Influence of volatile metabolites of the rhizobacterium *Serratia plymuthica* 4Rx13 on soilborne fungi

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Dedicated to my Father

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Abstract

Biologically, the rhizosphere is the most influenced part of the soil. In the last decades, microbial interactions in the rhizosphere and plant-microbial interactions have been extensively studied. These studies revealed that the plant growth promoting rhizobacteria (PGPR) can produce volatile secondary metabolites that function like antibiotics and inhibit or kill many phytopathogenic fungi. The mechanisms, by which bacterial volatiles inhibit the fungal growth or the fungal reaction to these volatiles, have been scarcely studied. The focus of the present study was to understand this interaction more intensively. A better understanding of these bacteria-fungi interactions might offer a possibility to find biological control agents.

Studies revealed that the rhizobacterium *Serratia plymuthica* 4Rx13 is able to inhibit the growth of the phytopathogenic fungi *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Phoma eupyrena*, and the non-pathogenic fungus *Neurospora crassa*. Rhizobacterial volatile metabolites were responsible for this effect. Here the physiological and biochemical response of these fungi to the rhizobacterial volatiles were investigated. Anti-oxidants systems by which fungi protect themselves from bacterial volatiles of *S. plymuthica* 4Rx13 were shown to be important. As an *in-vitro* test system, split Petri dishes were used allowing only volatile components to pass from one side to another to exclude direct contact between the fungi and the bacterium.

The initial physiological experiments indicated that in response to the bacterial volatiles, fungi showed pigmentation and growth inhibitions. The analysis of oxidative stress parameters revealed in presence of bacterial volatiles, enzymes of the anti-oxidant system like the superoxide dismutase, catalase, and laccase were activated indicating the presence of reactive oxygen species (ROS) in fungal cells. These results were supported by findings of elevated non-enzymatic lipid peroxidation in the fungal cell membrane suggesting that the fungal membrane integrity was affected. Together these observations indicate clearly oxidative stress symptoms in fungi.

The analysis of volatiles emitted by *S. plymuthica* 4Rx13 confirmed the already known spectrum, however, upon co-cultivation with the fungus *S. sclerotiorum*, the production of an additional volatile, 1-tridecanol, was observed, which was either released by the bacterium or the fungus. The fungus itself produced only two volatiles, most noticeable was the labda-8 (20), 12, 14-triene.

In addition, the emission and influence of ammonia emitted by *S. plymuthica* 4Rx13 was investigated. Ammonia indicated also oxidative stress reactions since the fungal growth was inhibited and the fungal membrane integrity as well as respective enzymes activities were affected.

I List of contents I

II List of figures VI

III List of tables X

IV List of abbreviations XI

I List of contents

1 Introduction 1

1.1 Rhizosphere and rhizodeposition 1

1.2 Root exudates 1

1.3 Key relationships in the rhizosphere 3

1.4 Beneficial plant growth promoting rhizobacteria and their mechanism of actions 3

1.5 Volatile-mediated antagonism between bacteria and fungi in the rhizosphere 4

1.6 Aim of the work 5

1.6.1 Objectives 5

1.6.2 Selected microorganisms 7

1.6.2.1 Selected bacterium 7

1.6.2.2 Selected fungal candidates 7

1.6.3 Schematic presentation of work 8

2 Material and methods 10

2.1 Microbial strains used for the experiments 10

2.2 Material 10

2.3 Maintainance of fungi 10

2.3.1 Long-term storage of fungi 10
2.3.2 Short-term maintainance of fungi 10

2.4 Maintainance of the bacterium 11

2.4.1 Long-term maintainance of the bacterium 11
2.4.2 Short-term storageof the bacterium 12

2.5 Bacterial preculture 12

2.6 Dual culture test 13

2.7 Experiments 14

2.7.1 Influence of bacterial volatiles on fungal morphology 14
2.7.2 Influence of bacterial volatiles on fungal radial growth 14
2.7.3 Influence of bacterial volatiles on fungal biomass production 14
2.7.4 Influence of fungal volatiles on bacterial growth 14
2.7.5 Biochemical assays 14

2.7.5.1 Quantification of non-enzymatic lipidperoxidation 14
2.7.5.2 Superoxide dismutase assay 16
2.7.5.3 Catalase assay 17
2.7.5.4 Intracellular laccase 17
2.7.5.5 Determination of protein concentrations 18

2.7.6 Adaptation experiment 18

2.7.7 Qualitatave Analysis of emitted fungal and bacterial volatiles in mono- and dual cultures 20

2.7.8 Quantification of ammonia produced by *S. plymuthica* 4Rx13 21

2.7.9 Determination of the pH value of fungal growth media 22

2.7.10 Influence of synthetic ammonia on fungal radial growth and biomass production 23

2.7.11 Influence of synthetic ammonia on fungal membrane integrity and enzyme activities indicating oxidative stress 23

2.7.12 Influence of 0.85 % o-phosphoric acid on the ammonia concentration in dual culture tests 24

2.8 Statistical analysis 25

3 Results 26

3.1 Setting up the test system of co-cultivation 26

3.2 Influence of volatile metabolites of *Serratia plymuthica* 4Rx13 on fungal growth and morphology 29

3.2.1 Fungal growth 29

3.2.1.1 Radial growth 29

3.2.1.2 Dry biomass 31

3.2.2 Fungal morphology 32

3.3 Influence of volatile metabolites of *Serratia plymuthica* 4Rx13 on membrane integrity and enzyme activities indicating oxidative stress 33

3.3.1 Non-enzymatic lipidperoxidation 33

3.3.2 Acitvity of the fungal superoxide dismutase (SOD) 34

3.3.3 Activity of the fungal catalase 35

3.3.4 Activity of the intracellular fungal laccases 36

3.3.5 Summary 37

3.4 Influence of fungal volatiles on the growth of *Serratia plymuthica* 4Rx13 38

3.5 Analysis of bacterial and fungal volatile metabolites emitted from mono- and dual- cultures 39

3.5.1 Volatile organic compounds 39

3.5.1.1 VOC emission by *Serratia plymuthica* 4Rx13 39
3.5.1.2 VOC emission by *Sclerotinia sclerotiorum* 42
3.5.1.3 VOC emission by *Serratia plymuthica* 4Rx13 and *Sclerotinia sclerotiorum* in dual culture 43

3.5.2 Ammonia production by the bacterium *Serratia plymuthica* 4Rx13 44

3.6 Influence of Ammonia on assay condition 45

3.6.1 Ammonia as a constituent of the bacterial volatile blend 45

3.6.1.1 pH values of the fungal medium in a bacterial monoculture 45
3.6.1.2 pH values of the fungal medium in a fungal monoculture 46
3.6.1.3 pH values of the fungal medium in a dual culture 46

3.6.2 Synthetic ammonia applied as a single compound 47

3.6.2.1 pH values of the fungal medium in absence of fungi 47
3.6.2.2 pH values of the fungal medium in presence of fungi 48

3.6.3 Summary 49

3.7 Effects of synthetic Ammonia on fungi 49

3.7.1 Preliminary experiments 49

3.7.1.1 Effect of synthetic ammonia on fungal growth 49
3.7.1.2 Influence of ortho-phosphoric acid on the inhibitory effect of ammonia 51

3.7.1.3 Conclusion 52

3.7.2 Influence of synthetic ammonia on fungal membrane integrity and enzyme activities indicating oxidative stress 53
3.7.2.1 Non-enzymatic lipidperoxidation (LPRX) 53
3.7.2.2 Activity of the fungal superoxide dismutase 54
3.7.2.3 Activity of the fungal catalase 55
3.7.2.4 Activity of the fungal intracellular laccase 56
3.7.2.5 Summary 56

3.8 Adaptation experiment 57

3.8.1 Initial exposure of the fungus *Sclerotinia sclerotiorum* to bacterial volatiles and hydroquinone (I) 57

3.8.2 Re-exposure of the fungus *Sclerotinia sclerotiorum* to bacterial volatiles and hydroquinone (II) 58

3.8.3 Summary of the adaptation experiment 60

4 Discussion 62

4.1 The test system 62

4.2 Growth and morphology 63

4.3 Oxidative stress 65

4.4 Volatile emission 69

4.4.1 Volatile organic compound emission 69

4.4.2 Inorganic compound ammonia produced by *Serratia plymuthica* 4Rx13 70

4.5 Adaptation experiment 72

4 Conclusion 74

5 List of literatures 75

6 Supplement 94

Curriculum vitae 119

Declaration 120

II List of figures

Figure 1: Schematic presentation of a root showing the six major regions of rhizodeposits. 2

Figure 2: Complex interactions mediated by root exudates. 2

Figure 3: Inorganic and organic bacterial volatiles. 4

Figure 4: Schematic presentation of oxidative stress situation and anti-oxidants in fungi. 6

Figure 5: Disease formation by phytopathogenic and nonpathogenic fungi. 8

Figure 6: Schematic presentation of objectives. 9

Figure 7: Schematic presentation of fungal short-term maintenance. 11

Figure 8: Schematic presentation of bacterial short-term maintenance. 12

Figure 9: Schematic presentation of dual culture and monoculture. 13

Figure 10: Schematic presentation of using cellophane membrane in test systems. 13

Figure 11: Mechanism of non-enyzmatic lipidperoxidation. 15

Figure 12: Schematic presentation of the adaptation experiment. 19

Figure 13: Scheme of the headspace system. 21

Figure 14: Split Petri dish showing a slot to insert a microliter syringe. 21

Figure 15: Quantification of ammonia using Quantofix ammonia test sticks in presence of bacteria and different ammonia solutions. 22

Figure 16: Measurements of the pH value in presence of an ammonia solution (A) or *Serratia plymuthica* 4Rx13 (B). 23

Figure 17: Setup showing *Sclerotinia sclerotiorum* exposed to different concentrations of synthetic ammonia. 23

Figure 18: Impact of o-phosphoric acid on fungal growth inhibition due to ammonia. 24

Figure 19: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of selected fungi. 27

Figure 20: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the biomass of selected fungi. 27

Figure 21: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the catalase activity of the fungus *Sclerotinia sclerotiorum.* 28

Figure 22: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the pH of the fungal media of *Sclerotinia sclerotiorum.* 28

Figure 23: Utmost influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of selected fungi. 29

Figure 24: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of selected fungi. 30

Figure 25: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the biomass of selected fungi. 31

Figure 26: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the coloration of the fungi *Rhizoctonia solani* and *Phoma eupyrena*. 32

Figure 27: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the cell membrane integrity (non-enzymatic lipidperoxidation) of selected fungi. 34

Figure 28: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the superoxide dismutase activity of selected fungi. 35

Figure 29: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the cell (catalase activity) of selected fungi. 36

Figure 30: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the intracellular laccase activity of selected fungi. 37

Figure 31: Influence of fungal volatiles on the growth of *Serratia plymuthica* 4Rx13. 38

Figure 32: VOC emission of *Serratia plymuthica* 4Rx13 at 30 °C. 40

Figure 33: VOC emission of *Serratia plymuthica* 4Rx13 at 20 °C. 41

Figure 34: VOC emission of *Sclerotinia sclerotiorum* at 20 °C. 42

Figure 35: VOC emission of *Serratia plymuthica* 4Rx13 and *Sclerotinia sclerotiorum* at 20 °C. 43

Figure 36: Influence of volatiles emitted by *Serratia plymuthica* 4Rx13 on the pH value of the fungal growth medium. 45

Figure 37: pH values of the fungal medium in fungal monocultures. 46

Figure 38: Influence of volatiles emitted by *Serratia plymuthica* 4Rx13 on the pH value of the fungal growth medium during co-cultivation with selected fungi. 47

Figure 39: Influence of ammonia on pH value of the fungal medium. 48

Figure 40: Influence of 15 mM ammonia on pH value of the fungal media in presence of fungal mycelium. 48

Figure 41: Influence of synthetic ammonia on the radial growth and biomass production of *Sclerotinia sclerotiorum*. 50

Figure 42: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 and synthetic ammonia on the growth of *Sclerotinia sclerotiorum* and pH value of the fungal media in presence/absence of o-phosphoric acid. 52

Figure 43: Influence of ammonia on the non-enzymatic lipid peroxidation in *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in comparison to effect of volatile metabolites of *Serratia plymuthica* 4Rx13. 53

Figure 44: Influence of ammonia on the superoxide dismutase activity in *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in comparison to effect of volatile metabolites of *Serratia plymuthica* 4Rx13. 54

Figure 45: Influence of ammonia on the cell (catalase activity) in *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in comparison to effect of volatile metabolites of *Serratia plymuthica* 4Rx13. 55

Figure 46: Influence of ammonia on the intracellular laccase activity in *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in comparison to effect of volatile metabolites of *Serratia plymuthica* 4Rx13. 56

Figure 47: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 and hydroquinone on the pH of fungal media and the radial growth, biomass production, and laccase activity in *Sclerotinia sclerotiorum*. 58

Figure 48: Influence of bacterial volatiles of *Serratia plymuthica* 4Rx13 and hydroquinone on pH value of the fungal medium and the radial growth, biomass production, and laccase activity in *Sclerotinia sclerotiorum* after re-exposure. 59

Figure 49: Comparison of DNH (top) and L-dopa (bottom) melanin synthesis pathways. 65

Figure 50: Cooperative SOD/Cat action for ROS detoxification. 66

Figure 51: The typical structure of laccase. 68

III List of tables

Table 1: Qualitative analysis of main VOCs emitted by *Serratia plymuthica* 4Rx13. 41

Table 2: Qualitative analysis of main VOCs emitted by *Sclerotinia sclerotiorum*. 42

Table 3: Qualitative analysis of main VOCs emitted in a dual culture of the bacterium *Serratia plymuthica* 4Rx13 and the fungus *Sclerotinia sclerotiorum.* 44

Table 4: Initial and re-exposure: overview on the impact of bacterial volatiles of *Serratia plymuthica* 4Rx13 and hydroquinone on radial growth, biomass production, and laccase activity in *Sclerotinia sclerotiorum.* 61

IV List of abbreviations

%	percent
°C	degree Celsius
x g	gravity-force
&	and
+/-	plus/minus
Aqua dest.	distilled water
CAT	catalase
CFU_i	initial colony forming units
cm	centimetre
EDTA	ethylenediaminetetraacetate
e. g.	exempli gratia
et al.	et alia (lat. and others)
g	gram
GC-MS	gas chromatography-mass spectrometer
h	hours
HCl	hydrogen chloride
HMW	high molecular weight
L	litre
LMW	low molecular weight
M	molar
$\mu E\ m^{-2}\ s^{-1}$	microeinsteins per second per square meter
mg	milligram

min	minute
µkat	microkatal
µl	microlitre
mL	millilitre
mM	millimolar
mm	millimeter
NA	Nutrient agar
nkat	nanokatal
nLprx	non-enzymatic lipid peroxidation
nm	nanometre
nmol	nanomole
OD_{600}	optical density by 600 nm wave length
pH	*potentia Hydrogenii*
ROS	reactive oxygen species
s	seconds
SA	Sabouraud-agar
SOD	superoxide dismutases
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
VOC	volatile organic compound
w/v	weight/volume

1 Introduction

1.1 Rhizosphere and rhizodeposition

The soil is dwelled by extremely diverse communities of macro- and microorganisms (**1**). The rhizosphere represents the most active portion of the soil boasting an intense biological, physical, and chemical activity influenced by compounds secreted by the root and microorganisms that feed on these exudates (**2, 3**). In 1904, the German agronomist and plant physiologist Lorenz Hiltner first coined the term "**rhizosphere**" to describe the plant-root interface, a word that is originated in part from the greek word "rhiza", that means root (**4, 5**). Hiltner described the rhizosphere as the area around a plant root that is inhabited by a unique population of microorganisms (**4**). Today it is defined as the narrowest part of the root where microorganisms are influenced by the root (**1**). The rhizosphere is subdivided into two parts, endo-rhizosphere (root cortex, epidermis, and root-hair) and the ecto-rhizosphere (**6**).

Newman examined a variety of plant species and estimated that roots can release between 10 and 250 mg carbon/g roots or about 10-40 % of their total photosynthetically fixed carbon (**7**). The carbon is released in both organic and inorganic forms. The organic compounds, having the most varied composition, might influence the chemical, physical, and biological processes in the rhizosphere (**8**). The composition and amount of the released compounds are determined by many factors. These factors include plant type, climactic conditions, insect herbivory, nutrient deficiency or toxicity, and the chemical, physical, and biological properties of the surrounding soil (**9)**. The root products exudated into the surrounding soil are generally called **rhizodeposits** (Figure 1).

1.2 Root exudates

Root exudates include both secretions like ions, free oxygen, water, enzymes, mucilage, and a diverse array of carbon containing primary and secondary metabolites as well as diffusates. The secretions are released actively while diffusates are released passively (**10, 11**). The organic compounds released through these processes can be further divided into high and low molecular weight compounds (HMW and LMW, respectively) (**10**). HMW exudates include mucilages (polysaccharides) and proteins that make up the majority of carbon released from the root. The list of specific LMW compounds is very long including organic acids, amino acids, sugar, phenolics, and other metabolites (**12**). These compounds comprise the majority root-exudates (**12**). They are generally easily utilized by the microbes (**13**).

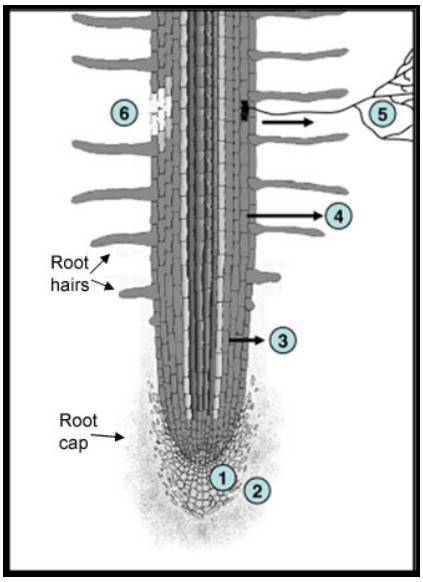

Figure 1: Schematic presentation of a root showing the six major regions of rhizodeposits (9).

1.Loss of cap and border cells, **2**.Loss of insoluble mucilage, **3**.Loss of soluble root exudates, **4**.Loss of volatile organic carbon, **5**.Loss of carbon to symbionts, **6**.Loss of carbon due to death and lyses of root epidermal and cortical cells.

Root exudates may act as chemical signals to attract symbiotic partners (e.g. rhizobia and legumes) or promote beneficial microbial colonization on root surfaces (e.g. *Bacillus subtilis*, *Pseudomonas florescence*). This may facilitate the acquisition of nutrients (e.g. Fe and P) and may protect the plant from invasiveness (e.g. allelopathy) or herbivory. Very little is known about mechanisms how LMW compounds influence rhizosphere processes (Figure 2) (**10-12**).

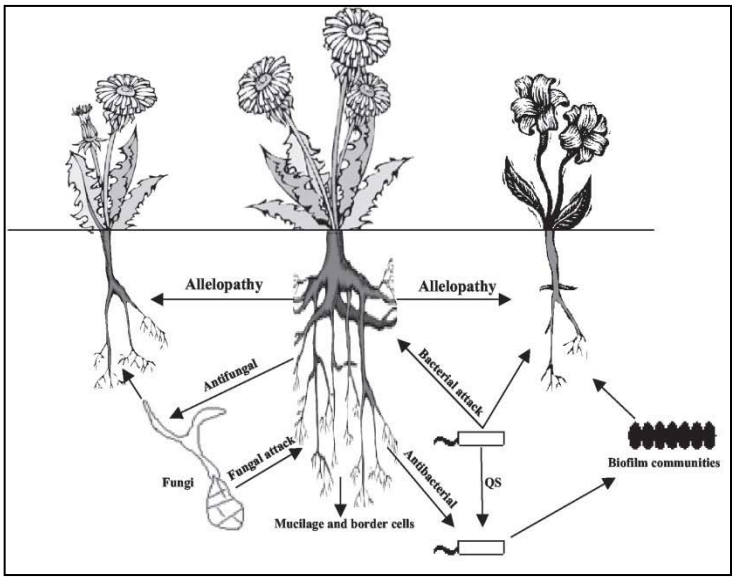

Figure 2: Complex interactions mediated by root exudates (12).

1.3 Key relationships in the rhizosphere

Root exudates make the rhizosphere a desirable niche for microbial communities to proliferate (**13**). These microbes compete for the nutrients, where some are more successful than others. These microbes interact with each other as well as with the plants. Bacteria and fungi are the major inhabitants of the soil rhizosphere, with either beneficial or deleterious interactions (**14, 15**). The relationship between bacteria and plants can be mutualistic (*Rhizobium* sp. and legume etc.), beneficial or harmful. Plant growth promoting bacteria (*Pseudomonas* sp.) can inhibit the growth of harmful bacteria (*Agrobacterium* sp.) or fungi (*Sclerotinia sclerotiorum* etc.). The relationship between fungi and plants can be also mutualistic (mycorrhizal fungi associations), beneficial or harmful (**16**). Plant growth promoting fungi (*Trichoderma* sp.) e.g. inhibit the growth of the harmful fungus *S. sclerotiorum*. These plant growth promoting microorganisms can directly or indirectly promote the plant growth (**16**).

1.4 Beneficial plant growth promoting rhizobacteria and their mechanism of actions

Plant growth promoting rhizobacteria (PGPR) are naturally occurring bacteria that colonize in the rhizosphere and support plant growth. When these bacteria were artificially applied to the root, they enhanced the growth and controlled plant diseases (**17, 18**). In addition, substances produced by these bacteria can improve the interaction between PGPR and other microorganisms, e.g. many PGPRs deal with biotic and abiotic stresses by producing secondary metabolites that are useful in competition and cooperation (**19, 20**). These bacteria may also produce secondary metabolites that are volatile and organic or inorganic in nature (**20-23**). Till now 356 bacterial species are known to produce volatile organic compounds (VOCs) and rhizobacteria are known to emit 209 different volatiles (**21**).

The volatiles produced by the rhizobacteria are the products or by-products of metabolic pathways. Chemically, these compounds are hydrocarbons, aliphatic alcohols, ketones, or acids in nature (**21**). Many bacterial volatiles are produced by catabolic pathways including glycolysis, proteolysis, and lipolysis but also specific biosynthesis is also required to produce terpenes, phenylpropanes, N- and S-containing compounds (Figure 3) (**22-24**).

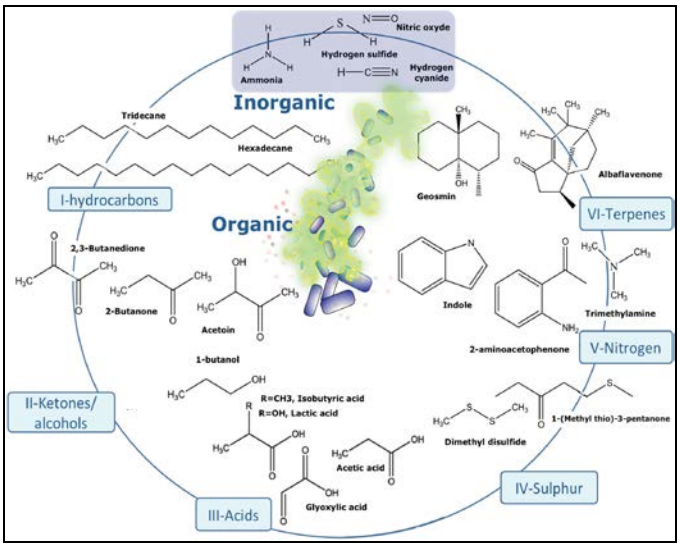

Figure 3: Inorganic and organic bacterial volatiles (24).

These rhizobacterial volatile compounds have a low molecular weight, high vapor pressure and low boiling point and they may affect other organisms of the same habitat (**25-27**). Many volatiles function as signal molecules or antibiotics (**28, 29**). They interact with plant-pathogenic microorganisms and host plants. For example, the PGPRs *Bacillus subtilis* and *B. amyloliquefaciens* produce acetoin and 2, 3-butanediol that trigger the growth of the plant *Arabidopsis thaliana* (**25**). The VOCs of *Pseudomonas* and *Serratia* strains can inhibit the growth of different microorganisms e.g. bacteria, fungi, nematodes, and *Drosophila* (**30, 26-28**) and, subsequently, support the growth of the plants (**26, 31-33**).

1.5 Volatile-mediated antagonisms between bacteria and fungi in the rhizosphere

In the rhizosphere, PGPR can inhibit the fungal growth (26). By antagonising phytopathogenic fungi, bacteria obtain plant exudates as well as reduce competition for those exudates (**26**).

The negative influence of bacterial volatiles on growth and sporulation of soil-borne fungi was also reported in the literature. It has been observed that the bacterial volatiles can efficiently repel phytopathogenic fungi (**34**). McCain (1966) presented that volatiles produced by *Streptomyces griseus* induced early sclerotia formation in *Sclerotium cepivorum* and *Rhizoctonia solani* (**35**). In another investigation, from 1.018 randomly selected bacterial strains, 32 % turned out to produce fungistatic volatiles. Bacterial species like *Burkholderia*, *Pseudomonas*, *Serratia*, *Xanthomonas*, *Pectobacterium*, and *Agrobacterium* species displayed a potent antifungal activity (**36**). Bacteria isolated from canola and soybean plants produced antifungal VOCs, which inhibited sclerotia and ascospores germination, as well as

mycelial growth of *Sclerotinia sclerotiorum* in *in vitro* and soil test systems (**37**). Under laboratory conditions, *Bacillus subtilis* inhibited the growth of different phytopathogenic fungi like *R. solani*, *Pythium ultimum*, *Botrytis cinerea*, *Fusarium avenacerum*, *F. solani*, *F. culmorum, S.sclerotiorum, Alternaria brassicola,* and *Leptosphaeria maculans* (**38**). The inhibition of the growth of *R. solani* by different bacterial species like *Serratia plymuthica, Stenotrophomonas maltophilia, S. rhizophila, Pseudomonas fluorescens*, and *P. trivialis* was also observed (**30**). The growth inhibition by 40% or more of *Microdochium bolleyi*, *Paecilomyces carneus, Phoma betae, S. sclerotiorum* or other fungal species by bacterial isolates was also demonstrated (**26**). All these reports indicated that volatile-based bacteria-fungi interactions are species specific. Possible reasons for specific interactions might be that (1) different fungi may respond to different components of the volatile mixture, or (2) the reaction sites of the fungi may be different or (3) the fungi might possess different abilities to cope with and/or to detoxify the volatile metabolites (**30**).

1.6 Aim of the work

1.6.1 Objectives

Following the fact that rhizobacteria can inhibit the fungal growth, the main objective was to understand the volatile-mediated interaction between rhizobacteria and fungi in more detail. The main concern was to understand what happens in fungal cells when the mycelium experiences growth inhibition as consequence of an exposure to harmful bacterial volatiles. How do they response to these volatiles? Free-living fungi often experience different environmental stresses simultaneously like suboptimal temperatures, insufficient nutrient supply, changing osmolarities, humidity, or pH values or chemical stresses (**39**). In principal, when bacterial volatiles influence fungal growth negatively, they can also be considered as an environmental stress. Bacterial volatiles can be assumed as a chemical stress, which may have an impact on the internal cellular conditions of these fungi, interrupting cell homeostasis and normal physiology (**39**). The environmental stress response (ESR) was studied in ascomycete fungi like *Candida albicans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* by Nikolaou (**40**) and Gasch (**39**). Nikolou (2008) presented that environmental stresses can generate three different general reaction patterns in the fungi, including reactions to osmotic, oxidative, and cell wall stress (**40**). The main concern was given to oxidative stress.

In oxidative stress situation, short-lived and highly reactive radicals as well as non-radical molecular species like singlet oxygen, the superoxide radical anions, hydrogen peroxide, and hydroxyl radicals can be formed in the cell membrane as well as inside the cell (Figure 4) (**41**). Followed by oxidative stress, non-enzymatic lipidperoxidation takes place inside the cell

membrane (**42**). To cope such stresses, fungi activate rapidly their defense systems (**39, 40**). Main anti-oxidants enzymes inside the cytosol include catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, thioredoxin reductases (**43**). Cells that fail to adapt adequately or withstand a severe stress may die (**44, 45**).

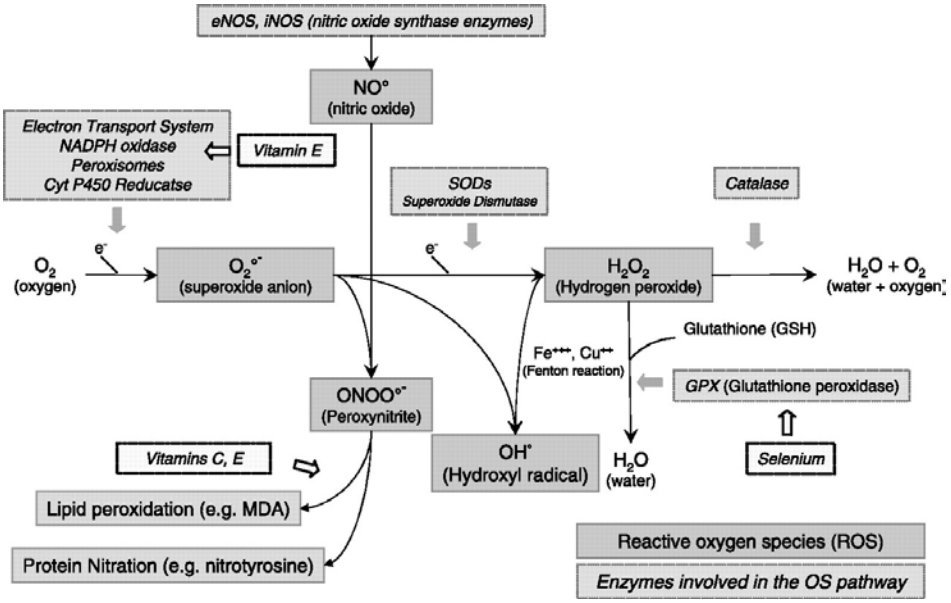

Figure 4: Schematic presentation of oxidative stress situation and anti-oxidants in fungi (41).

The onset of oxidative stress can occur quickly as illustrated by the oxidative burst (**46**). *Candida albicans,* a major fungal pathogen of human, generates toxic reactive oxygen species such as superoxides and hydrogen peroxides. The detoxification of these compounds takes place by activating catalase and superoxide dismutase (**47**). The expression of laccase in yeast was also found to confer a strong ability to scavenge intracellular H_2O_2 and to protect cells from lipid oxidative damage (**48**). Fungal laccases were also induced when the plant pathogenic fungus *Rhizoctonia solani* was paired with the PGPR *Pseudomonas fluorescence* (**49**). In order to understand if bacterial volatiles evoke oxidative stress in fungi followed by growth inhibition, antioxidant enzymes were interesting candidates to study. Since volatile-mediated bacteria-fungi interactions were investigated, it was also important to study the volatile emission profile in monoculture of bacteria or fungi as well as in dual culture where bacteria and fungi both were present. This could give a hint, which volatiles could be responsible for growth inhibition of fungi in the particular test system used.

1.6.2 Selected microorganisms

1.6.2.1 Selected bacterium

The bacterial candidate chosen was *Serratia plymuthica* 4Rx13. This bacterium is a potential biological control agent against selected fungi (**26**). It belongs to the genus *Serratia*, which is composed of facultative anaerobic, rod-shaped gammaproteobacteria with peritrichous flagellation. They appear ubiquitously (**50**). This genus produces chitinases as well as the plant hormone indole-3-acetic acid, which influences directly the growth of phytopathogenic fungi (**51**, **52**).

The species *S. plymuthica* 4Rx13 has the potential to release more than 100 volatile compounds (**53**). These compounds include dimethyl disulfide, dimethyl trisulfide, terpenoids, methanethiol, 2-phenylethanol, and other organic compounds. The main compound is sodorifen, which is a bicyclic polymethyloctadiene. Inorganic compounds include ammonia (**53**). The genome of this bacterium was sequenced (**50**), which is an additional reason to choose this bacterium.

1.6.2.2 Selected fungal candidates

In order to observe the antifungal activity of the volatiles released by *S. plymuthica* 4Rx13, four fungal species were chosen. Two fast-growing fungi were *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. These two fungi are agriculturally important plant pathogens with a wide host range and are distributed worldwide. Early reports showed that many rhizobacteria produce antifungal volatiles resulting in fungistasis against these two fungi by inhibiting fungal growth and germination (**26, 30, 36, 37, 38, 54** and **55**). As two slow-growing fungi, *Phoma eupyrena* and *Neurospora crassa* were selected. These two different groups of fungi were chosen because it was anticipated that fast-growing fungi might react differently than slow-growing fungi. *R. solani* belongs to the phylum basidiomycota while other three fungi belong to the phylum ascomycota. Except *N. crassa*, all other fungi are soil borne pathogens.

S. sclerotiorum causes white mold diseases in a wide range of host. It belongs to the so called Sac-fungi and it produces sclerotia (**56**) (Figure 5A). *R. solani* causes various plant diseases like collar rot, root rot, damping off, and wire stem (**57**) (Figure 5B). Since in previous own investigations it was observed that, many bacteria reduced the growth of these two fungi, they were chosen for the experiments performed in this thesis.

Figure 5: Disease formation by phytopathogenic fungi.

A. Rot formation by *Sclerotinia sclerotiorum* on cucumber fruit (**58**).
B. *Rhizoctonia solani* causes black scurf on tubers (**59**).
C. Blight in terminal bud of a true fir seedling produced by *Phoma eupyrena*(**60**).
D. Red conidiation of *Neurospora crassa*(**61**).

Phoma is a genus of common coelomycetous species. *P. eupyrena* is primarily responsible for phoma blight disease (Figure 5C) in different plants like douglas-fir, red and white fir, mugopines, lodgepole, ponderosa pines, and Engelmann spruce (**60**). Therefore *P. eupyrena* was an interesting fungal candidate to explore. *N. crassa* is a red bread mold (**62**) (Figure 5D) which is a widely used model organism in genetics (**63**). It can be found in tropical and sub-tropical areas (**62**). *N. crassa* was not a phytopathogenic fungus and therefore was selected to see how the bacterial volatiles affect the morphology, growth, and biochemistry of this fungus.

1.6.3 Schematic presentation of work

The first aim of this project was to observe whether the volatiles of *Serratia plymuthica* 4Rx13 affect the morphology, radial growth, and biomass production of the selected fungi. Different numbers of bacterial CFUs were tested to select an optimum bacterial concentration that could be used for further biochemical experiments. The second aim was to study whether bacterial volatiles would act as ROS or would cause a generation of ROS which would lead to peroxidation in the fungal cell membrane. Subsequently, the third aim was to investigate the anti-oxidative enzymes catalase, superoxide dismutase, and intracellular laccase enzymes in fungi exposed to *S. plymuthica* 4Rx13 volatiles. After pinpointing *S. plymuthica* 4Rx13 volatiles as biologically active compounds that affect morphology and growth of fungi, the volatile spectra should be analyzed in detail. Since bacterial volatiles may influence fungal volatile emission and *vice versa*, monocultures and dual cultures should be investigated separately. Since *S. plymuthica* 4Rx13 is able to produce also inorganic volatiles, the involvement of inorganic ammonia in altering growth and biochemistry in fungi should also be investigated (Figure 6).

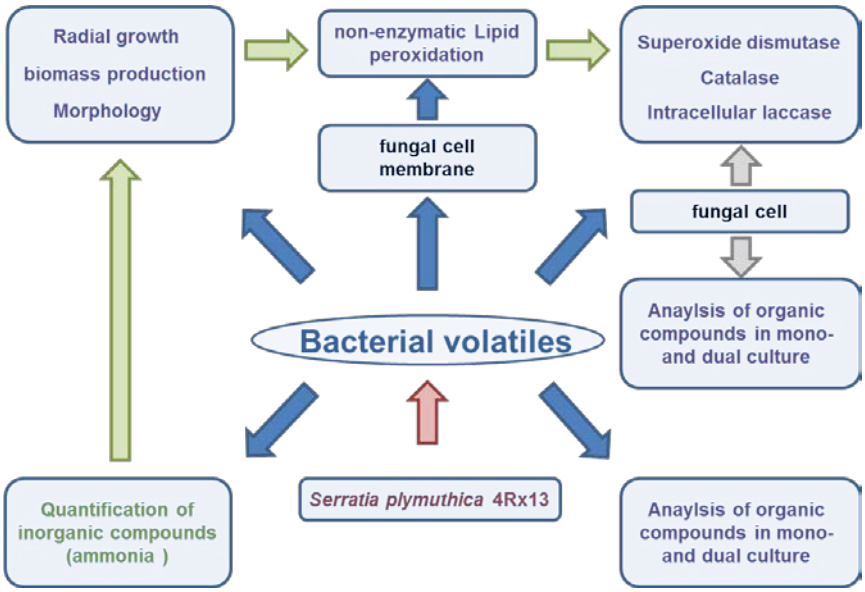

Figure 6: Schematic presentation of objectives.

2 Material and methods

2.1 Microbial strains used for the experiments

The rhizobacterial strain *Serratia plymuthica* 4Rx13 was isolated from the rhizosphere of *Brassica napus* L. subsp. *oleifera* by Berg *et al*. (**64**). The fungal strains *Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Phoma eupyrena* were collected by Prof. Dr. G. Berg (University of Graz, Austria). *Neurospora crassa* was provided by T. Rönneberg (University of Munich, Germany) (**26**).

2.2 Material

Unless otherwise stated, most of the chemicals and materials were purchased from the supplier Carl Roth (Karlsruhe, Germany). All sterilplasticware were obtained from Sarstedt (Nümbrecht, Germany).

2.3 Maintenance of fungi

2.3.1 Long-term storage of fungi

For long-term storage, a small agar plug with mycelia was taken from the margin of an actively growing fungal colony using two sterile tips and submerged in a glycerol-mix.

Glycerol-Mix (100 mL):

Glycerol:	60 mL
Glucose (50 %):	20 mL
Peptone from casein (20 %):	10 mL
Yeast extract (10%):	10 mL

All substances apart from glucose were mixed and autoclaved. The glucose solution was sterile filtered and added to the glycerol-mix after autoclaving.
Samples were initially stored at -20 °C for one day and finally preserved at -70 °C.

2.3.2 Short-term maintenance of fungi

For short-term use, the mycelial agar plug conserved in glycerol-mix was inoculated on a Sabouraud agar (SA) plate with the help of a sterile tip. Plates were kept at 20 °C under darkness. After 7 days, a small plug was taken from the margin of the fungal mycelium and inoculated on a new SA plate. The fungi were again grown for 7 days at 20 °C under darkness and were then ready to use in the experiments (Figure 7).

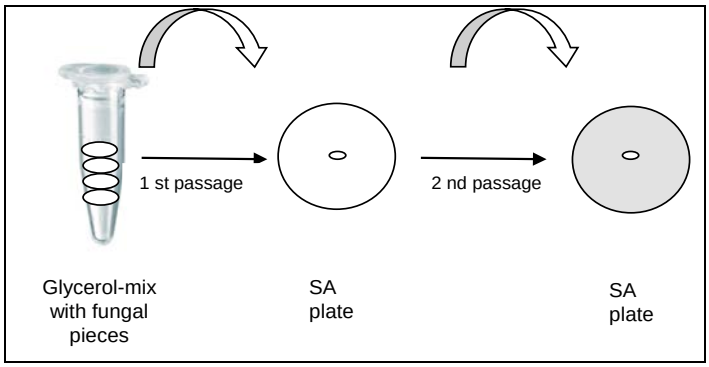

Figure 7: Schematic presentation of fungal short-term maintenance.

Sabouraud-Agar (1.5 %) (SA):

Glucose:	20 g
Casein peptone:	5 g
Meat peptone:	5 g
Agar-agar:	15 g
Aqua dest.	ad 1000 mL

Glucose was dissolved in 50 mL distilled water and sterile filtered. The other substances were dissolved in 950 mL water and autoclaved (Tischautoclav CV-EL18 L, Certoclav, Traun Austria). The glucose solution was added after sterilization. The medium should present a pH value of 5.7 (± 0.2)

2.4 Maintenance of the bacterium

2.4.1 Long-term maintenance of the bacterium

In a 2 mL Eppendorf tube, 500 µl of an overnight culture, 500 µl glycerol, and 500 µl nutrient broth II (NB II) were thoroughly mixed. The samples were stored at -70 °C.

Nutrientbroth II (gL^{-1}) (NB II):

(SIFIN Institute für Immunpräparate und Nährmedien GmbH Berlin, Germany):

Peptone from casein:	3.5 g
Peptone from meat:	2.5 g
Peptone from gelatin:	2.5 g
Yeast extracts:	1.5 g
NaCl:	5.0 g

15 g of the mix was dissolved in 1 L distilled water. The medium should show a pH value of 7.2 (± 0.2). It was sterilized by autoclaving.

2.4.2 Short-term storage of the bacterium

A bacterial inoculum was taken from bacterial stock with the help of a sterile loop. It was streaked onto nutrient agar II plates, incubated for 12-24 h at 30 °C under constant dim light (1.5 $\mu Em^{-2}s^{-1}$) and finally stored at 4 °C. Single colonies were used to prepare bacterial precultures. The plates were stored for a maximum of four weeks.

Nutrient agar (NA):

NBII:	15 g
Agar-agar:	15 g
Aqua dest.:	ad 1000 mL

After preparation, the medium was autoclaved and plates were prepared using 20 mL of the semi-cooled medium.

2.5 Bacterial preculture

NB II (6 mL) was poured in a plastic test tube and a single colony from a plate conserved at 4 °C was added with the help of a sterile loop. The tube was agitated for 12-16 h at 170 rpm, 30 °C under constant dim light (1.5 $\mu Em^{-2}s^{-1}$). The culture was ready to use as soon as the optical density reached 0.8 at 600 nm (OD_{600}) (Figure 8).

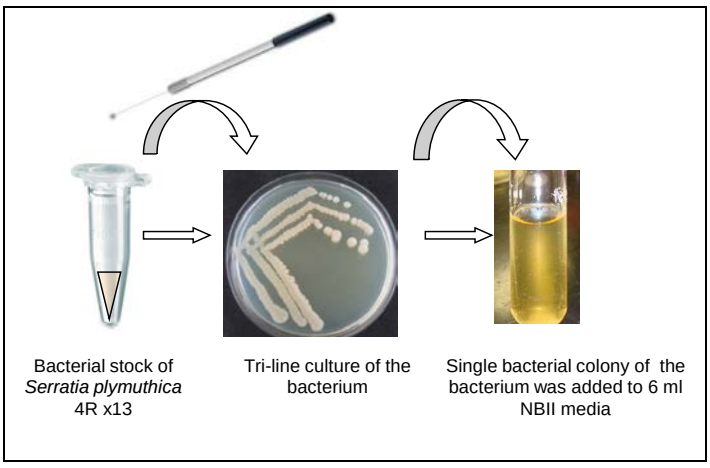

Figure 8: Schematic presentation of bacterial short-term maintenance.

2.6 Dual culture test

A volume of 10 µl, 50 µl, 100 µl, and 200 µl of a bacterial preculture corresponding to initial Colony Forming Units (CFU_i) of about 3-46*10^7, respectively, was point inoculated onto the NA side of a split Petri dish. The plates were kept at 30 °C for 48 h. Thereafter, a small mycelial agar plug of the desired fungus was inoculated onto the SA side of the dish (dual culture). Plates without bacterial suspension at the NA side served as control (monoculture). The plates were kept at 20 °C for a maximum of 7 days (Figure 9). The SA used for dual culture experiments contained 0.75 % agar-agar.

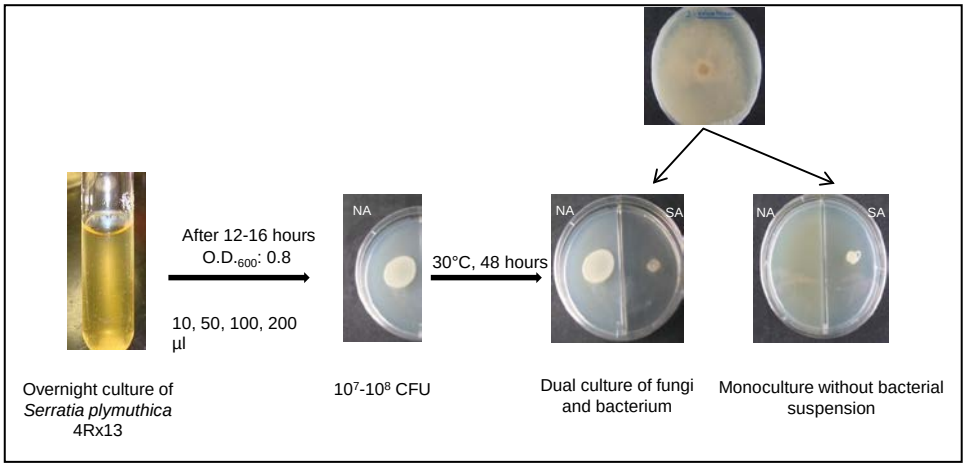

Figure 9: Schematic presentation of dual culture and monoculture test systems.

In order to allow the harvest of young fungal mycelia, a cellophane membrane was introduced. The membranes (85 mm diameter, HARRINGER e. K.) were divided into two equal parts and sterilized in 70 % non-denatured alcohol for two h. Each cellophane membrane was extensively rinsed two times with sterilized water. After cleaning, the membranes were placed onto the fungal side using a forceps. After drying the plates for a few minutes at room temperature, the experiment was started by inoculating the bacterial preculture (Figure 10).

