Early responses of *Medicago truncatula* roots after contact with the symbiont *Rhizophagus irregularis* or the pathogen *Aphanomyces euteiches*

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Abbreviations

ABA	Abscisic acid
AC	Accession number
ACC	1-Aminocyclopropane-1-carboxylic acid
AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhizal fungi
AVR	Avirulence protein
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
ERF	Ethylen Responsive Factors
eV	Electronvolt
CO	Chitosaccharide
CSSP	Common Symbiosis Signaling Pathway
CMA	Corn meal agar
DCA	Hexadecanediol
DMAPP	Dimethylallyl diphosphate
DMI	Does not Make Infection
dpi	Days post infection
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetra acetic acids
EIC	Extracted ion count
ERF	Ethylen Responsive Factors
ESI	Electrospray ionization
ETI	Effector triggered immunity
FDA	Fluorescein diacetate
FL	Flavonoids
forw	Forward
FPP	Farnesyl diphosphate
GA	Gibberellic acid
GABA	γ-Aminobutyric acid
GBP	Glucan-binding protein
GC-MS	Gas chromatography-mass spectrometry
GFP	Green fluorescent protein
GH	Glycoside hydrolases
GlcNAc	N-acetylglucosamine
(G)GPP	(Geranyl)geranyl diphosphate
HPLC	High performance liquid chromatography

HR	Hypersensitive response
HT	Yeast extract - tryptone
IAA	Indole-3-acetic acid
IPP	Isopentenyl diphosphate
IVOC	Induced volatile organic compounds
JA	Jasmonic acid
kDa	Kilodalton
KPI	Kunitz-type proteinase inhibitor
LC-MS	Liquid chromatography-mass spectrometry
LCO	Lipochitooligosaccharides
LYK	LysM domain containing receptor like kinases
LYR	LYK related genes
LysM	Lysin motif
MAMP	Microbe-Associated Molecular Patterns
MeJA	Jasmonic acid methyl ester
MeOH	Methanol
MEP	Methylerythritol phosphate
MSR	Modified Strullu-Romand medium
MtGEA	Medicago truncatula Gene Expression Atlas
m/z	Mass per charge
Mya	Million years ago
NCBI	National Center for Biotechnology Information
NFP	Nod Factor Perception
NSP	Nodulation Signaling Pathway
OD	Optical density
OHFA	Hydroxyhexadecanoic acid
P	Phosphate
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PPA	Prepenetration apparatus
ppm	Parts per million
PR	Pathogen related
PTGS	post transcriptional gene silencing
PTI	PAMP-triggered immunity
QS	Quorum sensing

qRT-PCR	Quantitative Real-Time PCR
rDNA	Ribosomal DNA
rev	Reverse
RG	Reference gene
RIN	RNA Integrity Number
RLK	Receptor-like kinases
ROS	Reactive oxygen species
rpm	Rounds per minute
Rt	Retention time
RT	Room temperature
SA	Salicylic acid
SARDI	South Australian Research and Development Institute
SL	Strigolactones
SPE	Solid phase extraction
TG	Target gene
TF	Transcription factor
VOC	Volatile organic compounds
WGA	Wheat germ agglutinin
2,4-D	2,4-Dichlorophenoxyacetic acid

1 Introduction

In this thesis the interaction between *Medicago truncatula* (*M. truncatula*), a model plant for legumes with one symbiotic partner, the arbuscular mycorrhizal fungus (AMF) as well as the interaction with a pathogenic oomycete *Aphanomyces euteiches* (*A. euteiches*) was analyzed. A focus was thereby set on the very early reaction of the plant roots after recognition of the particular microorganism but yet before the penetration of those organisms into the root tissue.

1.1 *Medicago truncatula*

M. truncatula belongs to the family of Fabaceae and represents the third largest family of higher plants including more than 20,000 species and 700 genera. [Doyle and Luckow, 2003]. Fabaceae offer unique research opportunities in the area of plant–microbe interactions including the symbiotic nitrogen fixation, mycorrhizal interactions and legume–pathogen interactions. In fact, the intriguing parallels and likely differences between these legume–microbe interactions put legumes in a focus of research on plant– microbe interactions generally [Cook, 1999]. The genus *Medicago* originates from the Fertile Crescent and its species have conquered the Mediterranean countries and its neighboring steppes. During XIXth century, they invaded other parts of the world, mainly America and Australia [Delalande et al., 2007]. *M. truncatula* is a close relative of *Medicago sativa*, a widely cultivated crop with limited genomic tools and complex autotetraploid genome. The *M. truncatula* genome sequence provides significant opportunities to expand the alfalfa's genomic toolbox [Young et al., 2011]. Key attributes of *M. truncatula* include its small, diploid genome (2 x 8 chromosomes, with about 500 Mbp); its self-fertile nature and its rapid generation time. The genome of *M. truncatula* was analyzed, capturing 94 % of all *M. truncatula* genes [Young et al., 2011] yet functional assignment stays unknown for most of the genes. Databases like the "J. Craig Venter Institute - Medicago Truncatula Genome Project v 4.0" (http://jcvi.org/medicago/), the Medicago truncatula Gene Expression Atlas (MtGEA - http://mtgea.noble.org/v3/) and the NCBI database (http://www.ncbi.nlm.nih.gov/guide/) help to attain available data. Numerous ecotypes of *M. truncatula* have been collected throughout the Mediterranean Basin, and the considerable phenotypic variation for features such as growth habit, flowering time, symbiotic specificity, and disease resistance represent an important resource to examine the genetic basis of legume functions [Cook, 1999]. Previously, most studies on *M. truncatula* have focused on single references or a limited number of populations. The ecotype collection used in the present work is based on a survey about the genetic diversity in a collection of 346 inbred lines spanning the bulk of diversity that has been collected throughout the species range to date. 13 microsatellite markers were used to assign genetic relationships and select a nested core collection of 32 inbred lines, representing the genetic diversity of the complete collection [Ronfort et al., 2006].

1.2 Mycorrhiza: a symbiosis between plants and fungi

The definition of symbiosis (greek: „living together“) is depicted controversial in literature. In some cases symbiosis is only referred to persistent mutualisms, while others include any type of persistent biological interactions (i.e. mutualistic, commensalistic, or parasitic) [Douglas, 2010]. Mutualism is the living together of two or more different organisms in an association that is mutually advantageous [Raven et al., 2006]. One example of symbiosis in the narrow sense of mutualism is the mycorrhiza. Mycorrhiza (greek: myces = fungus; rhiza = root) is a symbiotic interaction between fungi and roots of higher plants [Frank and Trappe, 2005]. Mycorrhiza can be divided according to their anatomical aspects in two broad categories, the ectomycorrhiza, colonizing the root intercellular spaces, and the endomycorrhiza, that penetrate and colonize the root cortex cells itself [Bonfante and Genre, 2010]. Endomycorrhiza can be further classified into orchid, ericoid, arbutoid, monotropoid and arbuscular mycorrhizas (AM) [Cardon, 2007]. AM is the most abundant class of endomycorrhiza [Smith and Read, 2008].

