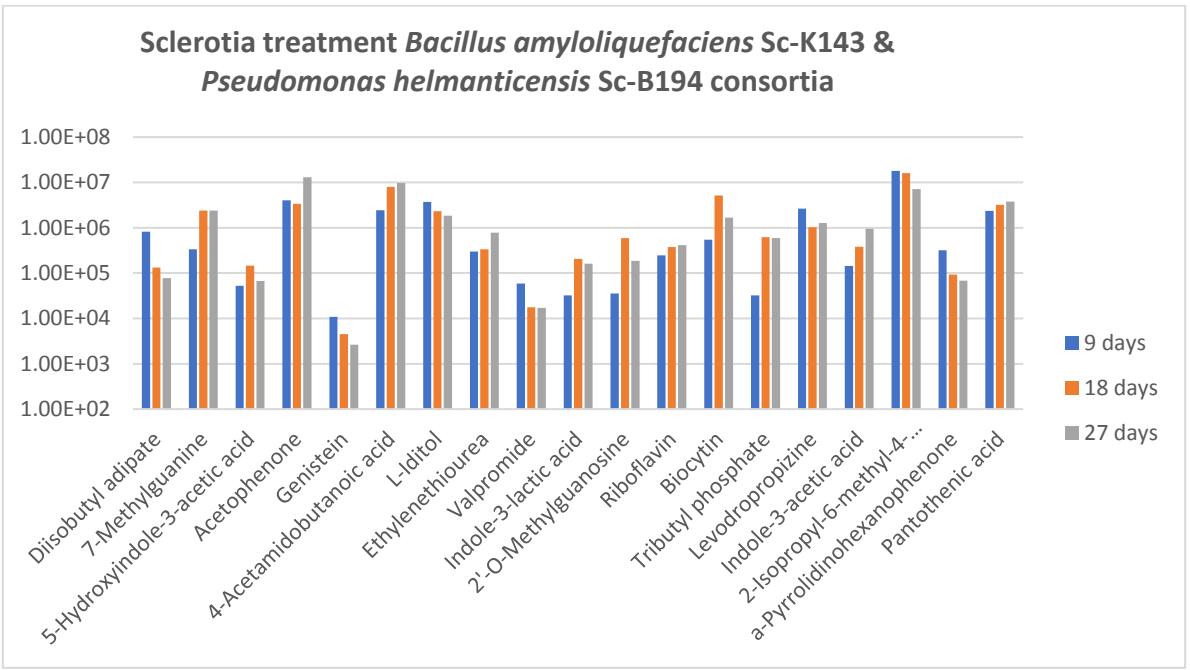


Figure 39. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B194 consortia. Increase in area was observed in the case of 7-Methylguanine, 4-Acetamidobutanoic acid, Ethylenethiourea, Riboflavin, Pantothenic acid and Indole-3-acetic acid. In contrast, Diisobutyl adipate, Genistein and L-Iditol were found in decreased concentration over time.