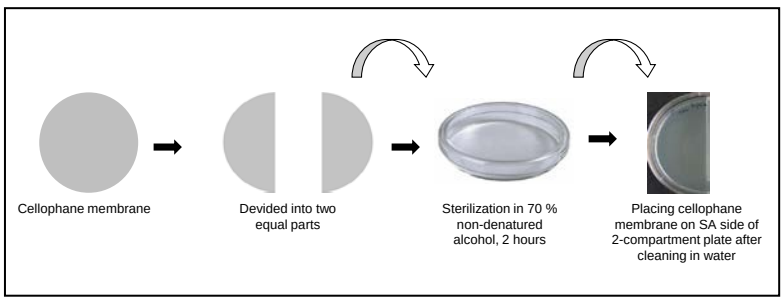

Figure 10: Schematic presentation of using cellophane membrane in test systems.

2.7 Experiments

2.7.1 Influence of bacterial volatiles on fungal morphology

After inoculation of fungal agar-plug, every 24 h images of monoculture and dual culture plates were recorded from anterior and posterior side using a digital camera CX4 (RICOH, Japan).

2.7.2 Influence of bacterial volatiles on fungal radial growth

Total area covered with fungal mycelium was calculated every 24 h in monoculture and dual culture plates using the software ImageJ (**65, 66**) which is a java-based image processing program. The data (pixel) were calculated using Microsoft excel sheets.

2.7.3 Influence of bacterial volatiles on fungal biomass production

Fungal mycelia cultivated on agar were peeled off using two tips. They were placed onto the opened lid of the Petri dish and since 0.75 % agar was used, it was easy to remove leftover agar. The mycelia were partly drained off moisture between sterilized filter papers for 1 h followed by complete drying at 80 °C for 24 h using pre-weighed Petri dishes. Mycelia grown of cellophane membranes were removed using a sterile forceps. They were immediately transferred to pre-weighed Petri dishes and dried at 80 °C for 24 h.

2.7.4 Influence of fungal volatiles on bacterial growth

This control experiment was performed to exclude an influence of fungal volatiles on the bacterial growth. Bacterial CFUs were calculated in presence (dual culture test) and absence of fungi (monoculture). An initial CFU of 42-52*10^7 ($O.D._{600}$=0.8) were point-inoculated on the bacterial NA side. The plates were placed at 30 °C for 2 days. A mycelial plug of the fungus *Sclerotinia sclerotiorum* was inoculated on the SA side of a split Petri plate. The plates were placed at 20 °C for 7 days. Bacterial CFU were measured every 24 h during co-cultivation and monoculture.

2.7.5 Biochemical assays

2.7.5.1 Quantification of non-enzymatic lipidperoxidation

Principle

The level of non-enzymatic lipidperoxidation (nLPRX) in the fungal tissue was measured by quantifying the amount of malondealdehyde (MDA), which is the end product of peroxidation of unsaturated fatty acids due to the presence of peroxidizing agents (Figure 11).

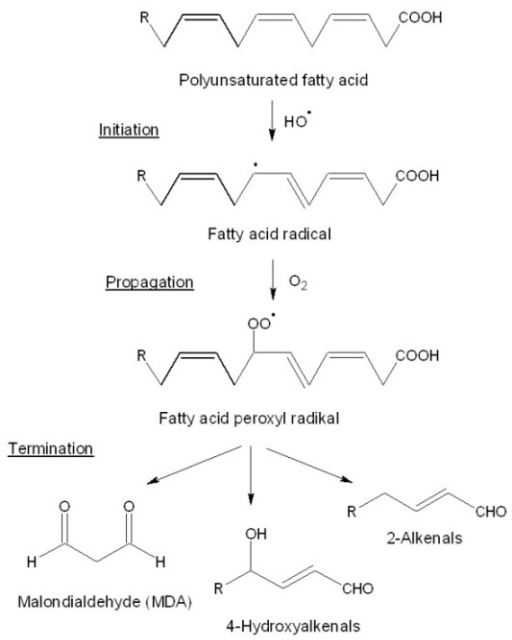

Figure 11: Mechanism of non-enyzmatic lipidperoxidation (67).

Thiobarbituric acid (TBA) was employed to form MDA-TBA adducts under high temperatures (90-100 °C), which can be colorimetrically analyzed. The method described by Dhinsa *et al.* was used with minor modifications (**68**).

Method

The fungal mycelia were harvested as described before (see 2.7.3) and 0.25 g of fresh mycelia were washed with 0.1 % trichloroacetic acid (TCA; E. Merck, Darmstadt, Germany) solution and homogenized under liquid nitrogen using a sterilized and pre-cooled mortar. The homogenate was suspended in 5 mL of 0.1 % TCA solution and centrifuged (HeraeusBiofuge fresco, UK) at 10.000 x g for 5 minutes. 1 mL supernatant was transferred to a glass test tube and mixed with 1 mL 20 % TCA containing 0.5 % thiobarbituric acid (TBA; Sigma-Aldrich, Steinheim, Germany). This mixture was boiled at 95 °C for 20 minutes. After cooling down in an ice bath for 10 minutes, the mixture was again centrifuged (HeraeusBiofuge fresco, UK) at 10.000 x g for 10 minutes. The absorbance of the supernatant was read at 532 nm (UltroSpec 3000, Pharmacia, Sweden) and the value for the non-specific absorbance at 600 nm was subtracted. The MDA amount was calculated in MDA equivalents in nmol/mL using the extinction coefficient ε of 155000 $M^{-1}cm^{-1}$according to the equation $[(A_{532}- A_{600})/155000\ M^{-1}]*10^6$.

2.7.5.2 Superoxide dismutase assay

Principle

Superoxide dismutases (SOD) catalyse the dismutation of superoxide radicals to hydrogen peroxide.

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2$$

Indirect spectrophotometric assays for measuring SOD activities depend on (i) controlled generation of $O_2^{\cdot -}$ by a donor molecule via autoxidation and (ii) subsequent scavanging of $O_2^{\cdot -}$ by a detector molecule**.** This scavange will be inhibited due to dismutation of the generated $O_2^{\cdot -}$ in the presence of SOD (**69-71**). The method used followed the descriptions of Marklund *et al.* (**72**) with some variations mentioned below, e.g. using pyrogallol as donor (**73**).

Method

The mycelium was harvested (see 2.7.3) and washed with ice-cold 50 mM potassium phosphate buffer (pH 7.5). 0.5 g of the mycelium was homogenized under liquid nitrogen using a sterilized and pre-cooled mortar. The homogenate was suspended in 8 mL (absence of cellophane membrane) or 5 mL (presence of cellophane membrane) of the same buffer. The suspension was centrifuged at 2.376 x g (HeraeusBiofuge fresco, UK) for 10 minutes at 4 °C. The supernatant represents the crude protein extract. The SOD activity was measured in a reaction consisting of 2.8 mL of 50 mM Tris–HCl buffer (pH 9) supplemented with 1 mM EDTA and 0.1 mL of the protein extract. The mixture was incubated at 30 °C for 5 min. The reaction was initiated by the addition of 0.05 mL of 10 mM pyrogallol in 10 mM HCl. The changes in absorbance were recorded at 420 nm for 5 min at room temperature (UltroSpec 3000, Pharmacia, Sweden). One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the generated $O_2^{\cdot -}$ and is expressed in U per mg of protein (**71, 72**).

2.7.5.3 Catalase assay

Principle

The enzyme catalase is responsible for the decomposition of hydrogen peroxide to water and oxygen.

$$2\ H_2O_2 + \text{Catalase} \rightarrow 2\ H_2O + O_2$$

The assay was performed following Chance and Maehly (**74**) with little modification.

Method

After harvesting (see 2.7.3), the mycelium was washed with an ice-cold 50 mM sodium phosphate buffer (pH 7). 0.5 g of the sample was homogenized under liquid nitrogen using a sterilized and pre-cooled mortar. The homogenate was suspended in 8 mL (absence of cellophane membrane) or 5 mL (presence of cellophane membrane) of the ice-cold sodium phosphate buffer. The suspension was centrifuged at 16.060 x g for 20 min at 4 °C. The supernatant (protein extract) was used for the assays. The 3 mL reaction mixture contained 2.95 mL of a 30 mM H_2O_2 (stock solution = 30% H_2O_2) in 50 mM sodium phosphate buffer (pH 7). The reaction was initiated by adding 50 µl of protein extracts. The enzyme activity was determined by measuring the initial rate of disappearance of H_2O_2 at 240 nm (UltroSpec 3000, Pharmacia, Sweden) for 60 seconds at room temperature. The catalase activity was calculated using the Lambert-Beer law (molar extinction coefficient of H_2O_2: 43.6 $M^{-1}cm^{-1}$).

2.7.5.4 Intracellular laccase

Principle

Laccases are the phenol-oxidase enzymes. Fungi often produce this enzyme when they interact with other microorganisms (**49**). Fungal laccases might be involved in the defence against oxidative stress. Laccase can significantly increase the resistance of yeast to H_2O_2 (**48**).

2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) or ABTS (Sigma-Aldrich, Germany) was used as substrate in order to determine a laccase activity. Laccase oxidizes this substrate, by forming the stable cation radical $ABTS^{\cdot+}$. The concentration of the intensely green-blue colored cation radical can be correlated to the activity of laccase (**75, 76**).

Method

After harvesting, 0.5 g of fresh mycelia was washed with ice-cold Britton-Robinson-buffer (pH 5). This was followed by the homogenization of the mycelia under liquid nitrogen using a sterilized and pre-cooled mortar. The homogenate was suspended in 5 mL of the same buffer. The samples were centrifuged (HeraeusBiofuge fresco, UK) at 16.088 x g for 20 min at 4 °C. The supernatant was used for the assay. 950 µl of supernatant were filled into a plastic cuvette and incubated at room temperature for 5 min. The cuvette was transferred to a spectrophotometer (UltroSpec 3000, Pharmacia, Sweden), and the reaction was initiated by adding of 50 µl of 20 mM ABTS, the samples were thoroughly mixed and the time dependent change of absorbtion was read at 405 nm (UltroSpec 3000, Pharmacia, Sweden) for 60 seconds. The laccase activity was calculated using the Lambert-Beers law (molar extinction coefficient: 35 $mM^{-1}cm^{-1}$).

Britton-Robinson buffer (pH 5)

A solution of 0.1 M boric acid, 0.1 M acetic acid, and 0.1 M o-phosphoric acid in distilled water was adjusted to pH 5 using NaOH.

2.7.5.5 Determination of protein concentrations

The concentrations of the total protein in all crude extracts in all four biochemical assays were determined using the method provided by Bradford (**77**).

2.7.6 Adaptation experiment

In order to prove effect specificity and the possibility of an adaptation of the fungal mycelium to the deleterious impact of bacterial volatiles, a special experiment was designed. In case of a specific effect, the fungal growth should recover after repeated exposure due to adaptation.

To examine this hypothesis, the fungus *Sclerotinia sclerotiorum* was initially exposed to *Serratia plymuthica* 4Rx13 in presence of a CFU_i of $40\text{-}48*10^7$ (Figure 12C). A monoculture of *S. sclerotiorum* served as negative control (Figure 12B). 5 mM hydroquinone (Sigma-Aldrich, Germany) was used as a positive control (Figure 12D). The radial growth, biomass production, the pH value of the fungal medium, and the activity of the intracellular laccase were measured after 24 h of incubation. After 2 days, mycelia originating from the negative control (Figure 12B) experienced the first time a treatment with bacterial volatiles and hydroquinone (Figure 12F+G). Fungal cultures originating from the dual culture (Figure 12C) and from the positive control (Figure 12D) were re-exposed to bacterial volatiles (Figure 12I) and hydroquinone (Figure 12K), respectively. For every treatment, a monoculture of *S. sclerotiorum* served as negative control (Figure 12E, H, and J). The plates were kept at

20 °C for 3 days. Again, every 24 h the radial growth, biomass production, the pH value, and intracellular laccase activity of the all mycelia (Figure 12E-K) were measured and related to the control that was never exposed to volatiles (Figure 12E). According to the hypothesis, adapted cultures (12H-K) should exhibit better growth in comparison to non-adapted cultures (12E-G).

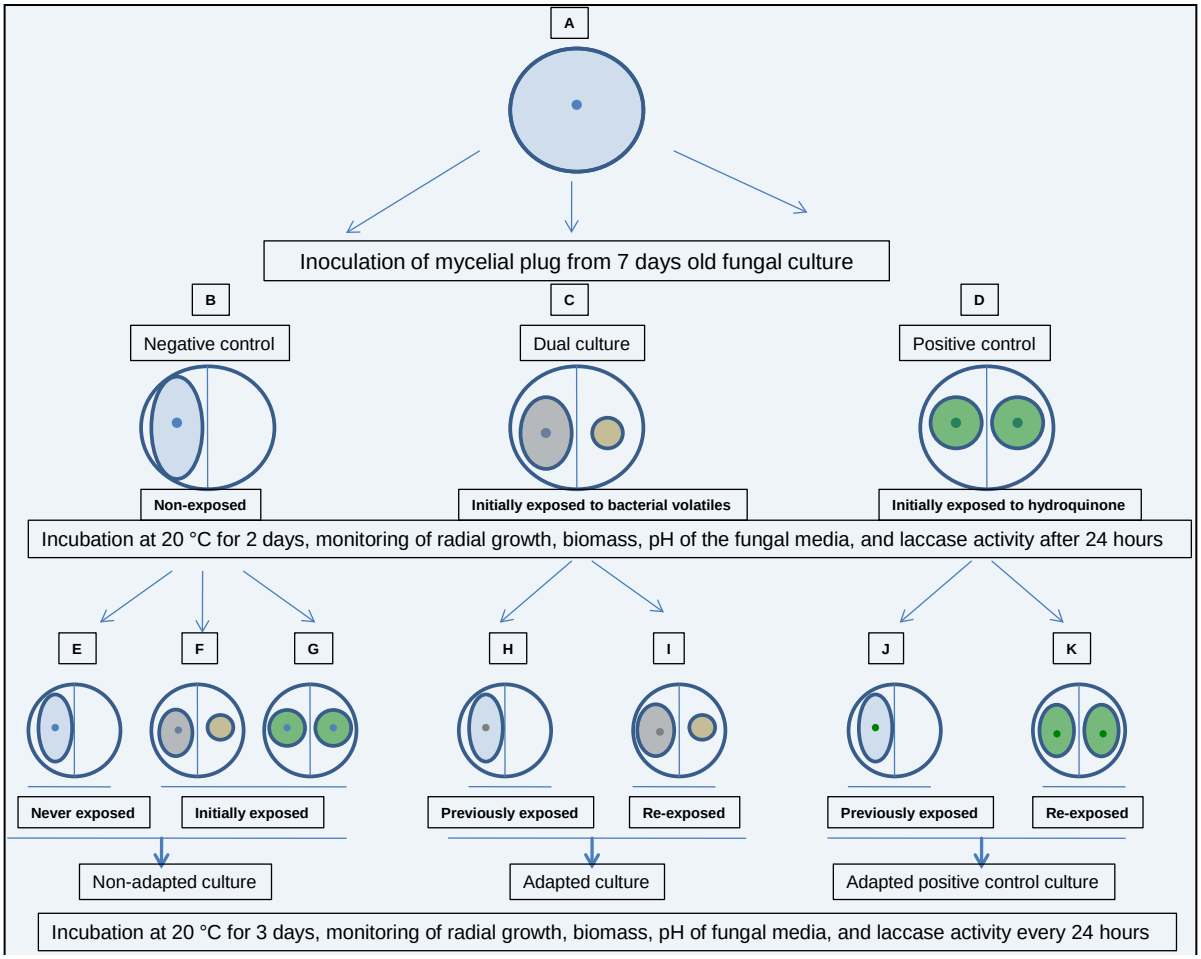

Figure 12: Schematic presentation of the adaptation experiment.

A. Culture of the fungus *Sclerotinia sclerotiorum* (7 days old); **B**. Negative control, monoculture of the fungus; **C**. Dual culture with the bacterium *Serratia plymuthica* 4Rx13; **D**. Positive control, the fungus was grown on 5 mM hydroquinone on both sides; **E-G**. Non-adapted cultures: **E**. Master negative control, this fungal culture had never contact with bacterial volatiles; **F**. Dual culture (initial exposure); **G**. Positive control (initial exposure); **H+I**. Adapted dual cultures: **H**. Negative control (previously exposed); **I**. Dual culture (re-exposed); **J+K**. Adapted positive control: **J**. Negative control (previously exposed); **K**. Positive control (re-exposed).

2.7.7 Qualitative analysis of emitted fungal and bacterial volatiles in mono- and dual cultures

In a first step, the volatile organic compounds (VOCs) of the bacterium *Serratia plymuthica* 4Rx13 and the fungus *Sclerotinia sclerotiorum* were individually analyzed. In a second step, the VOCs produced in the dual culture of these two microorganisms were analyzed. The dual culture test was carried out as described (2.6). 40-48*10^7 CFU_i of the bacterium *S. plymuthica* 4Rx13 were used in all cases.

A headspace system was constructed which is illustrated in the following scheme (Figure 13). In this system, two pumps had been used. One pump pushed the air through a charcoal filter, cotton filter, and a 0.65 % NaCl solution with airflow of 0.75 l/min. The NaCl solution was used for moisture in the system. The air passed through an analysis chamber holding a monoculture or dual culture plate and a second pump pulled the air out of this chamber with a flow of 0.5 l/min over an adsorbent column (100 mg SuperQ, Alltech Associates, Deerfield, IL, USA). Volatiles produced by the microorganisms were adsorbed on SuperQ. Contaminations of the system were prevented by using a higher inlet than outlet airflow. Access air escaped through the lid of the analysis chamber. The system was always incubated for two days at 30 °C followed by an incubation of three days at 20 °C which corresponds with all dual culture test setups. VOCs were collected in intervals of 24 h. Empty plates without any media and plates with bacterial or fungal media without any microorganisms served as control. Since the media may also produce volatiles they were independently measured and subtracted from the volatile profiles. After adding the internal standard nonyl acetate (1.5 µg in 10 µl) on column, the adsorbed compounds were eluted with 300 µl dichloromethane. Samples were analyzed using a gas chromatograph combined with a mass spectrometer (GC/MS-QP5000 from Shimadzu; 70 eV; Kyoto, Japan). It was equipped with a DB5-MS column (60 m X 0.25 mm X0.25 µm; J&W Scientific, Folsom, CA, USA). Analyses started with splitless liquid injection of 1 µl sample at 200 °C with a sampling time of 2 min using an autosampler (CTC Analytics, Zwingen, Switzerland). The initial column temperature was fixed to 35 °C, followed by an increase of 10 °C/min upto 280 °C which was held for 15 minutes. Helium was the standard carrier gas. The column flow was fixed at 1.1 mL/min with a linear velocity of 28 cm/s (**30**).

Mass spectra were obtained and compounds were identified by comparing their spectra with the spectra compiled the library of the National Institute of Standards and Technology (NIST 107) and by comparison of Kovats indices.
Experiments were performed 3 times. Compounds produced in all three experiments are presented in the result section.

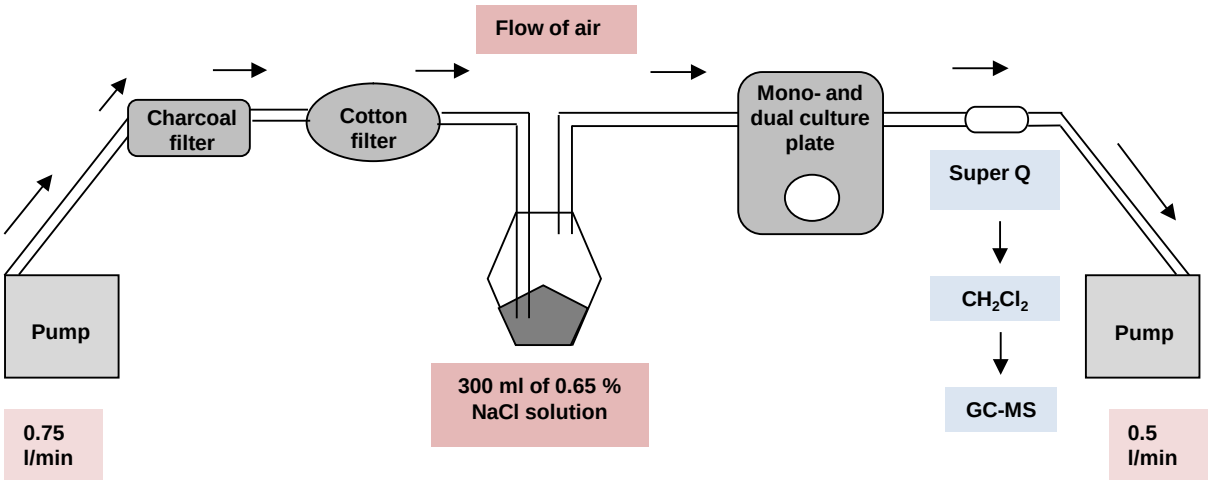

Figure 13: Scheme of the headspace system.

2.7.8 Quantification of ammonia produced by *S. plymuthica* 4Rx13

The amount of ammonia produced by the bacterium was quantified using Quantofix ammonium test sticks (Macherey & Nagel, Düren, Germany). Split Petri dishes were supplied with NA medium on one side. A small slot was drilled at the empty side of the plate, so that a microliter syringe could be inserted (Figure 14). The slot was closed using Nescofilm to avoid contaminations and any loss of volatiles. The empty half of the plate was fitted with an ammonia teststick and an inoculum of 40-48*10^7 CFU_i of *S. plymuthica* was spotted onto the NA medium (Figure 15A). The plates were incubated for 2 days at 30 °C and 3 days at 20 °C. Every 24 h, a microliter syringe was inserted through the slit and the Nessler reaction on the test stick was initiated with a droplet of distilled water (15 µl). After 30 seconds, the reaction was stopped by adding 15 µl of a 28 % NaOH solution. The colors of the sticks (Figure 15B) were related to a calibration conducted in parallel with synthetic ammonia solutions of 3 mM, 6 mM, 15 mM, 30 mM, 60 mM, and 300 mM ammonia. Ammonia solutions of these concentrations were poured into one side of split Petri dishes instead of bacterial cultures (Figure 15C). They were renewed every day because of the constant ammonia production by bacteria.

The simultaneous quantification of ammonia originating from bacterial cultures and synthetic ammonia solutions allowed a rough definition of that concentration of ammonia solutions evaporating an amount of ammonia into the headspace equivalent to those naturally emitted by *S. plymuthica* 4Rx13 (40-48*10^7 CFU_i) during the tests.

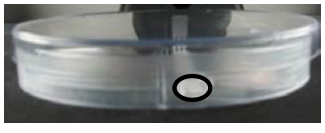

Figure 14: Split Petri dish showing a slot to insert a microliter syringe.

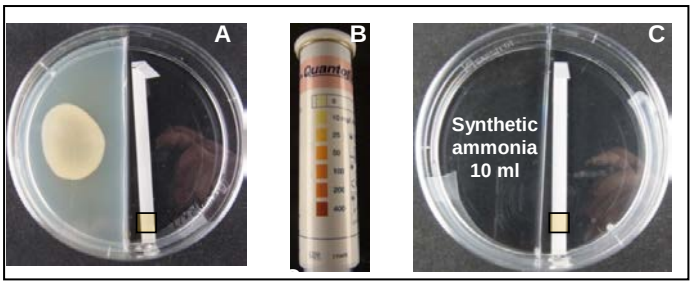

Figure 15: Quantification of ammonia using Quantofix ammonia test sticks in presence of bacteria and different ammonia solutions.

A. Inoculation of *Serratia plymuthica* 4Rx13 (CFU_i: 40-48*10^7); **B**. Container carrying ammonia Quantofix test sticks; **C**. Application of 3-300 mM synthetic ammonia solution.

3-300 mM ammonia solution

A 0.584 M stock was prepared using a 20 % synthetic ammonia solution. This stock solution was diluted with distilled water to prepare desired molar ammonia concentrations.

2.7.9 Determination of the pH value of fungal growth media

Previous research from our working group showed that ammonia produced by *Serratia plymuthica* 4Rx13 caused an alkalization of plant growth media due to the formation of ammonium ions (**78**). According to this knowledge, the pH values of the growth media were monitored during culture assays in two-compartment plates. Bacterial (CFU_i: 40-48*10^7) and fungal monocultures as well as dual cultures were investigated. Plates, where only media were present, served as control. The plates were incubated as described (see 2.6) for 2 days at 30 °C and subsequently for 3 days at 20 °C. The pH value was measured every 6 h at 30 °C and every 12 h at 20 °C. Analyses were conducted using pH indicating paper sticks (MACHEREY-NAGEL, Düren, Germany) which were placed directly onto the solid media (Figure 16B).

The influence of different concentrations of synthetic ammonia (3-300 mM) on the pH value was analyzed in a similar way (Figure 16A). 10 mL of the desired concentration were applied to one compartment of a split Petri dish. The second compartment was filled with fungal medium. After an incubation of 2 days at 30 °C, mycelial plugs were inoculated onto the SA. The plates were kept at 20 °C for additional 3 days. Plates without fungal inoculums served as control. The pH value of the fungal medium was measured every 24 h. The ammonia solutions were renewed every 24 h.

The correlation of the effects of the naturally emitted ammonia with the corresponding amount of artificially evaporated ammonia on the pH value was double-checked.

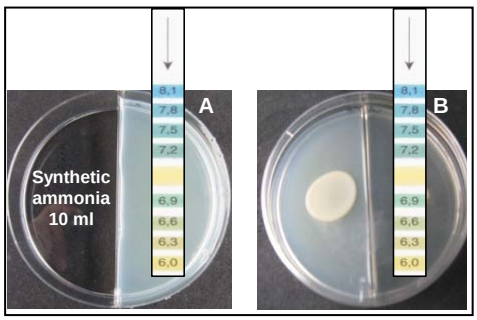

Figure 16: Measurements of the pH value in presence of an ammonia solution (A) or *Serratia plymuthica* 4Rx13 (B).

2.7.10. Influence of synthetic ammonia on fungal radial growth and biomass production

A volume of 10 mL of different ammonia concentrations (3 mM, 6 mM, 15 mM, 30 mM, 60 mM, and 300 mM) was poured into one compartment of split Petri dishes. After an incubation of 2 days at 30 °C, a plug of the fungus *Sclerotinia sclerotiorum* was inoculated onto the SA in the other compartment of the split plate (Figure 17). The plates were kept at 20 °C for three days. Ammonia was renewed every 24 h. The radial growth and biomass production were determined every 24 h (see 2.7.2).

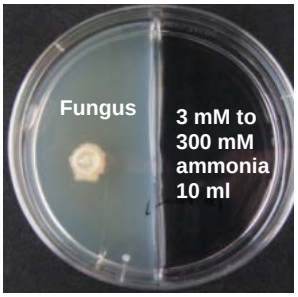

Figure 17: Setup showing *Sclerotinia sclerotiorum* exposed to different concentrations of synthetic ammonia.

2.7.11 Influence of synthetic ammonia on fungal membrane integrity and enzyme activities indicating oxidative stress

The concentration of the ammonia solution used for this experiment should (a) evaporate an amount of ammonia into the headspace of split Petri dish which is equivalent to the amount emitted by *S. plymuthica* 4Rx13 (40-48*10^7 CFU_i and (b) should cause a similar growth inhibition of the fungal mycelium. Based on the resultsobtained from preliminary experiments (2.7.8 and 2.7.10), a concentration of 15 mM ammonia was chosen. This concentration was checked regarding an impact on non-enzymatic lipidperoxidation in fungal membranes and enzyme activities indicating oxidative stress.

A volume of 10 mL of a 15 mM ammonia solution was filled into one side of split Petri dish. The other side contained SA. The plates were kept at 30 °C for 48 h. Mycelial plugs of 7 days old fungal cultures of *Sclerotinia sclerotiorum* and *Rhizoctonia solani,* respectively, were inoculated onto the SA. The plates were kept at 20 °C for three days. The ammonia solution was changed every 24 h.

The mycelia were harvested every 24 h and the non-enzymatic lipidperoxidation as well as the catalase, superoxide dismutase, and intracellular laccase assays were performed (see chapter 2.7.5). These data were compared with data obtained with *S. plymuthica* 4Rx13 growing in the second compartment (Chapter 3.3).

2.7.12 Influence of 0.85 % o-phosphoric acid on the ammonia concentration in dual culture tests

Phosphoric acid was used to confirm that *Serratia plymuthica* 4Rx13 produces ammoniasince it is known to form ammonium phosphate thereby eliminating ammonia from the Petri dish headspace (**79**). One compartment of a tripartite Petri dish was filled with 10 mL bacterial NA, the second compartment was filled with 10 mL fungal SA. *S. plymuthica* 4Rx13 was inoculated onto the NA with a CFU_i of 12-16*10^7. The inoculum had to be plated in this case in order to obtain a total fungal growth inhibition (Figure 18B). The third compartment was either filled with 5 mL of distilled water (control) or with 5 mL of 0.85 % o-phosphoric acid. After incubating for 2 days at 30 °C, mycelial plugs of the fungi *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, respectively, were placed onto the SA and the plates were kept at 20 °C for 3 days. Monoculture plates without bacterial suspension served as a control (Figure 18A). Every 24 h, the radial growth of the fungi (ImageJ) and pH value of the fungal growth medium was measured in each case (see 2.7.2 and 2.7.9).

An identical setup was used replacing the bacterial culture by a 60 mM ammonia solution. 60 mM ammonia was used here since total growth inhibition of the fungus was desired (Figure18C+D).

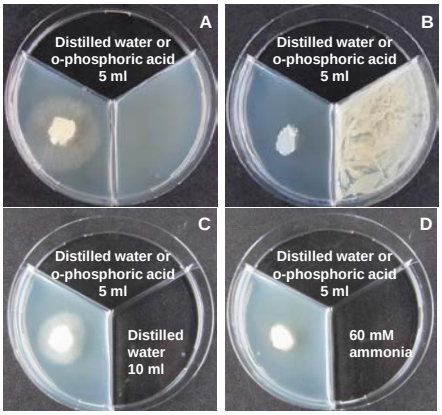

Figure 18: Impact of o-phosphoric acid on fungal growth inhibition due to ammonia.

A. Monoculture of the fungus (absence of bacteria); **B**. Dual culture between the fungus and the bacterium *Serratia plymuthica* 4Rx13; **C**. Monoculture of the fungus (absence of ammonia); **D**. Cultivation of the fungus in presence of 60 mM ammonia.Distilled water served as negative control.

2.8 Statistical analysis

The Wilkoxon signed-rank test was used to determine the statistical significance. It is a non-parametric statistical hypothesis test (**80**).

3 Results

The aim of this study was to understand the interaction between rhizobacteria and soil borne fungal plant pathogens. The fact that the rhizobacterium *Serratia plymuthica* 4Rx13 can produce volatile metabolites that reduce the growth of fungi as *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Phoma eupyrena*, and *Neurospora crassa* (**26**) brought up the question how bacterial volatiles evolve their efficacy and how fungi would defend themselves from these harmful volatiles. One of the hypothesis proposed here was that bacterial volatiles produce oxidative stress in the fungal cell. In order to remove the emerging reactive oxygen species (ROS), fungi might activate their anti-oxidant system. Four oxidative stress parameters were tested in fungi co-cultivated with *S. plymuthica* 4Rx13. Volatile metabolites emitted in mono- and dual cultures were analyzed in order to get an impression which volatiles might be responsible for the effect. Experiments were made in order to pin down ammonia produced by the bacterium as one of the volatiles affecting fungal growth, morphology, and biochemistry. Accordingly, in the following section, the results are described in different chapters. The abbreviation S indicates a supplementary chapter.

3.1 Setting up the test system of co-cultivation

Four fungal species were dual-cultivated with different initial numbers of colony forming units (CFU_i) of *S. plymuthica* 4Rx13 in order to observe any concentration dependent effects and to find the optimal CFU_i for upcoming experiments. Soon evolved the problem that the fungal mycelium that was cultivated on agar, could not be harvested after a short-termed incubation. On the first two days of incubation, the mycelia of *Sclerotinia sclerotiorum* and *Rhizoctonia solani* were too fragile and the amount was too little to cleanly and quantitatively remove it from the agar. For the slower growing species *Phoma eupyrena* and *Neurospora crassa*, this problem was even more pronounced. However, this early time of co-cultivation was assumed to be most important for investigations. In order to solve this problem, a cellophane membrane was introduced. This membrane was placed onto the agar before inoculating the mycelial plug. Now it was possible to harvest the fungal mycelia from day 1 onwards. However, it had to be assured that the introduction of this membrane did not make any difference in setup conditions as well as in fungal growth and behavior. Therefore, the development of the pH value of the fungal growth medium as well as the fungal growth and catalase activity were determined in presence and absence of the cellophane membrane and compared.

In a first experiment, a bacterial inoculum of $44\text{-}52*10^7$ CFU_i was used to determine the radial growth as well as biomass of the fungi *S. sclerotiorum* and *R. solani* after day 1 to day 5 grown on cellophane. The results obtained on day 3, 4, and 5 were compared with the

growth on plain agar. It could be observed that the inhibition of the radial growth (Figure 19) was in fact not exactly the same, but the patterns of results were definitely comparable.

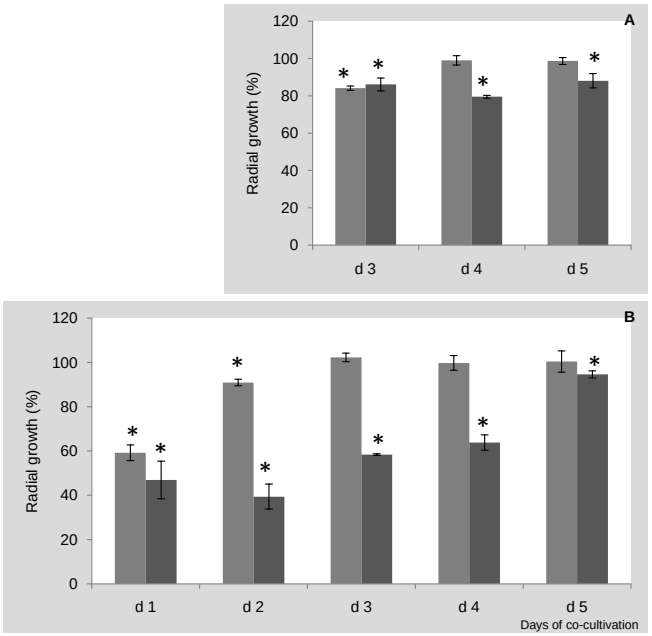

Figure 19: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of selected fungi.

A: plain agar; **B:** cellophane membrane.

Sclerotinia sclerotiorum; *Rhizoctonia solani*;

An initial CFU of 44-52*10^7 were point-inoculated on the bacterial side (NA)**.** The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on the fungal side. The dishes were placed at 20 °C. The radial growth was determined every 24 h starting with day 3 (**A**) and day 1 (**B**). The growth was expressed in pixel (software ImageJ). The control was equivalent to 274617 (d 3), 2810729 (d 4) and 2884807 (d 5) (*S. sclerotiorum*), 2392553 (d 3), 2856171 (d 4), 2855675 (d 5) (*R. solani*) (**A**), 292663 (d 1), 2332330 (d 2), 2354221 (d 3), 2494895 (d 4) and 2502220 (d 5) (*S. sclerotiorum*), 369191 (d 1), 1508437 (d 2), 2090937 (d 3), 2301340 (d 4), 2459472 (d 5) (*R. solani*) (**B**).
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

The biomass results (Figure 20) were also not same but were also comparable.

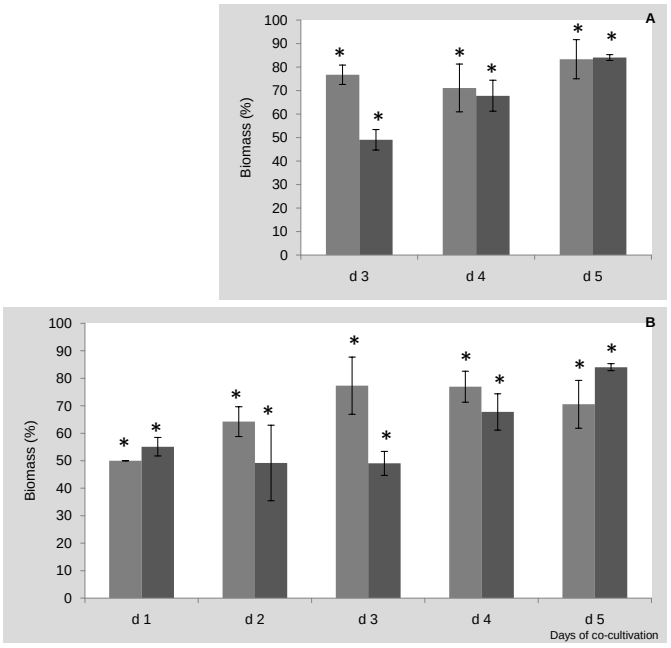

Figure 20: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the biomass of selected fungi.

A: plain agar; **B:** cellophane membrane.

Sclerotinia sclerotiorum; *Rhizoctonia solani*.

An initial CFU of 44-46*10^7 were point-inoculated on the bacterial side (NA)**.** The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on the fungal side. The dishes were placed at 20 °C. The biomass was determined every 24 h for starting with day 3 (**A**) and day 1 (**B**). The control in mg was equivalent to 80 (d 3), 90 (d 4) and 120 (d 5) (*S. sclerotiorum*), 70 (d 3), 110 (d 4) and 150 (d 5) (*R. solani*) (**A**), 5 (d 1), 30 (d 2), 80 (d 3), 120 (d 4) and 150 (d 5) (*S. sclerotiorum*), 4 (d 1), 17 (d 2), 40 (d 3), 80 (d 4) and 147 (d 5) (*R. solani*) (**B**).
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

The catalase was chosen in order to check and compare an enzyme activity in fungi grown on plain agar and on a cellophane membrane. *S. sclerotiorum* was exemplarly used in this experiments. Results exhibited that the catalase activities were comparable under both situations (Figure 21).

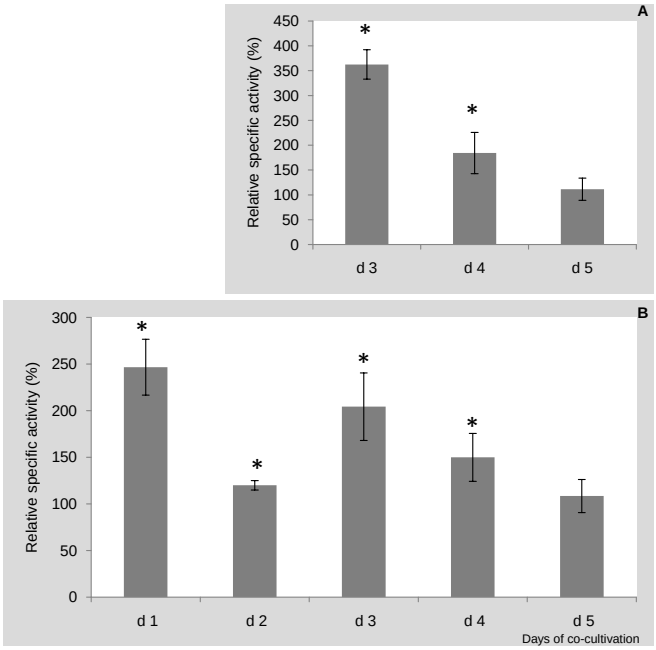

Figure 21: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the catalase activity of the fungus *Sclerotinia sclerotiorum.*

A: plain agar; **B:** cellophane membrane.

An initial CFU of 44-48*10^7 were point-inoculated on the bacterial side (NA)**.** The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on the fungal side (SA). The dishes were placed at 20 °C. The catalase activity was determined every 24 h starting with day 3 (**A**) and day 1 (**B**). The control in µkat/mg protein was equivalent to 2.42 (d 3), 2.32 (d 4) and 2.46 (d 5) (**A**), 0.74 (d 1), 1.35 (d 2), 1.80 (d 3), 1.67 (d 4) and 1.91 (d 5) (**B**).
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

Since bacterial volatiles can alkalize the fungal agar medium, the pH value of the cellophane membrane was also monitored during the co-cultivation at 20 °C. Results are presented in figure 22. It could be shown that the pH value of the cellophane membrane displayed the same development as the plain agar medium during the co-cultivation.

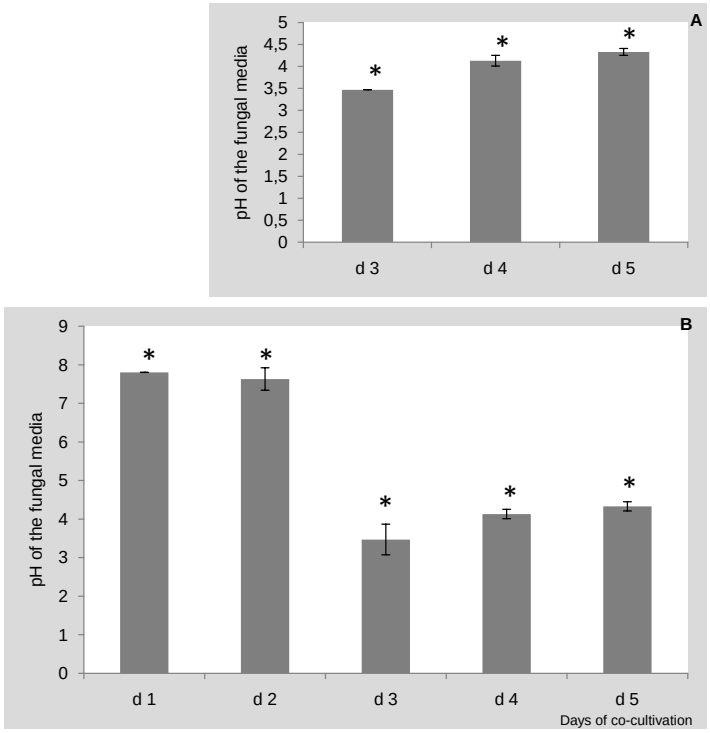

Figure 22: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the pH of the fungal media of *Sclerotinia sclerotiorum.*

A: plain agar; **B:** cellophane membrane.

An initial CFU of 44-48*10^7 were point-inoculated on the bacterial side (NA)**.** The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on the fungal side (SA). The dishes were placed at 20 °C. The pH was determined every 24 h starting with day 3 (**A**) and day 1 (**B**).
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding to the control.

Summarizing these four data sets, it can be concluded that the use of cellophane membranes did not make much difference. The cellophane membrane was partly employed in some experiments for the initial 2 days of co-cultivation with bacteria (3.2.1.2, 3.3.1-3) and completely employed for all other experiments.

3.2 Influence of volatile metabolites of *Serratia plymuthica* 4Rx13 on fungal growth and morphology

3.2.1 Fungal growth

In order to confirm the results shown by Vespermann *et al.* 2007 (**26**), the fungal radial growth was determined. Additionally, the biomass of the fungi was determined in order to monitor radial growth related biomass changes. After day 1, slow-growing fungi (*Phoma eupyrena* and *Neurospora crassa*) did not show a measurable growth. For these two fungi the growth was observed from day 2 onwards.

3.2.1.1 Radial growth

In general, *Sclerotinia sclerotiorum* (Figure S1A), *Rhizoctonia solani* (Figure S1B), and *Neurospora crassa* (Figure S1D) exhibited radial growth inhibition whereas *Phoma eupyrena* (Figure S1C) showed a slight enhancement that was contradictory from what Vespermann *et al.* presented in 2007 (**26**). The growth inhibition or promotion was dependent on the CFU_i and it increased with an increasing CFU_i. In presence of (44-46)*10^7 CFU_i, a maximum growth inhibition or promotion was observed (Figure S1). As a summary, figure 23 shows the maximum radial growth inhibition or promotion of all tested fungi.

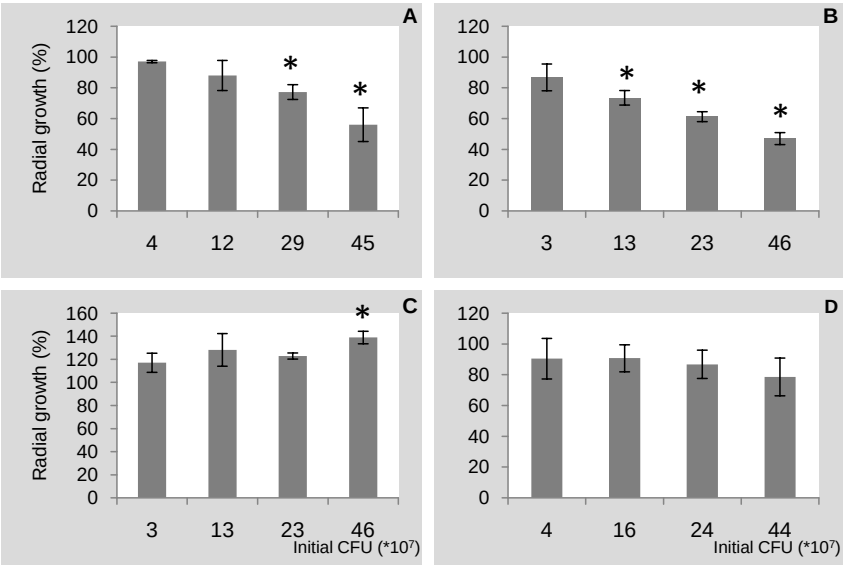

Figure 23: Utmost influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of selected fungi.

A: *Sclerotinia sclerotiorum*; **B:** *Rhizoctonia solani*; **C:** *Phoma eupyrena*; **D:** *Neurospora crassa*.