1.2.1 The Arbuscular Mycorrhiza

AM represent a unique interaction between two eukaryotes, an obligate biotrophic fungus and its host plant, leading to an improved fitness of the interacting partners [Bonfante and Genre, 2008]. The host plant supplies the fungus with photoassimilates (hexose) which is either metabolized to glycogen or lipids as energy storage [Varma and Hock, 1995]. The beneficial effect for the plant in return is more diverse and will be discussed in detail in section 1.2.3. Due to the ubiquitous occurrence and its widespread interaction with plants and the rhizosphere, AMF play an enormous role in terrestrial ecosystems. Nonetheless their biodiversity and all functional aspects are not yet understood in detail. Within the kingdom of fungi, AMF are classified to a separate phylum called *Glomeramycota*. This phylum comprises four orders: *Diversisporales*, *Glomerales*, *Archaeosporales* and *Paraglomerales*. The Glomerales comprise two families: *Claroideoglomeracea* and *Glomeraceae*. The *Glomeraceae* include the newly classified genus of *Rhizophagus*, that comprises the species used in this project: *Rhizophagus irregularis* (DAOM197198) (Fig.1) [Krüger et al., 2012].

Domain:	Eucaryotes
Kingdom:	Fungi
Phylum:	*Glomeromycota*
Class:	*Glomeromycetes* (AM)
Order:	*Glomerales*
Family:	*Glomeraceae*
Genus:	*Rhizophagus*
Species:	*Rhizophagus irregularis*

Figure 1: Phylogenetic classification of *R. irregularis*

Two major morphological patterns can be found in AMF, differentiating the *Paris* and *Arum* type. The Arum type penetrates the root cortex and fungal hyphae grow inter- and intracellularly to differentiate to arbuscules inside the cells. The Paris type develops intracellular hyphal coils while arbuscules are few in numbers, or completely absent [Smith and Smith, 1997].
The earliest fossils of spores and hyphae, which resemble spores of modern glomalean fungi, were found in the Middle Ordivician (460-455 Mya) [Redecker et al., 2000]. The oldest accepted fossils of land plants originate from the same era [Wellman and Gray, 2000]. These findings lead to the prominent hypothesis that plants coevolved with their fungal partners since they invaded the land. The association between plant roots and AMF has proven to be an evolutionary successful strategy since more than 80 % of all terrestrial plant species live in symbiosis with AMF [Schüssler, 2004].

1.2.2 Structures and life cycle of AMF

AMF can form different structures depending on the life cycle they are in. Each structure has a different function in the completion of the fungals life cycle.
Spores (greek: spore = germ) are produced by extraradical hyphae and are the major reproductive organs of AMF. Waiting for a host plant, they can survive several years in the soil even under unfavorable conditions. Spores are filled with cytoplasm and storage lipids which are used as energy source for presymbiotic growth. They are multinucleate containing up to several hundred nuclei per spore, hence single spores exhibit high genetic variation for ribosomal DNA as well as for protein-coding genes [Hijri and Sanders, 2005].
Hyphae The first morphological structure appearing from germinating spores are germ tubes, an early form of hyphae evolving the mycelium. Hyphae mediate the first contact between fungus and roots by developing hyphopodia (hyphal organs, forming an infection peg to enter the host). Inter- and intracellular hyphae in roots transport substances absorbed by extraradical hyphae from soil to arbuscules or directly to root cells of the host plant. The thin (2-10 µm) and highly branched structures allow to forage the adjacent soil for nutrients. Hyphal strands are able to penetrate several meters into the soil and to connect different plants [Varma and Hock, 1995]. The hyphal network of AMF is usually aseptate and coenocytic (multiple nuclei within the same cell), with hundreds of nuclei sharing the same cytoplasm [Parniske, 2008].
Vesicles are hyphal swellings containing cytoplasm and storage lipids that are formed intra- or intercellularly. Formation of vesicles is typical for the order of *Glomerales* [Neumann, 2007].
Arbuscules After penetration into the root cortex, highly branched haustoria, so called arbuscules can be formed within the cells. The typical treelike shape of arbuscules enable the fungi to occupy a large volume of the host cell. Arbuscules have a large surface area and form the major site of exchange between AMF and the host plant [Neumann, 2007]. AMF are considered to be ancient asexual organsims. Despite the absence of sexual reproduction, the process of anastomosis (hyphal fusion with a cytoplasmic connection), facilitates exchange and recombination of genetic material between hyphae [Parniske, 2008].

Life cycle AMF have three growth phases: asymbiotic, pre-symbiotic and symbiotic. During the asymbiotic growth stage, AMF spores can germinate several times in the absence of plants in order to explore their surrounding, but only short hyphae will develop and retract again as long as there are no plant exudates present. In the presence of plant-derived signals, the hyphal growth changes dramatically. In the beginning of their life cycle, during spore germination,