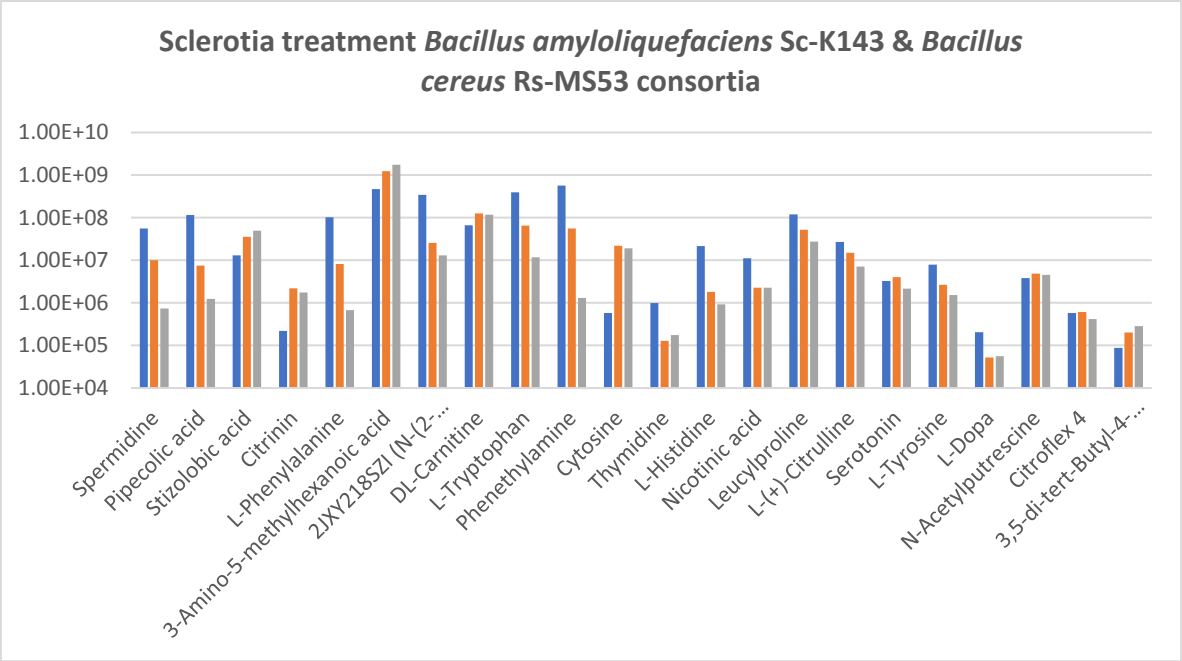


Figure 40. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143 and *Bacillus cereus* Rs-MS53 consortia. Significant fall in area value was observed in the case of Spermidine, Pipecolic acid, L-Phenylalanine, L-Tryptophan, Phenetylamine, L-Histidine, Leucylproline and L-Tyrosine. On the contrary, the area of Stizolobic acid, DL-Carnithine, Cytosine and 3-Amino-5-methylhexanoic acid was noticed to rise during the experiment time.