An initial CFU of 3-46*10^7 were point-inoculated on the bacterial side (NA). The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on the fungal side (SA). The dishes were placed at 20 °C. The radial growth was determined after day 1 (**A** and **B**) and 4 (**C** and **D**). The growth was expressed in pixel (software ImageJ). The control was equivalent to 550545 (**A**), 314290 (**B**), 540046 (**C**), 965626 (**D**).
n=3. Error bars presented standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

Radial growth inhibition or promotion was also dependent on the time of co-cultivation. The time dependent influence of (44-46)*10^7 CFU_i on fungal radial growth is shown in figure 24. The complete set of data is presented in figure S1. *Sclerotinia sclerotiorum* (Figure S1A and 24A) and *Rhizoctonia solani* (Figure S1B and 24B) exhibited a significant growth inhibition after day 1 and day 2. After that the growth was comparable to the control. *Phoma eupyrena* (Figure S1C and 24C) displayed a insignificant growth inhibition after day 2 but after that it showed an inconsistant growth promotion till day 7. Finally, the growth of *Neurospora crassa* (Figure S1D and 24D) was inhibited between day 2 and 4 in dual culture, after that it started growing as in the control experiment.

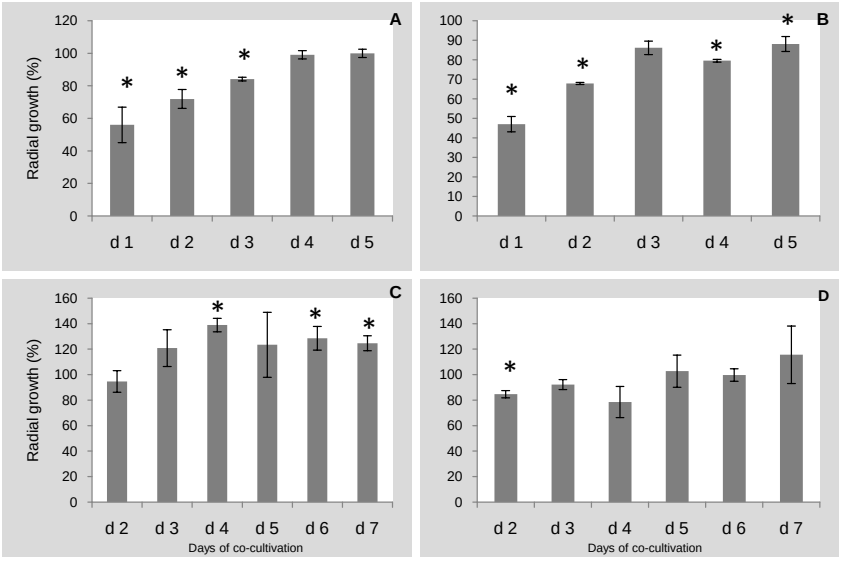

Figure 24: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of selected fungi.

A: *Sclerotinia sclerotiorum*; **B:** *Rhizoctonia solani*; **C:** *Phoma eupyrena*; **D:** *Neurospora crassa*.

An initial CFU of 44-46*10^7 were point-inoculated on the bacterial side (NA)**.** The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on fungal side (SA). The dishes were placed at 20 °C. Radial growth was determined every 24 h for 5 days starting with day 1 (**A** and **B**) and 6 days starting with day 2 (**C** and **D**). The growth was expressed in pixel (software ImageJ). The control was equivalent to 550545 (d 1), 1301689 (d 2), 2737247 (d 3), 3737249 (d 4), 3843218 (d 5) (**A**), 314290 (d 1), 1560714 (d 2), 2392553 (d 3), 2856171 (d 4), 2855675 (d 5) (**B**), 229086 (d 2), 439598 (d 3), 540046 (d 4), 943467 (d 5), 1178659 (d 6), 1473805 (d 7) (**C**), 174720 (d 2), 346760 (d 3), 965626 (d 4), 779612 (d 5), 1095549 (d 6), 1258540 (day 7) (**D**)
n=3. Error bars represent standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

3.2.1.2 Dry biomass

The biomass of the fungi was determined under the same experimental setup. The fungal mycelia of dual cultures were harvested, dried, and compared with the mycelium grown in monoculture.

All four fungi unveiled a decrease in biomass production depending on CFU_i and time of incubation. With an increasing CFU_i, an elevation in biomass loss was monitored and 44-46*10^7 CFU_i exhibited a maximum biomass decrease. The highests decrease was observed on initial days of co-cultivation. The complete data set is presented in the supplementary chapter in figure S2.

Figure 25 displays a summary of the time dependent effect of a (44-52)*10^7 CFU_i on biomass production. *Sclerotinia sclerotiorum* exhibited a maximum inhibition of 50 % after day 1 that was decreased to 40 % after day 2 (Figure 25A). *Rhizoctonia solani* showed at the first three days an inhibition of biomass production of 50 % (Figure 25B). *Phoma eupyrena* (Figure 25C) and *Neurospora crassa* (Figure 25D) exhibited 50 % biomass reduction after day 2. As they are slow-growing fungi, they exhibited biomass inhibition till day 6. Notable is the fact that the biomass production recovered over incubation time.

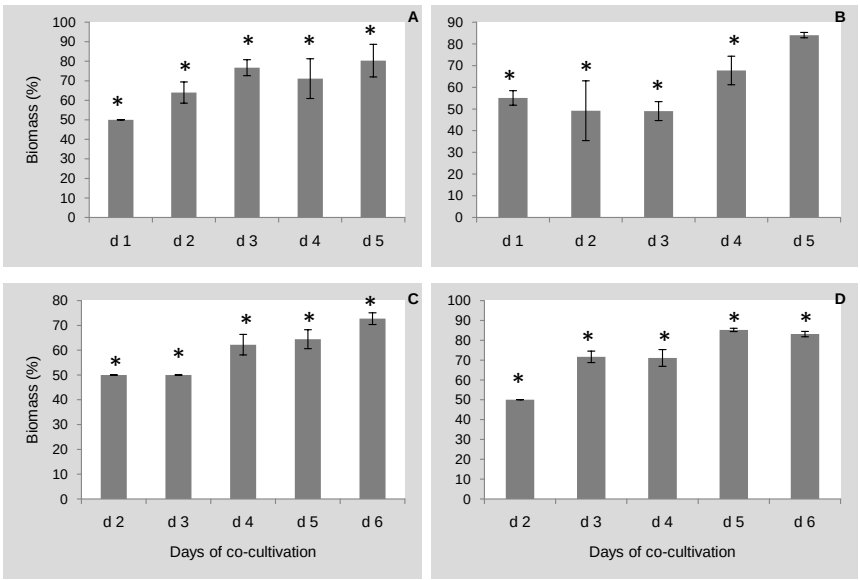

Figure 25: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the biomass of selected fungi.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani*; **C.** *Phoma eupyrena*; **D.** *Neurospora crassa*.

An initial CFU of 44-52*10^7 were point-inoculated on the bacterial side (NA)**.** The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on the fungal side (SA). The dishes were placed at 20 °C. The biomass was determined every 24 h for 5 days starting with day 1 (**A** and **B**) and 6 days starting with day 2 (**C** and **D**). The control in mg was equivalent to 5 (d 1), 30 (d 2), 80 (d 3), 90 (d 4) and 120 (d 5) (**A**), 7 (d 1), 30 (d 2), 160 (d 3), 170 (d 4) and 190 (d 5) (**B**), 5 (d 2), 80 (d 3), 120 (d 4), 140 (d 5) and 170 (d 6) (**C**), 5 (d 2), 80 (d 3), 120 (d 4), 140 (d 5) and 170 (d 6) (**D**).
n=3. Error bars present standard deviation. (*) presents statistical significance related to the corresponding control (100 %).

This leads to the conclusion that the maximal influence of bacterial volatiles on radial growth as well as biomass production occured at the initial days of co-cultivation in presence of the maximal number of bacterial CFU_i (40-52*10^7). Although *Phoma eupyrena* showed a slight and partly insignificant radial growth promotion, it produced less biomass. The inhibition of growth and biomass production was dependent on CFU_i and time incubation. Both growth and biomass production recovered after the initial inhibition.

3.2.2 Fungal morphology

Besides a changing growth behavior, some morphological changes like alteration of color are already visible to the naked eye. During the growth experiments, mono- and dual cultures were monitored for those changes and images were taken. As a reaction to bacterial volatiles, *Rhizoctonia solani* (Figure S3B) and *Phoma eupyrena* (Figure S3C) displayed changes in coloration in dual culture. These changes were observed when images were taken from posterior side. It seemed that the mycelia secreted a colored substance into the medium. The images showed that with an increasing CFU_i, the coloration gained intensity. *Rhizoctonia solani* showed a reddish coloration between day 2 and day 4 and *Phoma eupyrena* developed a brown coloration between day 2 and day 7. In Figure 26, the maximum effects are shown.

Sclerotinia sclerotiorum (Figure S3A) and *Neurospora crassa* (Figure S3D) did not show any changes in coloration.

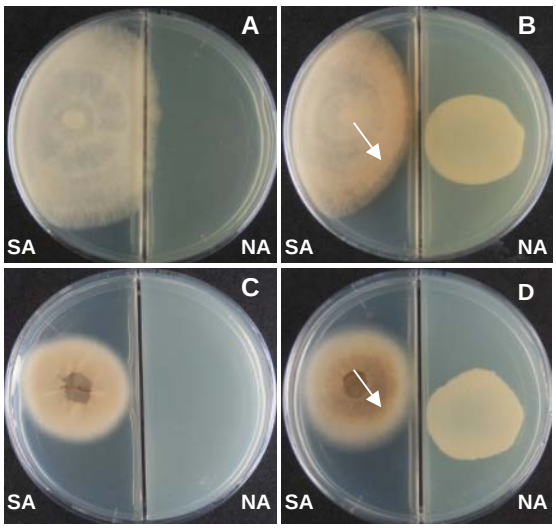

Figure 26: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the coloration of the fungi *Rhizoctonia solani* and *Phoma eupyrena*.

A: Monoculture of *Rhizoctonia solani*;
B: Dual culture of the fungus with *Serratia plymuthica* 4Rx13;
C: Monoculture of *Phoma eupyrena*;
D: Dual culture of the fungus with *Serratia plymuthica* 4Rx13.

An initial CFU of 46*10^7 were point-inoculated on the bacterial side (**NA**). The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs of the fungi were inoculated on fungal side (**SA**). The dishes were placed at 20 °C for 3 (**A** and **B**) and 5 (**C** and **D**) days. Images were taken every 24 h using a digital camera (Ricoh). n=3.

Summarising these data it can be said that in response to bacterial volatiles fungi are capable to exhibit changes in their mycelial color. This coloration was dependent on the CFU_i and fungal species.

3.3 Influence of volatile metabolites of *Serratia plymuthica* 4Rx13 on membrane integrity and enzyme activities indicating oxidative stress

It was postulated that bacterial volatiles might produce free radicals resulting in oxidative stress situations in fungi. Four oxidative stress parameters were investigated. In the following sections, selected results are described. The complete data set is presented in the supplementary chapter.

3.3.1 Non-enzymatic lipidperoxidation

Unsaturated fatty acids in fungal cell membranes are normally prone to oxidizing agents. They can form peroxyl radicals and the final product of such peroxidation is malondealdehyde (MDA) (**81**). Bacterial volatiles have to be considered to cause such non-enzymatic lipidperoxidation (nLPRX).

As presented in figure 27, all four fungi exhibited enhanced nLPRX when exposed to bacterial volatiles (CFU_i of 32-56*10^7). In *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, an increase in MDA formation was observed on day 1 and 2 during co-cultivation (Figure 27A and 27B, respectively). The MDA amount reached a maximum in *S. sclerotiorum* after day 2 of exposure (8-fold), whereas *R. solani* exhibited a maximum (6-fold) after day 1. *Phoma eupyrena* showed between day 2 and 4 an elevated level of MDA (Figure 27C). For *Neurospora crassa*, the maximum of 10-fold MDA generation was observed after 2 days of exposure (Figure 27D). In all fungi except in *P. eupyrena*, the amount of MDA dropped after 3 days of volatile exposure and then slowly declined. In fast-growing fungi, (*S. sclerotiorum* and *R. solani*) the final MDA level between 2.8-3.7 nmol/mL TCA equivalents was comparable to the control after 3 days.

In summary, all fungi showed in co-cultivation with *Serratia plymuthica* 4Rx13 enhanced nLPRX. Younger mycelia were most vulnerable to be affected by bacterial volatiles. A pronounced dependency from the number of CFU_i could not be observed. The complete data set is presented in the supplementary chapter in figure S4.

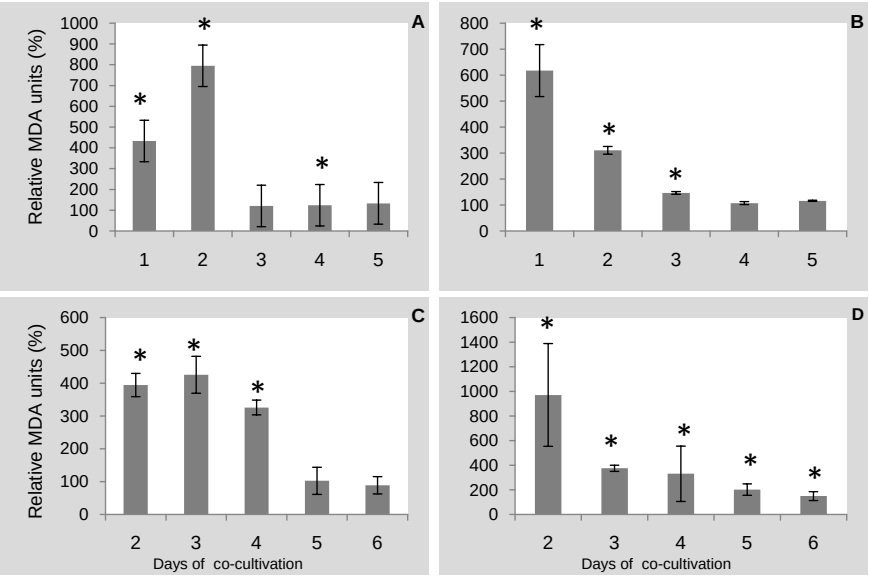

Figure 27: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the cell membrane integrity (non-enzymatic lipidperoxidation) of selected fungi.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani;* **C**. *Phoma eupyrena*; **D**. *Neurospora crassa*.

An initial CFU of 32-56*10^7 were point-inoculated on the bacterial side (NA)**.** The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on fungal side (SA). The dishes were placed at 20 °C. nLPRX was measured every 24 h for 5 days beginning with day 1 (**A** and **B**) and 6 days beginning with day 2 (**C** and **D**). MDA equivalents of the control in nmol/mL: 0.84 (d 1), 0.51 (d 2), 2.85 (d 3), 2.03 (d 4) and 1.6 (d 5) (**A**), 0.35 (d 1), 1.63 (d 2), 3.72 (d 3), 2.89 (d 4) and 3.12 (d 5) (**B**), 2.99 (d 2), 1.99 (d 3), 2.58 (d 4), 1.77 (d 5) and 1.71 (d 6) (**C**), 0.18 (d 2), 0.64 (d 3), 1.18 (d 4), 2.74 (d 5) and 4.23 (d 6) (**D**).
n=3. Error bars represent the standard deviations. (*) represents statistical significance related to the corresponding control (100 %).

3.3.2 Activity of the fungal superoxide dismutase (SOD)

The most important enzymatic antioxidants include the SOD, a metalloenzyme which catalyzes the disproportion of superoxide ($O_2^{\cdot -}$) to H_2O_2 (**72**). Measurements of this enzyme indicated free radicals inside the fungal cell presumably produced by bacterial volatiles.

Results revealed that all fungi showed increased SOD activity. *Sclerotinia sclerotiorum* was the most sensitive fungus because it experienced a 10-fold increase in SOD activity after day 1 (Figure 28A). Subsequently, the activity was reduced. The other 3 fungi did not exhibit such an elevation of SOD activity (Figure 28B-28D). *Rhizoctonia solani* showed a 2-fold increase for the first 2 days which was then gradually reduced (Figure 28B). *Phoma eupyrena* and *Neurospora crassa* exhibited the maximum of activity after day 2 (3-fold and 2-fold, respectively) that was also gradually reduced (Figure 28C and Figure 28D, respectively).

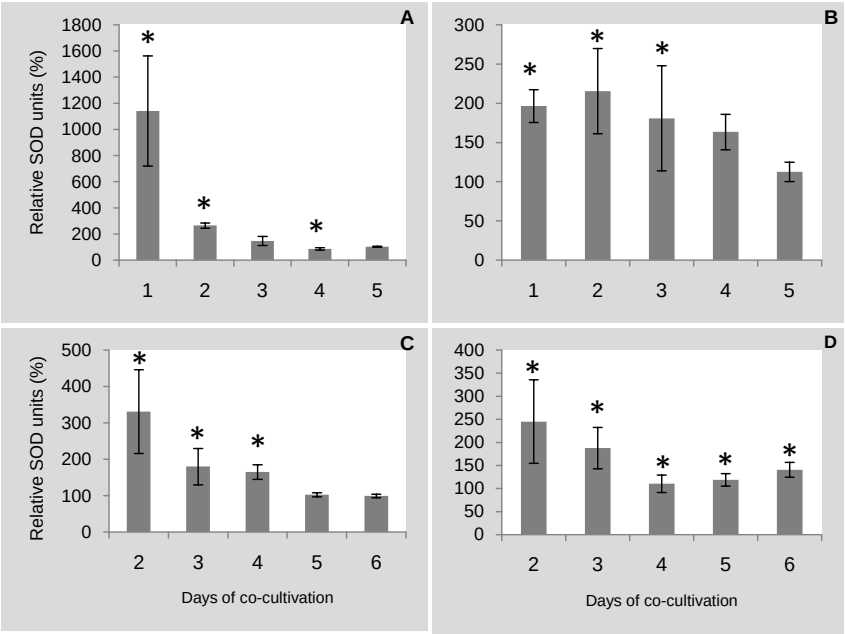

Figure 28: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the superoxide dismutase activity of selected fungi.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani*; **C**. *Phoma eupyrena*; **D**. *Neurospora crassa*.

An initial CFU of 39-52*10^7 were point-inoculated on bacterial side (NA). The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on fungal side (SA). The dishes were placed at 20 °C. SOD was measured every 24 h for 5 days beginning with day 1 (**A** and **B**) and 6 days beginning with day 2 (**C** and **D**). The control in units/mg protein was equivalent to 0.59 (d 1), 0.43 (d 2), 1.15 (d 3), 1.92 (d 4) and 2.38 (d 5) (**A**), 2.01 (d 1), 2.74 (d 2), 2.81 (d 3), 1.23 (d 4) and 1.3 (d 5) (**B**), 1.55 (d 2), 2.44 (d 3), 3.79 (d 4), 3.90 (d 5) and 5.3 (d 6) (**C**), 0.47 (d 2), 1.2 (d 3), 3.02 (d 4), 3.63 (d 5) and 3.49 (d 6) (**D**).
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

3.3.3 Activity of the fungal catalase

The up-regulation of the superoxide dismutase implies the investigation of the catalase (CAT). It is another important antioxidant enzyme that detoxifies H_2O_2 into H_2O and O_2. Measuring this enzyme acitvity, it was observed that all fungi had an up-regulation of the enzyme activity with an almost two-phase progression. All four fungi exhibited an increase in CAT activity between 2-3-fold, after day 1 or 2. The activity then more or less briefly declined, and subsequently climbs up again in older mycelia (Figure 29A-C). Only *Neurospora crassa* did not show any significant elevation of the catalase activity (Figure 29D). Additional results are presented in supplementary chapter (Figure S5A-5D).

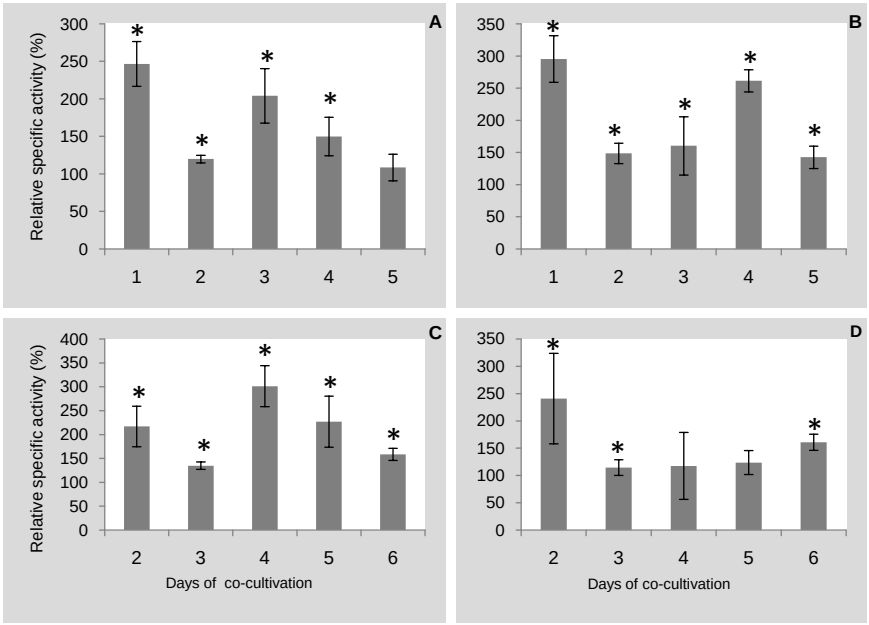

Figure 29: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the cell (catalase activity) of selected fungi.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani*; **C**. *Phoma eupyrena*; **D**. *Neurospora crassa*.

An initial CFU of 44-56*10^7 were point-inoculated on bacterial side (NA)**.** The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on fungal side (SA). The dishes were placed at 20 °C. CAT was measured every 24 h for 5 days beginning with day 1 (**A** and **B**) and 6 days beginning with day 2 (**C** and **D**). The control in µkat/mg protein was equivalent to 0.74 (d 1), 1.35 (d 2), 2.42 (d 3), 2.32 (d 4) and 2.46 (d 5) (**A**), 0.034 (d 1), 0.5 (d 2), 0.61 (d 3), 2.26 (d 4) and 2.48 (d 5) (**B**), 0.049 (d 2), 0.106 (d 3), 0.43 (d 4), 1.08 (d 5) and 1.91 (d 6) (**C**), 0.044 (d 2), 2.96 (d 3), 0.44 (d 4), 1.08 (d 5) and 1.92 (d 6) (**D**).
n=3. Error bars represent standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

3.3.4 Activity of the intracellular fungal laccase

The fourth parameter investigated was intracellular fungal laccase. Laccase belongs to the small group of enzymes called blue multi-copper oxidases. The expression of laccase in yeast was found to confer a strong ability to scavenge intracellular H_2O_2 and to protect cells from lipid oxidative damage (**48**).

Results indicated that in response to an exposure to bacterial volatiles, all fungi except *Phoma eupyrena* expressed an up-regulation of the laccase activity (Figure 30). *Sclerotinia sclerotiorum* exhibited a 5-fold laccase activity after day 1 which was gradually decreased to 2.5-fold after day 5 (Figure 30A). *Rhizoctonia solani* was the most sensitive fungus and displayed a maximum of a 35-fold activity compared to the control after day 1, which decreased towards 2.5-fold after day 2 and towards 1.5-fold after day 5 (Figure 30B). *Phoma eupyrena* showed rather a decrease in laccase activity (Figure 30C), whereas *Neurospora crassa* presented a late maximum activity after day 3 with an 8-fold increase. From day 5 onwards, the laccase activity gradually decreased (Figure 30D).

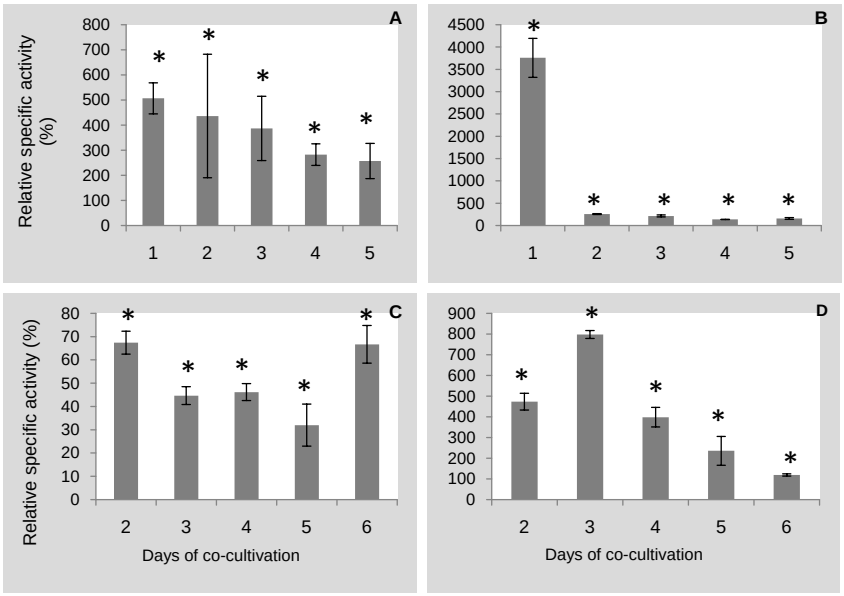

Figure 30: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the intracellular laccase activity of selected fungi.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani*; **C**. *Phoma eupyrena*; **D**. *Neurospora crassa.*

An initial CFU of 44-47*10^7 were point-inoculated on the bacterial side (NA). The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on the fungal side (SA). The dishes were placed at 20 °C. Laccase was measured every 24 h for 5 days beginning with day 1 (**A** and **B**) and 6 days beginning with day 2 (**C** and **D**). The control in nkat/mg protein was equivalent to 0.011 (d 1), 0.0383 (d 2), 0.0139 (d 3), 0.011 (d 4) and 0.013 (d 5) (**A**), 0.013 (d 1), 1.24 (d 2), 0.85 (d 3), 1.11 (d 4) and 0.53 (d 5) (**B**), 1.47 (d 2), 2.44 (d 3), 0.86 (d 4), 1.11 (d 5) and 0.17 (d 6) (**C**), 0.057 (d 2) , 0.071 (d 3), 0.04 (d 4), 0.073 (d 5) and 0.12 (d 6) (**D**).
n=3. Error bars represent standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

3.3.5 Summary

Summarizing all data on fungal growth and oxidative stress parameter, it can be concluded that bacterial volatiles of *S. plymuthica* 4Rx13 were able to inhibit radial growth as well as reduce the biomass of fungi. The maximum inhibition of growth appeared after day 1. Subsequently, the fungal growth gradually recovered. Furthermore, in response to the exposure to volatiles of *S. plymuthica* 4Rx13, the fungi also activated enzymes that may be responsible for overcoming deleterious effects caused by oxidative stress. This might confirm the assumption that bacterial volatiles produce oxidative stress in a fungal cell. The increase of nLPRX in fungal cell membranes during co-cultivation with *S. plymuthica* 4Rx13 supports this opinion. Since most of the effects were transient, the question is raised whether the alteration in enzyme activities helps the fungus in growth recovery.

3.4 Influence of fungal volatiles on the growth of *Serratia plymuthica* 4Rx13

Up to now, it has been known that volatile metabolites of the bacterium *Serratia plymuthica* 4Rx13 are able to reduce the fungal growth. Now the question was considered if fungal volatiles may affect the bacterial growth in the experimental setup used. To analyze that, the bacterium was grown in the presence and absence of the fungus *Sclerotinia sclerotiorum* and the bacterial CFU was determined during co-cultivation and compared with the control (no fungus). The results showed that fungal volatiles had no effect on bacterial growth (Figure 31).

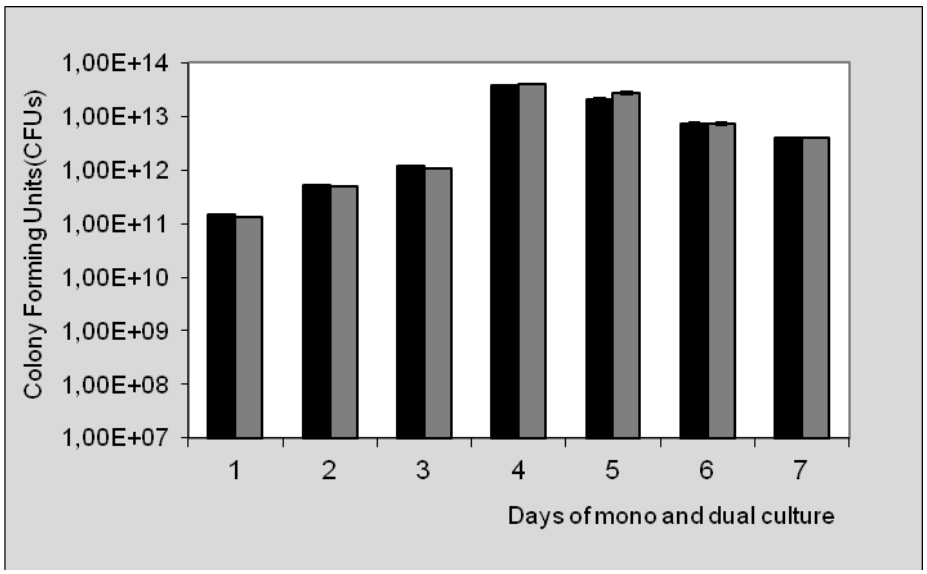

Figure 31: Influence of fungal volatiles on the growth of *Serratia plymuthica* 4Rx13.

■ bacterial growth in absence of the fungus *Sclerotinia sclerotiorum*; ■ bacterial growth in co-cultivation with the fungus *Sclerotinia sclerotiorum*.

An initial CFU of 42-52*10^7 were point-inoculated on the bacterial side (NA). The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on the fungal side (SA). The dishes were placed at 20 °C. The bacterial growth was monitored every 24 h.
n=3. Error bars indicate the standard deviation.

3.5 Analysis of bacterial and fungal volatile metabolites emitted from mono- and dual- cultures

Since the fungal-bacterial interaction was mediated by volatiles produced by the bacterium, it was of enormous interest to know which volatile compounds might be involved in the interaction. Two questions should be answered. (a) What was emitted by the bacterium and the fungus in monoculture? (b) Do both organisms influence each other concerning volatile emission in dual culture? In order to answer these questions, bacterial and fungal volatiles were analyzed referring to the experimental setting of co-cultivation.

3.5.1 Volatile organic compounds

For this experiment, a special VOC analysis chamber had been designed which ideally should not produce any volatiles itself. A chromatogram obtained from the empty system is exemplary shown in the supplementary chapter. Within 24 h no compound was produced (S7A). An example of chromatogram obtained from the bacterial/fungal media is presented in figure S7B. Up to eleven compounds could be detected but mostly not identified because the amount was too little. The most prominent volatiles present in this control were benzaldehyde and benzeneacetaldehyde.

3.5.1.1 VOC emission by *Serratia plymuthica* 4Rx13

In presence of an initial CFU of 40-48*10^7, the production of volatiles was monitored for two days at 30 °C and for three days at 20 °C. The qualitative spectrum of VOCs emitted by the bacterium supported the data shown by Kai *et al.* (**30, 53**). Two chromatograms are exemplarily presented in Figure 32 (30 °C) and 33 (20 °C). Other chromatograms are presented in the supplementary chapter (Figure S7C-7E).

The main compound was sodorifen (3) that was produced under both temperature conditions. Another typical compound could be identified as dimethyl trisulfide (1). In addition, ten other compounds were emitted that were either unknown or not identified but were also observed before (**30, 53**). These compounds could be assumed as isomers of sodorifen. They were characterized by calculating the retention indices (RI). Compound H (10) was detected only at 30 °C. All other compounds could be observed at both temperatures although; compound G (9) were produced till day 1 at 20 °C (Table 1). The quantity of VOCs produced by *S. plymuthica* 4Rx13 was greater at 30 °C than at 20 °C (Figure S7F).

VOCs not specified by numbers in Figure 31 and 32 were either compounds already found in the controls or could not be detected in all 3 individual experiments. Compounds that

appeared in less than 3 experiments are mentioned in the supplementary chapter (Figure S7F-M [compound K] and Table S1).

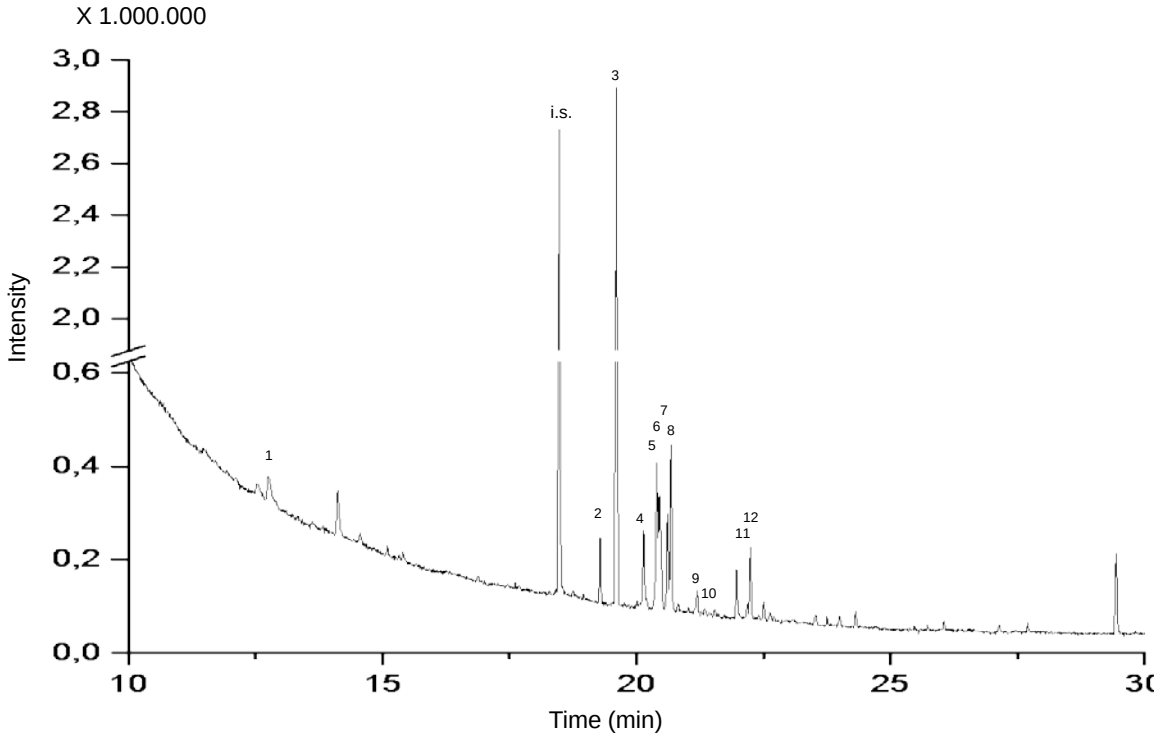

Figure 32: VOC emission of *Serratia plymuthica* 4Rx13 at 30 °C.

An initial CFU of 40-48*10^7 were point-inoculated onto bacterial NA side of a split Petri dish**.** The Petri dishes were kept in the VOC collecting chamber at 30 °C. Collection time for VOC analysis: 0-24 h (day 1, 30 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **1** (dimethyl trisulfide), **i.s.** (internal standard), **2** (compound A), **3** (sodorifen), **4** (compound B), **5** (compound C), **6** (compound D), **7** (compound E), **8** (compound F), **9** (compound G), **10** (compound H), **11** (compound I) and **12** (compound J). n=3.

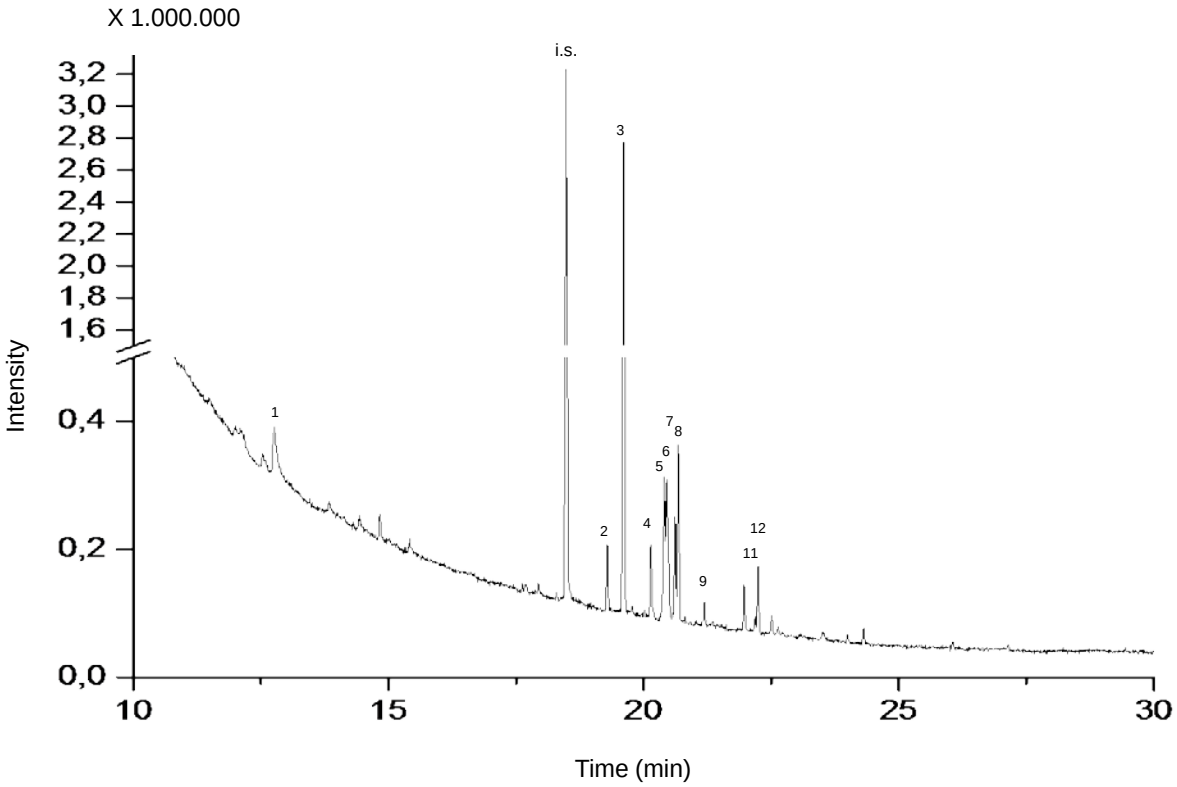

Figure 33: VOC emission of *Serratia plymuthica* 4Rx13 at 20 °C.

An initial CFU of 40-48*10^7 were point-inoculated onto bacterial NA side of a split Petri dish**.** The Petri dishes were incubated in the VOC collecting chamber at 30 °C for 2 days, subsequently at 20 °C for 3 days. Collection time for VOC analysis: 0-24 h (day 1, 20 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **1** (dimethyl trisulfide), **i.s.** (internal standard), **2** (compound A), **3** (sodorifen), **4** (compound B), **5** (compound C), **6** (compound D), **7** (compound E), **8** (compound F), **9** (compound G), **11** (compound I) and **12** (compound J). n=3.

Table 1: Qualitative analysis of main VOCs emitted by *Serratia plymuthica* 4Rx13.

■ 30 °C; ■ 20 °C. (+) VOC present. (–) VOC absent. n=3. Only VOCs appearing consistently in all 3 analyses were considered.

No	Compound (PubChem CID)	RI	day 1	day 2	day 1	day 2	day 3
1.	Dimethyl trisulfide (19310)	985	+	+	+	+	+
2.	Compound A (Not identified)	1366	+	+	+	+	+
3.	Sodorifen	1388	+	+	+	+	+
4.	Compound B (Not identified)	1413	+	+	+	+	+
5.	Compound C (Not identified)	1435	+	+	+	+	+
6.	Compound D (Not identified)	1439	+	+	+	+	+
7.	Compound E (Not identified)	1448	+	+	+	+	+
8.	Compound F (Not identified)	1454	+	+	+	+	+
9.	Compound G (Not identified)	1487	+	+	+	–	–
10.	Compound H (Not identified)	1517	+	+	–	–	–
11.	Compound I (Not identified)	1559	+	+	+	+	+
12.	Compound J (Not identified)	1562	+	+	+	+	+

3.5.1.2 VOC emission by *Sclerotinia sclerotiorum*

The production of fungal volatiles was monitored for three days at 20 °C. One chromatogram is exemplarily presented in Figure 34 (20 °C). Other chromatograms at 20 °C are presented in the supplementary chapter (S7G-7H). The fungus emitted only a very few number of VOCs. The diterpene labda-8(20),12,14-triene (14) was most obvious although it was produced only on day 3 in all three individual experiments (Table 2) in a quantity of 159 ng per 100 mg mycelium (Table S2). The compound 2-ethyl-hexanal (15) appeared only in two out of three individual experiments (Table S1), but it was emitted in high amounts at day 1 (3.6 µg/100 mg mycelium) and in less amount at day 2 and 3 (Table S2).

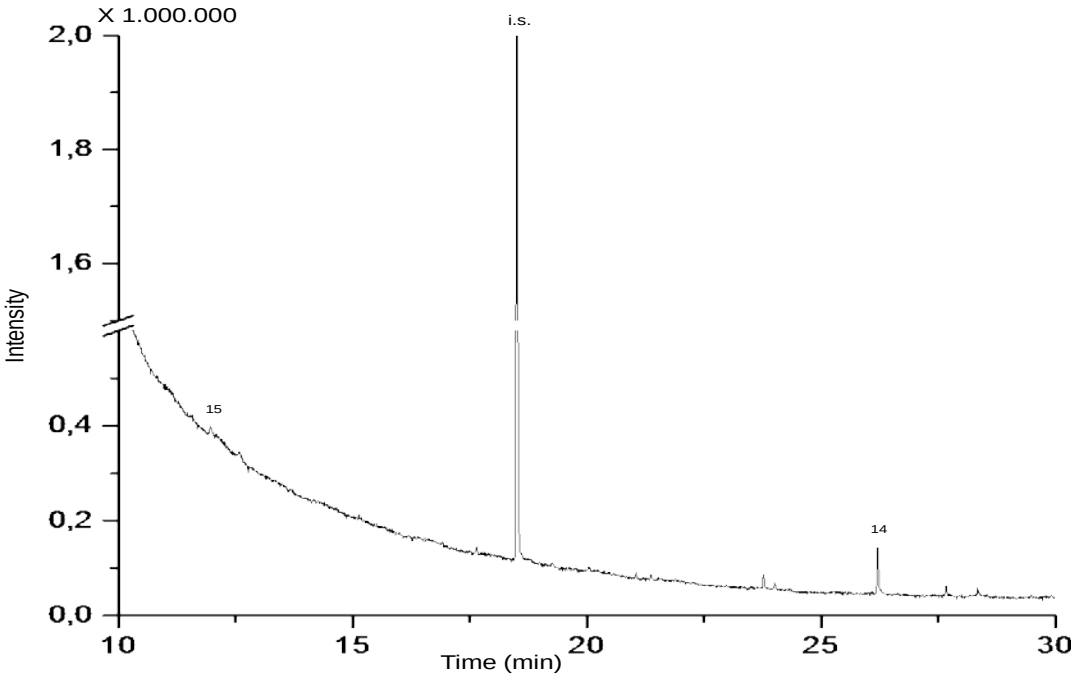

Figure 34: VOC emission of *Sclerotinia sclerotiorum* at 20 °C.

The split Petri dishes were kept in the VOC collecting system at 30 °C for 2 day. Mycelial plugs were inoculated and the VOC collecting system was kept at 20 °C for 3 days. Collection time for VOC analysis: 48-72 h, d 3, 20 °C. VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: i.s. (internal standard), **14** (labda-8 (20), 12, 14-triene), **15** (2-ethyl-hexanal. n=3.

Table 2: Qualitative analysis of main VOCs emitted by *Sclerotinia sclerotiorum*.

20 °C. (+) VOC present. (–) VOC absent. n=3. Only VOCs appearing consistently in all 3 analyses were considered.

No.	Compound (PubChem CID)	RI	d 1	d 2	d 3
14.	Labda-8(20),12,4-triene (6432579)	1977	–	–	+

3.5.1.3 VOC emission by *Serratia plymuthica* 4Rx13 and *Sclerotinia sclerotiorum* in dual culture

In dual culture of the bacterium and the fungus all already known volatiles could be found again (Table 3), however; one new volatile could be discovered. This compound might be 1-tridecanol (16) (Figure 35). It could be produced because of their interaction, either by the bacterium or the fungus. Unfortunately, this compound was only detected in 1 experiment out of 3 and is, therefore, mentioned in the supplementary chapter (Table S1). VOCs not specified by numbers in Figure 35 were either compound already found in the controls or could not be detected in all 3 individual experiments. The amount of volatiles produced by the bacterium was comparable to like that produced in monocultures (data not shown). A maximum of emission was reached at 30 °C after 24 hours (Figure S7F). Other chromatograms at 20 °C are shown in supplementary chapter (Figure S7I-S7J).