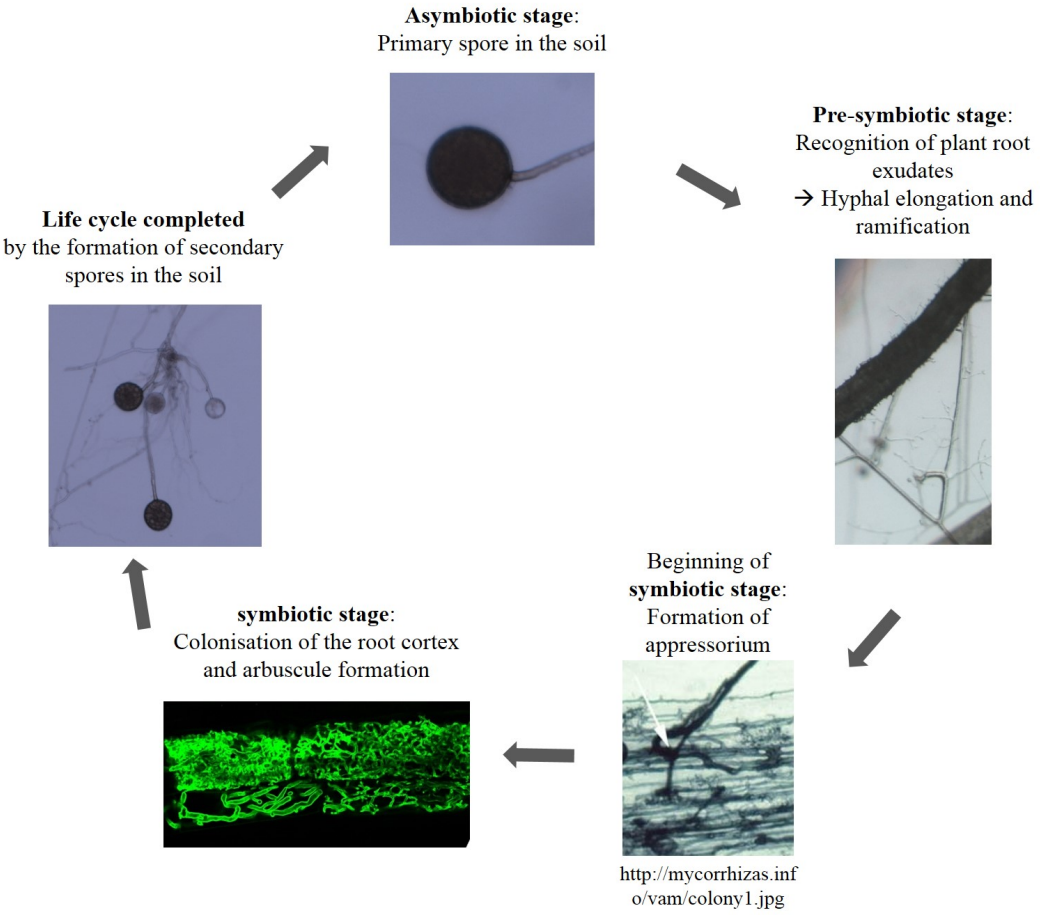

Figure 2: Life cycle of AMF

AMF grow under the consumption of stored carbohydrates in the spore. The AM development begins with a presymbiotic phase in presence of a host root. Reaching the plant root, hyphopodia are formed on epidermal cells. On behalf of the plant, this fungal chemical and mechanical stimulation induces the formation of a prepenetration apparatus (PPA), that facilitates the entrance of fungal hyphae and guides the fungus through root cells towards the cortex, leading to the establishment of the symbiotic phase. Reaching the cortex, the fungus enters the apoplast, where it branches and grows laterally along the root axis. PPA-like structures are induced by the fungal presence in inner cortical cells, where hyphae branch to form arbuscules. Carbohydrates received from the host are stored in vesicles, located in the apoplast. At this stage, the fungal life cycle can be completed by formation of secondary spores [Parniske, 2008].

1.2.3 Plant benefits from AMF

Phosphate (P) belongs to the most limiting nutrients for plant growth. In most soils, a large proportion of P exists in a plant unavailable form. P has a poor solubility and a very slow diffusion in the soil. Roots affect the P concentration of the soil solution by active phosphate uptake, hence creating a P depletion zone around the root [Kraus et al., 1987]. The extraradical fungal mycelium widely expands the P- depletion zone and due to the minor hyphal diameter, smaller soil pores can be exploited by a larger absorbing surface. Hence the symbiosis with AMF displays a powerful tool for plants to increase P availability. AMF have also shown to contribute to the acquisition of nitrogen (N) and micronutrients like zinc (Zn) and copper (Cu) [Govindarajulu et al., 2005].

AM fungal hyphae excrete glomalin, a glycoprotein with a high persistence in soil, that increases soil aggregate stability and thus improves water infiltration, aeration and resistance to erosion [Wright and Upadhyaya, 1998]. The consequently increasing water holding capacity leads to an improved water supply of plants. Furthermore plants colonized by AMF have a higher resistance to soil borne pathogenic fungi [Neumann, 2007]. Among the main mechanisms for disease suppression are competition for colonization sites, induction of plant defense responses (priming) and an improved nutritional status. Direct inhibitory effects of mycorrhiza have also been reported [Marschner, 2012].

AMF have shown to reduce the toxicity of harmful elements. Even if AMF increase the uptake of some heavy metals that are essential for plant growth (Cu, Zn), they protect their host plant from uptake of toxic amounts of those metals into the cytosol [Joner and Leyval, 2008].

1.3 *Aphanomyces euteiches* - an oomycete pathogen of legumes

A. euteiches belongs to the kingdom of Chromalveolata and the class of Oomycota. Oomycete organisms resemble fungi through morphological and physiological traits, but are phylogenetically not related to Mycota but to the Heterokont/ Stramenopile kingdom [Gaulin et al., 2007]. The most basal lineages of Oomycota are marine parasites, proposing its marine origin [Thines and Kamoun, 2010]. The genus *Aphanomyces* is further classified to the order Saprolegionales and the family Leptolegniaceae (see Fig.3). *A. euteiches* was found to be pathogenic to several weed species and legumes (e.g. *Pisum sativum, Medicago sativa, Phaseolus vulgaris, Vicia faba, Trifolium pratense, Trifolium repens, M. truncatula, Lens culinaris*). It causes seedling damping off and root rot diseases and is the most devastating disease in *Pisum sativum* in several countries. While a lot of research was devoted to the oomycete *Phytophtora* in the last decade, *A. euteiches* received little attention, although being an interesting parasite to study plant-oomycete interactions as it is pathogenic on the model legume *M. truncatula*. [Gaulin et al., 2007].

A

Domain	Eucaryotes
Kingdom	Chromalveolata
Phylum	Heterokontophyta
Class	Oomycota
Order	Saprolegniales
Family	Leptolegniaceae
Genus	*Aphanomyces*
Species	*A. euteiches*

B

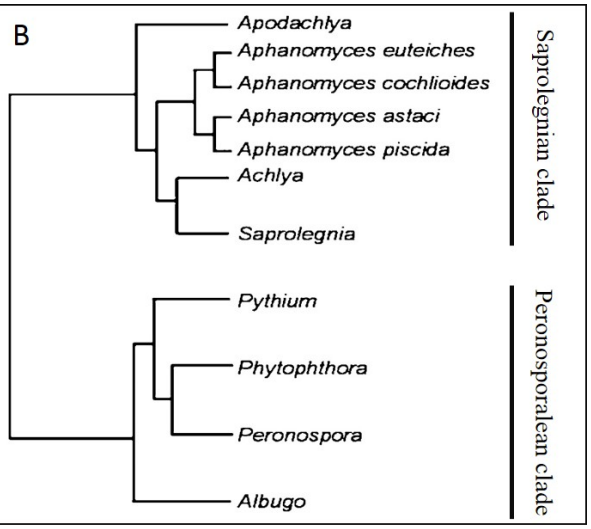

Figure 3: **A**: Phylogenetic classification of *A.euteiches*; **B**: Phylogenetic tree illustrating relationships within Oomycota [Gaulin et al., 2007]

One specific characteristic of the *Phytophtora* genus, is the presence of cellulose and the absence of chitin in the cell wall, which provided a good discriminating taxonomic feature towards the Mycota. Yet it was recently demonstrated that chitin could be detected in the cell wall of some oomycete species. According to this feature, oomycetes have been divided into two groups. The Leptomitales, containing both cellulose and chitin and the later evolved Peronosporales having only cellulose. It has been shown that the major cell wall components of *A. euteiches*, belonging to the Saprolegionales (which share common ancestry with the Leptomitales), are non-cristalline chitosaccharides. Those chitosaccharides comprise either 1,6-linked or 1,4-linked N-acetylglucosamine (GlcNAc) residues. GlcNAcs might be recognized by the plants via specific receptors and thus elicit a Pathogen-Assosiated Molecular Pattern (PAMP) reaction, that will be discussed in more detail later. GlcNAcs are sources of oligoglucosides

or chitooligosaccharides (COs). COs, inducing a very strong PAMP reaction, are perceived by plants via receptor-like proteins, containing extracellular lysin motif (LysM) domains, which have been shown to mediate the binding of GlcNAc-containing ligands [Nars et al., 2013].