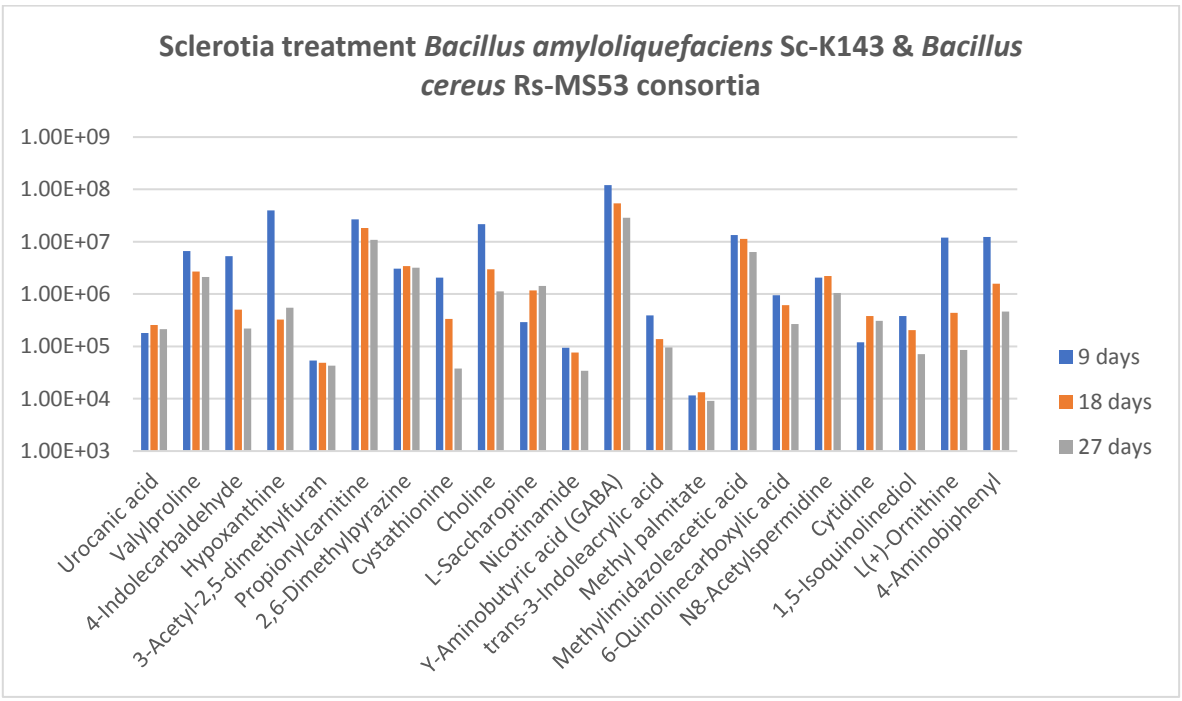


Figure 41. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143 and *Bacillus cereus* Rs-MS53 consortia. Stable area could be observed in the case of Methyl palmitate, 3-acetyl-2,5 dimethylfuran, Urocanic acid and 2,6-Dimethylpyrazine. 4-Indolecarbaldehyde, Hypoxanthine, Choline, L-Ornithine and 4-Aminobiphenyl had significant decrease in area value.

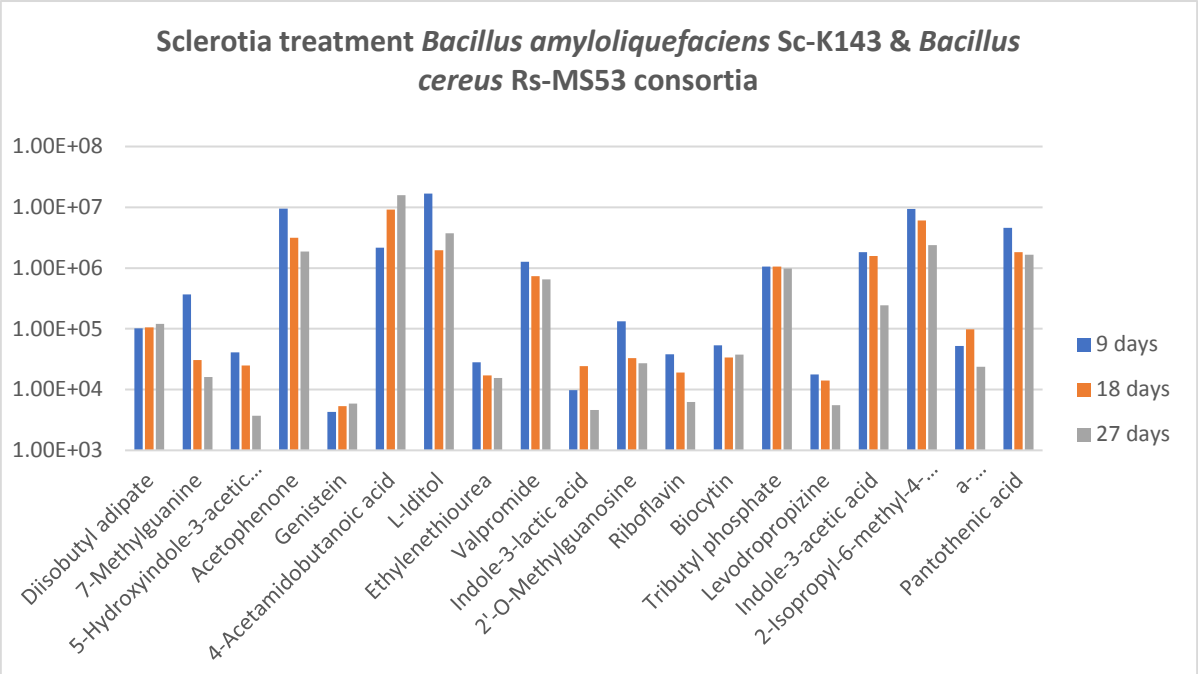


Figure 42. Area of compounds detected from sclerotia exudates stimulated with *Bacillus amyloliquefaciens* Sc-K143 and *Bacillus cereus* Rs-MS53 consortia. Stable compound area was observed in the case of Genistein and Tributylphosphate. Significant decrease was notified with 7-Methylguanine, Acetophenone, 2-O-Methylguanosine, Indole-3-acetic acid and Pantothenic acid.