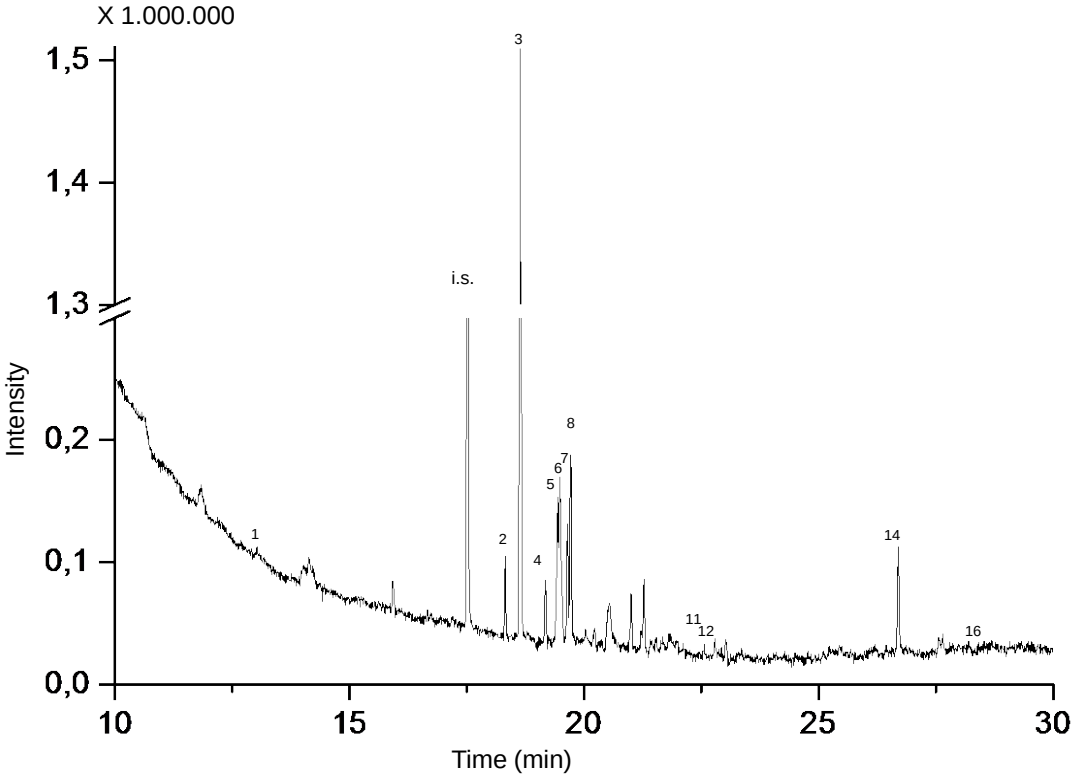

Figure 35: VOC emission of *Serratia plymuthica* 4Rx13 and *Sclerotinia sclerotiorum* at 20 °C.

An initial CFU of 40-48*10^7 were point-inoculated onto the bacterial NA side of a split Petri dish**.** The Petri dishes were kept in the VOC collecting chamber at 30 °C for 2 days. Mycelial plugs were inoculated and the system was kept at 20 °C. Collection time for VOC analysis: 48-72 h, d 3, 20 °C. VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS.Main peaks:**1** (dimethyl trisulfide), **i.s.**(internal standard), **3** (sodorifen), **4** (compound B), **5** (compound C), **6** (compound D), **7** (compound E), **8** (compound F), **11** (compound I), **12** (compound J),**14** (labda-8 (20),12, 14-triene) and **16** (1-tridecanol). n=3.

Table 3: Qualitative analysis of main VOCs emitted in a dual culture of the bacterium *Serratia plymuthica* 4Rx13 and the fungus *Sclerotinia sclerotiorum.*

30 °C; 20 °C. (+) VOC present. (–) VOC absent. n=3. Only VOCs appearing consistently in all 3 analyses were considered.

No	Compound (PubChem CID)	RI	day 1	day 2	day 1	day 2	day 3
1.	Dimethyl trisulfide (19310)	985	+	+	+	+	+
2.	Compound A (Not identified)	1366	+	+	+	+	+
3.	Sodorifen	1388	+	+	+	+	+
4.	Compound B (Not identified)	1413	+	+	+	+	+
5.	Compound C (Not identified)	1435	+	+	+	+	+
6.	Compound D (Not identified)	1439	+	+	+	+	+
7.	Compound E (Not identified)	1448	+	+	+	+	+
8.	Compound F (Not identified)	1454	+	+	+	+	+
9.	Compound G (Not identified)	1487	+	+	+	–	–
10.	Compound H (Not identified)	1517	+	+	–	–	–
11.	Compound I (Not identified)	1559	+	+	+	+	+
12.	Compound J (Not identified)	1562	+	+	+	+	+
14.	Labda-8(20),12,4-triene (6432579)	1977	–	–	–	–	+

3.5.2 Ammonia production by the bacterium *Serratia plymuthica* 4Rx13

Since the cultivation of bacteria on rich media can provoke ammonia production, the emission of bacterial ammonia had been investigated already in the past (**78**). This investigation was refined and adapted within the scope of the presented work here. The ammonia produced by a CFU_i of 40-48*10^7 was quantified in correlation to the assay conditions. *S. plymuthica* 4Rx13 was cultivated for 2 days at 30 °C and subsequently for 3 days at 20 °C. Every 24 hours, the amount of ammonia emitted by the bacterium was quantified and related to known amounts of ammonia by using reference solutions (3-300 mM, see chapter 2.7.8). At 30 °C the bacterium produced 2.1 mM ammonia within 24 h and at 20 °C it produced 1.4 mM ammonia within 24 h. This was comparable to the amount of gaseous ammonia evaporated by a 60 and 30 mM ammonia reference solution, respectively that served as source for ammonia instead of the bacterium.

3.6 Influence of ammonia on assay conditions

3.6.1 Ammonia as a constituent of the bacterial volatile blend

Previous research showed that ammonia produced by *Serratia plymuthica* 4Rx13 could alkalize the plant medium of *Arabidopsis thaliana* resulting in the growth inhibition of the plant (**78**). The pH of the growth medium, however, is also an important requirement for fungal growth. Fungi mostly prefer an acidic pH value. Alkalization has to be considered being responsible for growth inhibition of fungi. For this reason, the influence of bacterial volatiles on the pH value of the fungal growth medium was investigated. Following the procedure of dual-culturing, the pH was measured every 6 hours for 2 days at 30 °C and every 12 hours for 3 days at 20 °C. The pH of the control (media in a split Petri dish) possess a slightly acidic pH (5.9-6.1) (Figure S8A).

3.6.1.1 pH values of the fungal medium in a bacterial monoculture

Different CFU_i (3-54*10^7; $O.D._{600}$=0.8) of *S. plymuthica* 4Rx13 were point inoculated on the NA side of dual culture plates. With an elevated number of CFU_i, the pH value of the fungal medium increased compared to the control (Figure S8B). The strongest effects could be observed using a CFU_i of 54*10^7. These results are presented in Figure 36. Beginning with a slightly acidic pH value at 30 °C, the exposure to bacterial volatiles caused an increasing alkalization proceeding to a final alkaline pH of 8 after 2 days (Figure 36A). At 20 °C, the fungal medium persisted to display an alkaline pH value (Figure 36B).

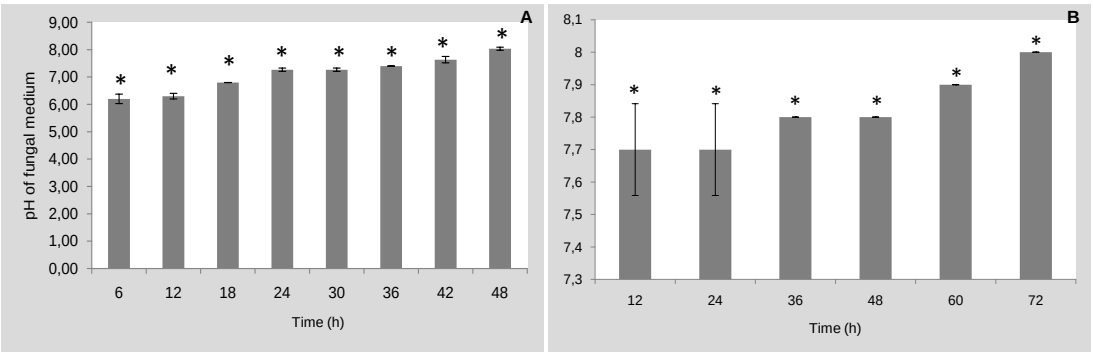

Figure 36: Influence of volatiles emitted by *Serratia plymuthica* 4Rx13 on the pH value of the fungal growth medium.

A: at 30 °C; **B:** at 20 °C;

An initial CFU of 54*10^7 were point inoculated on the bacterial side (NA). The split Petri dishes were kept first 2 days at 30 °C, next 3 days at 20 °C. The pH was measured every 6 h at 30 °C, every 12 h at 20 °C.
n=3. Error bars represent the standard deviations. (*) represents statistical significance related to the corresponding control.

3.6.1.2 pH values of the fungal medium in a fungal monoculture

At 30 °C, the pH of the fungal medium of course resembled the pH values of the control (Figure S8A). At the beginning of the incubation at 20 °C, the fungal medium displayed a slightly acidic pH value of 6. After 2 days in presence of *Sclerotinia sclerotiorum*, the pH value was consistently reduced to 3 (Figure 37A). This acidification could not be observed for the other three fungi. The fungal growth medium showed an almost constant acidic pH between 5 and 6 (Figure 37B-D).

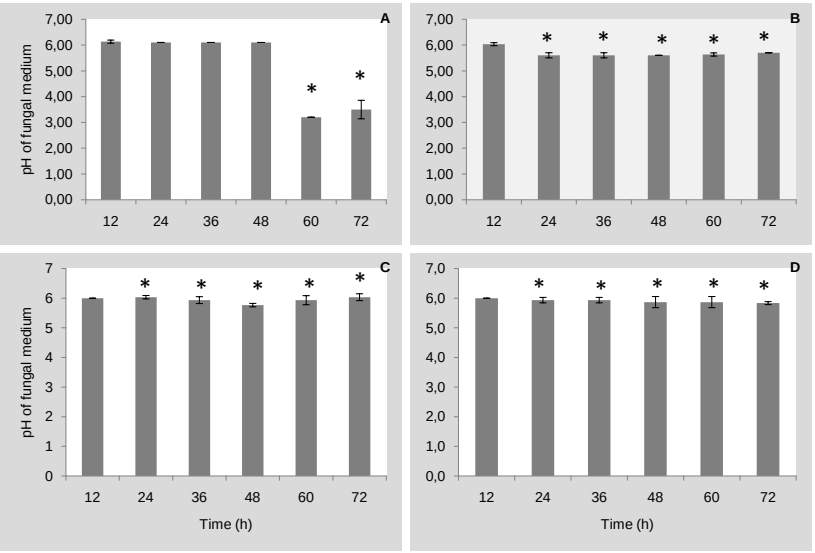

Figure 37: pH values of the fungal medium in fungal monocultures.

A: *Sclerotinia sclerotiorum*;
B: *Rhizoctonia solani*;
C: *Phoma eupyrena*;
D: *Neurospora crassa*.

The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated at fungal side (SA). The plates were kept at 20 °C for 3 days. The pH was measured every 12 hours at 20 °C.
n=3. Error bars represent standard deviations. (*) represents statistical significance related to the corresponding control.

3.6.1.3 pH values of the fungal medium in a dual culture

All four fungi were co-cultivated at a time with *S. plymuthica* 4Rx13. The results showed that the pH value of the fungal medium turned alkaline at 30 °C as observed in the bacterial monoculture (Figure 36A and S8B-A). The presence of fungal mycelia at 20 °C, however, considerably influenced the pH value of the fungal medium. The effect was CFU_i and time dependent (Figure S8C). The influence of the highest CFU_i is shown in Figure 38. *Sclerotinia sclerotiorum* decreased the pH after 3 days to a value of 3 (Figure 38A). *Rhizoctonia solani* exhibited a time dependent slow reduction to 7.5 (Figure 38B). *Phoma eupyrena* and *Neurospora crassa* did not cause much alteration. They grew on a pH value between 7.8 and 8. The complete data set is shown in the supplementary chapter (Figure S8C).

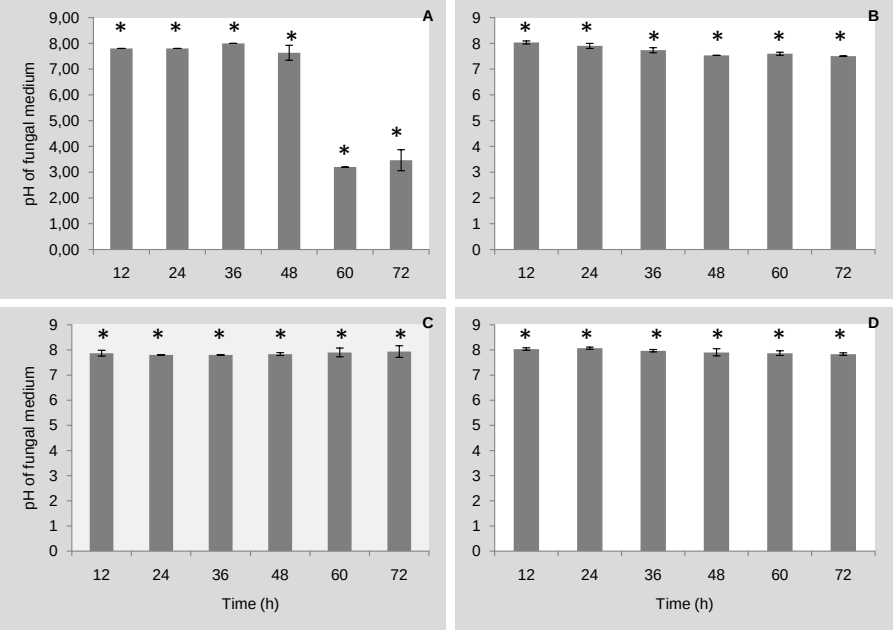

Figure 38: Influence of volatiles emitted by *Serratia plymuthica* 4Rx13 on the pH value of the fungal growth medium during co-cultivation with selected fungi.

A: *Sclerotinia sclerotiorum*;
B: *Rhizoctonia solani*;
C: *Phoma eupyrena*;
D: *Neurospora crassa*.

An initial CFU of $54*10^7$ CFU_i were point inoculated on the bacterial side (NA). The split Petri dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on fungal side (SA). The dishes were kept at 20 °C for 3 days. pH was measured every 12 hours at 20 °C.
n=3. Error bars represent the standard deviation. (*) signifies statistical significance related to the corresponding control.

3.6.2 Synthetic ammonia applied as a single compound

3.6.2.1 pH values of the fungal medium in absence of fungi

Referring to the ammonia concentration of the reference solutions (see 3.5.2), the influence of gaseous ammonia on the pH value of the fungal medium was monitored. The ammonia solutions (3-300 mM) replaced *S. plymuthica* 4Rx13 in a split Petri dish (see 2.7.9). The mono-culture of the bacterium, however, served as positive control. Figure 39A shows summarized findings including all values at 20 °C (2 days) and 30 °C (3 days), since the alkalization of the medium did not depend of the incubation time neither it depended on temperature. However, the alkalization appeared to be concentration dependent. Amounts of ammonia evaporated from solutions with low concentrations (3 and 6 mM) did not show a pronounced effect, whereas solutions with a concentration of 15 to 60 mM evaporated amounts that caused alkaline pH values around 8 which was comparable with the effect of $40\text{-}48*10^7$ CFU_i of *S. plymuthica* 4Rx13 (Figure 39B). These results indirectly confirmed the results of ammonia quantification (3.5.2) where the amount of naturally emitted ammonia could be related to a 30 and 60 mM reference solution at 20 and 30 °C, respectively. Ammonia evaporated from a 300 mM solution increased the pH value up to 10.

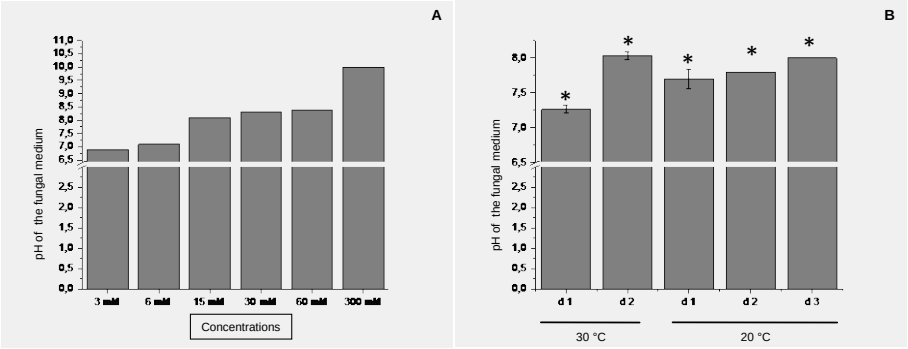

Figure 39: Influence of ammonia on pH value of the fungal medium.

A: Influence of selected ammonia solutions; **B:** Influence of volatiles of *Serratia plymuthica* (40-48*10^7 CFU_i).

10 ml of every ammonia solution was poured in one side of split Petri dish. The dishes were placed at 30 °C for 2 days, transferred to 20 °C for 3 days. The pH value was measured every 24 hours and compared with value produced by 40-48*10^7 CFU_i. The ammonia solution was changed every day.
n=3. Error bars represent standard deviations. (*) signifies statistical significance related to the corresponding control.

3.6.2.2 pH values of the fungal medium in presence of fungi

The experiment was conducted using the15 mM ammonia solution which alkalized the fungal medium in absence of fungal mycelium up to a pH value of 8 (3.6.2.1). *S. sclerotiorum* and *R. solani* were representatively chosen as fungal objects of examination. Both fungi re-influenced the pH value of their growth medium when exposed to *S. plymuthica* 4Rx13 (3.6.1.3). This phenomenon could also be observed when the fungal mycelium was exposed to synthetic ammonia (Figure 40). The effects were even a bit more pronounced. *S. sclerotiorum* lowered the pH value of its growth medium already after 2 days of incubation and finally acidified it (pH 3) after 3 days (Figure 40A). *R. solani* neutralized the alkaline pH of its growth medium after 2 days of incubation and finally lowered it to a slightly acidic pH value after 3 days (Figure 40B).

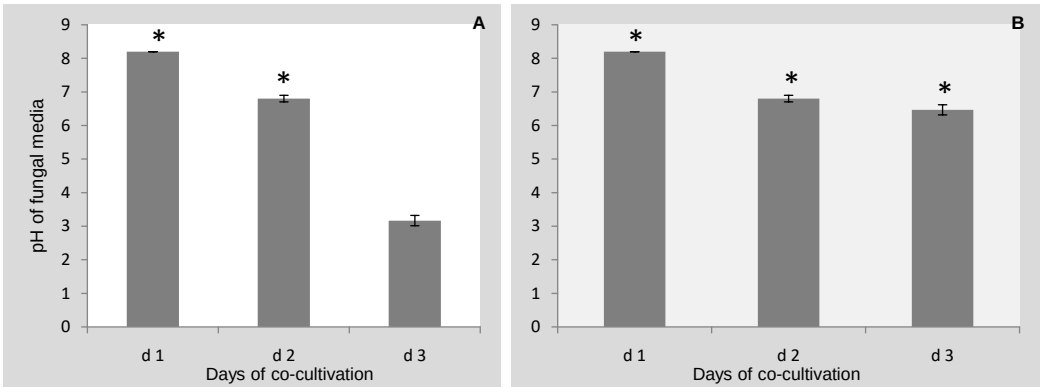

Figure 40: Influence of 15 mM ammonia on pH value of the fungal media in presence of fungal mycelium.

A: *Sclerotinia sclerotiorum*; **B:** *Rhizoctonia solani*.

10 ml of 15 mM ammonia solution was poured in one side of split Petri dish. The dishes were placed at 30 °C for 2 days. Mycelial plugs were inoculated on fungal SA side, the plates were transferred to 20 °C for 3 days. The pH was measured every 24 hours. Ammonia solution was changed every day.
n=3. Error bars represent standard deviation. (*) signifies statistical significance related to the corresponding control.

3.6.3 Summary

Volatiles produced by the bacterium *S. plymuthica* 4Rx13 were responsible for an alkalization of the fungal medium. This effect could be mimicked by an exposure to synthetic ammonia where solutions of 15–60 mM evaporated a sufficient amount of gaseous ammonia to obtain an alkalization comparable to the effect of an exposure to 40-48*10^7 CFU_i of *S. plymuthica* 4Rx13. Fungi like *R. solani* and especially *S. sclerotiorum* were able to re-acidify their growth medium during exposure to both bacterial volatiles and synthetic ammonia.

3.7 Effects of synthetic ammonia on fungi

The bacterium *Serratia plymuthica* 4Rx13 emitted a considerable amount of ammonia. This was subsequently accompanied by an alkalization of the fungal growth medium which could be mimicked by synthetic ammonia. Fungi had to cope with this alteration of growth conditions and it had to be considered that ammonia might be one of the bioactive components emitted by the bacterium. In order to varify this hypothisis, fungal cultures were exposed to synthetic ammonia and growth, membrane integrity, and enzyme acitivities were monitored. For these tests, the two fast growing fungi *Sclerotinia sclerotiorum* and *Rhizoctonia solani* were selected as subjects of investigations because of their different abilities to lower the pH value of their growth medium (Figure 40). The amount of ammonia applied in upcomming experiments should be equivalent to the amount naturally produced by *S. plymuthica* 4Rx13 at 20 °C. Therefore, before proceeding with bioassays, the fungus *S. sclerotiorum* was exposed to all ammonia reference solutions in order to assure that the concentration of 30 mM (Figure 39) represents the proper source for such an equivalent of gaseous ammonia causing a fungal growth inhibition similar to that of 40-48*10^7 CFU_i of *S. plymuthica* 4Rx13. The effect shall be reversible upon elimination of ammonia from the headspace in the test system.

3.7.1 Preliminary experiments

3.7.1.1 Effect of synthetic ammonia on fungal growth

S. sclerotiorum was exposed to ammonia solutions at 6 concentrations (3-300 mM). The effect on the radial growth and biomass production was only partly concentration and time dependent (Figure 41A+B). An increasing concentration between 3 and 15 mM caused a decrease in radial growth as well as biomass production and with an increasing incubation time, the effect was declining. However, in presence of ammonia solutions, in concentrations between 30 and 300 mM, almost no fungal growth was observed. In addition, these effects

did not show pronounced time and concentration dependencies. In the supplementary chapter pictures are shown (Figure S8D)

Although the concentration of 30 mM ammonia was (a) found to evaporate an equivalent amount of ammonia compared with the amount emitted by *S. plymuthica* 4Rx13 at 20 °C (3.5.2) and did (b) cause an alkalization comparable to that caused by the bacterium (3.6.2.1), it was very surprising to notice that this concentration of synthetic ammonia did not inhibit the fungal growth to an extent comparable to that of the bacterium. Moreover, it almost completely inhibited the radial growth and biomass production, whereas the exposure to 6 mM and 15 mM ammonia solutions influenced the fungal growth to an extent comparable with(40-48)*10^7 CFU_i of *S. plymuthica* 4Rx13 (3.2.1).

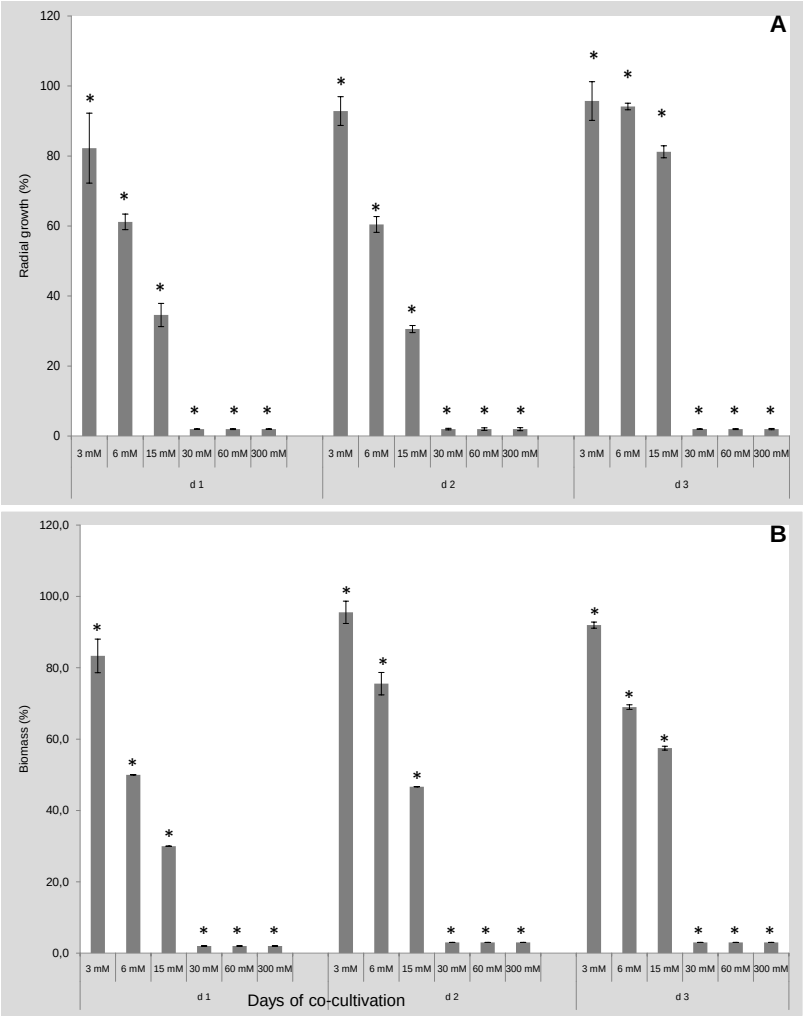

Figure 41: Influence of synthetic ammonia on the radial growth and biomass production of *Sclerotinia sclerotiorum*.

A: Radial growth
B: Biomass production

10 ml of every ammonia solution was poured into one compartment of a split Petri dish. The dishes were placed at 30 °C for 2 days. The mycelial plugs were inoculated on the SA side. Plates were placed at 20 °C for 2 days. Ammonia was renewed everyday. The radial growth was expressed in pixel (software ImageJ) and biomass was measured in mg. The control was equivalent to 1302392 (**A**) and 30 mg (**B**).
n=3. Error bars represent standard deviations. (*) represents statistical significance related to the corresponding control (100 %).

3.7.1.2 Influence of ortho-phosphoric acid on the inhibitory effect of ammonia

Ortho-phosphoric acid was used in order to prove the specificity of the ammonia effect. This concerns both the ammonia produced by *S. plymuthica* 4Rx13 as well as the synthetic ammonia. Mycelia of *S. sclerotiorum* and *R. solani* were co-cultivated with *S. plymuthica* 4Rx13 in presence and absence of o-phosphoric acid (0.85 %) in three-compartment plates. The acid is known to neutralize ammonia (**79**) and could therefore compensate the harmful effect of ammonia on the fungal growth by eliminating it from the headspace. Experiments using synthetic ammonia instead of the bacterium were conducted the same way. In the following subsection, experimental data obtained with *S. sclerotiorum* will be shown. The complete data set is shown in the supplementary chapter (Figure S9A-9H). *P. eupyrena* and *N. crassa* were also exposed to ammonia and o-phosphoric acid but they grew so slow that hardly any growth inhibition in three-compartment plates could be noticed (data not shown).

The results obtained with *S. sclerotiorum* after day 2 at 20 °C are shown in Figure 42. In absence of ortho-phoshoric acid, the fungal growth was totally inhibited in the presence of *S. plymuthica* 4Rx13 (CFU_i: $13*10^7$) (Figure 42B). The bacterial suspension was plated in order to obtain this significant gowth inhibiton. The addition of ortho-phosphoric acid to the system compensated for this deleterious effect (Figure 42D). The growth of the fungus exposed to the acid alone (Figure 42C) was comparable to their respective control (Figure 42A). Figure 42E quantitatively illustrates the effect of *S. plymuthica* 4Rx13 in absence and presence of ortho-phosphoric acid. The presence of the acid mediated an almost complete recovery from a 90 % inhibition. In the second data set shown in figure 42F-J, the bacterial suspension was replaced by a solution of synthetic ammonia. A concentration of 60 mM was used, again, in order to obtain a sufficient growth reduction. In absence of ortho-phosphoric acid, ammonia inhibited the fungal growth (Figure 42G) by 50 % (Figure 42J), whereas in presence of the ortho-phosphoric acid, growth inhibition was almost completely abolished (Figure 42I). The growth was only inhibited by 10 % (Figure 42H). Additionally, as shown in figure 42A and C, the fungal growth in presence of ortho-phosphoric acid alone (Figure 42H) was comparable to the control (Figure 42F).

In order to gain a complete data set, the pH values of the fungal medium were similarly monitored. They appeared to be consistantly acidic (Figure 42C, D, H and I) in precencs of ortho-phosphoric acid, which was completely comparable to the control (Figure 42A and 42F). Only the presence of bacterial volatiles (Figure 42B) and artifical ammonia (Figure 42G) in absence of ortho-phosphoric acid shifted the pH value towards an alkaline range between 7.4 and 7.8. This also implies a neutralization of ammonia, which indirectly pointed

towards an involvement of ammonia as one of the effective components in the bacterila volatile mixture.

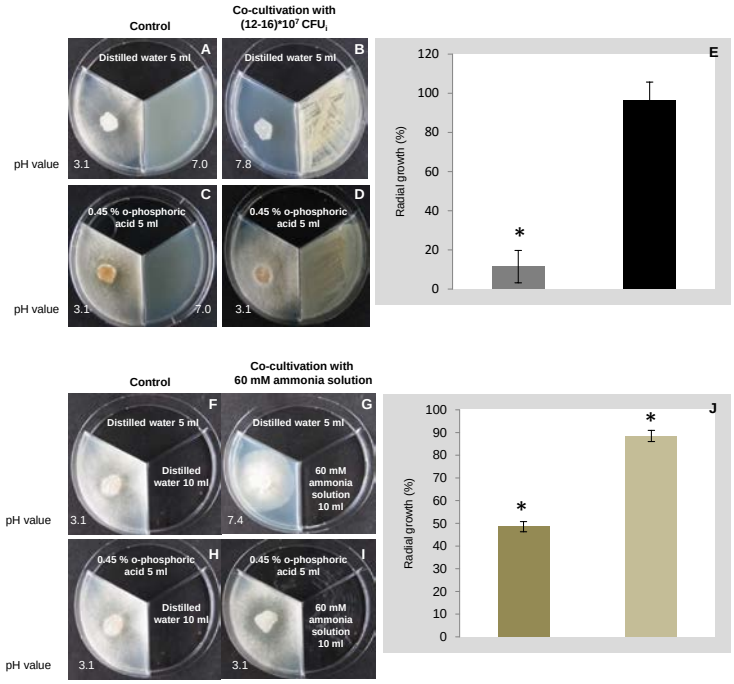

Figure 42: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 and synthetic ammonia on the growth of *Sclerotinia sclerotiorum* and pH value of the fungal media in presence/absence of o-phosphoric acid.

A. Monoculture of *Sclerotinia sclerotiorum*; **B**. dual culture of *Sclerotinia sclerotiorum* with *Serratia plymuthica* 4Rx13; **C**. monoculture of *Sclerotinia sclerotiorum* in presence of o-phosphoric acid; **D**. dual culture between *Sclerotinia sclerotiorum* and *Serratia plymuthica* 4Rx13 in presence of ortho-phosphoric acid; **E**. influence of *Serratia plymuthica* 4Rx13 on growth of *Sclerotinia sclerotiorum* in absence and presence of ortho-phosphoric acid. **F**. monoculture of *Sclerotinia sclerotiorum*; **G**. co-culture of *Sclerotinia sclerotiorum* with 60 mM ammonia; **H**. monoculture of *Sclerotinia sclerotiorum* in presence of o-phosphoric acid; **I**. exposure of *Sclerotinia sclerotiorum* to 60 mM ammonia in presence of o-phosphoric acid; **J**. influence of 60 mM ammonia on the growth of *Sclerotinia sclerotiorum* in absence and presence of ortho-phosphoric acid.

absence of o-phosphoric acid; presence of o-phosphoric acid.

An initial CFU of 13*10^7 were plated on bacterial NA or the compartment was filled with 10 ml of a 60 mM ammonia solution. The dishes were placed at 30 °C for 2 days. The mycelial plugs were placed on SA in a second compartment. The third compartment was filled with ortho-phosphoric acid if desired. In absence of ortho-phosphoric acid, distilled water was used. The growth and the pH value were measured after an incubation time of 2 days at 20°C. The growth was expressed in pixel (software ImageJ): controls were equivalent to 1815519 (-), 1826004 (+) and 1696543 (-), 1375432 (+) ortho-phosphoric acid for **(E)** and (**J**), respectively.
n=3. Error bars represent standard deviations. (*) presents statistical significance related to the corresponding control (100 %).

3.7.1.3 Conclusion

Results shown in the two previous chapters varified (a) that ammonia can inhibit fungal growth and biomass production and (b) that this effect can be prevented by simultanious application of ortho-phosphoric acid. The working solution of 30 mM ammonia could not be confirmed. Finally, the concentration of 15 mM was chosen for further experiments regarding membrane integrity and enzyme activity despite the fact that the inhibitory effect on the growth and biomass production was slightly more pronounced. The choice was decisively influenced by: (a) the impact on the fungal growth was after all comparable to the effect obtained with 40-48*10^7 CFU$_i$ of *S. plymuthica* 4Rx13 and (b) even more important: the pH value of the fungal medium matched the value generated by the naturally produced amount.

The influence of the 6 mM ammonia solution on the pH value was insufficient. It did not alkalize the medium (see 3.6.2).

3.7.2 Influence of synthetic ammonia on fungal membrane integrity and enzyme activities indicating oxidative stress

3.7.2.1 Non-enzymatic lipidperoxidation

In response to exposure to a 15 mM ammonia solution, both fungi exhibited elevated nLPRX after day 1 and 2. Figure 43 presents the result for both fungi. In *S. sclerotiorum*, the MDA formation was 4-fold higher compared to the control after day 1. This was comparable to the impact of volatiles of *S. plymuthica* 4Rx13 (Figure 43A). While the effect of ammonia ceased with increasing time of incubation, the impact of the bacterial volatiles reached a maximum after 2 days of exposure. After day 3, the level of MDA of any exposed mycelia was comparable with the control. For *R. solani,* the picture was different (Figure 43B). The nLPRX caused by ammonia dropped behind the impact of bacterial volatiles. It presented only an increment by 2-fold after 1 day of exposure. The increase in nLPRX compared to bacterial volatiles after 2 days of exposure was insignificant. After day 3, the MDA level caused by ammonia appeared to be even less than the control. In summary, the pattern of nLPRX caused by ammonia did not match those caused by bacterial volatiles. Especially when bacterial volatiles caused a maximum of nLPRX, the effect of ammonia was not that pronounced.

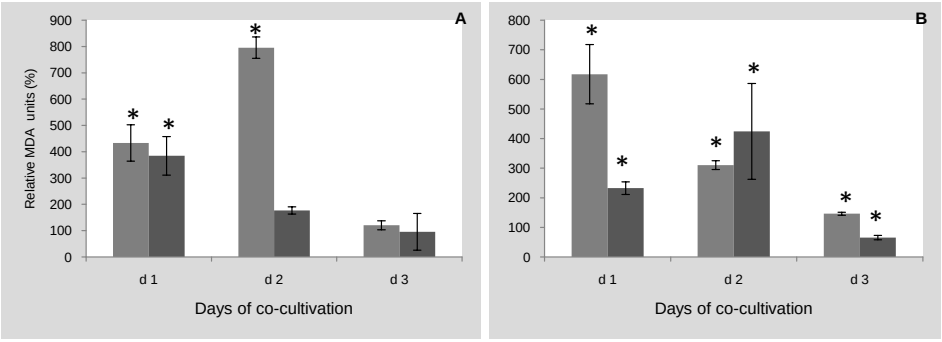

Figure 43: Influence of ammonia on the non-enzymatic lipid peroxidation in *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in comparison to effects of volatile metabolites of *Serratia plymuthica* 4Rx13.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani*.

S. plymuthica 4Rx13 (CFU_i: 32-51*10^7); 15 mM ammonia solution.

10 ml of 15 mM ammonia solution was poured in one side of split Petri dish. The dishes were placed at 30 °C for 2 days. Mycelial plugs were inoculated on SA. The plates were incubated at 20 °C for 3 days. The MDA level was measured every 24 h. The ammonia solution was changed every day. MDA equivalents of the control in nmol/mL: 0.84 (d 1), 0.51 (d 2), 2.85 (d 3) caused by *Serratia* and 1.1 (d 1), 0.4 (d 2), 0.4 (d 3) caused by ammonia (**A**). 0.35 (d 1), 1.63 (d 2), 3.72 (d 3) caused by *Serratia* and 1.03 (d 1), 0.32 (d 2), 0.78 (d 3) caused by ammonia (**B**).
n=3. Error bars present standard deviations. (*) represents statistical significance related to the corresponding control (100 %).

3.7.2.2 Activity of the fungal superoxide dismutase

Ammonia caused in both the fungi increased SOD activity (Figure 44). In case of *S. sclerotiorum* (Figure 44A), the SOD activity was 7-fold higher compared to the control after day 1 and was reduced to 1.6-fold after day 2. After day 3, the activity was similar to the control. This pattern of SOD activation caused by ammonia corresponded to that caused by bacterial volatiles, however, at all time points the SOD activity was lower. The response of *R. solani* is shown in Figure 44B. Ammonia increased the SOD activity 3-fold after day 1. This surpassed the bacterial effect. After day 2 and 3, the activity appeared to be similar to the control but less than the effect of bacterial volatiles.

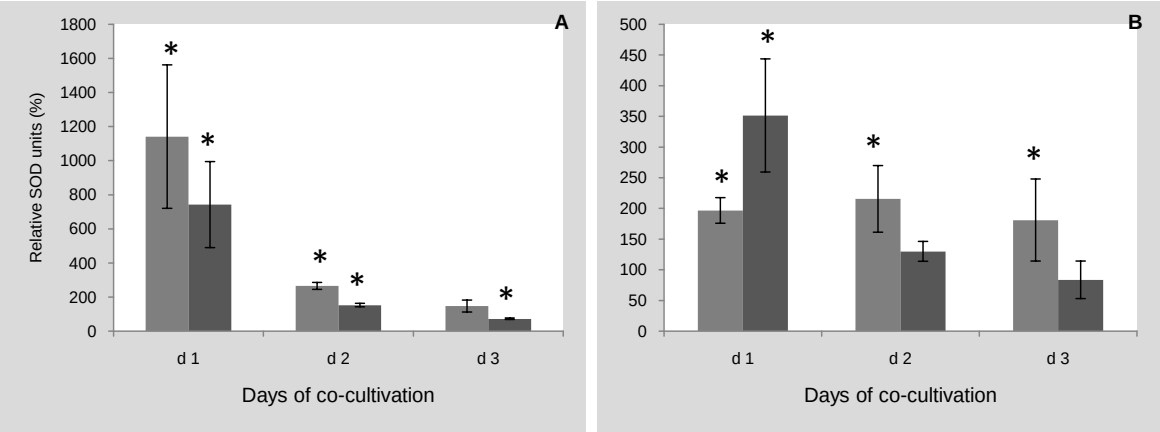

Figure 44: Influence of ammonia on the superoxide dismutase activity in *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in comparison to effects of volatile metabolites of *Serratia plymuthica* 4Rx13.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani*.

S. plymuthica 4Rx13 (CFU_I: 32-51*10^7); 15 mM ammonia solution.

10 ml of 15 mM ammonia solution was poured in one side of split Petri dish. The dishes were placed at 30 °C for 2 days. Mycelial plugs were inoculated on SA. The plates were incubated at 20 °C for 3 days. The SOD activity was measured every 24 h. The ammonia solution was changed every day. The control in units/mg protein was equivalent to 0.59 (d 1), 0.43 (d 2), 1.15 (d 3) caused by *Serratia* and 6.57 (d 1), 3.26 (d 2), 2.35 (d 3) caused by ammonia (**A**). 2.01 (d 1), 2.74 (d 2), 2.81 (d 3) caused by *Serratia* and 2.58 (d 1), 3.78 (d 2), 0.79 (d 3) caused by ammonia (**B**).
n=3. Error bars present standard deviations. (*) represents statistical significance related to the corresponding control (100 %).

3.7.2.3 Activity of the fungal catalase

In response to ammonia, both the fungi exhibited an elevated but rather weak catalase activity in comparison to the control (Figure 45). In *S. sclerotiorum* a noticeable increase (3-fold) could only be observed after day 1. This increment was similar to the impact of bacterial volatiles. With increasing incubation time, the activity was similar to the control (day 3) or even less (day 2), but always less in correlation to bacterial volatiles (Figure 45A). *R. solani* (Figure 45B) exhibited only after day 2 the increase in catalase activity (2-fold). After day 1 and 3, the activity was similar to the control with a most obvious decrease in activity compared to bacterial volatiles after day 1.

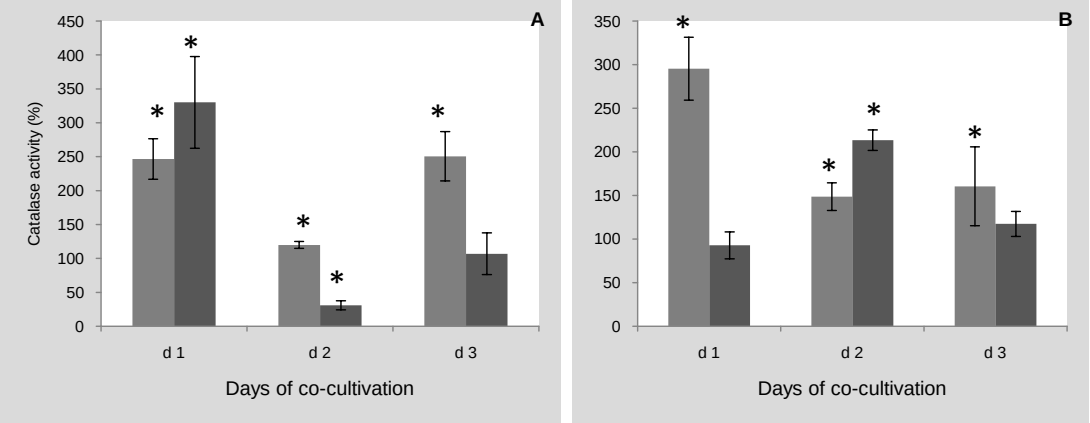

Figure 45: Influence of ammonia on the catalase activity in *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in comparison to effects of volatile metabolites of *Serratia plymuthica* 4Rx13.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani*.

S. plymuthica 4Rx13 (CFU_i: 32-51*10^7); 15 mM ammonia solution.

10 ml of 15 mM ammonia solution was poured in one side of split Petri dish. The dishes were placed at 30 °C for 2 days. Mycelial plugs were inoculated on SA. The plates were incubated at 20 °C for 3 days. The catalase activity was measured every 24 h. The ammonia solution was changed every day. The control in µkat/mg protein was equivalent to 0.74 (d 1), 1.35 (d 2), 2.42 (d 3) caused by *Serratia* and 0.75 (d 1), 3.82 (d 2), 3.81 (d 3) caused by ammonia (**A**). 0.034 (d 1), 0.5 (d 2), 0.61 (d 3) by *Serratia* and 0.0034 (d 1), 2.32 (d 2), 2.98 (d 3) caused by ammonia) (**B**).
n=3. Error bars present standard deviations. (*) represents statistical significance related to the corresponding control (100 %).

3.7.2.4 Activity of the fungal intracellular laccase

The laccase activity was the only parameter that exhibited in response to ammonia pronounced different patterns of influence compared to bacterial volatiles. In *S. sclerotiorum* the activity was 10-fold higher compared to the control after day 1, which insignificantly increased to 14-fold after day 2 and returned to control values after day 3. This pattern differed from the rather uniform increment of laccase activity after exposure to bacterial volatiles (Figure 46A), *R. solani* showed enhanced laccase activity (Figure 46B) on all three days of incubation. Ammonia caused a 5-fold elevation after day 1, which increased continuously to 14-fold after day 3. This activity pattern in *R. solani* was reverse to that caused by bacterial volatiles.

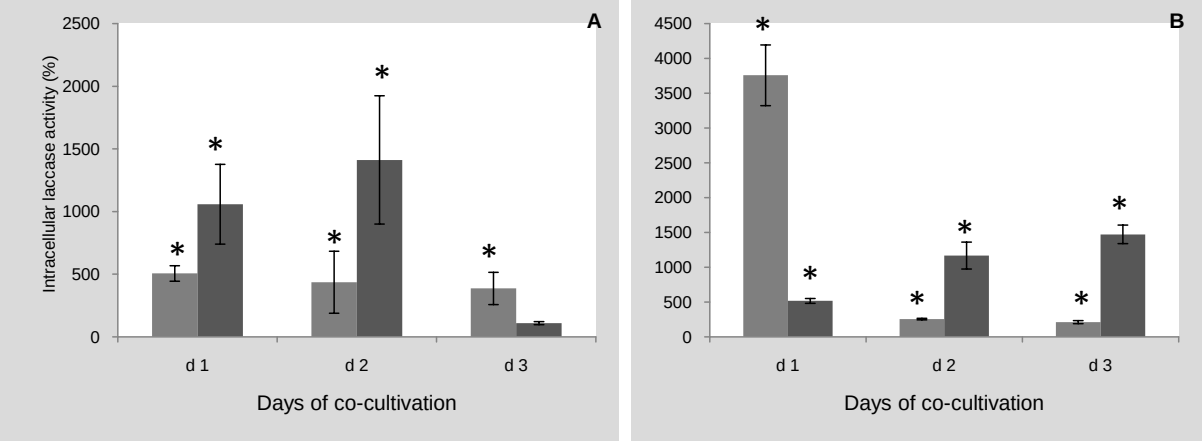

Figure 46: Influence of ammonia on the intracellular laccase activity in *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in comparison to effects of volatile metabolites of *Serratia plymuthica* 4Rx13.

A. *Sclerotinia sclerotiorum*; **B**. *Rhizoctonia solani*.

S. plymuthica 4Rx13 (CFU_i: 32-51*10^7); 15 mM ammonia solution.

10 ml of 15 mM ammonia solution was poured in one side of split Petri dish. The dishes were placed at 30 °C for 2 days. Mycelial plugs were inoculated on SA. The plates were incubated at 20 °C for 3 days. Laccase was measured every 24. Ammonia solution was changed every day. The control in nkat/mg protein was equivalent to 0.011 (d 1), 0.038 (d 2), 0.013 (d 3) caused by *Serratia* and 0.038 (d 1), 0.0011 (d 2), 0.0085 (d 3) caused by ammonia (**A**) and 0.013 (d 1), 1.24 (d 2), 0.85 (d 3) caused by *Serratia* sp. and 0.0084 (d 1), 0.019 (d 2), 0.025 (d 3) caused by ammonia) (**B**).
n=3. Error bars present standard deviations. (*) represents statistical significance related to the corresponding control (100 %).