The life cycle of *A. euteiches* includes asexual and sexual stages (see Fig. 4). Infection starts by oospore germination adjacent to a host root. Oospores form a germ tube and a terminal zoosporangium, that may release over 300 primary zoospores. The biflagellate motile zoospores adhere to the host tissue. After root penetration, coenocytic hyphae develop mainly extracellularly. After a few days, haploid antheridia (male organ) and oogonia (female organ) are formed. With the help of fertilization tubes, antheridia penetrate oogonia and deliver nuclei, resulting in the formation of diploid oospores [Scott, 1961]. The formation of oospores is the completion of their life cycle. The oospores are 20-35 μM in diameter and have a thick protective wall and energy reserves allowing them to survive for many years in the soil. Upon germination, hundreds of zoospores can be released again via a germ sporangium (short mycelial strand) [Papavizas and Ayers, 1974]. The germination of oospores is probably stimulated by chemical signals exuded by the host roots [Shang et al., 2000]. Primary disease symptoms in infected roots are water-soaked, softened brown lesions followed by root rotting resulting in significant reductions of root mass. Secondary symptoms like chlorosis, necrosis and wilting of the foliage might follow [Hughes et al., 2014].

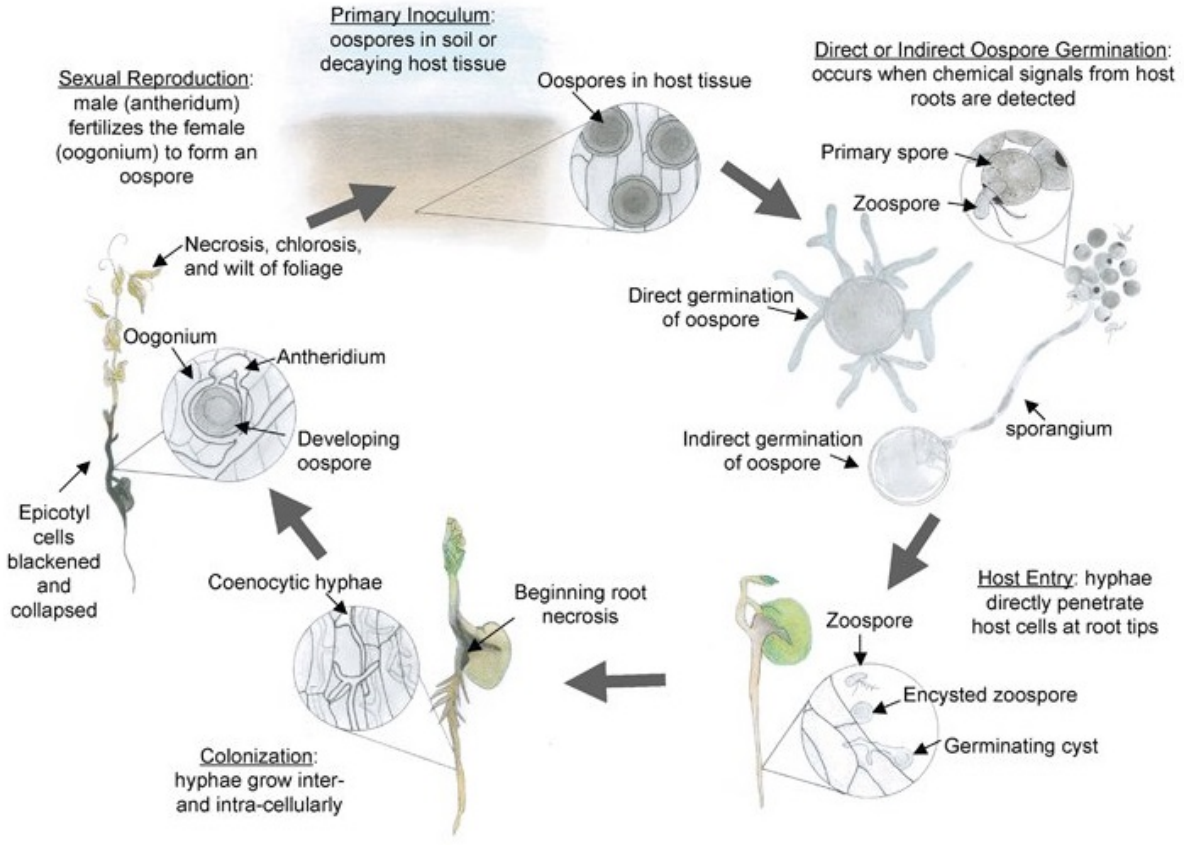

Figure 4: Life cycle of *Aphanomyces euteiches* [Hughes et al., 2014]

1.4 Communication between plant and rhizosphere microorganisms - friend or foe

Plants are sessile organisms and can not evade attacks of different organisms by rapid relocation, nor do they posses such complex immune system against bacteria, fungi, viruses or parasites as animals have. Instead plants highly invest in the synthesis of a huge diversity of secondary metabolites with a wide range of toxicological properties [Wink, 2013]. Via the exudation of this wide range of compounds, roots may regulate the soil microbial community in their close vicinity to cope with herbivores, encourage beneficial symbioses, change the chemical and physical properties of the soil, and inhibit the growth of competing plant species and communicate with other species [Steeghs et al., 2004]. Some compounds may attract beneficial organisms, while other compounds serve as defense tools against pathogenic organisms [Theis and Lerdau, 2003]. Those so called secondary metabolites are not necessary for growth, survival or reproduction but are beneficial to plants, by mediating the interaction with environmental influences and are thus essential for successful competition and reproduction [Ratzinger, 2008].
The plants innate immune system employs mechanism of specific signaling, hormonal changes and transcriptional reprogramming [Parker and Ellis, 2010]. This immune reaction towards pathogens implies costs for plants. In turn, beneficial organisms help plants to improve their nutritional status, protect from diseases and ameliorate general plant health. Consequently it is of crucial importance for plants and their energy balance to recognize the identity of present organisms as early as possible and to react accordingly [Gilbert, 2001].
The evolution of signaling between mutually beneficial organisms is a process driven by both partners, but due to the abuse of this sophisticated chemical language by pathogenic organisms, significant overlaps in communication with different organisms exist [Hirsch et al., 2003]. One example is the signaling of strigolactones towards the symbiotic organism of AMF that is abused by parasitic weeds like *Striga* [Bouwmeester et al., 2007]. Another example was proposed concerning the role of RAM2 (**R**equired for **A**rbuscular **M**ycorrhization), a gene that is involved in the production of cutin monomers. It was shown that *ram2* plants are not only unable to be colonized by AMF, but also showed defects in the colonization by an oomycete pathogen (lacking appressoria formation), thus indicating a signaling function for the AMF- plant interaction, that has been recruited by pathogens for their own host invasion [Wang et al., 2012].