4 Discussion

4.1 Characterization of isolates using BOX-PCR

BOX-PCR is a fingerprinting method used to evaluate the dispersion of microbial species or lineages in species, to connect specific genotypes to certain environment conditions or to analyse certain microbial type endemicity (Brusetti L. *et al.,* 2008). Precisely, in this thesis there was an intention to characterize bacterial isolates and analyse similarity between them within one species. 4 sub-groups of isolates were identified to be similar in the group of *Bacillus amyloliquefaciens spp.* The whole group of isolates of *B. cereus spp.* had shown to share same fingerprint while the rest of *Bacillus spp.* and *Pseudomonas spp*. groups did not display similarity between isolates. These results will help in the future trials which will be based on the choice of bacteria for further analysis. BOX-PCR effectiveness in a genetic study of various bacteria had been examined by many researchers. Lanoot *et al.* (2004) used BOX-PCR as a fingerprinting analysis to investigate 473 *Streptomyces* species. Marques *et al.* (2008) obtained results which show that this powerful tool can discriminate between 120 bacterial strains of the *Pseudomonas syringae - P. viridiflava* group at a level of species. BOX-PCR method was also used to reveal differences in the quantity and bacterial repetitive sequence distribution in the clinical isolates of *Pseudomonas aeruginosa* genomes (Wolska *et al.,* 2011). This method detected 38 genetic patterns, among which seven were main genotypes, containing 30 unique patterns and three to eight isolates.

4.2 Volatile assay against *S. sclerotiorum* and *R. solani*

Volatile assays were performed against two plant pathogens, S*. sclerotiorum* and *R. solani* for the investigation of mycelial growth inhibition by *Bacillus spp*. and *Pseudomonas spp.* In this trial, three single isolates and two combinations of bacteria were used. In the case of *S. sclerotiorum,* a combination of *Psuedomonas helmanticensis* Sc-B94 and *Bacillus amyloliquefaciens* Sc-K143 gave the best results and inhibited mycelium growth for 94%. The single application of each of these bacterial strains against the fungi achieved the inhibition of aproximately 90%. Volatile assay against *R. solani* gave even better results. There was a

complete inhibiton of fungal growth of *R. solani* in the cases of single application of *Pseudomonas helmanticensis* Sc-B94 and combination/consortia of *Pseudomonas helmanticensis* Sc-B94 and *Bacillus amyloliquefaciens* Sc-K143. In this case, the combination of two *Bacillus spp*. Rs-MS53 and Sc-K143 achieved a higher inhibition of mycelial growth than against *S. sclerotiorum* (Figure 11). Visualizing these results, it can be concluded that in both cases *Bacillus spp.* and *Pseudomonas spp*. worked well when combined together. Ferreira JH. *et al.,* (1991) stated that among 20 genera of bacteria, *Bacillus spp., Pseudomonas spp. and Streptomyces spp.* are important biological agents and that *Bacillus spp.* are known to produce several antibiotics. The screening with KING'S B confirmed previous results where a combination of bacteria resulted in an increased inhibition of phytopathogenic fungi via mVOCs.

4.3 In vitro dual culture assay with *Fusarium oxysporum*

Referring to the previously described BOX-PCR results, the number of 47 bacterial strains used was reduced to 40 because of the proved strain similarity and affiliation to the same species. Therefore, in order to avoid repetition of results and to save time and costs, 40 isolates were tested for *in vitro* inhibition of the mycelium growth of *F. oxysporum.* The bacteria and fungi were supposed to grow on the same Waksman agar plate. Inhibition of the mycelium growth was corresponding to no growth around bacteria. 25 of 40 bacteria tested gave positive results and inhibited mycelium growth while the rest of them gave no inhibitory effect. 17 bacteria that had the inhibition zone of 20 mm or higher belong to *Bacillus amyloliquefaciens spp.*. From the group of bacterial isolates which inhibited the mycelium growth of fungi for 10 mm or higher, 5 of them are the members of the *Bacillus amyloliquefaciens* group and one of them was *Bacillus aerius.* In their research, Yuan J. *et al.* (2012) showed the results of inhibitory effect of *Bacillus amyloliquefaciens* NJN-6 on *Fusarium* oxysporum f. sp. *cubense.* The plate was divided into two compartments; one for the growth of fungi and another for bacterial growth. The VOCs produced by *Bacillus amyloliquefaciens* NJN-6 were successful in reducing the mycelial growth of *F. oxysporum* as well as in germination of spores. Yuan J. and his colleagues (2012) were following the

experiment for some time and were unable to show that bacterial VOCs completely destroy *F. oxysporum* but they significantly affect the growth of fungal mycelium.