3.7.2.5 Summary

Summarizing these four sets of data it can be concluded that ammonia produced by the bacterium might be involved in growth impairment and oxidative stress production in fungi. However, the patterns of influence during the exposure and co-cultivation induced by synthetic ammonia and bacterial volatiles, respectively, differed considerably. It may be deduced that there are also other bacterial volatiles responsible for growth inhibition and oxidative stress production in fungi.

3.8 Adaptation experiment

From all experiments conducted so far, it could be concluded that bacterial volatiles are responsible for growth inhibition and oxidative stress production in fungi. Most effects reached a maximum at d 1 and/or day 2 of co-cultivation with *Serratia plymuthica* 4Rx13. The effects were obviously transient and over the course of co-cultivation the fungi seemed to overcome the inhibitory impact on growth. This raises the questions whether the alteration in enzyme activities specifically helped the fungi to gain growth recovery and, furthermore, whether these alterations were triggered as a truly specific response to bacterial volatiles. In order to answer these questions an experiment consisting of two steps was designed where the fungal mycelia were repeatedly exposed to bacterial volatiles. It was hypothesized that due to adaptation fungal cultures shall grow better and shall cope better with oxidative stress than non-adapted fungal cultures.

As a first step (I), mycelia of the fungus *Sclerotinia sclerotiorum* were initially exposed to bacterial volatiles for two days (dual culture). A fungal monoculture served as control. As step two (II), fungal mycelia of both the control and dual culture were subsequently transferred to new plates. Whereas the mycelium originating from the control was initially exposed to bacterial volatiles, the mycelium originating from the dual culture was re-exposed. Fungal cultures on hydroquinone (5mM) which was dissolved in SA served as a positive control and were treated as the dual culture. After day 1 of initial exposure and day 1- 3 of re-exposure, the pH of the fungal medium as well as the radial growth, biomass, and the activity of intracellular laccase of the fungus were determined. An overview of this experiment in pictures is displayed in the supplementary chapter (Figure S6A). The results obtained after day 1 of initial exposure and day 1 of re-exposure are presented in the following chapters. The complete data set of re-exposure is shown in Figure S6B-6E.

3.8.1 Initial exposure of the fungus *Sclerotinia sclerotiorum* to bacterial volatiles and hydroquinone (I)

After day 1 of the initial exposure to bacterial volatiles, *S. sclerotiorum* showed a 40 % inhibition of the radial growth (Figure 47A) and biomass production (Figure 47B), whereas in the presence of hydroquinone, the inhibition of growth and biomass production reached 90 % (Figure 47A and Figure 47B, respectively). The fungal medium (SA) exhibited in absence of the bacterium as well as in presence of hydroquinone a pH value of 6.6, whereas in presence of the bacterium, the fungal medium displayed a slightly increased pH value of 7.3 (Figure 47C). The laccase activity which was chosen as the proxy for enzyme activities in this experiment, was 5–fold up-regulated of in the presence of bacterial volatiles as well as hydroquinone (Figure 47D) in comparison to the control (fungal monoculture).

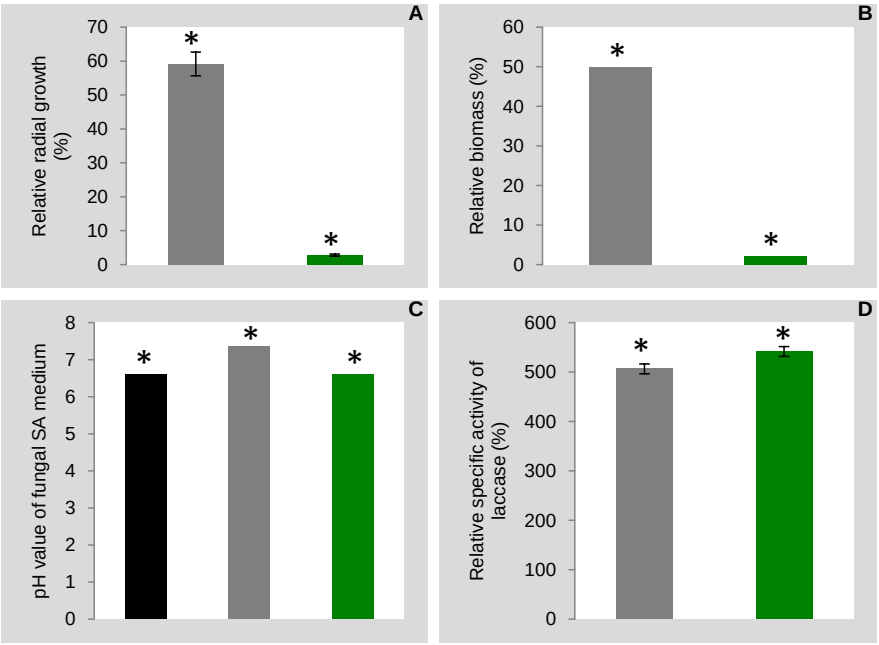

Figure 47: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 and hydroquinone on the pH of fungal media and the radial growth, biomass production, and laccase activity in *Sclerotinia sclerotiorum*.

A. radial growth; **B**. biomass; **C**. pH value of fungal media; **D**. intracellular laccase.

negative control (monoculture non-exposed); dual culture; positive control (5 mM hydroquinone).

An initial CFU of 40-48*10^7 were point-inoculated onto the bacterial nutrient agar of split Petri dishes. The dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on fungal SA side. The dishes were placed at 20 °C and parameters were measured after day 1 of incubation. The radial growth was expressed in pixel (software ImageJ). The controls were equivalent to 459433 (**A**), 5 mg (**B**), 0.016 nkat/mg protein (**D**). n=3. Error bars show standard deviations. (*) represents statistical significance related to the negative control (**A, B, D:** 100 %).

3.8.2 Re-exposure of the fungus *Sclerotinia sclerotiorum* to bacterial volatiles and hydroquinone (II)

After the initial exposure, the fungus was (a) re-exposed to bacterial volatiles and hydroquinone and (b) inoculated as monoculture (negative control, previously exposed). At the same time, the former negative control (fungal monoculture, non-exposed) was (a) initially exposed to both stressors and (b) inoculated as monoculture (master negative control, never exposed).

After re-exposure to bacterial volatiles as well as to hydroquinone, these fungal cultures indeed underwent a process of adaptation since they exhibited less susceptibility to bacterial volatiles compared to fungal cultures that were now initially exposed and subsequently non-adapted. Hereby, all results were related to the master negative control which represented 100 %.

Figure 48 presents the detailed picture of results obtained after day 1 of cultivation/exposure. Adapted re-exposed fungal cultures exhibited less growth inhibition in comparison to non-

adapted now initially exposed fungal cultures. These re-exposed fungal cultures showed even only little growth inhibitions (10-20 %) when compared to the master negative control. Adapted fungal cultures in monoculture (previously exposed to fungal volatiles and hydroquinone) showed growth comparable to the master control (Figure 48A). The same patterns could be observed for biomass productions (Figure 48B). Adapted fungal cultures showed significantly less inhibition than non-adapted cultures and their biomass productions recovered also significantly when compared with the master negative control.

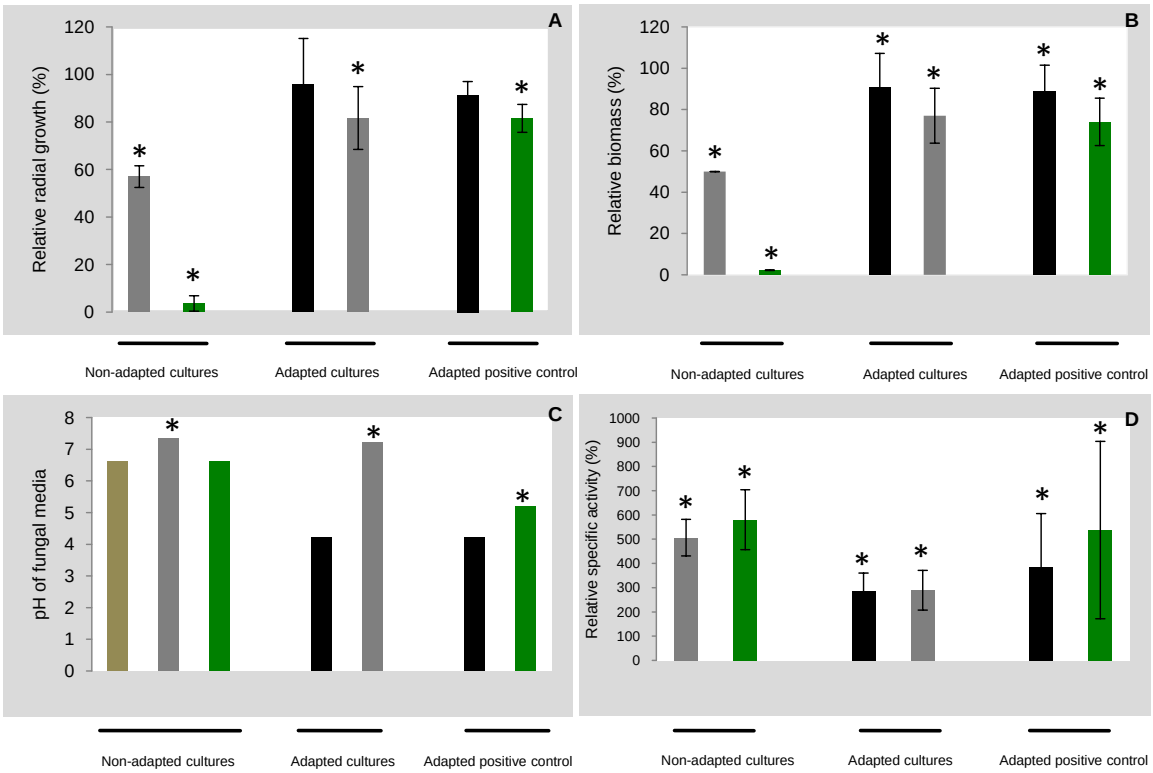

Figure 48: Influence of bacterial volatiles of *Serratia plymuthica* 4Rx13 and hydroquinone on pH value of the fungal medium and the radial growth, biomass production, and laccase activity in *Sclerotinia sclerotiorum* after re-exposure.

A. radial growth; **B**. biomass; **C**. pH value of fungal media; **D**.intracellular laccase.

master negative control (never exposed); negative control (previously exposed); dual culture; positive control (5 mM hydroquinone).

An initial CFU of 40-48*10^7 were point-inoculated onto the bacterial nutrient agar (NA) of split Petri dishes. The dishes were kept at 30 °C for 2 d. Mycelial plugs were inoculated on fungal SA side. The dishes were placed at 20 °C and parameters were measured after day 1 of incubation. The radial growth was expressed in pixel (software ImageJ). The master negative controls (100 %) were equivalent to 459836 (**A**), 5 mg (**B**), 0.019 nkat/mg protein (**D**).
n=3. Error bars show standard deviations. (*) represents statistical significance related to the master negative control.

The pH values of the fungal medium showed very interesting patterns (Figure 48C). Whenever exposed to bacterial volatiles, the value started to become alkaline. The recovery of the fungal growth in adapted fungal cultures obviously occurred independently from the pH value of the medium. When exposed to hydroquinone the first time (non-adapted), the value turned only slightly acidic which was comparable to the master negative control (never exposed). Most interestingly, the mycelia of negative controls (previously exposed)

generated a lower pH value compared to the master negative control indicating a different metabolic status since both the master negative control and the negative control of the adapted culture experienced the same incubation time but the adapted culture control showed a more pronounced acidification of the growth medium. The culture that was re-exposed to hydroquinone showed a similar phenomenon. Adaptation to hydroquinone resulted in a more eloquent acidification of the growth medium.

The activity of the intracellular laccase was still up-regulated in adapted fungal cultures, however, this increment was less pronounced in comparison with non-adapted cultures. Surprisingly, the negative control of the adapted dual cultures (previously exposed to volatiles) showed a similar specific activity as the re-exposed cultures. It could have been expected that the activity in re-exposed fungal cultures would have been at a similar level as the initially exposed culture in order to cope with the ongoing influence of bacterial volatiles. In contrast, fungal cultures re-exposed to hydroquinone still showed an elevated specific laccase activity compared to the previously exposed culture. Nevertheless, it could be deduced that an adaptation to bacterial volatiles caused a reaction of the fungal mycelia that subsequently lowered the need for a laccase activity.

3.8.3. Summary of the adaptation experiment

In conclusion, it can be argued that re-exposure of cultures of *Sclerotinia sclerotiorum* to bacterial volatiles revealed the advantage of adaptation. The key results are summarized in table 4. Re-exposed fungal cultures grew better than fungal cultures which were initially exposed (non-adapted) and were less derogated by the volatile compounds. The intracellular laccase activity remained elevated, indicating that the fungus still, but more efficiently, responded to the stress situation. Up-regulation of enzyme activities (in this case, the laccase activity) seemed to be required for the fungal growth recovery. The pH value of the fungal medium remained elevated in adapted dual cultures suggesting that the fungal mycelium resumed growing despite an unchanged environment for the fungus. The effect of bacterial volatiles and hydroquinone on initially exposed cultures, no matter if exposed in part I or part II, was always comparable (Figure S6F) which additionally supported the fact that the fungal mycelium truly responded to bacterial volatiles and that this reaction promoted an fungal growth recovery.

Table 4:
Initial and re-exposure: overview on the impact of bacterial volatiles of *Serratia plymuthica* 4Rx13 and hydroquinone on radial growth, biomass production, and laccase activity in *Sclerotinia sclerotiorum*

Inhibition of /Increase in	Bacterial volatiles			Hydroquinone		
	I	II		I	II	
	Initially exposed	non-adapted (initially exposed)	Adapted (re-exposed)	Initially exposed	non-adapted (initially exposed)	Adapted (re-exposed)
Radial growth	40 %	40 %	18 %	97 %	97 %	19 %
Biomass production	50 %	50 %	23 %	98 %	98 %	26 %
Laccase activity	5 fold	5 fold	3 fold	5 fold	5 fold	5 fold

4 Discussion

Fungi like *Sclerotinia sclerotiorum*, *Rhizoctonia solani, Neurospora crassa* were not only inhibited in growth and biomass production, they showed a pronounced increase in superoxide dismutase, catalase and laccases activities after exposure to bacterial volatiles produced by *Serratia plymuthica* 4Rx13. Although, *Phoma eupyrena* did not show much growth inhibition, it showed also alterations in pigmentation of the mycelium like *R. solani* and displayed also an increase in enzyme activities as the other three fungi. Bacterial volatiles obviously generated environmental stress, specifically oxidative stress, in cell membranes (non-enzymatic lipidperoxidation) as well as inside the cell (enzyme activities). In order to cope with deleterious effects of ROS, fungal cells developed rapid responses to repair and protect against these harmful volatiles. Enzymes like superoxide dismutase, catalase and laccases were up-regulated to remove ROS from the fungal cell. The analyses of VOC profile revealed that in contrast to the bacterium, the fungus *S. sclerotiorum* did not produce many volatiles. In dual culture, a new compound (1-Tridecanol) added to the VOC spectrum due to the interaction between both organisms. The inorganic volatile ammonia produced by the bacterium changed the pH value of the fungal medium and could be one of the compounds responsible for growth reduction and an oxidative stress response in fungi. Repeated exposure to bacterial VOCs provoked an adaptation and proved that bacterial volatiles might indeed be responsible for generating oxidative stress in fungi. In the following sections, main aspects of the results mentioned above are discussed.

4.1 The test system

The employment of split Petri dishes was useful to study volatile-mediated interactions between bacteria and fungi. The introduction of a cellophane membrane was very helpful in order to harvest fungal material. A cellophane membrane is a thin, transparent sheet made of regenerated cellulose (**82**). The use of cellophane membranes in fungal growth experiments has been reported before. It seemed to be a suitable support for mycelial growth (**83-85**). Due to the pore size, molecules such as proteins and other bioactive substances or nutrients can pass through this membrane (**86**). Nutrient agar (NAII) was chosen as a growth medium for the bacterium. It is a nitrogen rich medium and in previous research it had been observed that rhizobacteria produced antifungal volatile compounds in presence of nitrogen rich media (**37**).

4.2 Growth and morphology

All fungi exhibited growth inhibition when exposed to rhizobacterial volatiles. It could be speculated that fungal morphogenesis was also be affected. Hyphal growth is normally polarized and the hyphae extend only at the extreme apex. Extension or growth of hyphae is associated with the synthesis of new cell wall materials like glucan and chitin. A young hypha shows parts like vesicles, protoplasm and vacuoles. Branching is associated with the activation of genes as well as enzymes. Hydrolytic enzymes are needed to penetrate the substrates (**87**). In response to bacterial volatiles, the cell wall components as well as the protoplasm and vesicles might not have been synthesized. The gene expression and enzyme synthesis regarding the hyphal growth could also be affected. This might have been one of the principles which lead to a retardation of fungal radial growth as well as biomass production.

Although all fungi grew on the same growth medium, *S. sclerotiorum* and *R. solani* grew ahead of *N. crassa* and *P. eupyrena* and showed a maximum of growth inhibition at the early stage of co-cultivation with the bacterium. One could expect that phytopathogenic and actually many non-phytopathogenic fungi are fast growing assuming that they find the optimal substrate and optimal conditions. For *N. crassa* and *P. eupyrena*, Sabouraud-agar (SA) did not seem to fulfil this requirement. The very little inhibition of the radial growth of *N. crassa* and actually absent growth inhibition of *P. eupyrena* was contradictory to findings of Vespermann *et al.* (**26**) but might be simply attributed to the slow growth, however; the inhibition of biomass production showed that also *N. crassa* and *P. eupyrena* were affected by bacterial volatiles. In addition, the different reactions of different fungal species might be explained by the composition of the cell wall itself. The fungal cell wall is basically composed of chitin, glucan and glycoproteins. While glucan as the major structural polysaccharide and chitin as the stabilizing polymer provide an overall integrity and stability; glycoproteins with or without enzyme activity contribute to cell wall dynamics. Glycoproteins that lack enzyme activity (structural glycoproteins) are extensively modified by oligosaccharide side chains which can considerably differ amongst fungal species (**88**). Structural glycoproteins of the fungal cell wall show a balancing reaction when they are exposed to different stress conditions. For example, *Candida albicans* was exposed to a variety of environmental signals (pH, temperature), stresses (oxidative, nitrosative, and osmotic stress), and microbial imposed environments (quorum sensing molecules). In response to osmotic stress, a shortening of the acid-labile side chains took place, whereas oxidative stress induced an elongation of side chains rich in mannose (**89**). These structural glycoproteins containing side chains rich mannose are known as mannans (**90**). In addition, in pathogenic fungi the structural cell wall proteins are known to play a key role in fungal-host relationship (**91**).

Following these facts it can be assumed that the fungi experienced the oxidative stress differently and showed a distinct reaction in response to bacterial volatiles. This might have resulted in slight radial growth stimulation on one hand (e.g. *P. eupyrena*) and in growth inhibition on the other hand (e.g. *S. sclerotiorum*). This inhibition of the radial growth of *S. sclerotiorum* can be correlated with previous work, where a stacked and limited hyphal structure was observed in dual culture between the bacterium *Serratia plymuthica* 4Rx13 and the fungus *S. sclerotiorum*. In response to harmful bacterial volatiles, the inhibition of radial growth was characterized by a fungal mycelium which was tightly packed and the margin was sharply delimited (**92**).

Earlier reports showed the coloration of the fungal media when fungi were dual cultivated with rhizobacteria (**93**). In response to the volatile metabolites of the bacterium *S. plymuthica* 4Rx13, *R. solani* and *P. eupyrena* showed coloration. This coloration may be the result of melanin synthesis. Melanin is normally incorporated in the thickened cell walls of many fungal species. Fungi synthesize melanin to give a better rigidity, resistance to lytic enzymes and oxidative stresses, physical stresses, and protection against antagonistic activities from other organisms (**94**). The coloration was the first indication of the presence of stress in fungi in dual culture. In addition, melanin could be synthesized as reaction to electrolytic leakage or loss of water from the fungal cell when exposed to the bacterial volatiles (**87**). After a certain time even in the monocultures of these two fungi little melanin production was observed. This could be assigned to stress because of limitations of space or nutrients.

Fungi synthesize melanin from endogenous acetyl CoA/malonyl CoA via a 1, 8-dihydroxy-naphthalene (DHN) intermediate (**91, 95**). Alternatively, some fungi produce melanin from L-3, 4-dihydroxyphenylalanine (L-dopa) or tyrosine. If L-dopa is the precursor molecule, it is oxidized to dopaquinone by laccase (**94**) (Figure 49). An earlier report showed that fungi exposed to antagonistic bacteria up-regulated their tyrosinase or laccase activities (**97**). They are related to the fungal growth (**98, 99**).

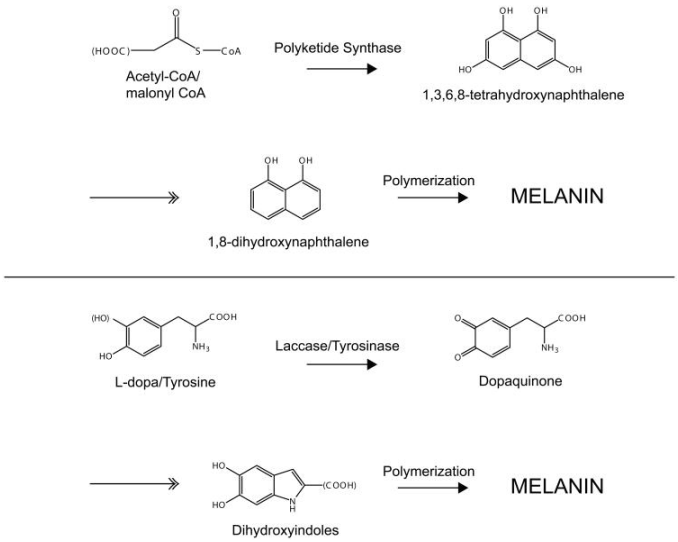

Figure 49: Comparison of DNH (top) and L-dopa (bottom) melanin synthesis pathways (99).

The coloration increased with increasing initial colony forming units (CFU_i) which indicated that fungi might experience more bacterial volatiles and for protection more pigments were synthesized in the fungal cell wall. Growth inhibition was also positively related with an increasing bacterial CFU_i. This suggests that the growth inhibition and the pigment production might be directly linked. From early reports it is known that *S. sclerotiorum* can produce melanin (**100**). But in this case, in response to the bacterial volatiles, it did not show any changes in mycelia coloration which means that the stress experienced by this fungus may have not been enough for an elevated melanin production.

4.3 Oxidative stress

Non-enzymatic lipidperoxidation (nLPRX)

Lipidperoxidation represents an indicator of oxidative stress in cell membranes. Peroxidation of membrane lipids is a complex process involving unsaturated fatty acids and, in particular, polyunsaturated fatty acids containing one or more ethylene groups positioned between *cis* double bonds. These ethylene groups are highly reactive to oxidizing agents and can form peroxyl radicals that can start a free radical chain reaction (propagation phase) and generate new radical species and peroxidation end-products such as malondialdehyde (MDA) (**101, 102**).

Lipid bilayers of fungal membranes are the one of the initial structures affected by bacterial volatiles. In the fungal cell membrane, NADPH oxidases (Nox) can be up-regulated in this case. The activated Nox enzyme complexes can generate superoxide, the precursor of other species of reactive oxygen by utilizing NADPH as an electron donor (**103**). Fungi, with higher

intrinsic unsaturated membrane fatty acids are vulnerable to peroxidation which could lead to the loss of membrane integrity and eventually lead to cell death. The amount of malondialdehyde, the end product of lipidperoxidation, was the measure for the decrease in membrane integrity. Generally, young mycelium showed more nLPRX than older mycelium. The membrane might restore the unsaturation in the late phase of growth. Lipidperoxidation was also dependent on the number of CFU_i. Some cells might experience death at higher numbers, which might be the reason for lower amount of MDA observed in presence of a higher CFU_i. The slow-growing fungi *N. crassa* and *P. eupyrena* were more sensitive than the fast-growing fungi *S. sclerotiorum* and *R. solani*. They showed lipidperoxidation even in presence of lower CFU_i. The cell membrane might be more sensitive in slow-growing fungi. A higher number of unsaturated fatty acids might explain the sensitivity of these fungi. Alternatively, repair mechanisms of slow growing fungi might not have been eloquent enough because by-products of lipidperoxidation could affect the mitochondrial respiratory chain of the fungal cell (**104**) or cause DNA damage (**102**). Lipidperoxidation can be nullified by the anti-oxidant system by breaking the propagation cycle (**105**).

Superoxide dismutase and catalase

Reactive oxygen species can damage DNA, proteins, and membrane lipids and can result in mutagenesis, inhibition of growth, and cell death (**106**). Data found in the literature suggest a relation between H_2O_2 production, activity of peroxidases, processes of loss of cell wall plasticity, and decrease in growth (**107**). Therefore, the investigation of a putative relation of growth inhibition and an activation of the antioxidant defense system in phytopathogenic and nonpathogenic fungi as response to bacterial volatiles was a very promising approach. Does the anti-oxidative system protect the fungal mycelium against bacterial volatiles?

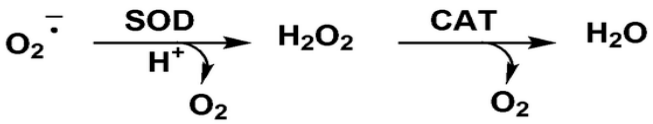

Figure 50: Cooperative SOD/Cat action for ROS detoxification (108).

The hypothesis was that these volatiles might generate not only H_2O_2 but also superoxide anions ($O_2^{\cdot-}$) and hydroxyl radicals (OH^-). In order to maintain ROS at physiological un-harmful levels, fungal cells possess enzymatic defense systems (**98**). Many enzymes like catalase, glutathione peroxidase and superoxide dismutase (SOD) participate in reactive oxygen species (ROS) elimination (**109**). The most important enzymatic antioxidants include SOD, a metalloenzyme which catalyzes the disproportion of superoxide ($O_2^{\cdot-}$) to H_2O_2 (**110**). SODs are widespread in nature. This enzyme is found in nearly all organisms, in all oxygen-

metabolizing cells, examined to date (**111-113**). According to their metal cofactor, SODs can be divided into three main isoforms: (i) Cu/ZnSOD, (ii) MnSOD, and (iii) FeSOD (**109**). Like other eukaryotic cells, the fungal cell has a homotetrameric MnSOD and a Cu/ZnSOD (**111**). The contribution of the fungal Cu/ZnSOD to the pathogenesis of human fungal infections has been extensively studied; MnSODs are less known (**114**). Some MnSODs are reported to play a role in cellular defense against oxidative stress (**115-121**). In some pathogenic fungi, the contribution of MnSODs to the pathogenesis was also observed (**109**).

Catalase (CAT) is another important antioxidant enzyme. Catalase detoxifies H_2O_2 and converts lipid hydro peroxides to nontoxic alcohols (**122**). CAT is a common enzyme found in all living organisms exposed to oxygen (e.g. bacteria, fungi, plants, animals) (**123**). CAT consists of four identical subunits (homo-tetramer) (**124**). Subsequently, four porphyrin heme (iron) groups are available to react with the hydrogen peroxide (**74**). These systems can be induced in response to oxidative stress caused by imbalances between the production and the detoxification of oxygen radicals (**125**). Broadly, there are three different types of monofunctional heme-catalases (heme A, heme B, and heme C) (**126-128**). Filamentous ascomytes have normally more than one catalase (**129**). Most microorganisms without catalase grow slowly and are stress sensitive (**129, 130-132**).

In *Neurospora crassa*, SOD and CAT were activated because of the presence of light stress (**133**). In *Fusarium* sp. internal environmental changes resulted in changes in these enzymes (**125**). Also bacterial volatiles affected the activities. *Sclerotinia sclerotiorum* was the most sensitive fungus that displayed 10-fold increase in SOD activity at the beginning of co-cultivation. However; ROS can also be involved in fungal development (**134**). They are signal molecules which play an important role in ageing (**135**). When fungi stop growing because of unfavorable conditions they produce a permanent form which is species dependent; usually resistant structures like sclerotia, asexual or sexual reproduction structures. In *N. crassa* it was observed that asexual development was triggered by up-regulation of ROS, redox imbalance, and rapid changes in enzyme activities (**136-138**). Catalase increased the development of sexual and asexual development (**134, 138**). *Rhizoctonia solani* and *S. sclerotiorum* experienced with aging a final limitation in growth because of too little space in the split Petri dish or limited nutrients. They needed to produce sclerotia or other structures to survive. That could be a reason why they showed an elevation in catalase activity in their late phase of co-cultivation. *N. crassa* was the most slow-growing species and probably experienced not any problems with space or nutrients. This was the only species that showed a gradual reduction in catalase activity that could be related with the SOD activity.

In dual culture, some fungal cells may die in presence of higher number of bacterial CFU_i. This might be the reason why in dual culture the catalase activity is negatively related with

the number of CFU_i. Slow-growing fungi are much more sensitive showing a catalase activity even in presence of lower CFU_i.

In general, the data regarding SOD and CAT suggest the involvement of these two enzymes in the defense against ROS produced by the bacterial volatiles.

Laccase

The fourth parameter regarding oxidative stress was laccase. Laccase belongs to the small group of enzymes called the blue multi copper oxidases.

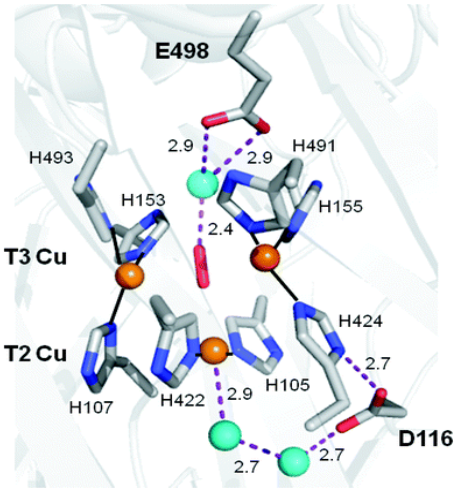

Figure 51:

The typical structure of a active form of laccase (139).

It is widely distributed in higher plants and fungi. In fungi, laccase is present in ascomycetes, deuteromycetes and basidiomycetes and it is particularly abundant in many white-rot fungi that degrade lignin (**75**). Laccases are also found in insects and bacteria (**140**). In insects, they are involved in cuticular sclerotization (**141, 142**). In bacteria, the enzyme might be involved in melanization (**143**). Previous research has indicated that fungal laccases play a role in lignin degradation, the development of fruiting bodies, fungal morphogenesis, fungal pathogenicity and the synthesis of melanin (**144-150**). Only lately, the expression of laccase in yeast was found to confer a strong ability to scavenge intracellular H_2O_2 and to protect cells from lipid oxidative damage (**48**).

The results revealed that in response to the bacterial volatiles, all fungi except *Phoma eupyrena* showed an elevation in laccase activity. This was supported by the finding that laccase activity was induced in *R. solani* after co-cultivation with *Pseudomonas fluorescens* (**49**). In the present investigation, *R. solani* was the most sensitive fungus and it exhibited 38-fold increase in laccase activity after day 1. The maximal laccase activity was observed at the beginning of co-cultivation. *P. eupyrena* did not show any laccase activity. In this fungus the up-regulation of catalase or glutathione peroxidase might be enough to scavenge H_2O_2 from cells; it did not require the activation of intracellular laccase. Combining these four sets

of data, it can be said that all fungi showed an indication of oxidative stress in presence of bacterial volatiles in the cell membrane as well as inside the cell. These parameters were mostly up-regulated at the beginning and decreased afterwards. Only catalase showed activities also in the later phase of co-cultivation. Bacterial volatiles probably played a role like fungicides inducing oxidative stress. Many papers report on the involvement of fungicides in inducing antioxidants in fungi. The imbalance between oxidants and anti-oxidants was assumed to be the reason of oxidative stress condition caused by fungicides (**151, 152**). The bacterial volatiles investigated in the present work were not lethal since fungi survived after growth inhibition. The imbalance between production and scavenging of ROS caused the oxidative stress situations and subsequently growth retardation. *P. eupyrena* and *N. crassa* were more sensitive and showed substantial development of radical species in the cell membrane (nLPRX) and inside the cell (CAT activity) even in presence of lowest CFU_i. This may partly explain their slow growth in the present test system.

4.4 Volatile emission

4.4.1 Volatile organic compound emission

Monocultures of *Serratia plymuthica* 4Rx13 grown on nutrient agar emitted less organic volatiles (VOCs) compared to the liquid medium (53). On the solid medium the bacterial culture was more stagnant than in the liquid medium. May be that was the reason why the bacterium produced less volatiles. In addition, at 20 °C the bacterium produced fewer amounts of volatiles compared to 30 °C. It is known from the literature that bacteria change their metabolism at lower temperature (**153**). The change in metabolism resulted in lower amounts of volatiles. As described in the literature, the VOC profile of the bacterium in both mono- and dual culture was dominated by sodorifen. Other VOCs emitted from liquid cultures included e.g. dimethyl disulfide (DMDS), dimethyl trisulfide, methanethiol, 2-phenyl alcohol, and other aromatic compounds (**53**), but only DMDS could be detected as a volatile emitted from monocultures and dual cultures.

In order to gain an impression of fungal VOCs, *Sclerotinia sclerotiorum* was investigated. Overall, this fungus emitted only very few VOCs. The most prominent compound was the bicyclic diterpenoid Labda-8(20),12,14-triene which was emitted in the late phase (48-72 h) of mono- and co-cultivation. This compound represents a so called labdane which in turn belongs to the class of diterpene lactones. It was not reported so far to be produced by fungi. Labdanes have been obtained from some plants like *Fritillaria ebeiensis* and *Cistrus incanus* (**154**). Labdane-type bicyclic diterpene synthases were isolated from Actinomycetales (**155**). Labdanes possess a variety of biological activities; among them antimicrobial properties (**156**). An early report described that the sclerotia of the fungus *S. sclerotiorum* emitted 2-

methylenebornane and 2-methylisoborneol (**157**). There are no reports on VOC emission of the other 3 fungi.

In the past many VOC profiles from bacteria and fungi were published (29). The VOC profiles were always obtained from individually grown species. Based on the bacterial-fungal interaction studies performed in this thesis an intriguing question arose: Does co-cultivation of *S. plymuthica* and *S. sclerotiorum* provoke the emission of additional volatile compounds? After comparing the VOC emission in monoculture and dual culture, only one compound, 1-Tridecanol, was detected in dual culture. However, it is not known whether the bacterium or the fungus held responsible for the emission. If produced by the bacterium, this new compound may have a role in ROS production in fungi at 20 °C. From the literature it is also known that in *in vitro* dual culture systems, DMDS showed growth inhibition of *Arabidopsis thaliana* **(53**). Therefore, DMDS could also be responsible for fungal growth inhibition. The amount of bacterial VOCs did not change in dual culture compared to the monoculture. This showed that the fungus has neither any influence on bacterial growth nor it can affect the volatile production of the bacterium.

4.4.2 Inorganic compound ammonia produced by *Serratia plymuthica* 4Rx13

Inorganic compounds produced by the bacterium include ammonia (**53)**. It is furthermore reported that *Serratia plymuthica* 4Rx13 and six other rhizobacteria emit ammonia, which was detected in the headspace with Nessler's reagent (**53, 78**). The ammonia production was dependent on the growth medium and was emitted when bacteria were grown on peptone-rich media (NB or LB). This phenomenon could be also shown by Gori *et al.* (**158**). Ammonia produced by yeasts may contribute to an increase in pH during ripening of cheeses and ammonia production is dependent on the growth medium. When yeasts were grown in presence of a nitrogen source (cheese agar), ammonia production was much higher compared to a cultivation in presence of a glycerol medium (carbon medium).

The genome of *S. plymuthica* 4Rx13 encodes more than 55 putative ammonia-producing enzymes, which strongly support the process of ammonia synthesis (**78**). Ammonia is normally not released but assimilated into organic compounds by glutamate dehydrogenase, glutamine synthetase, and glutamate synthase (**29**). In general, bacterial ammonia can be produced by nitrite ammonification (**159**), by the degradation of various amino acids utilized from media (**160**), by the decarboxylation of amino acids to produce biogenic amines as well as ammonia (**161**), by deamination, and by the urease-mediated hydrolytic degradation of urea (**162**). By altering the pH value in the rhizosphere due to ammonia emission, bacteria may influence organismal diversity and plant-microbe interactions (**20**).

Ammonia, detected in the atmosphere above alkaline soil appeared to be responsible for the inhibition of germination in conidia of *Botrytis cinerea* and *Penicillium nigricans* (**163**). Eno *et al.* injected anhydrous ammonia into Florida sand and they observed reductions in the populations of fungi, bacteria, and nematodes (**164**). Neal *et al.* have observed that ammonia killed rapidly the sclerotia of the fungus *Phymatotrichum omnivorum* (**165**). McCallan and Setterstrom have also observed that ammonia is toxic to many different fungi (**166**). Henis and Chet presented that an elevated pH value was accompanied by a fungicidal effect against sclerotia of *Sclerotium rolfsii* (**167**). Ammonia was also identified as antifungal compound in dual culture test using *Enterobacter cloacae*, *Rhizoctonia solani*, and *Pythium ultimum* (**168**). Excessive ammonia inhibits the growth and survival of submerged aquatic plants, but has also been directly attributed to the decline of numerous aquatic macrophytes (**169, 170**).

Ammonia is very much water-soluble and after penetrating the majority of biological membranes rapidly, ammonia can react with internal H^+ forming NH_4^+ ions (**78**). Ammonia doesn't need any transporters like ammonium ions. Ammonia enters through the membrane lipid bilayer depending on temperature, lipid composition of membranes, and thickness of the membrane (**171**). Once inside the fungal cell, ammonia accumulation can cause high cytosolic pH values followed by an increase of the cytoplasmic Ca^{2+} ions concentration due to increased permeability of the plasma membrane or due to Ca^{2+} release from internal storage. That alters the calcium homeostasis (**78**). Therefore, ammonia could work like a stress factor for the fungi, although lower concentration may function as a signaling factor (**29**). Ammonia induced fruit-body production in *Coprinus cinereus* in darkness (**172**). It functioned as a signal in *Candida albicans* that warns the incoming nutrient starvation (**173**).

The accumulation of external NH_4^+ ions elevated the pH value of media (**174, 175**). Previous experiments showed that ammonia produced by rhizobacteria changed the pH value of plant media to alkaline resulting in the growth inhibition of *Arabidopsis thaliana* (**76**). The fungal medium in dual culture was definitely alkalized by the ammonia produced by *S. plymuthica* 4Rx13, so that in addition $(NH_4)^+$ ions could also affect the fungal growth. Furthermore, as fungi always prefer acidic pH values, alkalization of the fungal media can be one of the causes of growth inhibition. It was interesting to notice that in dual culture in presence of different bacterial CFU_i, the pH value of the fungal medium differed from one fungus to another. All fungi had an almost neutral pH value at the beginning of co-cultivation. However, *Sclerotinia sclerotiorum* acidified its growth medium after day 2 of co-cultivation and it can be postulated that *S. sclerotiorum* produced oxalic acid which was responsible for acidification (**176, 177**). *Neurospora crassa* showed a final slightly acidic pH value. This fungus might also have produced some acids.

S. plymuthica 4Rx13 with a CFU_i of (3-4)*10^7 generated always an acidic pH value. This CFU_i did probably not produce enough ammonia to alkalize the fungal medium. Interestingly, in presence of this lowest CFU_i, slow growing fungi showed growth inhibition and oxidative stress. Obviously, other volatiles except ammonia could have been responsible for growth inhibition and oxidative stress production. Oxidative stress production by ammonia was supported by observation in plants and (**178**) and in the aquatic macrophyte *Myriophyllum mattogrossense* (**179**). In this context, it is interesting to note that synthetic ammonia could only partly mimic the effect of the bacterial volatile mixture. Ammonia evaporated from a 15 mM ammonia solution could evoke oxidative stress in the fungal cell membrane and inside the cell; however, its patterns of activity did not match the patterns of enzyme activities and nLPRX caused by bacterial volatiles. Mostly, in presence of ammonia, the effect on oxidative stress parameters was less than in dual culture. It can be assumed that some other volatiles should also be responsible for ROS production in fungi. However; ammonia is one of the active compounds in the bacterial volatile mixture since ortho-phosphoric acid was able to soften the effect of both bacterial volatiles and synthetic ammonia. Ortho-phosphoric acid is known to react with ammonia to form ammonium phosphate salts (**180**).

An inconsistency was observed when applying synthetic ammonia to fungal cultures. Although the amount of ammonia produced by the bacterium had been quantified, this amount of synthetic ammonia could not be used since the effect was too harsh on the fungal mycelium. One reason might be that the daily renewal of the ammonia solution was not equivalent to the way a living organism like *S. plymuthica* 4Rx13 produced ammonia. More ordinary is the fact, that different charges of commercial ammonia stock solutions had been used. Finally, considering the growth inhibition and medium alkalization, the amount of ammonia that evaporated from a 15 mM solution was assumed to be a comparable amount.

4.5 Adaptation experiment

Adaptation to a stress or certain environmental conditions would prove that (i) an organism responds specifically to these condition and (ii) that physiological and biochemical changes are truly related to a certain stress. An adaption to stress conditions can be accomplished by repeated exposure. In this case, the fungal mycelium of *Sclerotinia sclerotiorum* was repeatedly exposed to bacterial volatile and, indeed, re-exposed (adapted) mycelia of *S. sclerotiorum* grew better than non-adapted mycelia. The anti-oxidant system of adapted mycelia was already activated and subsequently, they showed significantly less growth inhibition since they could cope immediately with oxidative stress. Furthermore, the adapted monoculture of *S.sclerotiorum* (previously exposed) showed a growth that was comparable to the master control (never exposed). The mycelia obviously recovered very quickly. Their morphogenesis was retarded for a short period or new cell wall material could not be

synthesized for a short time. Surprisingly, re-exposed (adapted) mycelia grew better in dual culture although the pH value of the fungal media remained alkaline. Again, adapted mycelia could deal with the pH change and could grow better. Remaining elevated laccase activities in adapted mycelia also indicated an activated anti-stress response. It would be desirable to investigate the activities of the other oxidative stress induced enzyme activities.

Summarizing these data it can be stated that *S. sclerotiorum* specifically responded to oxidative stress provoked by bacterial volatiles. After an initial exposure, the fungus induced it's resistance ability against oxidative stress. When exposed a second time, this fungal culture could draw on already activated anti-stress mechanisms. The positive control (exposure to hydroquinone) impressively confirmed this result. These results were supported by observations using plants. Plants experience more stress tolerance after adaptation (**181**). They even deal better after adaptation with herbivores in comparison to non-adapted plants (**182**).

5 Conclusion

As concluding remarks it can be said that in response to bacterial volatiles fungi showed growth inhibition. By alkalizing the pH of the fungal media, ammonia inhibited the fungal growth. Further, adaptation experiment gave a signal that bacterial volatiles could also generate ROS in the fungal cell membrane (nonenzymetic Lipidperoxidation) and inside the cell. 1-Tridecanol could be responsible for fungal growth inhibition and oxidative stress production in *Sclerotinia sclerotiorum*. *Rhizoctonia solani* and *Phoma eupyrena* had probably up-regulated their melanin to protect themselves from the harmful affects of oxidative stress. Fungi also up-regulated enzymes like catalase, superoxide dismutase, laccase as a way to protect themselves from ROS. Slow-growing fungi were more sensitive than the fast-growing fungi.

6 List of literatures

1. **Narula, N., Kothe, E., Behl, R.K.** (2009) Role of root exudates in plant-microbe interactions. *Journal of Applied Botany and Food Quality*, **82**, pp. 122 – 130.

2. **Kamilova, F.K., Kravchenko, L.V., Shaposhinkov, A.I., Azarova, T., Makarova, N., Lugtenberg, B.** (2006) Organic acids, sugars and L-tryptophan in exudates of vegetables growing on stone wool and their effects on activities of rhizosphere bacteria. *Molecular Plant-Microbe Interactions*, **19**, pp. 250-256.

3. **Kumar, R., Bhatia, R., Kukreja, K., Behl, R.K., Dudeja, S.S., Narula, N.** (2007) Establishment of Azotobacter on plant roots: chemotactic response, development and analysis of root exudates of cotton (*G. Hirusitum* L.) and wheat (*T. aestivum* L.). *Journal of Basic Microbiology*, **47**, pp. 436-439.

4. **Hiltner, L.** (1904) Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie und unter besonderer Berücksichtigung der Grundungung und Brache. *Deutsche Landwirtshafts-gesellschaft*, **98**, pp. 59-78.

5. **Hartmann, A., Rothballer, M., Schmid, M.** (2008) Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant Soil*, **312**, pp. 7-14.

6. **Behl, R.K., Ruppel, S., Kothe, E., Narula, N.** (2007) Wheat x Azotobacter x VA mycorrhiza interactions towards plant nutrition and growth – a review. *Journal of Applied Botany and Food Quality*, **81**, pp. 95-109.

7. **Newman, E.I. (1985)** The rhizosphere: carbon sources and microbial populations. *In Ecological Interactions in Soil. A. H. Fitter. Oxford, Blackwell Scientific Publications,* pp. 107.

8. **Jones, D.L., Nguyen, C., Finlay, R.D.** (2009) Carbon flow in the rhizosphere: carbon trading at the soil-root interface. *Plant Soil*, **321**, pp. 5-33.

9. **Rovira, A.D.** (1969) Plant root exudates. *Botanical Review*, **35**(1), pp. 35-57.