1.4.1 The role of plant derived volatile organic compounds

More than 100,000 chemical products are produced by plants and at least 1,700 of these are known to be volatile. Volatile organic compounds (VOCs) are defined as any organic compound with a vapor pressures high enough to be vaporized into the atmosphere under normal conditions [Loreto and Schnitzler, 2010]. The importance of these compounds can be depicted by the fact that the estimated emission of VOCs by terrestrial plants accounts for 36% of all photosynthates [Kesselmeier et al., 2002].
VOCs are involved in the reaction towards a range of biotic influences like plant defense against

insects, pollinator attraction, plant-plant communication, plant-pathogen interactions, reactive oxygen species (ROS) removal and thermo-tolerance. However, VOCs are also emitted in response to abiotic stress perception and may play a role in the response towards stress such as physical damage, nutrient deficiency, salinity, drought and ozone exposure [Shulaev et al., 2008].

From foliar VOC synthesis it was described that some VOCs are stored in the tissue and may be volatilized into the atmosphere by healthy unwounded plants depending on their concentration and physiochemical properties [Niinemets et al., 2004]. In contrast, there are so called induced VOCs (IVOCs), that may be emitted hours or days after any kind of environmental stress, either locally or systemically [Pare et al., 2005]. The constitutive VOCs normally released from healthy plants may become inducible volatiles after foliar damage [Vuorinen et al., 2004]. Since IVOCs are produced only after biotic and abiotic stresses, carbon is only used when needed and plant fitness is not reduced generally (Dicke, 2000). A large variety of VOCs, emitted by plant shoots, has been extensively described and characterized, whereas VOCs from the root systems received less attention. Based on what is known about volatile-induced responses in aboveground plant parts, and the multitude of belowground organisms interacting with the roots in their natural environment, it may be expected that belowground volatile-induced responses are as common as aboveground induced responses [Harren and Cristescu, 2013].

Most VOCs reported are largely lipophilic products with molecular masses under 300 Da and can be mainly assigned to the following classes: terpenoids (based on terpenes), fatty acid derivatives, benzenoids and phenylpropanoids and various nitrogen and sulfur containing compounds [Dudareva et al., 2004].

Terpenes, as the largest class of plant secondary metabolites, have many volatile representatives: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), and some diterpenes (C20).

The formation of the C5 units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) is followed by two alternative pathways: the mevalonate pathway from acetyl-CoA and the methylerythritol phosphate (MEP) pathway from pyruvate and glyceraldehyde-3- phosphate. The MEP pathway, localized in the plastids, provides IPP and DMAPP predominantly for hemi-, mono- and diterpene biosynthesis, while the cytosol-localized mevalonate pathway provides C5 units mainly for sesquiterpene biosynthesis. In the second phase of terpene biosynthesis, IPP and DMAPP condense to form geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) as precursors of monoterpenes, sesquiterpenes, and diterpenes, respectively. In the third phase of terpene biosynthesis the conversion of the prenyl diphosphates, DMAPP (C5), GPP (C10), FPP (C15), and (GGPP) (C20), to hemi-, mono-, sesqui-, and diterpenes respectively, follows. These reactions, carried out by the large family of terpene synthases, produce the primary representatives of each skeletal type [Dudareva et al., 2004].

1.4.2 The role of root exudates on rhizosphere microorganisms

Root products are all substances produced by roots and released into the rhizosphere. Most root products are C compounds (rhizodeposition), but ions, O_2 and water are also included. Root exudates belong to one fraction of the rhizodeposition. While the lysates comprise all passively released deposits, exudates indicate the substances that are released from living root cells. In Fig. 5 an overview of rhizodeposits and a further classification of exudates is given. While the 1st category of exudates, the diffusates, comprise mainly low molecular weight organic compounds (such as sugars, amino acids, organic anions, phenolics) that can easily be assimilated by soil microorganisms, the 2nd category of exudates, the secretions, are the most interesting class concerning this study, since the secretions comprise substances with specific functions such as nutrient mobilization, detoxification, defense reactions, or signaling compounds released by controlled mechanisms [Marschner, 2012].

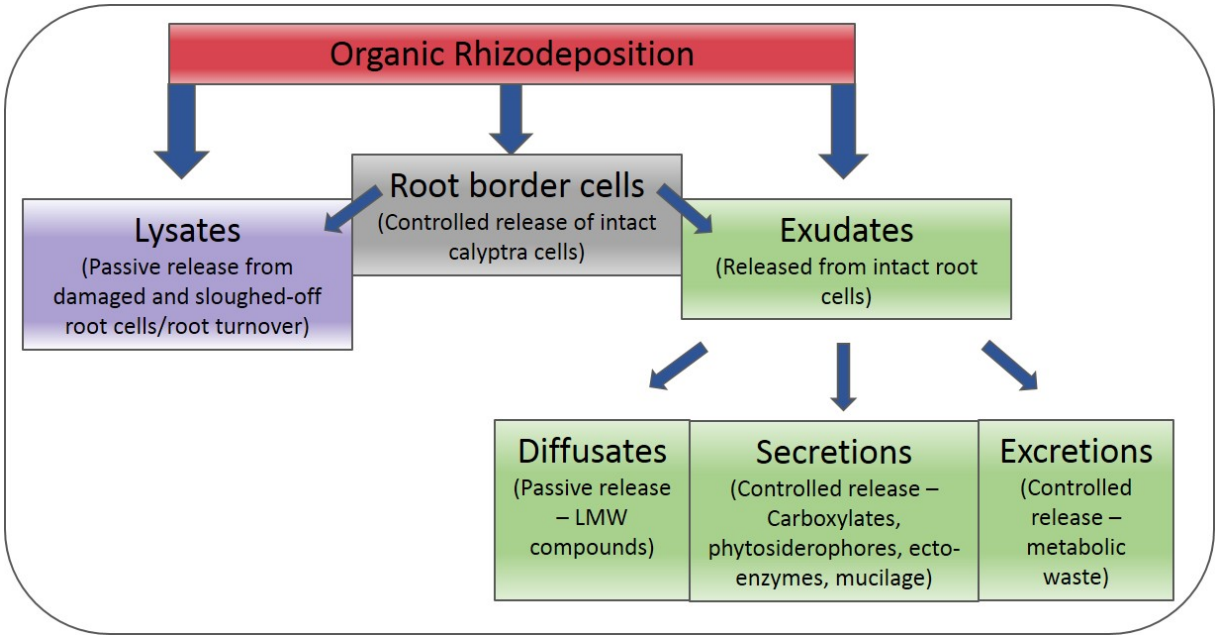

Figure 5: Classification of organic rhizodeposition. Adapted from Marschner (2012)

Growth and vitality of microorganisms in soil is largely dependent on the carbon availability. Due to the high stability of soil organic matter, the often easily available plant root exudates are a highly welcome source of energy for microorganisms [Nobili et al., 2001]. It has been described for the roots of *A. thaliana* that exudation of phytochemicals is an ATP-dependent active process involving different transporters [Loyola-Vargas et al., 2007]. The fact that exudates are actively released suggests that they play a major role in shaping the soil microbial community. Secondary metabolites of root exudates are not yet described in detail, and it seems that root exudates of different plant species differ in a dramatic way, as shown in the comparison of *A. thaliana* and *M. truncatula* root exudates [Broeckling et al., 2008].