4.4 Antagonistic screening assay against *Fusarium oxysporum*

Unlike the dual culture assay previously described, in this experiment bacterial isolates were tested individually against plant pathogen *F.oxysporum.* Separate NA and PDA plates were used in set up for bacteria and fungi, respectively. The results showed that 10 out of 25 tested bacteria did not have antagonistic potential and could not inhibit mycelial growth at all. 5 of them were most successful and succeeded to inhibit the growth for 15% or more. The rest were less successful and made the inhibition of up to 10% (Figure 15). One of the reasons for the lack of inhibition could be the difference in growth media used. There is a possibility that bacteria do not produce enough strong volatiles when being grown on NA media, unlike the Waksman agar used in the dual culture assay, or that the more effective antifungal compounds are non-volatile.

4.5 Bacterial VOCs production as a response to different nutrients

The aim of this experiment was to obtain insights into the difference of volatilome produced by bacteria in dependence of different nutrient media. Overall, the substantial difference can be noticed between volatilome profiles. It was interesting to see that the nutrients from the Artifical Root Exudate (ARE) media induce the production of acetoin which was mentioned in the work by Ryu C.M. *et al.* (2003). Precisely, acetoin is one of the volatiles produced by *Bacillus spp.* that positively affects the growth of *Arabidopsis thaliana* and is known to be plant growth-promoting factor. It can be noticed that for all growth media mostly produced compounds were alcohols, ketones, acids, sulphur-containing compounds, etc. There is obvious evidence that growth media influence the production of volatilome from different bacteria. In a microbiological research done on four *Lysobacter* strains by Lazazzara V. and colleagues (2017), it was stated that the bacterial volatilome composition and functional properties were affected by the growth substrate on which bacteria were grown. It was also shown that volatilome differences were mainly in connection to metabolic changes instead of formation of biomass. The functional properties and composition of the

bacterial volatilome indicate changes in metabolism in VOC production regarding to available nutrients and growth conditions in the soil.

4.6 Bacterial response to signal molecules produced by fungi

Even though bacteria and fungi are very often found to form complex consortia and to interact in nature, they have almost always been studied individually. However, it is well known that this interaction has important role in food industry, biotechnology as well as in overall ecosystem functioning. Very often these two kingdoms sense each other and either contest or cooperate in nature. There are a lot of researches known about bacterial volatiles and their effect on fungi and protection of plants but it is very little known about fungal volatilome and its role in bacterial-fungal interaction (Frey-Klett *et al.,* 2011). In the conducted experiment, volatiles from *R. solani* and *S. sclerotiorum* were used to stimulate the bacterial isolates. The aim of this stimulation was to investigate if there were changes in the bacterial volatilome after being exposed to 2-methyl-1-butanol, 3-methyl-1-butanol, acetone, acetic acid and 2-butanone as a mixture or as single substance. Very few new volatiles were identified when the bacteria (*Bacillus cereus* Rs-MS53, *Bacillus amyloliquefaciens* Sc-K143 and *Pseudomonas helmanticensis* Sc-B94) were subjected to the mixture of fungal volatiles. In their research, Bitas V. *et al*. (2013) stated that VOCs are known to work either in cooperation or antagonistically with other volatiles, which suggests that under some conditions the effects of one volatile can cancel out the effect of other volatile and cause different phenotypes. Therefore, in the case of stimulating bacteria with the mixture of fungal volatiles, it could be that they cancel out the activity of each other and the result was decreased production of volatile compounds from bacteria. When the isolates were subjected to individual stimulation with fungal metabolites, increased volatilome could be seen in each case. The highest number of volatiles was noticed to be produced after the bacteria were stimulated with acetic acid. Main groups identified in altered volatilomes contained alkanes, alkenes, ketones, alcohols, carboxylic acids, sulphurous- and aromatic compounds. Research done by Schmidt and colleagues (2016) presented that there are some changes in phenotypic responses of soil bacterial strains to fungal volatiles emitted. More

precisely, *Collimonas pratensis* and *Serratia plymuthica*, were shown to manifest changes in their motility to *Fusarium culmorum* produced volatiles. Therefore, this remarkable research shows that fungal volatiles have important role in bacterial-fungal interactions and should be investigated more.