10. **Bais, H.P., Park, S.W., Weir, T.L., Callaway, R.M., Vivanco, J.M.** (2004) How plants communicate using the underground information superhighway. *Trends in Plant Science*, **9**, pp. 26-32.

11. **Nardi, S., Concheri, G., Pizzeghellol, D., Sturaro, A., Rella, R., Parvoli, G.** (2000) Soil organic matter mobilization by root exudates. *Chemosphere,* **5**, pp. 653-658.

12. **Walker, T.S., Bais, H.P., Grotewold, E., Vivanco, J.M.** (2003) Root Exudation and Rhizosphere Biology. *Plant Physiology*, **132**, pp. 44-51.

13. **McNear, D.H.**(2013) the Rhizosphere - Roots, Soil and Everything In Between. *Nature Education Knowledge*, **4**(3), edition 1.

14. **Chaparro, J.M.** (2014) Rhizosphere microbiome assemblage is affected by plant development. *International Society for Microbial Ecology*, **8**, pp. 790–803.

15. **Schreiter, S.** (2014) Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. *Frontiers in Microbiology*, **5**(144).

16. **Whipps, J.M.** (2001) Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany*, **52**, pp. 487-511.

17. **Kloepper, J.W., Zablotowicz, R.M., Tipping, E.M., Lifshitz, R.** (1991) Plant growth promotion mediated by bacterial rhizosphere colonizers. *In: D. L. Keister and P. B. Cregan (ed.), The Rhizosphere and Plant Growth. Kluwer Akademic Publishers, Dordrecht, The Netherlands*, pp. 315–326.

18. **Kloepper, J.W., Leong, J., Teintze, M., Schroth, M.N.** (1980) Enhancing plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature*,**286**, pp. 885–886.

19. **Hughes, D.T., Sperandio, V.** (2008) Inter-kingdom signalling: communication between bacteria and their hosts. *Nature Reviews Microbiology*, **6**, pp. 111–120.

20. **Surette, M.G., Davies, J.** (2008) A new look at secondary metabolites. In: Winans SC, Bassler BL (eds). Chemical Communication Among Bacteria. *Washington, DC: ASM Press*, pp. 307–322.

21. **Schenkel, D., Lemfack, M.C., Piechulla, B., Splivallo, R.** (2015) A meta-analysis approach for assessing the diversity and specificity of belowground root and microbial volatiles. *Frontier in plant science*, **6**(707).

22. **Schulz, S., Dickschat, J.S.** (2007) Bacterial volatiles: The smell of small organisms. *Natural Product Reports*, **24**, pp. 814–842.

23. **Penuelas, J., Asensio, D., Tholl, D.,** (2014) Biogenic volatile emissions from the soil. *Plant Cell and Environment*, **37**, pp. 1866–1891.

24. **Audrain, B., Farag, M.A., Ryu, C., Ghigo, J.** (2015) Role of bacterial volatile compounds in bacterial biology. *FEMS Microbiology Reviews*, **39**, pp. 222–233.

25. **Ryu, C.M., Farag, M.A., Hu, C.H.** (2003) Bacterial volatiles promote growth in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, pp.4927–4932.

26. **Vespermann, A., Kai, M., Piechulla, B.** (2007) Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. *Applied and Environmental Microbiology*, **73**(17), pp. 5639–5641.

27. **Barret, M., Morrissey, J.P., O'Gara, F.** (2011) Functional genomics analysis of plant growth-promoting rhizobacterial traits involved in rhizosphere competence. *Biology and Fertility of Soils*, **47**(7), pp. 729–743.

28. **Santoro, M.V., Zygadlo, J., Giordano, W., Banchio, E.** (2011) Volatile organic compounds from rhizobacteria increase biosynthesis of essential oils and growth parameters in peppermint (*Mentha piperita*). *Plant Physiology and Biochemistry*, **49**(10), pp.1177–1182.

29. **Effmert, U., Kalderás, J., Warnke, R., Piechulla, B.,** (2012) Volatile mediated interactions between bacteria and fungi in the soil. *Journal of Chemical Ecology*, **38**(6) pp. 665–703.

30. **Kai, M., Effmert, U., Berg, G., Piechulla, B.** (2007) Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Archives of Microbiology*, **187**(5), pp. 351–360.

31. **Müller, H., Westendorf, C., Leitner, E.** (2009) Quorum-sensing effects in the antagonistic rhizosphere bacterium *Serratia plymuthica* HRO-C48. *FEMS Microbiology Ecology*, **67**(3), pp. 468–478.

32. **Dandurishvili, N., Toklikishvili, N., Ovadis, M.** (2011) Broad range antagonistic rhizobacteria *Pseudomonas fluorescens* and *Serratia plymuthica* suppress *Agrobacterium* crown gall tumours on tomato plants. *Journal of Applied Microbiology*, **110**(1), pp. 341–352.

33. **Popova, A.A., Koksharova, O.A., Lipasova, V.A., Zaitseva, J.V., Katkova-Zhukotskaya, O.A., Eremina, S.I.** (2014) Inhibitory and toxic effects of volatiles emitted by strains of *Pseudomonas* and *Serratia* on growth and survival of selected microorganisms, *Caenorhabditis elegans* and *Drosophila melanogaster*. *Hindawi Publishing Corporation BioMed Research International Volume,* Article ID 125704, 11 pages.

34. **Weisskopf, L.** (2013) The potential of bacterial volatiles for crop protection against phytophathogenic fungi, Microbial Pathogens and Strategies for Combating Them. *Science, Technology and Education*, ed. A. Méndez-Vilas (Badajoz: Formatex Research Center), pp. 1352–1363.

35. **Mccain, A.H. (1966)** A Volatile Antibiotic Produced by *Streptomyces Griseus*. *Phytopathology*, **56**(2), pp. 150.

36. **Zou, C-S., Mo, M-H., Gu, Y-Q., Zhou, J-P., Zhang, K-Q.** (2007) Possible contributions of volatile-producing bacteria to soil fungistasis. *Soil Biology and Biochemistry*, **39**, pp. 2371–2379.

37. **Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C.** (2005) Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biology and Biochemistry*, **37**, pp. 955–964.

38. **Fiddaman, P.J., Rossall, S.** (1993) The production of antifungal volatiles by *Bacillus subtilis*. *Journal of Applied Bacteriology*, **7**, pp. 119–126.

39. **Gasch, A.P.** (2007) Comparative genomics of the environmental stress response in ascomycete fungi. *Yeast,* **24**, pp. 961-976.

40. **Nikolaou, E., Agrafioti, I., Stumpf, M., Quinn, J., Stansfield, I., Brown, A.J.P.** (2009) Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evolutionary Biology*, **9**(44), pp. 1-18.

41. **Aung-Htut, M.T., Ayer, A., Breitenbach, M., Dawes, I.W.** (2012) Oxidative stresses and ageing. *Subcellular Biochemistry*, **57**, pp.13–54.

42. **Djamali, A.** (2007) Oxidative stress as a common pathway to chronic tubulointerstitial injury in kidney allografts. *American Journal of Physiology*, **293**(2), pp. 445-455.

43. **Breitenbach, M., Weber, M., Rinnerthaler, M., Karl, T., Breitenbach- Koller, L.** (2015) Oxidative Stress in Fungi: Its Function in Signal Transduction, Interaction with Plant Hosts, and Lignocellulose Degradation. *Biomolecules*, **5**, pp. 318-342.

44. **Hohmann, S.** (2002) Osmotic stress signalling and osmoadaptation in yeasts. *Microbiology and Molecular Biology Review*, **66**(2), pp. 300-372.

45. **Chen, D., Wilkinson, C.R.M., Watt, S., Penkett, C.J., Toone, W.M., Jones, N., Bahler, J.** (2008) Multiple pathways differentially regulate global oxidative stress responses in fission yeast. *Molecular Biology of the Cell*, **19**(1), pp. 308-317.

46. **Avery, S.V. (**2001) Metal toxicity in yeasts and the role of oxidative stress. *Advances in Applied Microbiology*, **49**, pp. 111–142.

47. **Dantas, A.D.S., Day, A., Mélanielkeh, K.I., Achan, B., Quinn, J.** (2015) Oxidative stress responses in the human fungal pathogen *Candida albicans*. *Biomolecules*, **5**, pp. 142-165.

48. **Yang, Y., Fan, F., Zhuo, R., Ma, F., Gong, Y., Wan, X., Jiang, M., Zhang, X.** (2012) Expression of the Laccase Gene from a White-rot Fungus in *Pichia pastoris* Can Enhance the Resistance of this Yeast to H_2O_2-Mediated Oxidative Stress by Stimulating the Glutathione-Based Antioxidative System. *Applied and Environmental Microbiology*, **78**(16), pp. 5845–5854.

49. **Crowe, J.D. and Olsson, S.** (2001) Induction of Laccase Activity in *Rhizoctonia solani* by Antagonistic *Pseudomonas fluorescens* Strains and a Range of Chemical Treatments. *Applied and Environmental Microbiology*, **67**(5), pp. 2088-2094.

50. Weise, T., Thürmer, A., Brady, S., Kai, M., Daniel, R., Gottschalk, G., Piechulla B. (2014) VOC emission of various *Serratia* species and isolates and genome analysis of *Serratia plymuthica* 4Rx13. *FEMS Microbiology Letters*, **352**, pp. 45-53.

51. Kalbe, C., Marten, P., Berg G. (1996) Members of the genus *Serratia* as beneficial rhizobacteria of oilseed rape. *Microbiological Research*, **151**, pp. 433–439.

52. Frankowski, J., Lorito, M., Scala, F., Schmid, R., Berg, G., Bahl H. (2001) Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. *Archieves of Microbiology*, **176**, pp. 421–426.

53. Kai, M., Crespo, E., Cristescu, S.M., Harren, F.J.M., Francke, W., Piechulla, B. (2010) *Serratia odorifera*: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*. *Applied Microbiology and Biotechnology*, **88**, pp. 965–976.

54. Liu, W., Wu, W., Zhu, B., Du, Y., Liu, F. (2008) Antagonistic activities of volatiles from four strains of *Bacillus* spp. and *Paenibacillus* spp. against soil-borne plant pathogens. *Agricultural Sciences in China, Beijing*, **7**(9), pp.1104-1114.

55. Avis, T.J., and Be´langer, R.R. (2001) Specificity and mode of action of the antifungal fatty acid *cis*-9-heptadecenoic acid produced by *Pseudozyma flocculosa*. *Applied and Environmental Microbiology*, **67**, pp. 956–960.

56. Bolton, M.D., Thomma, B.P.H.J, Nelson, B.D. (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Molecular Plant Pathology*, **7**(1), pp. 1-16.

57. Garcia, V.G., Onco, M.A.P., Susan, V.R. (2006) Review: Biology and Systematics of the form genus *Rhizoctonia*. *Spanish Journal of Agricultural Research* **4**(1), pp. 55-79.

58. Ji, S.H., Paul, N.C., Deng, J.X., Kim, Y.S., Yun, B., Yu, S.H. (2013) Biocontrol Activity of *Bacillus amyloliquefaciens* CNU114001 against Fungal Plant Diseases. *Mycobiology*, **41**(4), pp. 234-242.

59. Wharton, P., Kirk, W., Berry, D. and Snapp, S. (2007) *Rhizoctonia* stem canker and scurf of potato. Extension Bulletin E-2994.

60. James, R.L. (1989) Effects of fumigation on soil pathogens and beneficial microorganisms. *In: Landis, T.D., tech. coord. Proceedings: Intermountain Forest Nursery Association Meeting. GTR-RM-184. USDA Forest Service, Rocky Mountain Research Station,* pp. 29–34.

61. http://www.ux.uis.no/~ruoff/Neurospora_Rhythm.html

62. Perkins, D. (2002) *Neurospora*: a model of model microbes.*Nature Reviews Genetics,* **3**(5), pp. 397–403.

63. Trans-NIH *Neurospora* Initiative.

64. Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., Smalla, K. (2002) Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. *Applied and Environmental Microbiology*, **68**, pp. 3328–3338.

65. Schneider, C.A., Rasband, W.S., Elicieri, K.W. (2012) “NIH Image to ImageJ: 25 years of image analysis”. *Nat methods*, **9**(7), pp. 671-675.

66. Collins, T.J. (2007) ImageJ for microscopy. *Bio Techniques*, **43**(1), pp. 25-30.

67. Dukić, N.M., Simin, I.N., Svirčev, E., Orčić, D., Beara, I., Lesjak, M., Božin, B. (2012) The Effect of Plant Secondary Metabolites on Lipid Peroxidation and Eicosanoid Pathway. Chapter 9.

68. Dhindsa, R.S., Dhindsa, P.P., Thorpe, T.A. (1981) Leaf Senescence: Correlated with Increased Levels of Membrane Permeability and Lipid Peroxidation, and Decreased Levels of Superoxide Dismutase and Catalase. *Journal of Experimental Botany*, **32**(126), pp. 93-101.

69. Gray, B.H., Lee, L.H., Wyman, J.F. (1985) An Automated Analysis for Superoxide Dismutase Enzyme Activity. *Journal of Analytical Toxicology*, 9.

70. Gao, R., Yuan, Z., Zhao, Z., Gao, X. (1998) Mechanism of pyrogallol autooxidation and determination of superoxide dismutase enzyme activity. *Bioelectrochemistry and Bioenergetics*, **45**, pp. 41-45.

71. Li, X. (2012) Improved Pyrogallol Autoxidation Method: A Reliable and Cheap Superoxide-Scavenging Assay Suitable for All Antioxidants. *Journal of Agriculture and Food Chemistry*, **60**, pp. 6418-6424.

72. Marklund, S., Marklund, G. (1974) Involvement of superoxide anion radical in autoxidation of pyrogallol and a convenient assay for superoxide-dismutase. *European Journal of Biochemistry*, **47**, pp. 469–474.

73. Maciel, G.M., Inacio, F.D., Sa-Nakanishi, A.B.D., Haminiuk, C.W.I., Castoldi, R., Comar, J.F., Bracht, A., Peralta, R.M. (2013) Response of *Ganoderma lucidum* and *Trametes* sp. to the herbicide picloram: Tolerance, antioxidants and production of ligninolytic enzymes. *Pesticide Biochemistry and Physiology*, **105**, pp. 84-92.

74. Chance, B., Maehly, A. C. (1955) Assay of catalase and peroxidase. *Methods in Enzymology,* **2**, pp. 764-775.

75. Kunamneni, A., Ballesteros, A., Plou, F.J., Alcalde, M. (2007) Fungal laccase – a versatile enzyme for biotechnological applications. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*.

76. Scott, S.L., Chen, W.J., Bakac, A., Espenson, J.H. (1993) Spectroscopic parameters, electrode-potentials, acid ionization-constants, and electron-exchange rates of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) radicals and ions.*J.H. Journal of Physical Chemistry***, 97**, pp. 6710.

77. Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, **72**, pp. 248-254.

78. Weise, T., Kai, M., Piechulla, B. (2013) Bacterial ammonia causes significant plant growth inhibition. *PLOS ONE*, **8**(5), e63538.

79. Ryden, J.C., Mc Neil, J.E. (1984) Application of the micrometeorological mass balance method to the determination of ammonia loss from a grazed sward. *Journal of the Science and Food Agriculture*, **35**, pp. 1297–1310.

80. Richard, L. (2011) Concepts & Applications of Inferential Statistics.

81. Ayala, A., Muñoz, M.F., Argüelles, S. (2014) Review Article, Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative Medicine and Cellular Longevity*, 360438, 31 pages.

82. Subramanian, G. (2012) Biopharmaceutical Production. *WILEY-VCH*.

83. Saligjarias, I.D., Gravanis, F.T., Epton, H.A.S. (2002) Biological control of *Botrytis cinerea* on tomato plants by the use of epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182: II. A study on mode of action. *Biological Control*, **25**, pp. 151–161.

84. Hossain, M.B., Piotrowski, M., Lensing, J., Gau, A.E. (2009) Inhibition of fungal growth of *Venturia inaequalis* by the extracellular protein fraction from the antagonistic bacterium *Pseudomonas fluorescens* BK3. *Biological Control*, **48**, pp. 133–139.

85. Jones, W.T., Harvey, D., Sun, X.L., Greenwood, D.R., Al-Samarrai, T.H., Mesarich, C.H., Lowry, J., Templeton, M.D. (2009) Heterologous expression, isotopic-labeling and immuno-characterization of Cin1, a novel protein secreted by the phytopathogenic fungus *Venturia inaequalis. Protein Expression and Purification*, **65**, pp. 140–147.

86. Liu, Shu-Y., Feng, Pei-C., Lo, Chaur-T., Peng, Kou-C. (2010) Plate assay for fungal enzymes using cellophane membranes. *Analytical Biochemistry*, **397**, pp. 121–123.

87. Hudson, Harry J. (1986) Fungal Biology, II series. *Press syndicate of the University of Cambridge*.

88. Bowman S.M., Free, S.J. (2006) The structure and synthesis of the fungal cell wall. *BioEssays*, **28**, pp. 799-808.

89. Koyama, T., Makita, M., Shibata, N., Okawa, Y. (2009) Influence of oxidative and osmotic stresses on the structure of the cell wall mannan of *Candida albicans* serotype A. *Carbohydrate Research*, **344**, pp. 2195–2200.

90. Webster, J., Weber, R.W.S. (2007) "Introduction to Fungi" New York, NY: *Cambridge University Press*, 5-7.

91. Butler, M.J., Day A.W. (1998a) Fungal melanins: a review. *Canadian Journal of Microbiology*, **44**, pp. 1115–1136.

92. Baermann, G. (2014) Expressionsstudien an ausgewählten Genen des cell wall integrity pathwaysin *Sclerotinia sclerotiorum* in Reaktion auf flüchtige Metabolite von *Serratia plymuthica* 4Rx13.

93. Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., Piechulla, B. (2009) Bacterial volatiles and their action potential. *Applied Microbiology and Biotechnology*, **81**(6), pp. 1001–1012.

94. Fisher, R., Kües, U. (2006) The mycota: Growth, differentiation and sexuality (2 nd edition): Asexual sporulation in mycelia fungi.

95. Langfelder, K., Streibel, M., Jahn, B., Haase, G., Brakhage, A.A. (2003) Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genetics and Biology*, **38**, pp.143–158.

96. Eisenman, H.C., Casadevall, A. (2012) Synthesis and assembly of fungal melanin. *Applied microbiology and biotechnology*, **93**(3), pp. 931-940.

97. Mackie, A.E., Wheatley, R.E. (1999) Effects and incidence of volatile organic compound interactions between soil bacterial and fungal isolates. *Soil biology and Biochemistry*, **31**, pp. 375-385.

98. Lilly, V.G., Barnett, H.L. (1951) Enzymes and enzyme action. *In: Physiology of the Fungi, McGraw-Hill, London*, pp. 45±64.

99. White, N.A., Boddy, L. (1992) Differential extracellular enzyme production in colonies of *Coriolus versicolor*, *Phlebia radiata* and *Phlebia rufa*: effect of gaseous regime. *Journal of General Microbiology*, **138**, pp. 2589-2598.

100. Butler, M.J., Gardiner, R.B., Day, A.W. (2009) Melanin synthesis by *Sclerotinia sclerotiorum*. *Mycologia*, **101**, pp. 296-304.

101. Halliwell, B., Chirico, S. (1993) Lipid peroxidation: its mechanism, measurement, and significance. *American Journal of Clinical Nutrition*, **57**, pp. 715S–725S.

102. Marnett, L.J. (1999) Lipid peroxidation—DNA damage by malondialdehyde. *Mutation Research*, **424**, pp. 83–95.

103. Kim, H.J., Chen, C., Kabbage, M., Dickman, M.B. (2011) Identification and characterization of *Sclerotinia sclerotiorum* NADPH Oxidases. *Applied and environmental microbiology*, **77**(21), pp. 7721-7729.

104. Wedzicha, B.L. (1984) Chemistry of sulfur dioxide. *Elsevier Applied Science Publishers, New York, NY*.

105. Georgiou, C.D., Zervoudakis, G., Petropoulou, K.P. (2003) Ascorbic acid might play a role in the sclerotial differentiation of *Sclerotium rolfsii*. *Mycologia,* **92**, pp. 308–316.

106. Fridovich, I. (1978) The biology of oxygen radicals. *Science*, **201**(4359), pp. 875–880.

107. MacAdam, J.W., Nelson C.J., Sharp R.E. (1992 a) Peroxidase activity in the leaf elongation zone of tall fescue. I. Spatial distribution of ionically bound peroxidase activity in genotypes differing in length of elongation zone. *Plant Physiology*, **99**, pp. 872-878.

108. Grau, M., Rigodanza, F., White, A.J.P., Soraru, A., Carraro, M., Bonchio, M., Britovsek, G.J.P. (2014) Ligand tuning of single-site manganese-based catalytic antioxidants with dual superoxide dismutase catalase activity. *Chemical Communications*, **50**, pp. 4607-4609.

109. Fréalle, E., Noël, C., Viscogliosi, E., Camus, D., Dei-Cas, E., Delhaes, L. (2005) Manganese superoxide dismutase in pathogenic fungi: An issue with pathophysiological and phylogenetic involvements. *FEMS Immunology and Medical Microbiology*, **45**, pp. 411–422.

110. Santos, R., Bocquet, S., Puppo, A., Touati, D. (1999) Characterization of an Atypical Superoxide Dismutase from *Sinorhizobium meliloti. Journal of Bacteriology*, **181**, pp. 4509–4516.

111. Fridovich, I., (1995) Superoxide radical and Superoxide Dismutases. *Annual Review of Biochem*istry, **64**, pp. 97–112.

112. Touati, D. (1997) Oxidative Stress and Molecular Biology of Antioxidant Defences. *in: J.G. Scandalias (Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY,* pp. 447–493.

113. Gregory, E., Goscin, S., Fridovich, I. (1974) Superoxide Dismutase and Oxygen Toxicity in a Eukaryote. *Journal of Applied Bacteriology*, **117**, pp. 456.

114. Hamilton, A.J., Holdom, M.D. (1999) Antioxidant systems in the pathogenic fungi of man and their role in virulence. *Medical Mycology*, **37**, pp. 375–389.

115. Andersson, A., Rasool, O., Schmidt, M., Kodzius, R., Fluckiger, S., Zargari, A., Crameri, R., Scheynius, A. (2004) Cloning, expression and characterization of two new IgE-binding proteins from the yeast *Malassezia sympodialis* with sequence similarities to heat shock proteins and manganese superoxide dismutase. *European Journal of Biochemistry*, **271**, pp. 1885–1894.

116. Cox, G.M., Harrison, T.S., McDade, H.C., Taborda, C.P., Heinrich, G., Casadevall, A., Perfect, J.R. (2003) Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. *Infection and Immunity*, **71**, pp. 173–180.

117. Fluckiger, S., Fijten, H., Whitley, P., Blaser, K., Crameri, R. (2002a) Cyclophilins, a new family of cross-reactive allergens. *European Journal of Immunology*, **32**, pp. 10–17.

118. Fluckiger, S., Mittl, P.R., Scapozza, L., Fijten, H., Folkers, G., Grutter, M.G., Blaser, K., Crameri, R. (2002b) Comparison of the crystal structures of the human manganese superoxide dismutase and the homologous *Aspergillus fumigatus* allergen at 2-A resolution. *The Journal of Immunology*, **168**, pp. 1267–1272.

119. Giles, S.S., Batinic-Haberle, I., Perfect, J.R., Cox, G.M. (2005) *Cryptococcus neoformans* mitochondrial superoxide dismutase: an essential link between antioxidant function and high-temperature growth. *Eukaryotic Cell*, **4**, pp. 46–54.

120. Lamarre, C., Le May, J.D., Deslauriers, N., Bourbonnais, Y. (2001) *Candida albicans* expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. *The Journal of Biochemistry*, **276**, pp. 43784–43791.

121. Narasipura, S.D., Ault, J.G., Behr, M.J., Chaturvedi, V., Chaturvedi, S. (2003) Characterization of Cu, Zn superoxide dismutase (*SOD1*) gene knock-out mutant of *Cryptococcus neoformans* var. *gattii*: role in biology and virulence. *Molecular Microbiology*, **47**, pp. 1681–1694.

122. Jenkinson, S.G., Lawrence, R.A., Burk, R.F., Williams, D.M. (1982) Effects of Copper Deficiency on the Activity of the Selenoenzyme Glutathione Peroxidase and on Excretion and Tissue Retention of $^{75}SeO_3^{2-}$[12]. *The Journal of Nutrition*, **112**, pp. 197–209

123. Chelikani, P., Fita, I., Loewen, P.C. (2004) Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences,* **61** (2), pp. 192–208*.*

124. Boon, E.M., Downs, A., Marcey, D. (2007) Catalase: H_2O_2: H_2O_2 Oxidoreductase*. Catalase Structural Tutorial Text*. Retrieved*.*

125. Ayar-Kayali, H., Ozer, N., Tarhana, L. (2002) Intracellular superoxide dismutase, catalase, and glutathione peroxidase activities and membrane lipid peroxide levels in *Fusarium acuminatum* upon environmental changes in a defined medium. *Archives of Biochemistry and Biophysics*, **400**, pp. 265–272.

126. Johnson, C.H., Klotz, M.G., York, J.L., Kruft, V., McEwen, J.E. (2002) Redundancy, phylogeny and differential expression of *Histoplasma capsulatum* catalases. *Microbiology*, **148**, pp. 1129–1142.

127. Klotz, M.G., Loewen, P.C. (2003) The Molecular Evolution of Catalatic Hydroperoxidases: Evidence for Multiple Lateral Transfer of Genes Between Prokaryota and from Bacteria into Eukaryota. *The Molecular Evolution of Catalatic Hydroperoxidases*, **20**, pp. 1098–1112.

128. Zamocky, M., Gasselhuber, B., Furtmuller, P.G., Obinger, C. (2012) Molecular evolution of hydrogen peroxide degrading enzymes. *Archives of Biochemistry and Biophysics*, **525**, pp. 131-144.

129. Scandalios, J.G., Ruis, H., Koller, F. (1997) in: (Ed.) Oxidative Stress and the Molecular Biology of Antioxidant Defenses. *Cold Spring Harbour Laboratory Press, New York*, pp. 309–342.

130. Michán, S., Lledías, F., Hansberg, W. (2003) Asexual development is increased in *Neurospora crassa* cat-3-null mutant strains. *Eukaryotic Cell*, **2**, pp.798–808.

131. Loewen, P.C. (1984) Isolation of catalase-deficient *Escherichia coli* mutants and genetic mapping of kat E, a locus that affects catalase activity. *Journal of Bacteriology*, **157**, pp. 622–626.

132. Navarro, R.E., Stringer, M.A., Hansberg, W., Timberlake, W.E., Aguirre, J. (1996) catalase A, a new *Aspergillus nidulans* gene encoding a developmentally regulated catalase. *Journal of Current Genetics*, **29**, pp. 352–359.

133. Gessler, N.N., Sokolov, A.V., Bykhovsky, V.Y., Belozerskaya, T.A. (2002) Superoxide dismutase and catalase activities in carotenoid-synthesizing fungi *Blakeslea trispora* and *Neurospora crassa* under the oxidative stress. *Applied Biochemistry and Microbiology*, **38**(3), pp. 237-242.

134. Gessler, N.N., Aver'yanov, A.A., Belozerskaya, T.A. (2007) Reactive Oxygen Species in Regulation of Fungal Development. *Biochemistry (Moscow)*, **72**(10), pp. 1091-1109.

135. Osiewavz, H., Kimpel, E., (1999) Mitochondrial-nuclear interactions and lifespan control in fungi. *Experimental Gerontol*ogy, **34**, pp. 901–909.

136. Hansberg, W., Aguirre J. (1990) Hyperoxidant states cause microbial cell differentiation by cell isolation from dioxygen. *Journal of Theoretical Biology*, **142**, pp. 201–221.

137. Aguirre, J., Rios-Momberg, M., Hewitt, D., Hansberg, W. (2005) Reactive oxygen species and development in microbial eukaryotes. *Trends in Microbiology*, **13** pp. 111–118.

138. Hansberg, W., Aguirre, J., Rios-Momberg, M., Rangel, P., Peraza, L., Montes de Oca, Y., Cano-Domínguez, N. (2008) Stress in yeasts and filamentous fungi*. A.S.V.M. Stratford, P. van West (Eds.), Academic Press, Elsevier, London,* pp. 235–257.

139. Brissos, V., Chen, Z., Martins L.O. (2012) The kinetic role of carboxylate residues in the proximity of the trinuclear centre in the O_2 reactivity of CotA-laccase. *The Royal Society of Chemistry*, **41**, pp. 6247–6255.

140. Messerschmidt, A., Huber, R. (1990) Cell Membrane Functions. *European Journal of Biochemistry*, **187**, pp. 341.

141. Andersen, S.O., Peter, M.G., Roepstorff, P. (1996) Cuticular sclerotization in insects. *Comparative Biochemistry and Physiology*, **113**, pp. 689–705.

142. Kramer, K.J., Kanost, M.R., Hopkins, T.L., Jiang, H., Zhu, Y.C., Xu, R., Kerwin, J.L., Turecek, F. (2001) Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron*, **57**, pp. 385–392.

143. Faure, D., Bouillant, M.L., Bally, R. (1994) Isolation of *Azospirillum lipoferum* 4T Tn5 mutants affected in melanization and laccase activity. *Applied and Environmental Microbiology*, **60**, pp. 3413–3415.

144. Ardon, O., Kerem, Z., Hadar, Y. (1998) Enhancement of lignin degradation and laccase activity in *Pleurotus ostreatus* by cotton stalk extract. *Canadian Journal of Microbiology,* **44**, pp. 676–680.

145. Chen, S.C., Ge, W., Buswell, J.A. (2004) Molecular cloning of a new laccase from the edible straw mushroom *Volvariella volvacea*: possible involvement in fruit body development. *FEMS Microbiology Letters*, **230**, pp. 171–176.

146. Choi, G.H., Larson, T.G., Nuss, D.L. (1992) Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. *Molecular Plant-Microbe Interactions*, **5**, pp. 119 –128.

147. Nagai, M. (2003) Important role of fungal intracellular laccase for melanin synthesis, purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies. *Microbiology*, **149**, pp. 2455–2462.

148. Suguimoto, H.H., Barbosa, A.M., Dekker, R.F.H., Castro-Gomez, R.J.H. (2001) Veratryl alcohol stimulates fruiting body formation in the oyster mushroom *Pleurotus ostreatus*. *FEMS Microbiology Letters*, **194**, pp. 235–238.

149. Wei, D.S. (2010) Laccase and its role in production of extracellular reactive oxygen species during wood decay by the brown rot basidiomycete *Postia placenta*. *Applied and Environmental Microbiology*, **76**, pp. 2091–2097.

150. Zhao, J., Kwan, H.S. (1999) Characterization, molecular cloning, and differential expression analysis of laccase genes from the edible mushroom *Lentinula edodes*. *Applied Environmental Microbiology*, **65**, pp. 4908–4913.

151. Teisseire, H. Vernet., G. (2001) Effects of the fungicide folpet on the activities of antioxidative enzymes in duckweed (*Lemna minor*). *Pesticide Biochemistry and Physiology*, **69**, pp. 112–117.

152. Wu, Y-x., Tiedemann, A. (2002) Impact of fungicides on active oxygen species and antioxidant enzymes in spring barley (*Hordeum vulgare* L.) exposed to ozone. *Environmental Pollution*, **116**, pp. 37–47.

153. Barria, C., Malecki, M., Arraiano, C.M. (2013) Bacterial adaptation to cold. *Microbiology*, **159**, pp. 2437–2443.

154. Hanson, J.R. (2012) Diterpenoids of terrestrial origin. *Natural Product Reports*, **29**, pp. 890-898.

155. Yamada, Y., Komatsu, M., Ikeda H. (2016) Chemical diversity of labdane-type bicyclic diterpene biosynthesis in Actinomycetales microorganisms. *The Journal of antibiotic*s, **69**, pp. 515-523.

156. Singh, M., Pal, M., Sharma, R.P. (1999) Biological Activity of the Labdane Diterpenes. *Planta medica*, **65**, pp. 2-8.

157. Fravel, D.R., Connick, W.J., Grimm, C.C., Llyod, S.W. (2002) Volatile Compounds Emitted by Sclerotia of *Sclerotinia minor*, *Sclerotinia sclerotiorum*, and *Sclerotium rolfsii. Journal of Agricultural and Food Chemistry*, **50**(13), pp 3761–3764.

158. Gori, K., Mortensen, H.D., Arneborg, N., Jespersen, L. (2007) Ammonia production and its possible role as a mediator of communication for *Debaryomyces hansenii* and other cheese-relevant yeast species. *Journal of Diary Science*, **90**, pp. 5032–5041.

159. Simon, J. (2002) Enzymology and bioenergetics of respiratory nitrite ammonification. *FEMS Microbiology Reviews*, **26**, pp. 285–309.

160. Kanapka, J.A., Kleinberg, I. (1983) Catabolism of arginine by the mixed bacteria in human salivary sediment under conditions of low and high glucose concentration. *Archives of Oral Biology*, **11**, pp. 1007–1015.

161. Özogul, F., Özogul, Y. (2007) The ability of biogenic amines and ammonia production by single bacterial cultures. *European Food Research Technology*, **225**, pp. 385–394.

162. Kleiner, D., Traglauer, A., Domm, S. (1998) Does ammonia production by Klebsiella contribute to pathogenesis? *Bulletin de l'Institut Pasteur,* **96**, pp. 257–265.

163. Schippers, B., Meijer, J.W., Liem, J.I. (1982) Effect of ammonia and other soil volatiles on germination and growth of soil fungi. *Transactions of the British Mycological Soc*iety, **79**, pp. 253–259.

164. Eno, C.F., Blue, W.G., Good, J.M. (1955) The effect of anhydrous ammonia on nematodes, fungi, bacteria and nitrification in some Florida soils. *Soil Science Society of America Proceedings*, **19**, pp. 55-58.

165. Neal, D.C., Wester, R.E., Gunn, K.C.(1933) Growth of the cotton root-rot fungus in synthetic media, and the toxic effect of ammonia on the fungus. *Journal of Agricultural Research.*, **47**, pp. 107-118.

166. McCallan, S.E.A., Setterstorm, C. (1940) Toxicity of ammonia, chlorine, hydrogen cyanide, hydrogen sulphide and sulpher dioxide gases. *I. General methods and correlations, Contributions from Boyce Thompson Institute*, **11**, pp. 325-330.

167. Henis, Y., Chet, I. (1967) Mode of action of ammonia on *Sclerotium rolfsii*. *Phytopathology*, **57**, pp. 425-427.

168. Howell, C.R., Beier, R.C., Stipanovic, R.D. (1988) Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of *Pythium preemergence* damping-off by the bacterium. *Ecology and Epidemiology*, **78**, pp. 1075–1078.

169. Ni, L.Y. (2001) Effects of water column nutrient enrichment on the growth of *Potamogeton maackianus*. A. Been. *Journal of Aquatic Plant Management*, **39**, pp. 83–87.

170. Brun, F.G., Hernandez, I., Vergara, J.J., Peralta, G., Perez-Llorens, J.L. (2002) Assessing the toxicity of ammonium pulses to the survival and growth of *Zostera noltii*. *Marine Ecology Progress Series*, **225**, pp. 177–187.

171. Kikeri, D., Sun, A., Zeidel, M.L., Hebert, S.C. (1989) Cell membranes impermeable to NH_3. *Nature*, **339**, pp. 478-480.

172. Morimoto, N., Suda, S., Sagara, N. (1981) Effect of ammonia on fruit-body induction of *Coprinus cinereus* in darkness. *Plant and Cell Physiology*, **22**, pp. 247-254.

173. Palkova, Z., Vahova, L. (2003) Ammonia signaling in yeast colony formation. *International Review of Cytology*, **225**, pp. 229–272.

174. Kosegarten, H., Grolig, F., Wieneke, J., Wilson, G., Hoffmann, B. (1997) Differential ammonia-elicited changes of cytosolic pH in root hair cells of rice and maize as monitored by 29,79-bis-(2-car-boxyethyl)-5 (and -6)-carboxyfluorescein-fluorescence ratio. *Plant Physiology*, **113**, pp. 451–461.

175. Wilson, G.H., Grolig, F., Kosegarten, H. (1998) Differential pH restoration after ammonia-elicited vacuolar alkalization in rise and maize root hairs as measured by fluorescence ratio. *Planta,* **206**, pp. 154–161.

176. Dickman, M.B., Mitra, A. (1992) *Arabidopsis thaliana* as a model for studying *Sclerotinia sclerotiorum* pathogenesis. *Physiological and Molecular Plant Pathol*ogy, **41**, pp. 255-263.

177. Williams, B., Kabbage, M., Kim, H-J., Britt, R., Dickman, M.B. (2011) Tipping the Balance: *Sclerotinia sclerotiorum* Secreted Oxalic Acid Suppresses Host Defenses by Manipulating the Host Redox Environment. *PLoS Pathogens*, **7**(6): e1002107.

178. Bittsanszky, A., Pilinszky, K., Gyulai, G., Komives, T. (2015) Overcoming ammonium toxicity. *Plant Science*, **231**, pp. 184–190.

179. Nimptsch´, J., Pflugmacher, S. (2007) Ammonia triggers the promotion of oxidative stress in the aquatic macrophyte *Myriophyllum mattogrossense*. *Chemosphere,* **66**(4), pp.708.

180. Bonfante P., Anca I.A. (2009) Plants, Mycorrhizal Fungi, and Bacteria: A Network of Interactions. *Annual Review of Microbiology*, **63**, pp. 363-383.

181. Thoudal, M. (2012) Priming as a strategy to enhance stress tolerance in plants with focus on the role of Brassinosteroid related genes. *Swedish University of Agricultural Sciences, ISSN: 1651-5196 Nr 132*.

182. Christopher, J.F., Mescher, M.C., Carlson, J.E., De Moraes, C.M. (2008) Plant Defence Priming against Herbivores: Getting Ready for a Different Battle. *Plant Physiology*, **146**, pp. 818–824.

7 Supplements

Chapter 1: Influence of volatile metabolites of *Serratia plymuthica* 4Rx13 on the growth and morphology of the fungi *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Phoma eupyrena* and *Neurospora crassa*.

1.1 Radial growth (No cellophane membrane)

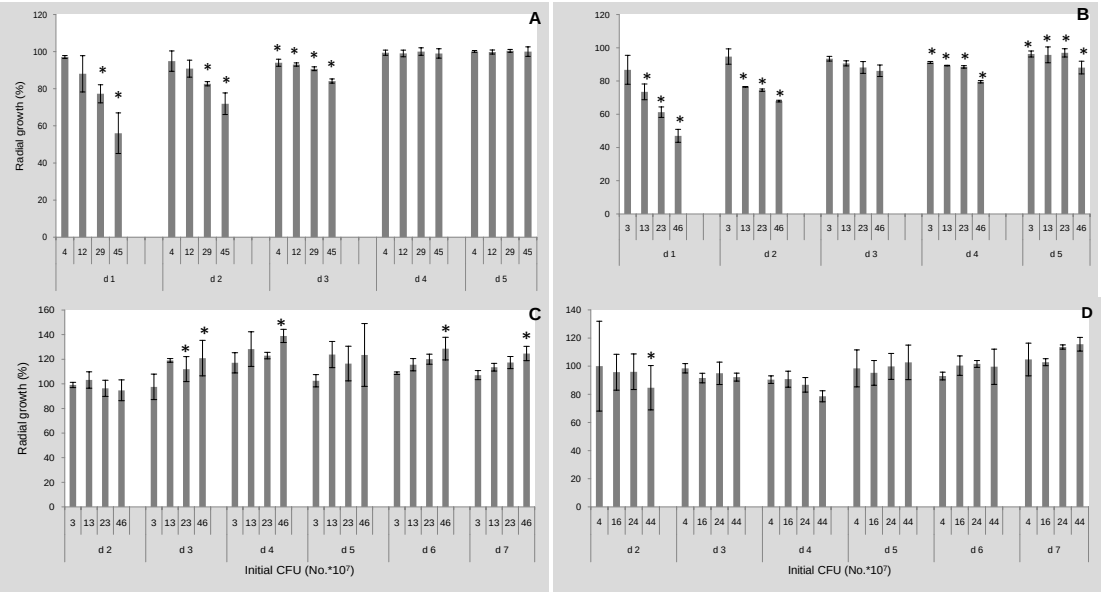

Figure S1: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of selected fungi.

A: *Sclerotinia sclerotiorum*; **B:** *Rhizoctonia solani*; **C:** *Phoma eupyrena*; **D:** *Neurospora crassa*.

The fungal growth was analyzed using the software ImageJ. The growth was expressed in pixel (software ImageJ). The control was equivalent to 550545 (d 1), 1301689 (d 2), 2737247 (d 3), 3737249 (d 4), and 3843218 (d 5) (**A**), 314290 (d 1), 1560714 (d 2), 2392553 (d 3), 2856171 (d 4), and 2855675 (d 5) (**B**), 229086 (d 2), 439598 (d 3), 540046 (d 4), 943467 (d 5), 1178659 (d 6), and 1473805 (d 7) (**C**)174720 (d 2), 346760 (d 3), 965626 (d 4), 779612 (d 5), 1095549 (d 6), and 1258540 (d 7) (**D**).
n=3. Error bars show standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

1.2 Biomass

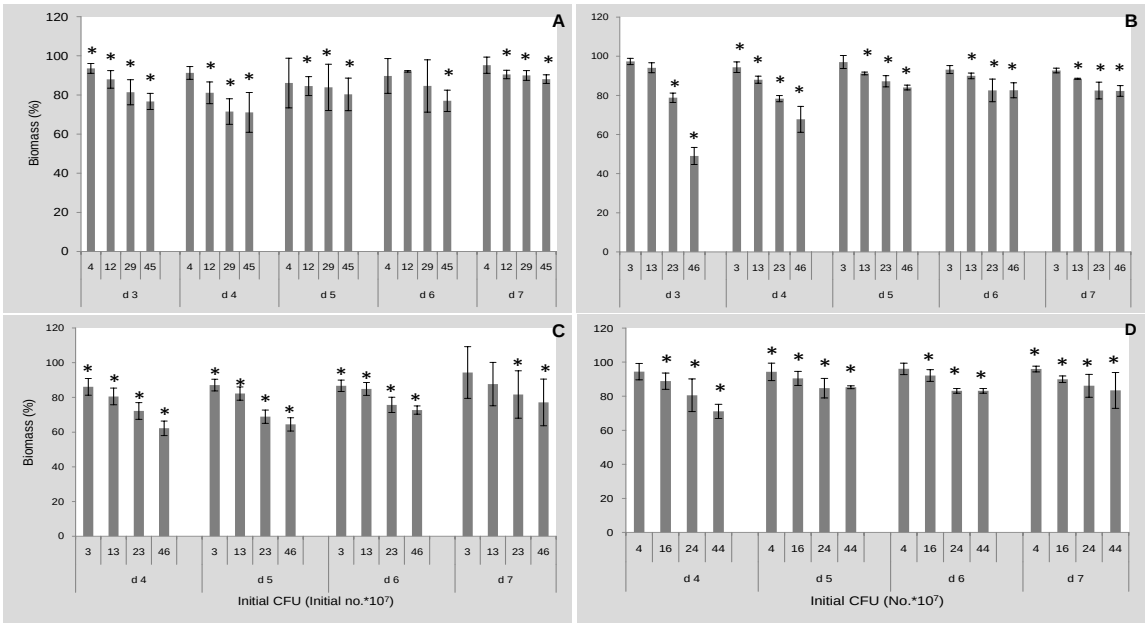

Figure S2: Influence of volatiles of *Serratia plymuthica* 4Rx13 on biomass of selected fungi.

A: *Sclerotinia sclerotiorum*; **B:** *Rhizoctonia solani*; **C:** *Phoma eupyrena*; **D:** *Neurospora crassa*.

The control in mg was equivalent to 80 (d 3), 90 (d 4), 120 (d 5), 130 (d 6), and 140 (d 7) (**A**), 160 (d 3), 170 (d 4), 190 (d 5), 230 (d 6), and 240 (d 7) (**B**), 120 (d 4), 150 (d 5), 180 (d 6), and 210 (d 7) (**C**), 120 (d 4), 140 (d 5), 170 (d 6), and 207 (d 7) (**D**) .
n=3. Error bars show standard deviation. (*) represents statistical significance corresponding to the control (100 %).