1.4.3 Communication between plants and AMF

For AMF as obligate biotrophic organism, it is crucial to encounter a host before spore resources are depleted [Bonfante and Genre, 2010]. Therefore AMF release soluble signals to inform the plant partner about its presence. It is known that AMF spores secrete chitin tetramers and pentamers (CO4/5), induced by fungal SL perception and triggering Ca^{2+} spiking in root cells. They also exude sulphated and non-sulphated lipochitooligosaccharides (LCOs), that are very similar to Nod factors, the signaling molecules of rhizobia, and trigger lateral root formation [Maillet et al., 2011]. The plant on the other hand, seems to attract the fungus with different substances, penetrating the rhizosphere stepwise as depicted in Fig 6. When the fungal spores are still rather distant from the roots, secreted flavonoids (FL) stimulate hyphal elongation and pre-symbiotic fungal growth. While approaching the roots, 2-hydroxy fatty acids (2OH-FA) exuded from the roots, induce low degree of hyphal ramification off the primary hyphae, that is followed by stronger branching as soon as plant derived strigolactones (SL) are sensed, promising a close proximity to the plant root. The last step before root penetration, hyphopodium developement is induced by the perception of cutin monomers, 1,16-hexadecanediol (16:0-DCA) and 16-hydroxyhexadecanoic acid (16:0-OHFA) [Nadal and Paszkowski, 2013].

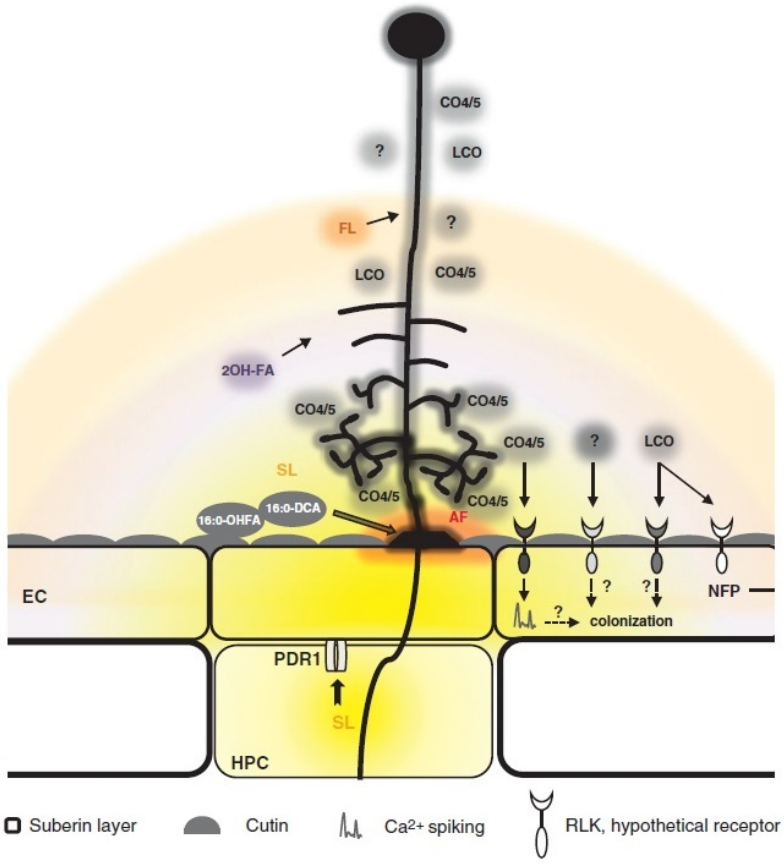

Figure 6: Schematic summary of pre-symbiotic communication of plant roots towards AMF. Flavonoids (FL), 2-hydroxy fatty acids (2OH-FA), strigolactones (SL), cutin monomers, 1,16-hexadecanediol (16:0-DCA)/ 16-hydroxyhexadecanoic acid (16:0-OHFA), chitin tetramers/ pentamers (CO4/5), lipochitooligosaccharide (LCO) [Nadal and Paszkowski, 2013].

The initial stage of the symbiosis, the signal perception of the plant, differs between fungal and bacterial symbionts (see review of Bonfante and Requena, 2011). While receptors of the Nod factor are known, receptors for the Myc factor are not yet identified. Rhizobia have a narrow host range, in contrast AMF are rather unspecific. One AMF species can infect several plant species and one plant species can be colonized by several AMFs [Smith and Read, 2008]. Perception of AMF signals induces the so called common symbiosis signaling pathway (CSSP). The CSSP shares a partially overlapping signal transduction with nodule symbiosis. In this pathway, microbial signals are translated into a calcium signal that determines the activation of essential symbiotic genes.

At least seven proteins that are required for both the AM symbiosis and the symbiosis with rhizobia have been identified in legumes. For *M. truncatula*, four have been identified: *MtDMI1* (*Doesn´t Make Infection1*), encoding a potassium-permeable cation channel in the nuclear envelope, *MtDMI2* encoding a Leucine-rich-repeat receptor kinase at the plasma membrane, *MtDMI3* encoding a calcium and calmodulin-dependent protein kinase and MtIPD3 encoding an unknown protein that features a nuclear localization signal and a carboxy-terminal coiled-coil domain [Parniske, 2008] (see Fig 7).