4.7 Stress response by *Sclerotinia sclerotiorum* sclerotia

Using LC-MS, it was possible to determine the content of sclerotia liquid when they are subjected to bacteria. The experiment was followed for 27 days and the liquid from the sclerotia was taken every 9 days. It could be visible that, with the time passing, less liquid could be seen on the plate and some of the sclerotia were starting to germinate and produce mycelium while others looked seriously damaged (Figure 27). 62 different compounds could be observed with some of them having interesting biological characteristics, mostly important in plant metabolism. Among the compounds detected from sclerotia exudates, there is spermidine, which is one of the major polyamines in the plant responsible for cell differentiation, proliferation and programmed cell death (Kusano *et al.,* 2007). Another interesting substance was citrinin, a metabolite revealed to inhibit the growth of *Sclerotinia minor* and other pathogens (Melouk and Akem, 1987). It was also interpreted that citrinin is mycotoxin found in *Aspergillus niveus* and *Penicillium citrinum* (He and Cox, 2016). Measurements revealed that the concentration of citrinin was enhanced with time. Pipecolic acid is a metabolite known to act as a critical regulator of inducible immunity in plants (Navarova *et al.,* 2012) and has so far not been identified in fungi. Phenethylamine is produced from certain fungi and bacteria and has a potential anti-microbial activity against some pathogenic strains of *E. coli* (Terence, 1977). Aliferis and Jabaji (2010) analysed sclerotia exudates of *Rhizoctonia solani* and stated that these exudates contain various phenolics, amino acids, carboxylic acids, fatty acids and carbohydrates. They believe that such metabolites give significant contribution to fungal physiology but are also potential bioactive source containing moderate toxicity.

5 Conclusion

Bacteria produce a wide range of volatile compounds which have antifungal activity and protect plants and crops from their enemies. These compounds are not just able to enhance plant growth, but also to promote fungal mycelium growth inhibition. Since the demand for sustainable plant protection is increasing, there is a need for improved biologicals that make use of microbial volatiles. In this study, the knowledge about microbial volatile organic compounds and their specific formation under different conditions is enhanced. The outcome displayed differences between volatilome profiles and compositions of isolates regarding to growth media. We can conclude that nutrients from growth media highly influence the volatile production of bacteria. The part of this study focused also on fungal produced metabolites which were used to stimulate bacterial isolates, *Bacillus amlyoliquefaciens* Sc-K143, *Bacillus cereus* Rs-MS53 and *Pseudomonas helmanticensis* Sc-B94, in the form of mixture and individually. Analysis with GC-MS confirmed altered volatilome profiles and production of novel compounds from bacterial isolates in both cases. This indicates that compounds produced by fungi can influence the volatilome of bacterial isolates. As the last experiment in this study, the liquid produced from *S. sclerotiorum* sclerotia was analysed with LC-MS after they were exposed to stress by bacteria. Various compounds were detected and few of them have important biological functions connected to fungi metabolism. To conclude, this study demonstrated that microbial volatile organic compounds are promising in the fight against phytopathogenic fungi, plant protection and application in agriculture in general.

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7 Appendix

7.1 List of abbreviations

µ	micro
cm	centimeter
%	percent
mM	milimolar
ml	mililiter
g	gram
min	minute
HS-SPME GC/MS	Headspace-Solid phase microextraction Gas chromatography/Mass spectrometry
UHPLC-MS	Ultra high performance liquid chromatography
HCl	Hydrogen chloride
SDS	Sodium dodecyl sulfate
Ph1	2,4-diacetylphloroglucinol
PCA	Phenazine1-carboxylic acid
WA	Water agar
ARE	Artificial Root Exudate
TSA	Tryptic Soy Agar
RI	Retention Index
ISR	Induced systemic resistence
EC	Electrical conductivity
HCN	Hydrogen cyanide
mVOC	Microbial volatile organic compound

7.2 List of figures

7.3 List of tables