1.3 Morphology (Morphological study by taking images in absence of cellophane membrane)

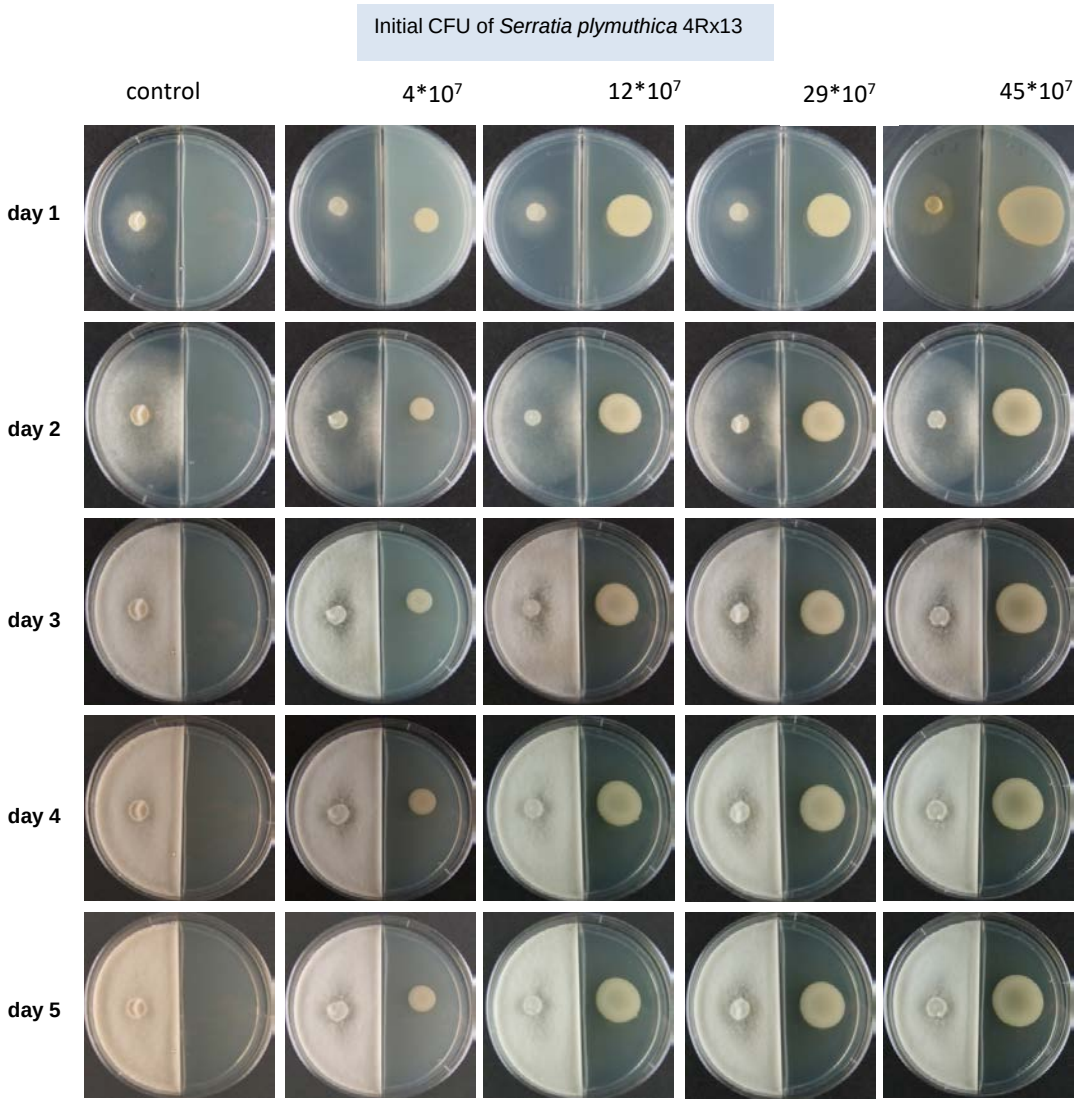

Figure S3A: Influence of volatiles of *Serratia plymuthica* 4Rx13 on morphology of the fungus *Sclerotinia sclerotiorum*. n=3.

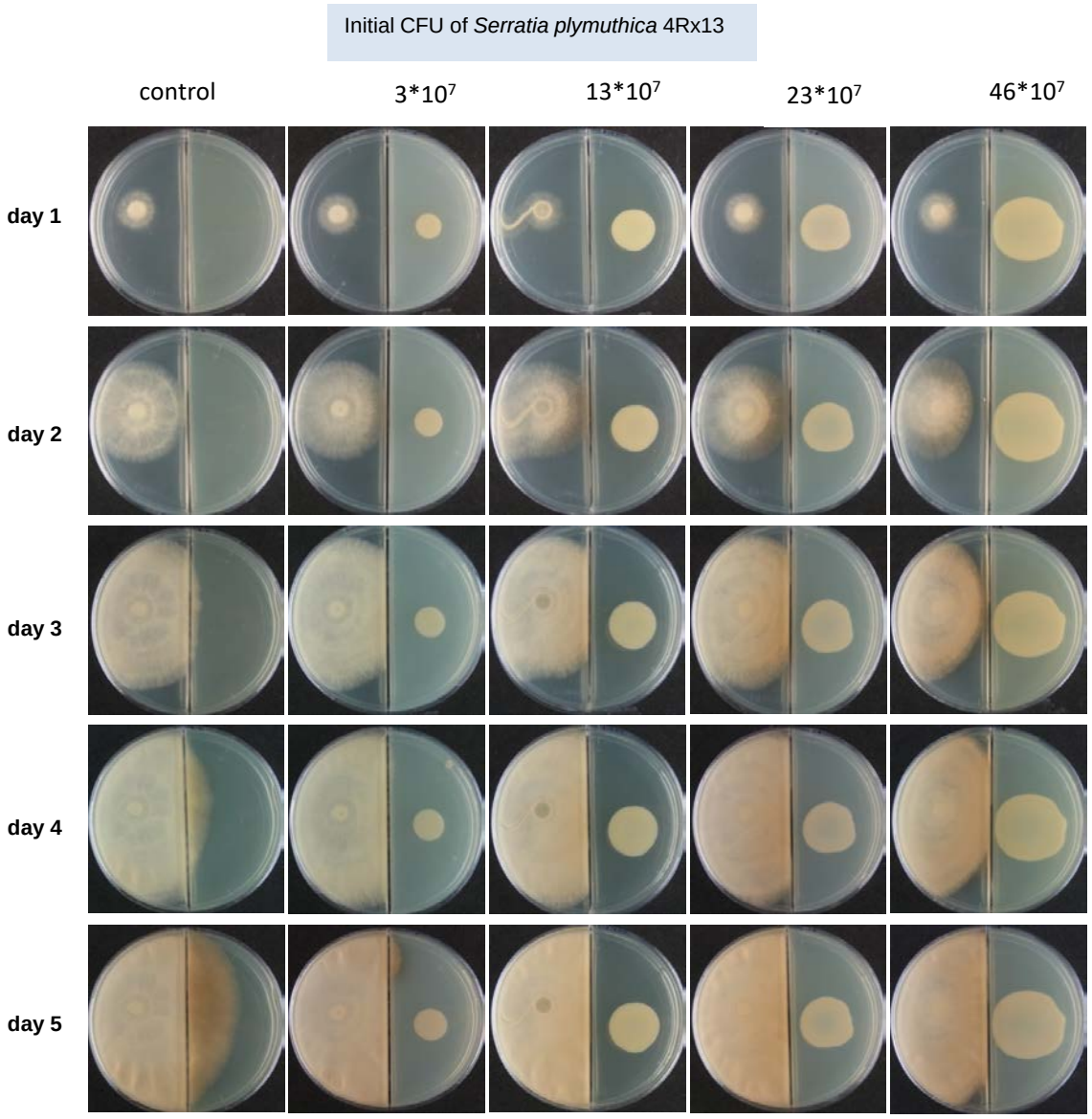

Figure S3B: Influence of volatiles of *Serratia plymuthica* 4Rx13 on morphology of the fungus *Rhizoctonia solani.* n=3.

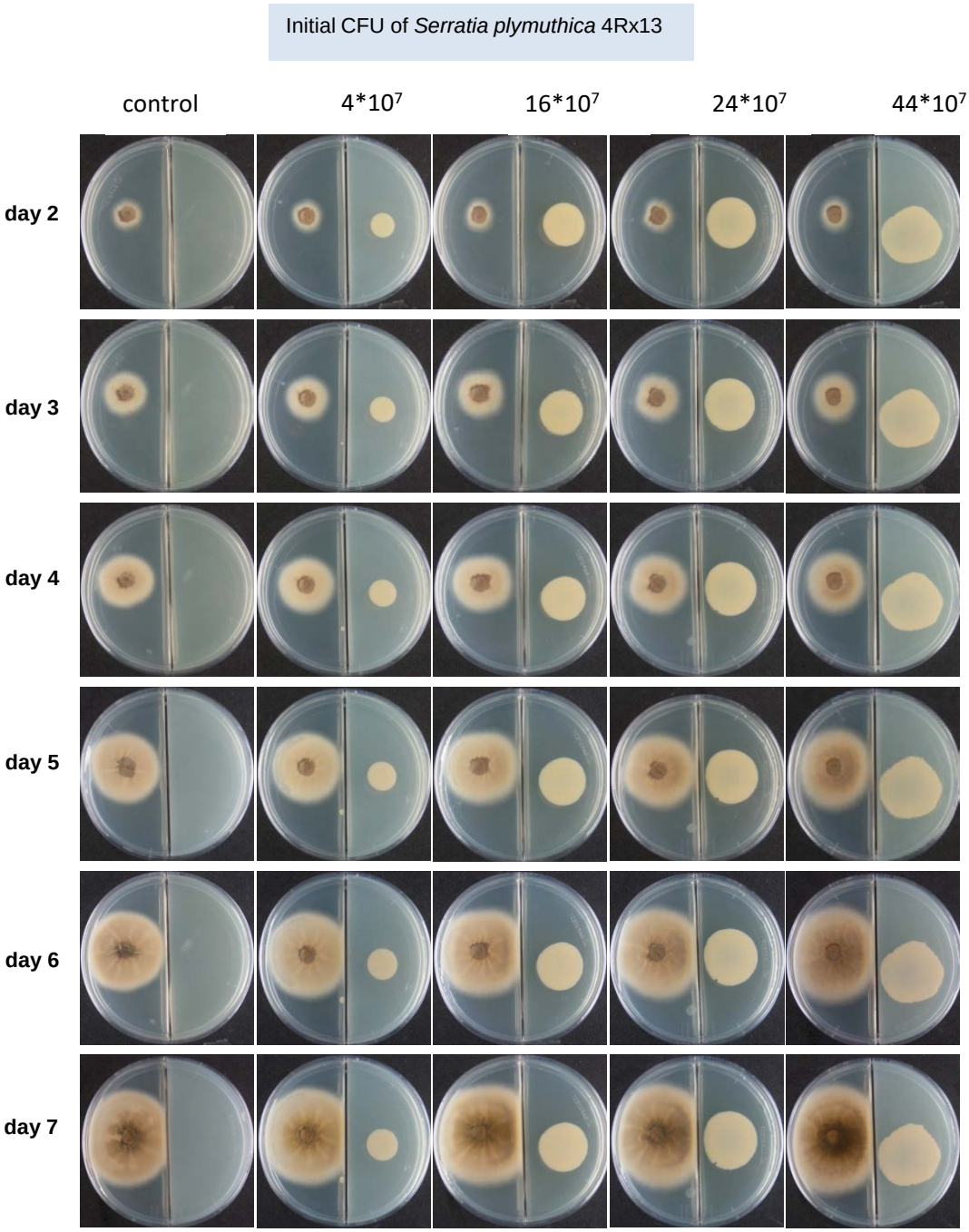

Figure S3C: Influence of volatiles of *Serratia plymuthica* 4Rx13 on morphology of the fungus *Phoma eupyrena.* n=3.

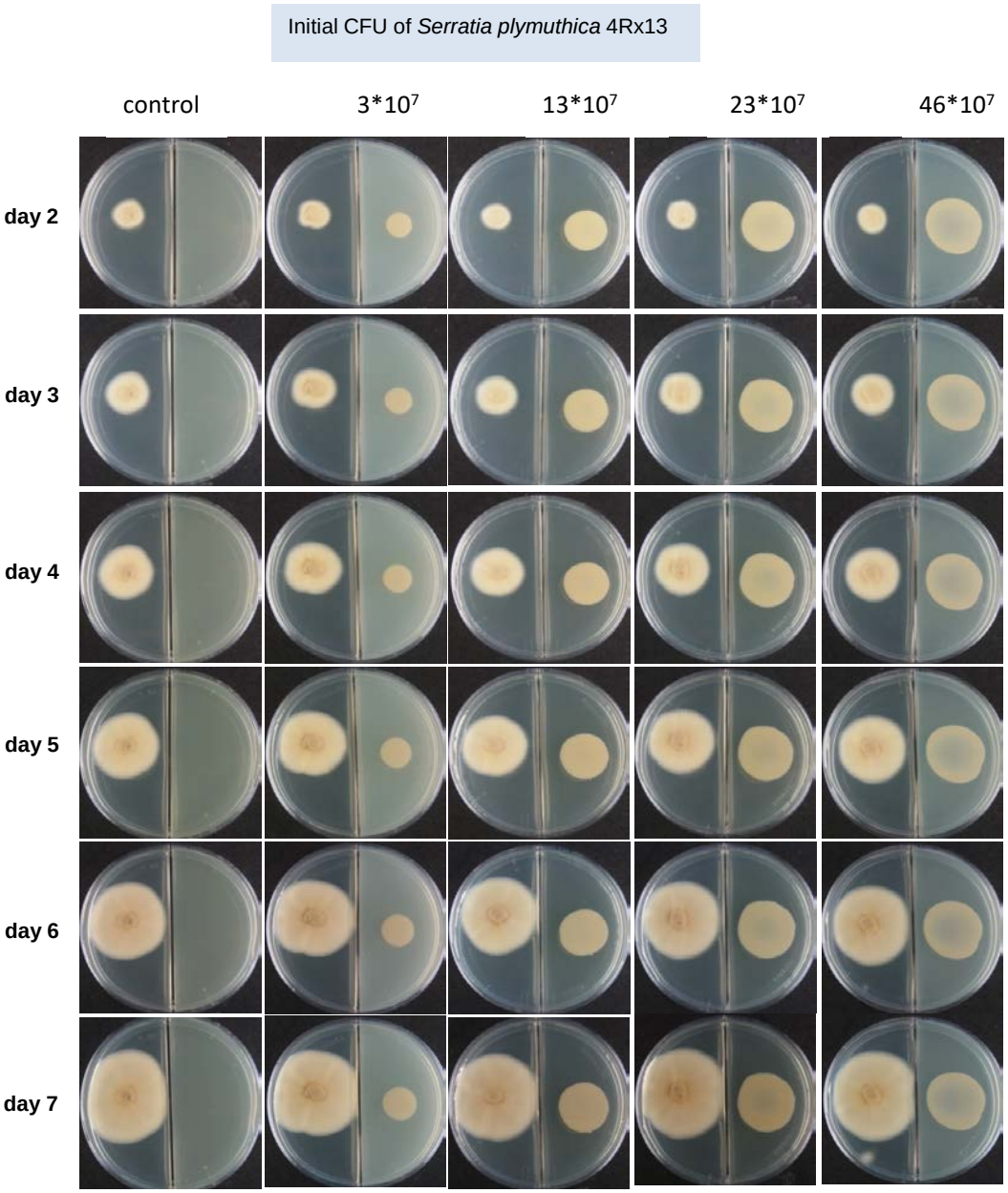

Figure S3D: Influence of volatiles of *Serratia plymuthica* 4Rx13 on morphology of the fungus *Neurospora crassa*. n=3.

Chapter 2: Influence of volatile metabolites of *Serratia plymuthica* 4Rx13 on the membrane integrity and enzyme activities indicating oxidative stress

2.1 Non-ezymatic lipidperoxidation

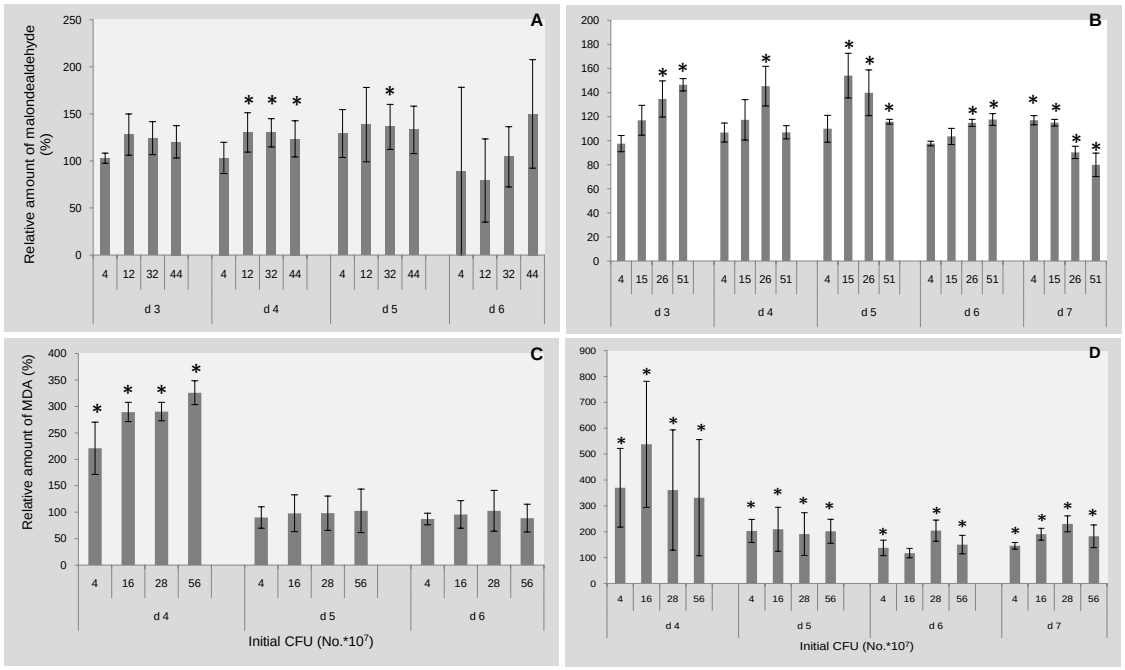

Figure S4: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the cell membrane integrity (non-enzymatic lipidperoxidation) of selected fungi.

A: *Sclerotinia sclerotiorum*; **B:** *Rhizoctonia solani*; **C:** *Phoma eupyrena*; **D:** *Neurospora crassa*.

MDA equivalents of the control in nmol/mL: 2.85 (d 3), 2.03 (d 4), 1.60 (d 5), and 0.88 (d 6) (**A**), 3.72 (d 3), 2.89 (d 4), 3.12 (d 5), 4.16 (d 6), and 3.20 (d 7) (**B**), 2.58 (d4), 1.77 (d 5), and 1.71 (d 6) (**C**), 1.18 (d 4), 2.74 (d 5), 4.23 (d 6), and 4.76 (d 7) (**D**).
n=3.Error bars show standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

2.2 Catalase

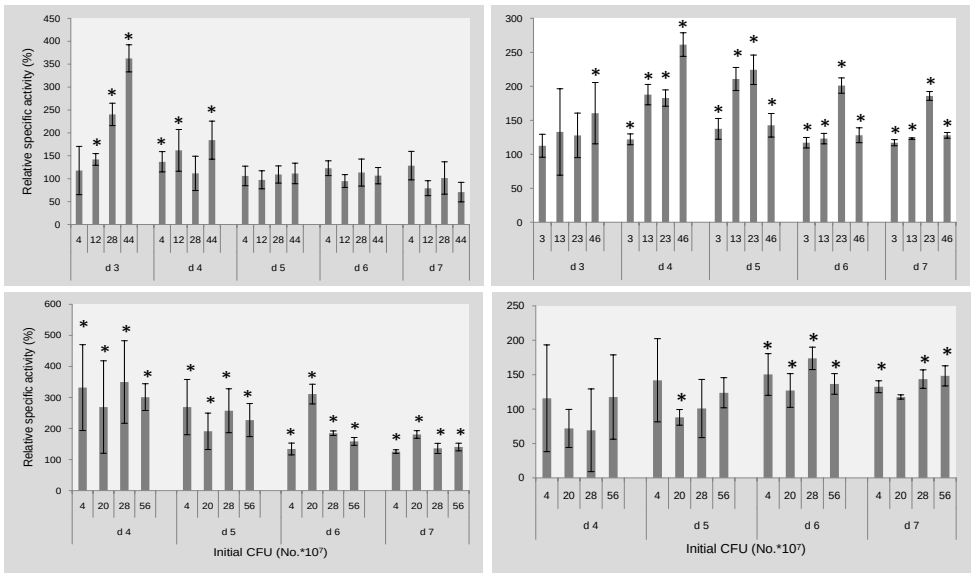

Figure S5: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the cell (catalase activity) of selected fungi.

A: *Sclerotinia sclerotiorum*; **B:** *Rhizoctonia solani*; **C:** *Phoma eupyrena*; **D:** *Neurospora crassa*.

The control inμkat/mg protein was equivalent to 2.42 (d 3), 2.32 (d 4), 2.46 (d 5), 2.6 (d 6), and 2.27 (d 7) (**A**), 0.61 (d 3), 2.26 (d 4), 2.48 (d 5), 4.42 (d 6), and 7.53 (d 7) (**B**), 0.43 (d 4), 1.08 (d 5), 1.91 (d 6), and 2.32 (d 7) (**C**), 0.44 (d4), 1.08 (d 5), 1.92 (d 6), and 2.32 (d 7). n=3. Error bars show standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

Chapter 3: Adaptation experiment

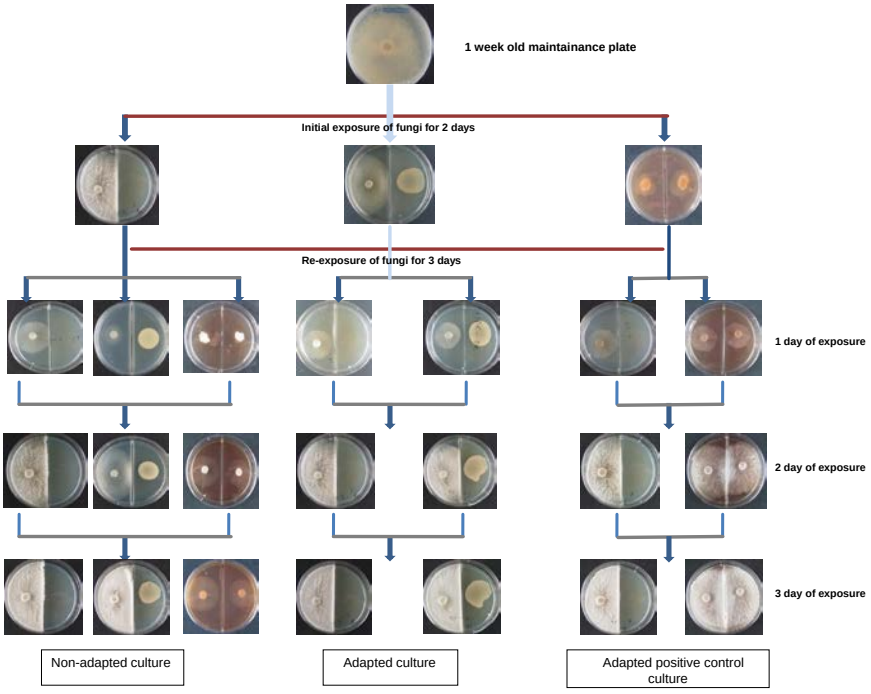

Figure S6A: An overview of adaptation experiment.

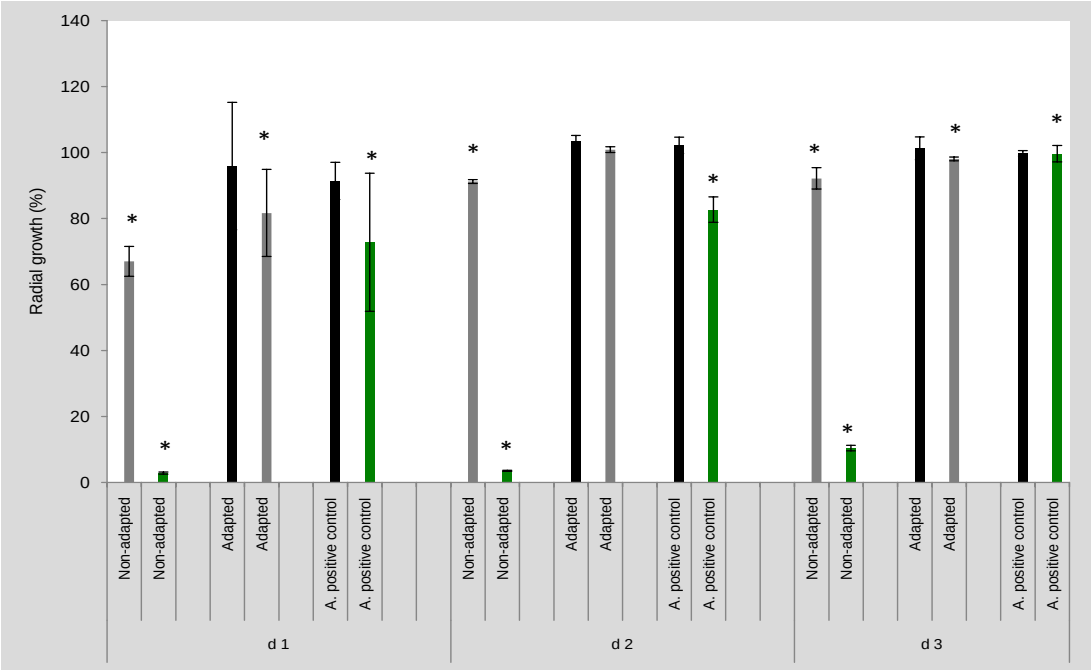

Figure S6B: An overview of radial growth experiment.

negative control; dual culture; positive control (hydroquinone). A. stands for adapted culture.

The growth was expressed in pixel (software ImageJ). The control was set 100 % corresponding to 103625 (day 1), 2330685 (day 2), 2552565 (day 3). n=3. Error bars show standard deviation. (*) represents statistical significance related to the corresponding master control (100 %).

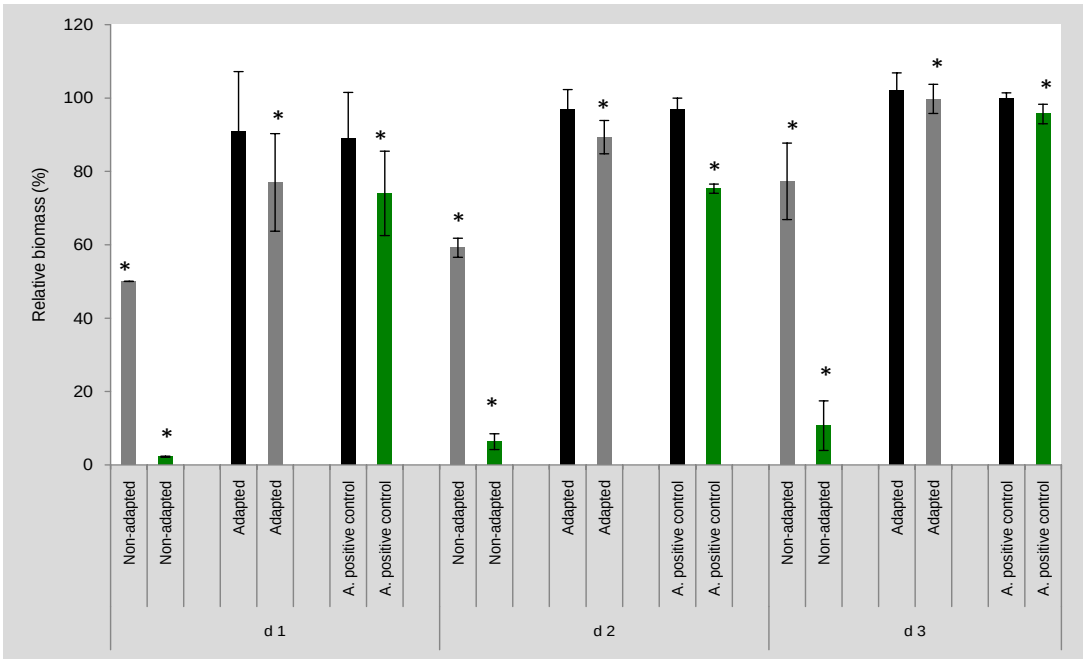

Figure S6C: An overview of biomass experiment.

negative control; dual culture; positive control (hydroquinone). A. stands for adapted culture.

The control in mg was equivalent to 5 (d 1), 30 (d 2), 80 (d 3). n=3. Error bars show standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

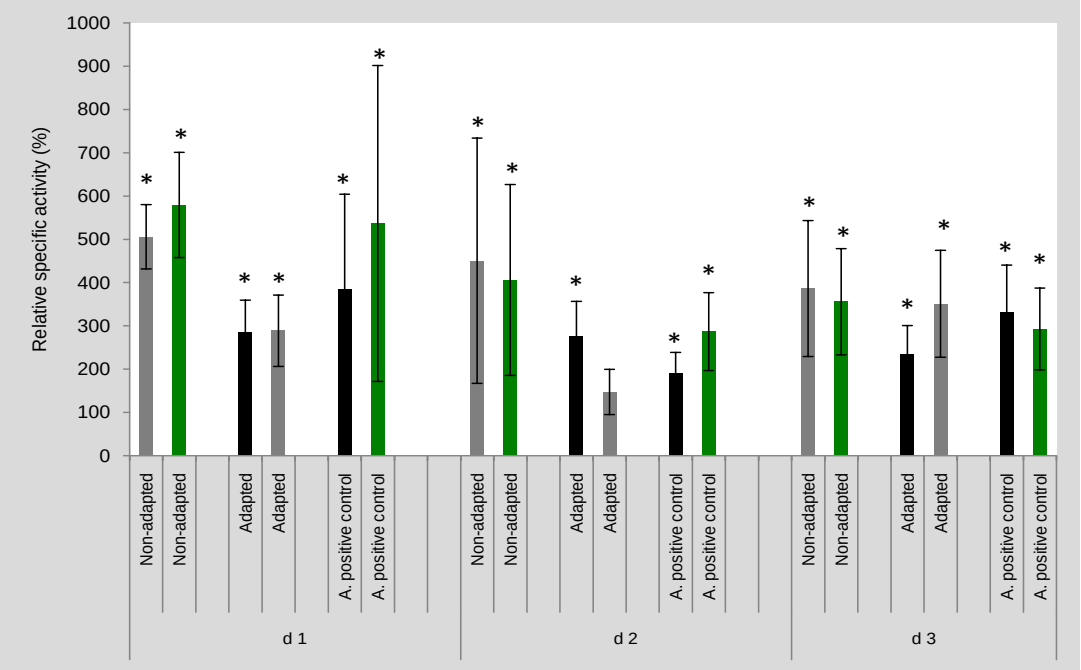

Figure S6D: An overview of intracellular laccase experiment.

negative control; dual culture; positive control (hydroquinone). A. stands for adapted culture.

The control in nkat/mg protein was equivalent to 0.010 (d 1), 0.016 (d 2), 0.013 (d 3). n=3. Error bars show standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

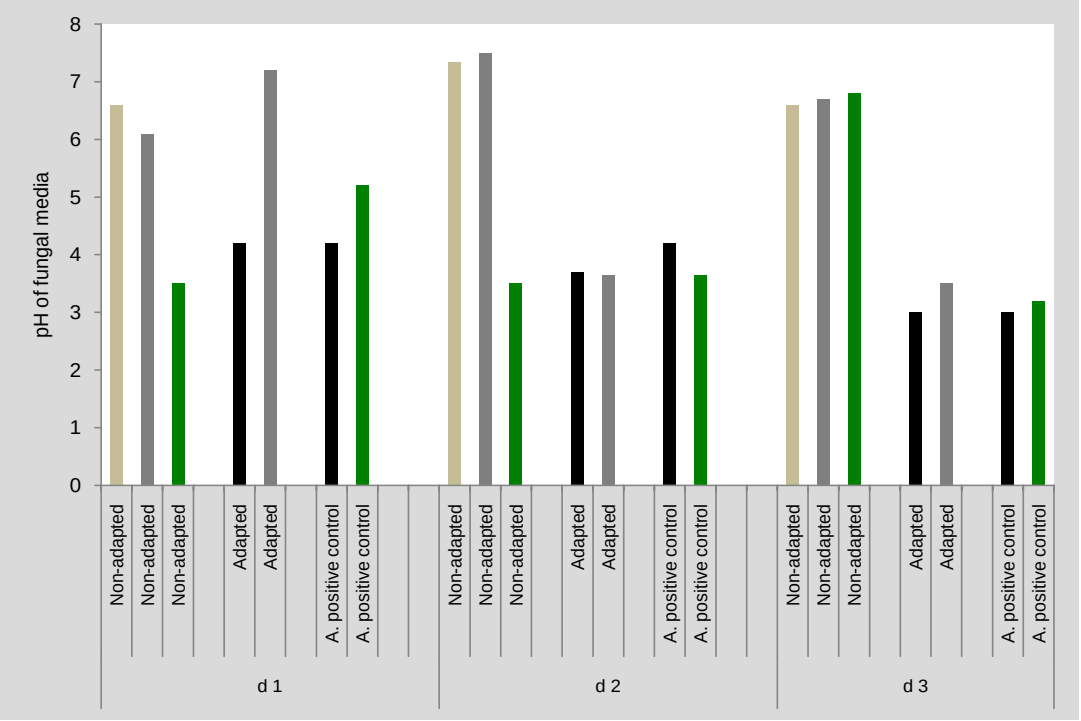

Figure S6E: An overview of pH of the fungal media.

master control; negative control; dual culture; positive control (hydroquinone). A. stands for adapted culture.

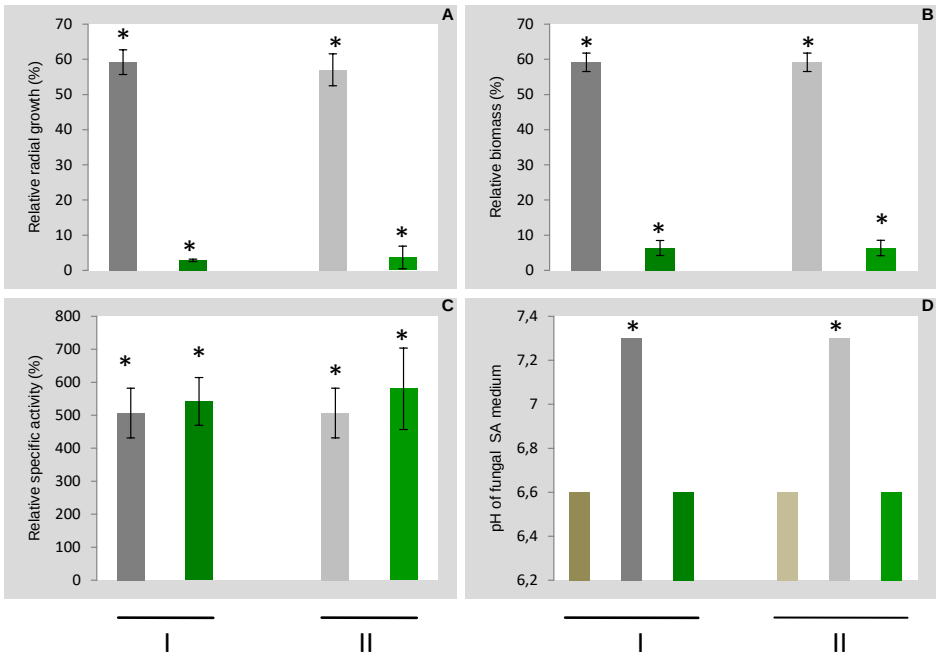

Figure S6F: Influence of bacterial volatiles of *Serratia plymuthica* 4Rx13 and hydroquinone on the radial growth, biomass, pH of the fungal medium, and laccase activity: Comparison of initially exposed and non-adapted fungal culture.

A.radial growth; **B**. biomass; **C**. pH of fungal media; **D**. intracellular laccase.

I: Initial exposure: initial exposure to the bacterial volatiles; initial incubation on hydroquinone; negative control.
II: Re-exposure: re-exposure to bacterial volatiles; re-exposure to hydroquinone; master control.

An initial CFUof 40-48*10^7 were point-inoculated onto the bacterial nutrient agar (NA) of split Petri dishes. The dishes were kept at 30 °C for 2 days. Mycelial plugs were inoculated on fungal SA side. Monocultures of the fungus served as negative control. The incubation on hydroquinone (5 mM) served as positive control. The dishes were placed at 20 °C and parameters were measured after day 1 of incubation. After incubation for 2 days, mycelia plugs were re-exposed to bacterial volatiles and the hydroquinone. The plates were placed at 20 °C. Again after day 1 all parameters were measured. The radial growth was expressed in pixel (software ImageJ). The control was equivalent to 459433 (**I**), 459836(**II**) (**A**), 5 mg (**I** and **II**) (**B**), 0.016 nkat/mg protein (**I**), 0.019 nkat/mg protein(**II**) (**D**).
n=3. Error bars show standard deviation. (*) represents statistical significance related to the corresponding master control.

Chapter 4: VOC emission in different systems

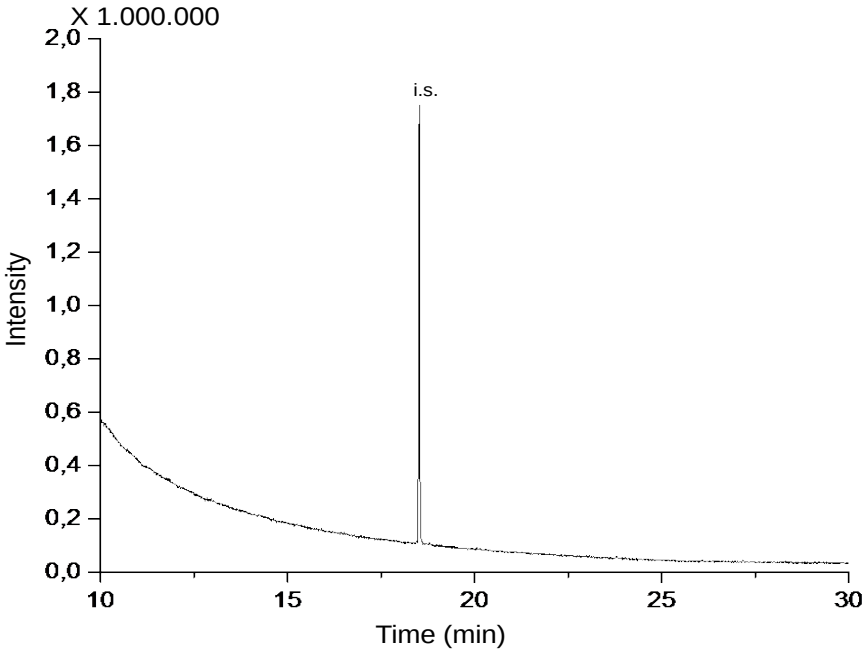

Figure S7A: VOC emission of empty system.

Collection time for VOC analysis: 0-24 h (day 1, 30 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks**: i.s.** (Internal standard).
n=3.

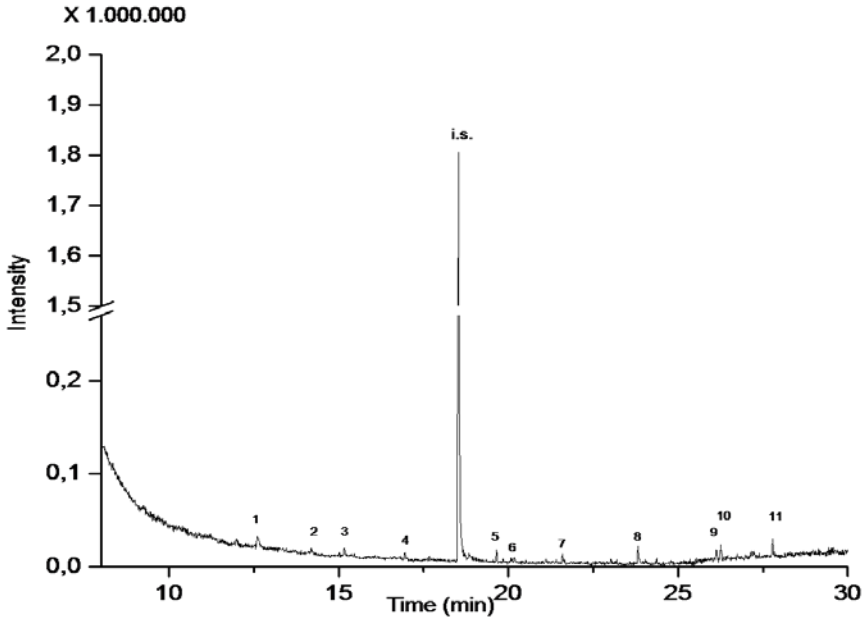

Figure S7B: VOC emission in presence of SA+NA media.

Collection time for VOC analysis: 0-24 h (day 1, 30 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: 1 (benzaldehyde), 2 (benzeneacetaldehyde), and **i.s.** (Internal standard).
n=3.

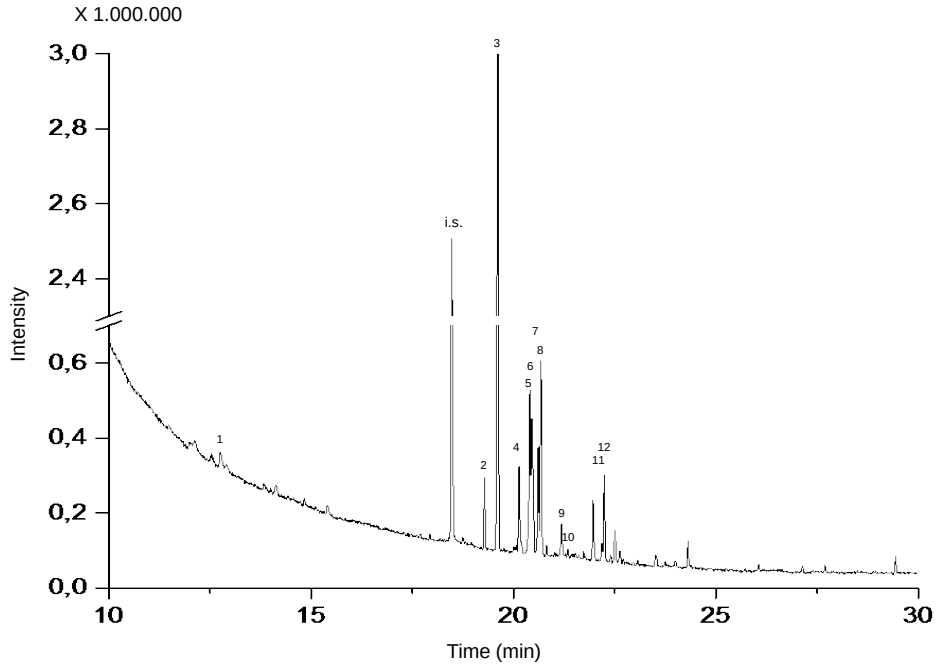

Figure 7C: VOC emission of *Serratia plymuthica* 4Rx13 at 30 °C.

Collection time for VOC analysis: 24-48 h (day 2, 30 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **1** (Dimethyl trisulfide), **i.s.** (Internal standard), **2** (compound A), **3** (sodorifen), **4** (compound B), **5** (compound C), **6** (compound D), **7** (compound E), **8** (compound F), **9** (compound G), **10** (compound H), **11** (compound I) and **12**(compound J). n=3.

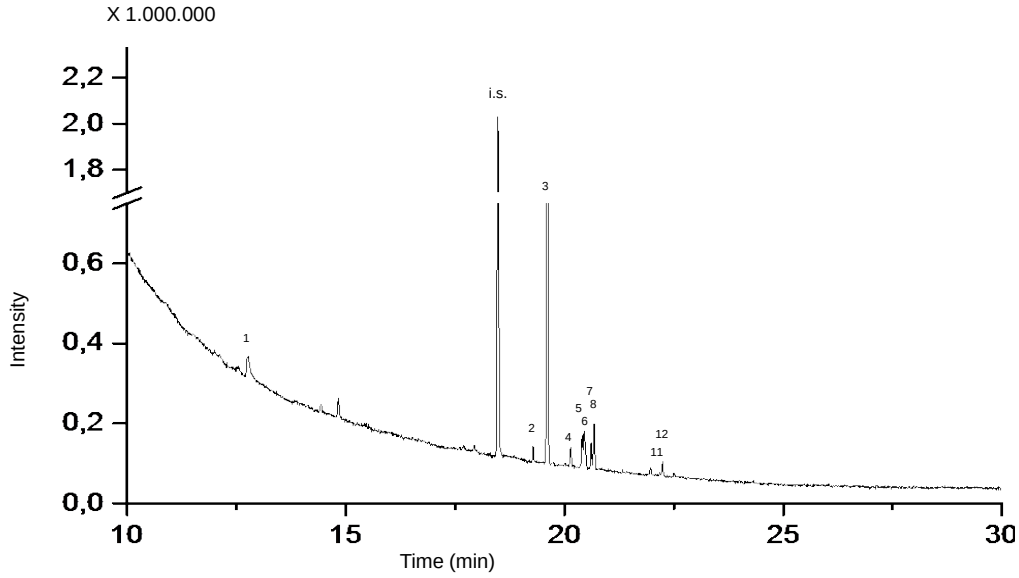

Figure 7D: VOC emission of *Serratia plymuthica* 4Rx13 at 20 °C.

Collection time for VOC analysis: 24-48 h (day 2, 20 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **1** (Dimethyl trisulfide), **i.s.** (Internal standard), **2** (compound A),**3** (sodorifen), **4** (compound B), **5** (compound C), **6** (compound D), **7** (compound E), **8** (compound F), **11** (compound I) and **12** (compound J). n=3.