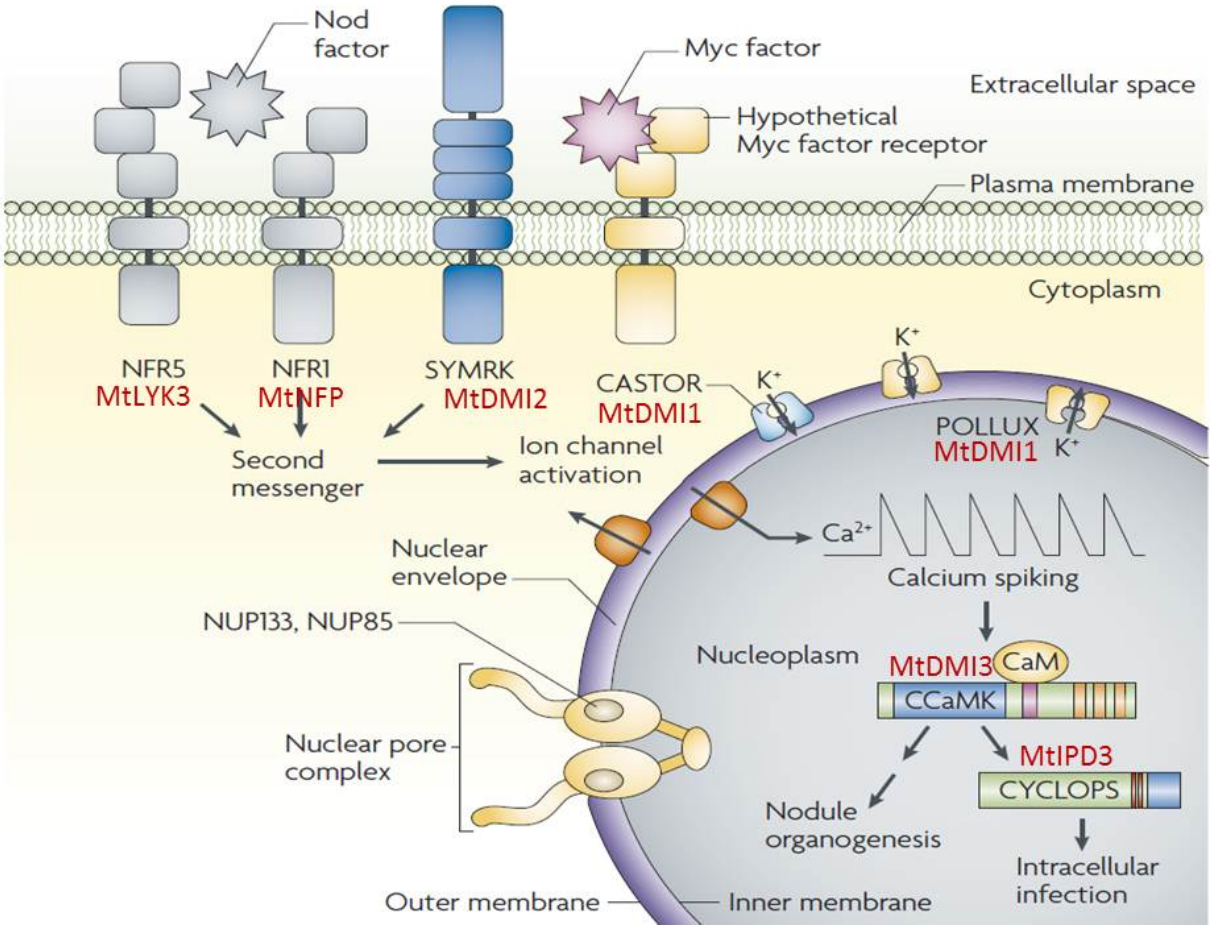

Figure 7: Common symbiotic signaling pathway for AM and root-nodule symbiosis. The orthologous gene names for *M. truncatula* are marked in red. Modified after Parniske, 2008

Despite similarities between the signaling molecules of AMF and rhizobia, specificity must be

maintained so that plants activate downstream processes that promote fungal colonization after Myc factor perception, and processes that promote bacterial invasion and nodule initiation after Nod factor perception. This specificity might be provided by parallel signaling mechanisms or cell-specific modes of response to the symbiosis signaling pathway [Oldroyd, 2013].
For the perception of the Nod factor, two LysM type receptor-like kinases (RLK) have been described. MtLYK3 (LysM domain containing RLK) /MtNFP (Nof factor perception) or LjNFR1/ LjNFR5 (Nod factor receptors 1/5) in *M. truncatula* and *L. japonicus* respectively [Radutoiu et al., 2003, Op den Camp et al., 2011].
RLKs of plants are involved in diverse signaling pathways, including the response to microbial signals in symbiosis and defense. The common overall structure of RLKs imply an extracytoplasmic domain, a transmembarane domain and an intracellular protein kinase domain, responsible for the downstream signal transduction. RLK´s with lysin-motifs (LysM) ectodomains exert a specific recognition for N-acetylglucosamine (GlcNAc)- containing signaling molecules, such as chitin, peptidoglycan, and LCOs [Antolin-Llovera et al., 2012]. LysM domain containing proteins of plants have attracted increasing attention, since the identification of NFR1 and NFR5, two LysM RLKs of *L. japonicus*, responsible for the recognition of the Nod factor [Zhang et al., 2007]. The signaling downstream of different RLKs is not yet understood in detail and it remains to be clarified, how the specificity of interaction towards different organisms is ensured [Antolin-Llovera et al., 2012].

1.4.4 Induced plant defense responses

In general plant defense responses are initiated by pathogen recognition via sensitive perception mechanisms. On the molecular level of plant pathogenesis, this initial process can be mediated either by gene-for-gene interaction or by pathogen derived elicitors, so called pathogen-associated molecular patterns (PAMPs) [Trapphoff et al., 2009]. A gene-for-gene response occurs in the plant cell when a plant possesses a dominant resistance (R) gene, that is complementary to a pathogen derived avirulence protein (AVR). This R-effector association activates resistance, leading to defense signaling in the host. Resistance is manifested as localized cell death at the infection site also called hypersensitive response (HR) and reduced spread of the pathogen [Chisholm et al., 2006]. PAMP- mediated resistance represents a system that adjusts the responses to the severity of infection [Tellstrom et al., 2007]. Once a pathogen has passed the plant cell wall, it will encounter extracellular surface receptors that recognize PAMPs. This perception induces PAMP- triggered immunity (PTI). Pathogens have evolved means to suppress PTI by interfering the host recognition or by secreting effector proteins into the plant cytosol to alter the PTI defense response. To counteract the suppression of primary defense response, plants evolved an effector triggered immunity (ETI). Pathogens seem to have counteracted by interfering with ETI by other effectors [Chisholm et al., 2006, Jones and Dangl, 2006].

1.5 Aim of the study

This thesis was implemented in a project called "'Chemical Communication in the Rhizosphere"' supported by the "'Pakt for Research and Innovation"'. The purpose of the project was to get a better insight of events in the rhizosphere, the narrow soil layer in vicinity of roots, which is chemically and biologically influenced by root deposits and which is characterized by its dense microbial activity. Plants are permanently confronted with this diverse surrounding and are deeply impacted by it. For agricultural plant production it is of major importance to understand mechanisms that induce tolerance/resistance towards pathogens or the capability to cooperate with beneficial organisms.

One aim of this thesis was to evaluate the SARDI core collection of *M. truncatula* ecotypes, collected in different parts of the world, regarding their symbiotic interaction with AMF. The colonization rate of all ecotypes should be evaluated and the root exudate composition of ecotypes, having a very strong/ weak interaction with AMF, should be analyzed in order to identify some key substances that might have a promoting effect on the symbiotic interaction with AMF.

A second aim of this study was to better understand the first steps of recognition and interaction of the model plant *M. truncatula* towards the symbiont *R. irregularis* or the pathogenic oomycete *A. euteiches*. The decisive mechanisms in the early interaction should be analyzed on transcriptional level with the help of a whole genome array. Additionally the emitted root volatiles and secondary metabolites of root exudates should be analyzed via GC/MS and LC/MS, respectively. These data sets should help to draw a picture of the first events induced in the plant not only by vicinity of symbiotic spores of *R. irregularis*, but also by contact with spores of the pathogenic oomycete *A. euteiches*. Genes that were specifically induced by only one of the two microorganisms, should be functionally analyzed regarding their impact on root colonization with RNA interference and overexpression of the target genes via transient root transformation in *M. truncatula*. The function of identified volatiles and exuded secondary metabolites should be more closely analyzed with bioassays, that should clarify the effect of the candidate substances on the different microorganisms.