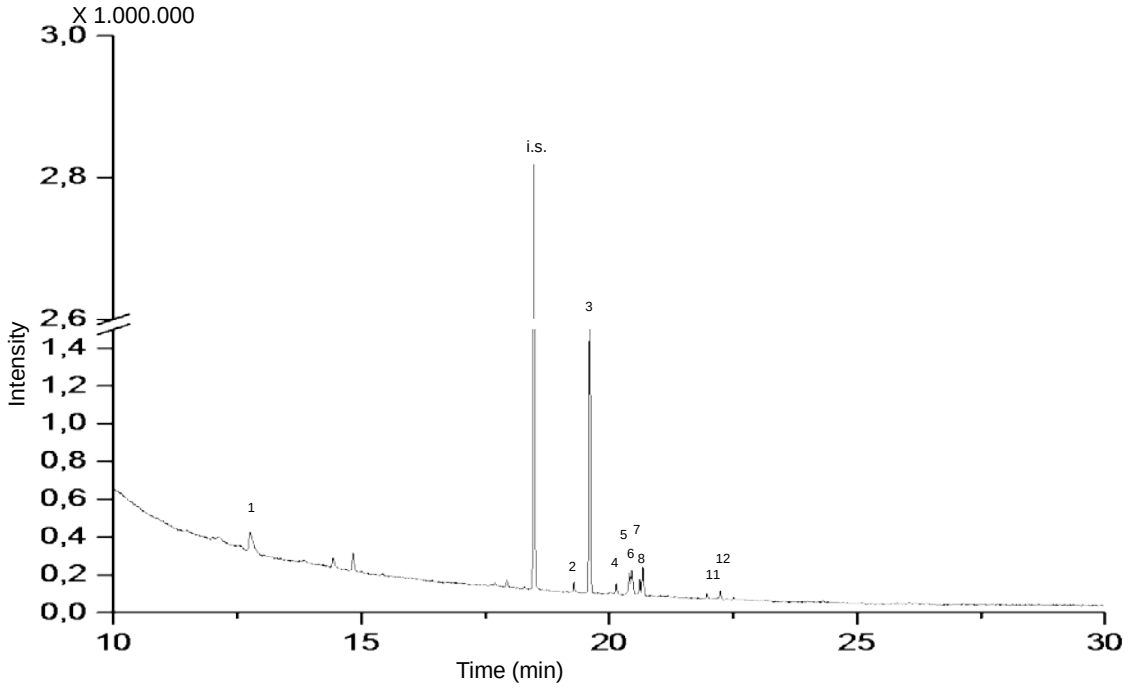

Figure 7E: VOC emission of *Serratia plymuthica* 4Rx13 at 20 °C.

Collection time for VOC analysis: 48-72 h (day 3, 20 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **1** (Dimethyl trisulfide), **i.s.** (Internal standard), **2** (compound A),**3** (sodorifen), **4** (compound B), **5** (compound C), **6** (compound D), **7** (compound E), **8** (compound F), **11** (compound I) and **12** (compound J). n=3.

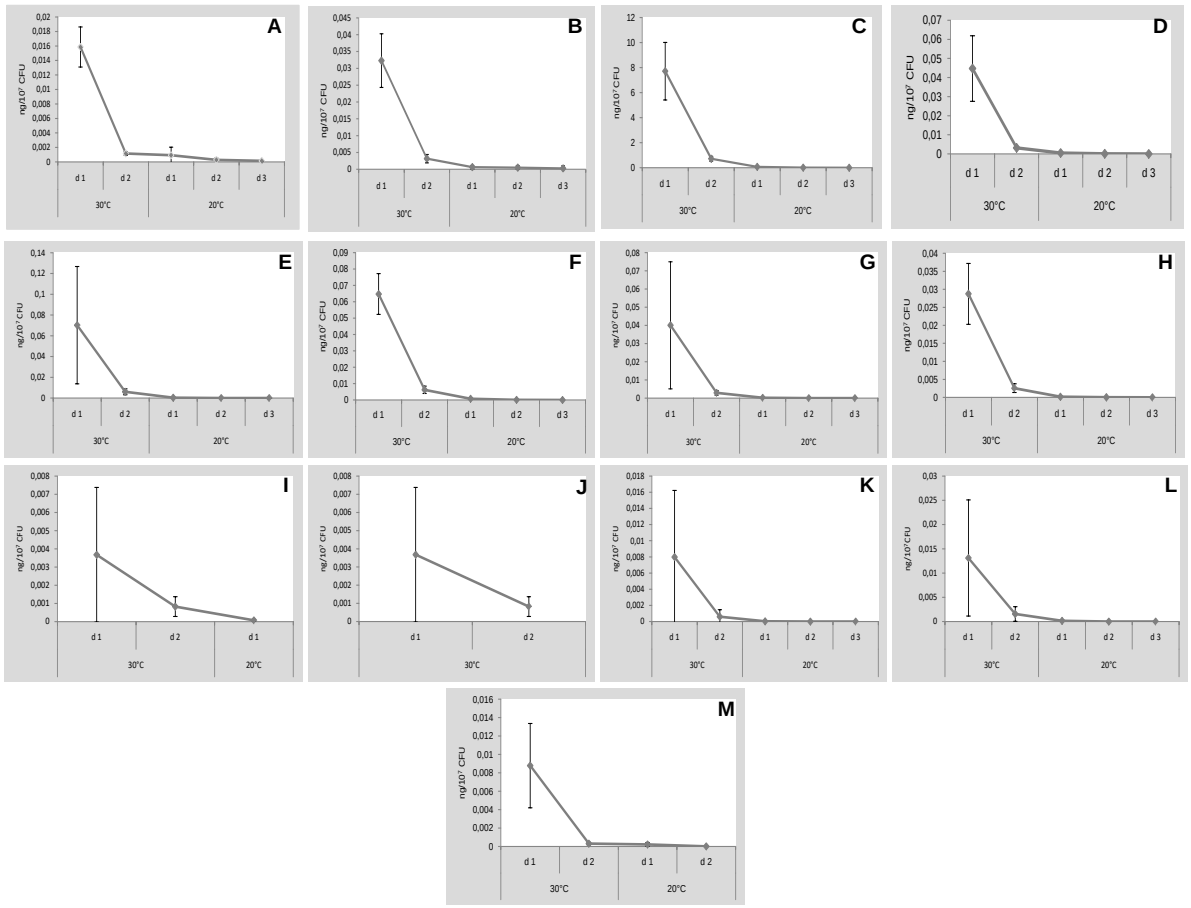

Figure S7F: Quantity of VOCs produced by *Serratia plymuthica* 4Rx13.

A: Dimethyl trisulfide; **B:** Compound A; **C:** Sodorifen; **D:** Compound B; **E:** Compound C; **F:** Compound D; **G:** Compound E; H**:** Compound F; **I:** Compound G; **J:** Compound H; **K:** Compound I; **L:** Compound J; **M:** Compound K.

Quantities are expressed in ng/10^7 bacterial CFU. Error bars present standard deviation. n=3.

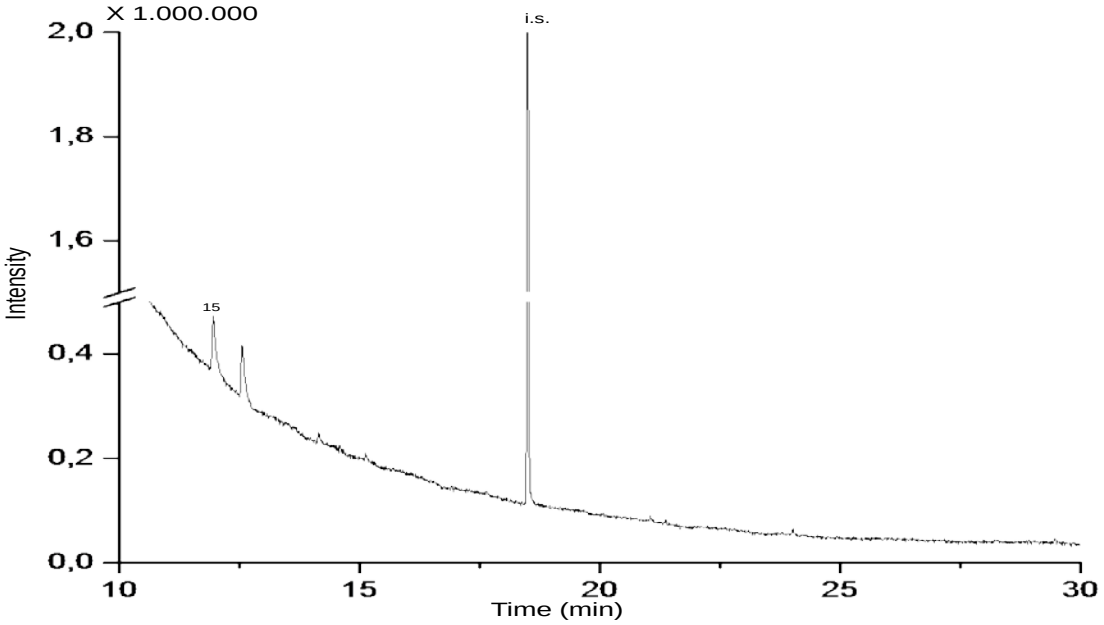

Figure 7G: VOC emission of *Sclerotinia sclerotiorum*at 20 °C.

Collection time for VOC analysis: 24 h (day 1, 20 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **15** (2-ethyl-hexanal), i.s**.** (Internal standard). n=3.

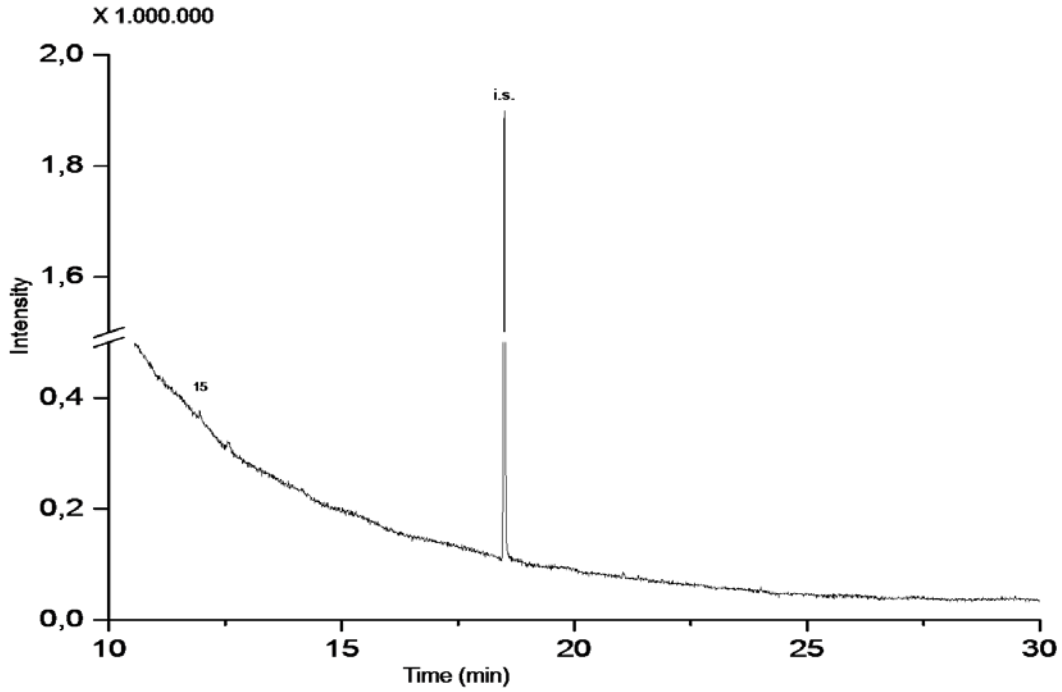

Figure 7H: VOC emission of *Sclerotinia sclerotiorum* at20 °C.

Collection time for VOC analysis: 24-48 h (day 2, 20 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **15** (2-ethyl-hexanal), i.s**.** (Internal standard). n=3.

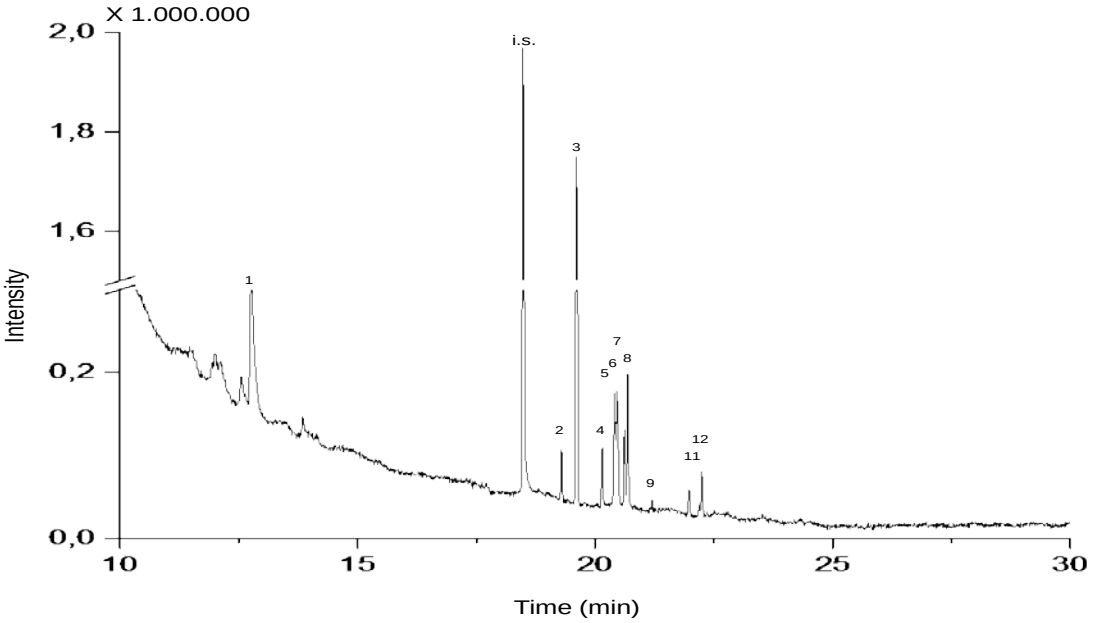

Figure 7I: VOC emission of *Serratia plymuthica* 4Rx13 and *Sclerotinia sclerotiorum* at 20 °C.

Collection time for VOC analysis: 0-24 h (day 1, 20 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **1** (Dimethyl trisulfide), **i.s.** (Internal standard), **2** (compound A), **3** (sodorifen), **4** (compound B), **5** (compound C), **6** (compound D), **7** (compound E), **8** (compound F), **9** (compound G), **11** (compound I) and **12** (compound J). n=3.

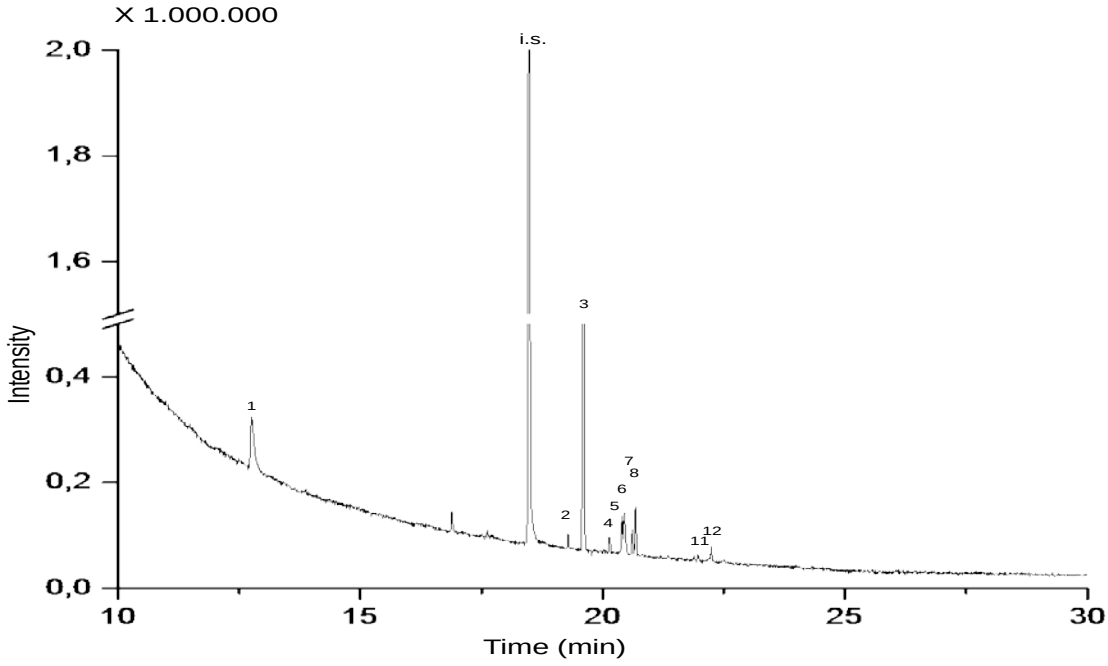

Figure 7J: VOC emission of *Serratia plymuthica* 4Rx13 and *Sclerotinia sclerotiorum*at 20 °C.

Collection time for VOC analysis: 24-48 h (day 2, 20 °C). VOCs were eluted from the collection column with 300 µl methylenechloride. 1 µL was applied to the GC-MS. Main peaks: **1** (Dimethyl trisulfide), **i.s.** (Internal standard), **2** (compound A),**3** (sodorifen), **4** (compound B), **5** (compound C), **6** (compound D), **7** (compound E), **8** (compound F), **11** (compound I) and **12** (compound J). n=3.

Table S1: VOC in different systems at 30°C and 20°C. represents the volatiles at 30°C and represents the volatiles at 20°C. + stands for presence and – stands for absence of a compound. n=3.

No	Compound (PubChem CID)	VOC system	Present in experiments	Retension index (RI)	d 1	d 2	d 1	d 2	d 3
13.	Compound K	*Serratia plymuthica* monoculture and in dual culture	2 times	1089	+	+	+	+	-
15.	2-ethyl-Hexanal (31241)	*Sclerotinia sclerotiorum*	2 times	958			+	+	+
16.	1-Tridecanol (8207)	Dual culture	1 time	2105	–	–	–	–	+

Table S2: Amount of main fungal compounds.

Compound name	ng/0.1 g mycelia
6 (Labda-8 (20), 12, 14-triene)	day 3=159
2-ethyl-Hexanal (31241)	day 1=3574, day 2=1017, day 3=252

Chapter 5: pH values of the fungal medium in different conditions

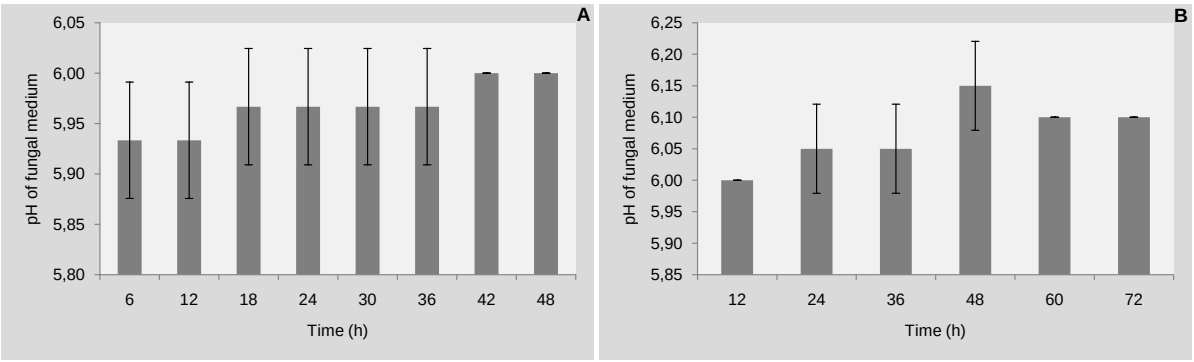

Figure S8A: pH measurement in empty system.

A: pH measurementat 30 °C ; **B:** pH measurementat 20 °C.

Fungal side had an acidic pH.n=3. Error bars present the standard deviation.

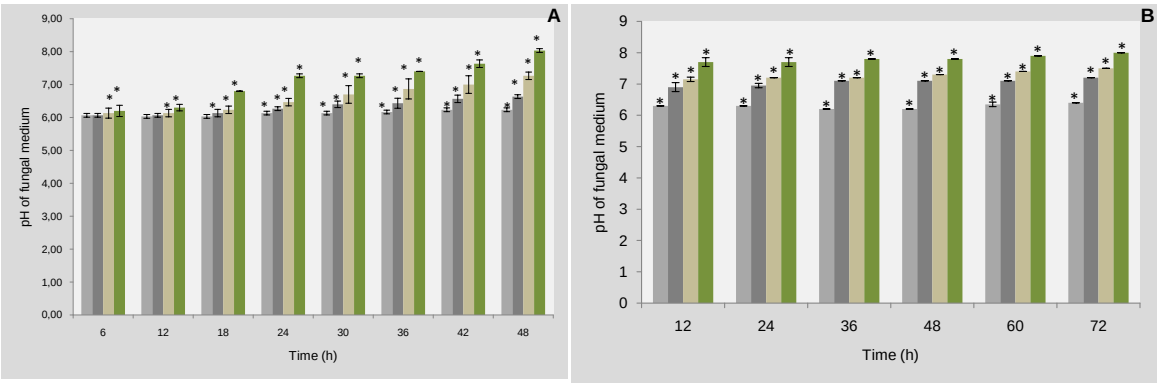

Figure S8B: pH measurement of fungal medium in presence of different initial CFUs.

A: pH measurementat 30 °C ; **B:** pH measurementat 20 °C.

$3*10^7$ CFU_i; $12*10^7$ CFU_i; $27*10^7$ CFU_i; $54*10^7$ CFU_i.

The results showed that at 30 °C the fungal media was acidic at the beginning but after 24 hours became basic. At 20 °C, the fungal media was basic (except $3*10^7$ CFU_i). n=3. (*) presents statistical significance related to the corresponding control.

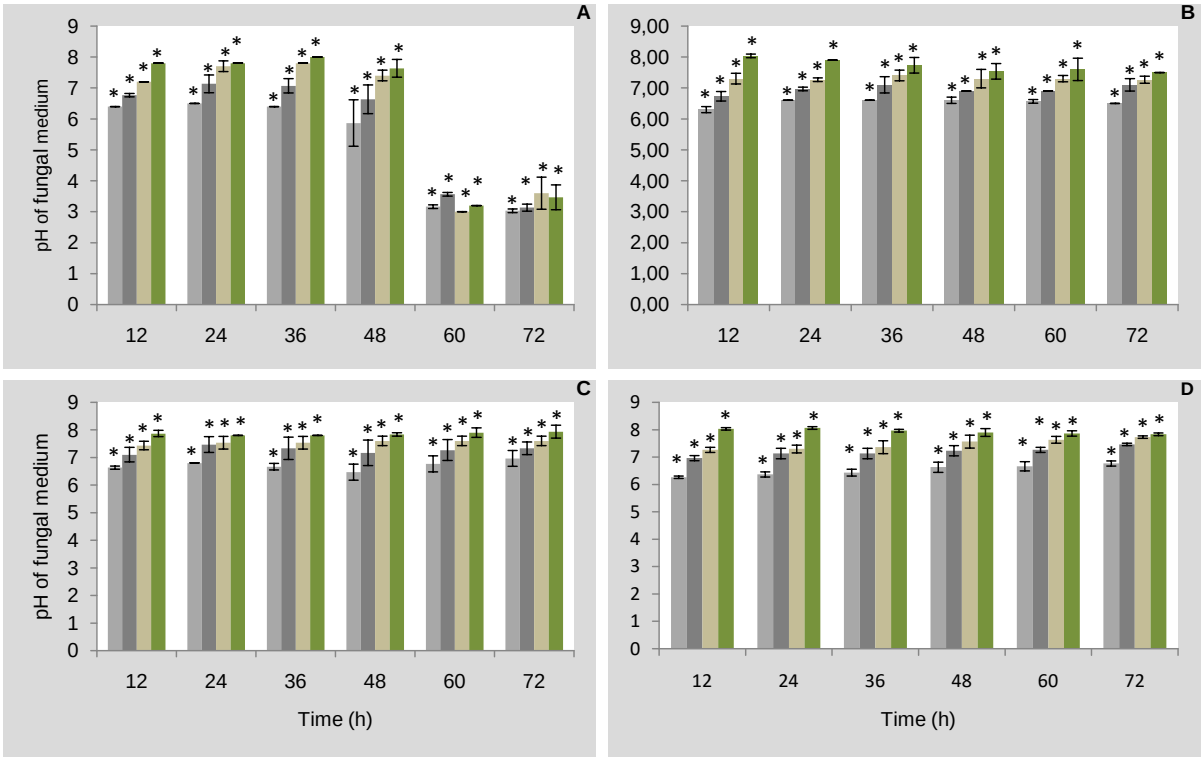

Figure S8C: pH measurement of fungal medium in presence of different initial CFUs and also fungi at 20 °C.

A: *Sclerotinia sclerotiorum*; **B:** *Rhizoctonia solani*; **C:** *Phoma eupyrena*; **D:** *Neurospora crassa*.

$3*10^7$ CFU_i; $12*10^7$ CFU_i; $27*10^7$ CFU_i; $54*10^7$ CFU_i.

The fungal media was basic (except $3*10^7$ CFU_i). *Sclerotinia sclerotiorum* had acidic pH after 2.5 days where other fungi showed a basic pH.
n=3. (*) presents statistical significance related to the corresponding control.

Chapter 6: Influence of synthetic ammonia on radial growth and biomass of the fungus *Sclerotinia sclerotiorum*

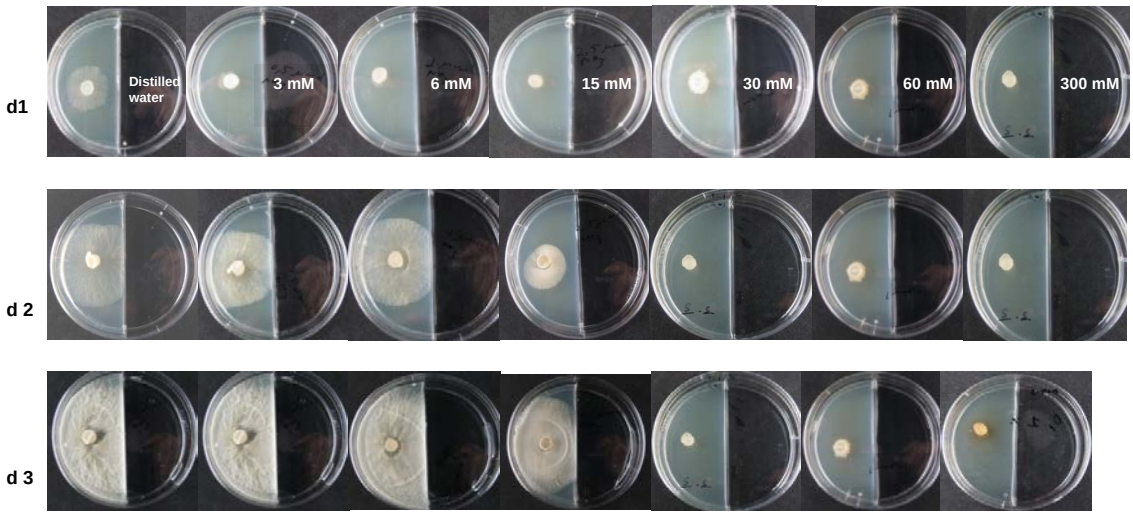

Figure S8D: Influence of 3-300 mM synthetic ammonia on radial growth of *Sclerotinia sclerotiorum* between day 1 and day 3 at 20 °C.

Chapter 7: Influence of ortho-phosphoric acid on the inhibitory effect of ammonia

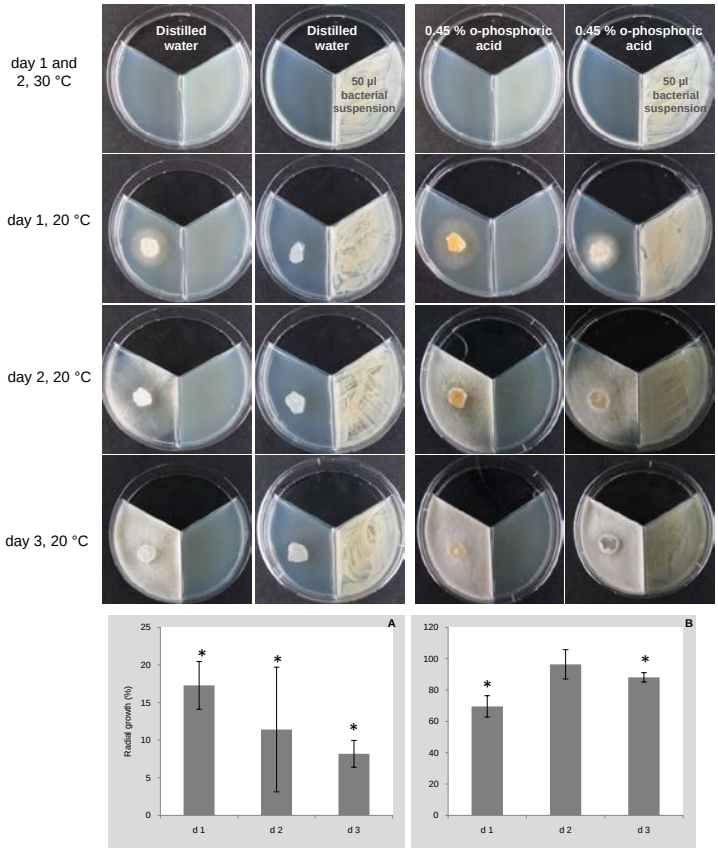

Figure S9A: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of *Sclerotinia sclerotiorum* in presence/absence of o-phosphoric acid.

A. absence of o-phosphoric acid.**B**. presence of o-phosphoric acid.

In absence of o-phosphoric acid, the fungal growth was less (**A**) where in presence of o-phosphoric acid fungus could grow better (**B**). The growth was expressed in pixel (software ImageJ). The control was set 100 % corresponding to 751743 (d 1), 1815519 (d 2), 1836048 (d 3) (**A**) and 687715 (d 1), 1826004 (d 2), 1820847 (d 3) (**B**).
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

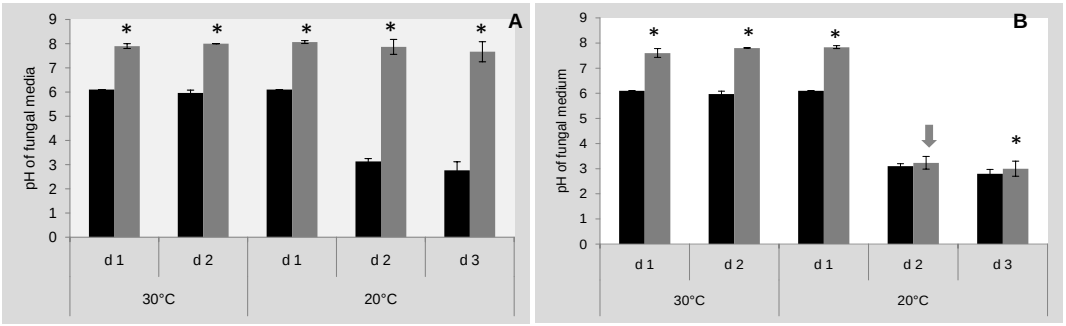

Figure S9B: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the pH value of the fungal media of *Sclerotinia sclerotiorum* in presence/absence of o-phosphoric acid.

represents control and represents bacterial suspension.

In presence of bacterial suspension (**A**) fungal media had a basic pH always where in presence of bacterial suspension and o-phosphoric acid (**B**) the fungal media showed an acidic pH after day 2 onwards.
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control.

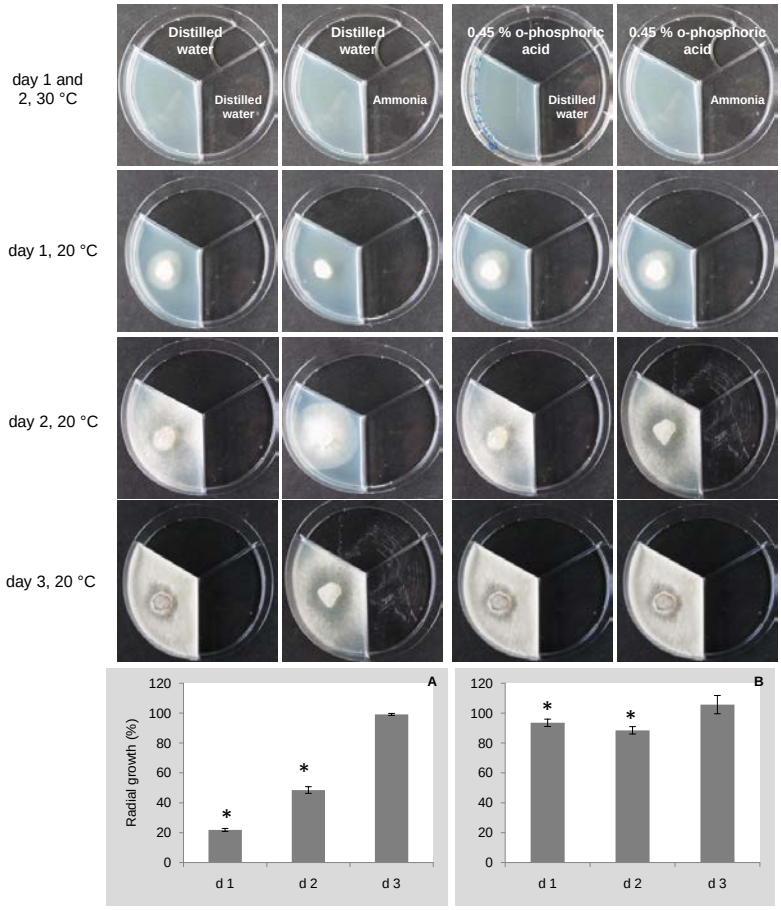

Figure S9C: Influence of synthetic ammonia on the radial growth of *Sclerotinia sclerotiorum* in presence/absence of o-phosphoric acid.

A. absence of o-phosphoric acid.**B**. presence of o-phosphoric acid.

In the first case (A), the fungal growth was less where in the second case (B) fungus could grow better. The growth was expressed in pixel (software ImageJ). The control was set 100 % corresponding to 751743 (d 1), 1815519 (d 2), 1836048 (d 3) (**A**) and 687715 (d 1), 1826004 (d 2), 1820847 (d 3) (**B**).
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control (100 %)

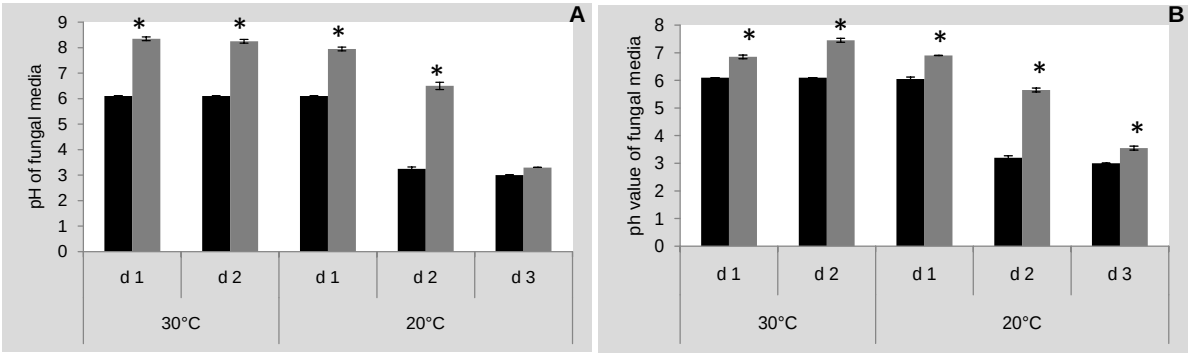

Figure S9D: Influence of synthetic ammonia on the pH value of the fungal media of *Sclerotinia sclerotiorum* in presence/absence of o-phosphoric acid.

represents control and represents bacterial suspension.

In presence of ammonia (**A**) fungal media showed a basic pH at the beginning that was decreased to 3.3 after day 3, where in the second case (**B**), the fungal media in presence of o-phosphoric acid had a reduction in the pH value to neutral or acidic pH.
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control.

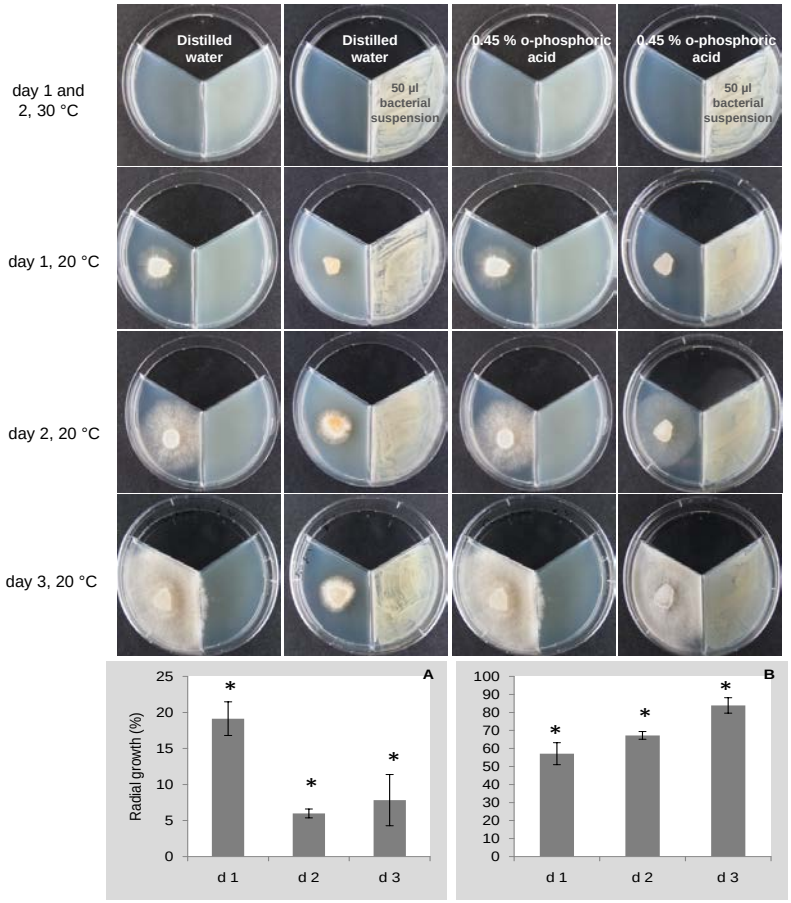

Figure S9E: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the radial growth of *Rhizoctonia solani* in presence/absence of o-phosphoric acid.

A. absence of o-phosphoric acid.**B**. presence of o-phosphoric acid.

In absence of o-phosphoric acid the fungal growth was almost inhibited (**A**) where in presence of o-phosphoric acid fungus showed better growth (**B**). The growth was expressed in pixel (software ImageJ). The control was set 100 % corresponding to 446542 (d 1), 1226984 (d 2), 1817040 (d 3) (**A**) and 425349(d 1), 1631367 (d 2), 1819328 (d 3) (**B**). n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

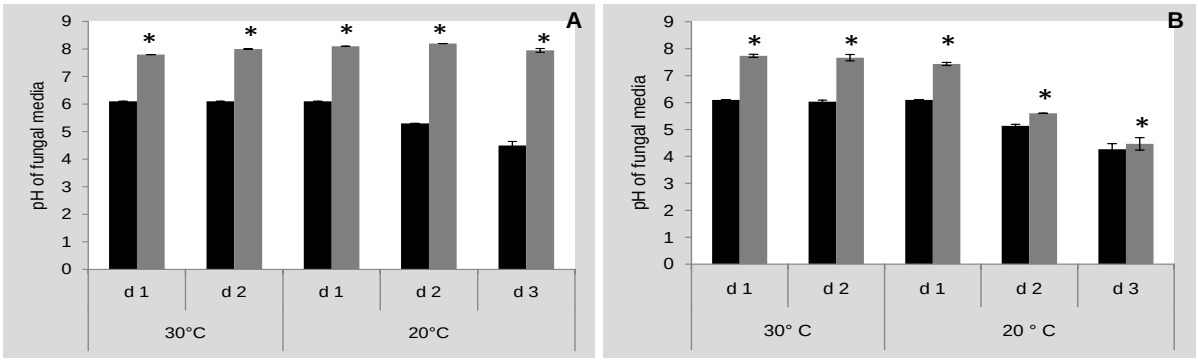

Figure S9F: Influence of volatiles of the bacterium *Serratia plymuthica* 4Rx13 on the pH value of the fungal media of *Rhizoctonia solani* in presence/absence of o-phosphoric acid.

represents control and represents bacterial suspension.

In presence of bacterial suspension (**A**) fungal media had a basic pH always where in presence of bacterial suspension and o-phosphoric acid (**B**) the fungal media showed a reduction in pH to acidic after day 2.
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control.

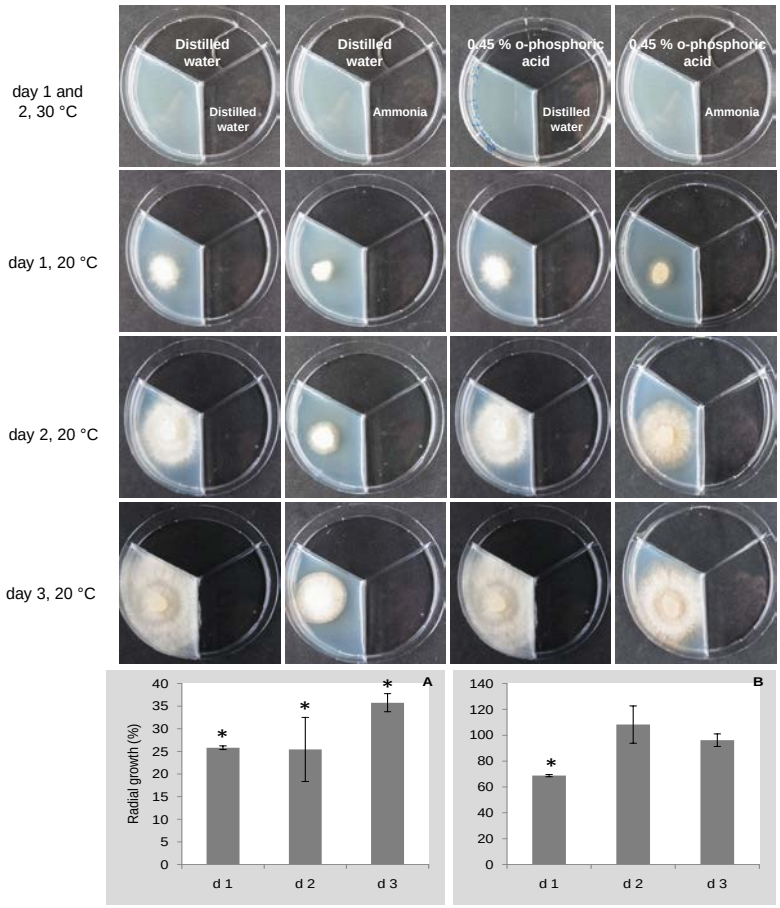

Figure S9G: Influence of synthetic ammonia on the radial growth of *Rhizoctonia solani* in presence/absence of o-phosphoric acid.

A. absence of o-phosphoric acid.**B**. presence of o-phosphoric acid.

In absence of o-phosphoric acid, (65-75) % fungal growth was inhibited (**A**) where in presence of o-phosphoric acid fungus showed almost no growth inhibition (**B**). The growth was expressed in pixel (software ImageJ). The control was set 100 % corresponding to452749 (d 1), 1072897 (d 2), 1764773 (d 3) (**A**) and 47025 (d 1), 114788 (d 2), 188723 (d 3) (**B**).
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control (100 %).

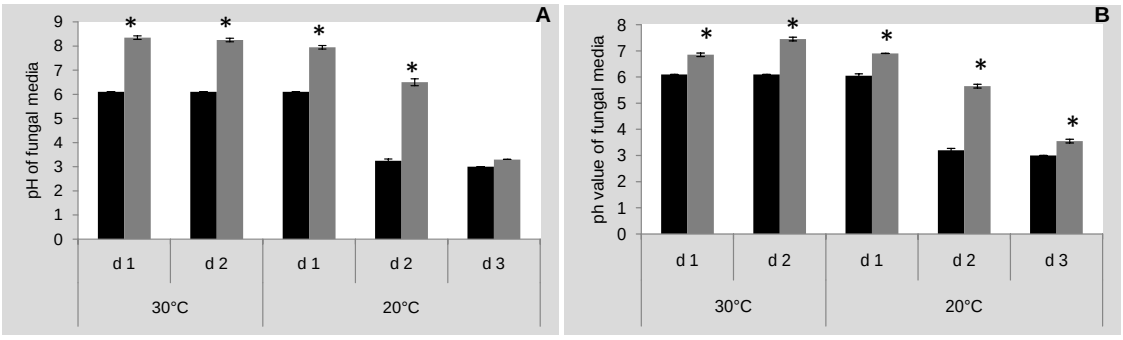

Figure S9H: Influence of synthetic ammonia on the pH value of the fungal media of *Rhizoctonia solani* in presence/absence of o-phosphoric acid.

represents control and represents bacterial suspension.

In presence of ammonia (**A**) fungal media showed a basic pH at the beginning that was decreased to 3.3 after day 3, where in the second case (**B**), the fungal media in presence of o-phosphoric acid and ammonia had a reduction in the pH value to 6.1.
n=3. Error bars present standard deviation. (*) represents statistical significance related to the corresponding control.

Curriculum vitae

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List of publications:

Das P., Effmert U., Piechulla B. (2016) Influence of Rhizobacterial volatiles on different properties of soilborne fungi (In prep.).

List of symposia contributions/participations:

1. **Das, P.**, Domik D., Lemfack C., Kai M., Magnus N., Effmert U., Piechulla B. (2015) Flüchtige mikrobielle Metabolite und deren biologische Funktion. ``$F^3$– Forschung trifft Forschung`` -Forschungscamp, Rostock, Germany (Poster presentation).

2. **Das P.**, Effmert U., Piechulla B. (2013) Influence of rhizobacterial volatiles on soilborne fungi. Conference on ,``Microbial interactions in complex eco-systems´´ in Turin, Italy (Poster presentation).

Declaration

I hereby declare that the work presented in this thesis entitled, "**Influence of volatile metabolites of the rhizobacterium *Serratia plymuthica* 4Rx13 on soilborne fungi**"is entirely original and was carried out by me independently at Department of Biochemistry, Institute for Biological Sciences, University of Rostock under the supervision of Prof. Dr. Birgit Piechulla. I further declare that this thesis has not formed the basis for the award of any degree or diploma, fellowship or similar title of any University or Institution.

Rostock, 22. August 2015

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Piyali Das

Selbstständichkeitserklärung

Ich gebe folgende Erklärung ab:

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Piyali Das