In order to relate the specificity of exudation patterns to the CSSP, that is induced by the presence of AMF, *dmi3* mutant plants (defect in the CSSP) should be implemented in the trial.

Some publications suggest that during the initial stages of AM development a defense response is activated that is suppressed in later stages of the symbiotic interaction [David et al., 1998,Spanu et al., 1989,Harrison, 1993,Kapulnik et al., 1996,Lambais and Mehdy, 1992,Volpin et al., 1995]. Yet those studies were done several days to weeks after the inoculation of plants with AMF and little is known about the defensive status of plants directly after microbial contact. According to the above mentioned observations, we hypothesize, that the very early reaction within the plant towards a symbiont, will look very similar to the contact with a pathogenic organism. Yet it remains to be answered, whether a general PAMP like reaction that we expect to appear in the first moments of AMF recognition, might be accompanied by a specific answer that allows an early switch from "'defense"' to "'symbiosis"' within the plant.

2 Material and methods

2.1 Material

2.1.1 Chemicals and supplies

All chemicals, enzymes and supplies needed were obtained, if not mentioned separately, from the companies Life Technologies, Carl Roth, Sigma-Aldrich, Serva, Roche and Merck.

2.1.2 Plants

Allium porrum L. cv. Elefant (Erfurter Samen- und Pflanzenzucht GmbH, Erfurt, Germany).
Medicago truncatula GAERTN. cv. Jemalong A17 (Perkiss Seeds, Australia)
Medicago truncatula lines from the SARDI core collection kindly offered by Jean-Marie Prosperi, INRA Montpellier, France: 163, 174, 544, 736, 734, 530, 368, 555, 154, 543, 239, 648, 542, 550, 49, 552, 337, 245, 321, 545, 679, 369, 554, 557, 263, 198, 310, 144, 290, 549, 213.
Medicago truncatula Tnt1 insertion lines, generated on the cultivar R108 from "'The Samuel Roberts Nobel Foundation"'.

2.1.3 Microorganisms

Rhizophagus irregularis: Sterile spores of the line DAOM181602 = DAOM197198 were kindly offered by Natalia Requena, KIT Karlsruhe, Germany.
G. intraradices: For the non-sterile inoculation on expanded clay, the former isolate 49 (new phylogenetic classification not known) was kindly offered from H. von Alten, University of Hannover, Germany.
Aphanomyces euteiches: GB I1 was kindly offered by Phillip Franken, IGZ, Großbeeren.
Echerichia coli The *E. coli* strain XL1 Blue was used for cloning.
Agrobacterium rhizogenes: The *Agrobacterium* mediated root transformation of *M. truncatula* was done with the strain *ARqua1*.
Agrobacterium tumefaciens: For the transformation of *Nicotiana benthamiana* leaves, the *Agr. tumefaciens* strain GV3101::pMP90 was used.
For longterm storage of *E. coli* and *Agrobacteria*, aliquots of liquid cultures were stored in 15% (v/v) glycerol at -80°C.

2.1.4 Oligonucleotides and Plasmids

All used oligonucleotides were ordered from Eurofins MWG Operon. For primer design the Primerfox-Homepage (http://www.primerfox.com/index.html) was used.
Oligo calc (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used to control specific properties of primers. All primer sequences are listed in the Appendix in Tab. 4) and the important plasmids are described in the corresponding chapters with an overview in the Appendix in Tab. 5.

2.2 Biological methods

2.2.1 Fertilizer and media for cultivation of plants and microorganisms

All media were sterilized by autoclaving for 20 min at 121°C and 2 bar if not mentioned differently. Antibiotics were added after cooling the media to about 60°C. Antibiotics were solved in H_2O_{dd}, if not mentioned differently and sterile- filtered (Rotilabo®- Spitzenfilter; 0,22 µm).

Table 1: Concentration of Antibiotics and X-Gal in bacterial selection medium

Antibiotic	stock solution	working concentration
Carbenicillin*	50 mg/ml**	50 µg/ml
Kanamycine	50 mg/ml	50 µg/ml
Streptomycine	50 mg/ml	50 µg/ml
Gentamycin	50 mg/ml	50 µg/ml
Spectinomycin	100 mg/ ml	100 µg/ml
X-Gal	50 mg/ ml	50 µg/ml

*equivalent to Ampicillin; **solved in ethanol

Long Ashton Fertilizer (20 % Phosphate)

For 1 l Long-Ashton fertilizer 10 ml of stock solution microelements A, 1 ml of stock solution microelements B, 0.22 g Fe-EDTA and the amounts of macroelements listed below, were solved in H_2O_{dest} [Hewitt, 1966].

Macroelements	Amount in [g]	Microelements A (for 1 l)	Amount in [g]
KNO_3	4.04	$MnSO_4 \times 1\ H_2O$	1.60
$Ca(NO_3)_2$	9.44	$CuSO_4 \times 5\ H_2O$	0.25
$MgSO_4 \times 7\ H_2O$	3.68	$ZnSO_4 \times 7\ H_2O$	0.33
$NaH_2PO_4 \times 1\ H_2O$	0.36	H_3BO_3	3.10
		NaCl	5.90
		Microelements B (for 1 l)	Amount in [g]
		$(NH_4)_6Mo_7O_{24} \times 4\ H_2O$	0.88

Luria- Bertani- Medium (LB):

1.0% (w/v) Tryptone; 0.5% (w/v) Yeast extract 1.0% (w/v) NaCl 1.5% (w/v) Agar*

1X Phosphate Buffered Saline (PBS)

NaCl 137 mM KCl 2.68 mM

Na2HPO4 10.144 mM KH2PO4 1.7635 mM pH: 7.4

Citrate buffer (1 l / 0.01 M)

18 ml citric acid stock solution: 0.1 M (autoclaved)

82 ml sodium citrate stock solution: 0.1 M (autoclaved)

The two compounds were mixed and adjusted to a volume of 1 l with H_2O and pH 6 and autoclaved [Declerck et al., 2005].

MSR- medium (Modified Strullu-Romand)
The protocol for MSR stocksolutions can be found in the Appendix in Tab. 65. Medium preparation (1 l): 10 ml of solution 1 (macro elements), 10 ml of solution 2 (calcium nitrate), 5 ml of solution 3 (vitamins), 5 ml of solution 4 (NaFeADTA), 1 ml of solution 5 (microelements) and 10 g of sucrose [Declerck et al., 2005]. The pH was adjusted depending on the intended use (pH 5.5 for AMF production in root cultures, pH 6.5 for cultivation of whole plants). For solid medium, 4 g/l phytagel were added before autoclaving for 15 min, 121°C, 1 bar. The medium had to be used directly, since phytagel can not be redissolved after cooling down.

Fahraeus- medium according to Boisson-Dernier et al., 2001 with slight modifications: Before addition of gelrite, the pH was adjusted to 7.4. The $CaCl_2$ as well as the antibiotics were added after autoclaving, when the temperature was cooled to about 60°C.

Substance	Final concentration
Macro elements	
$CaCl_2$	0.9 mM
$MgSO_4$	0.5 mM
KH_2PO_4	0.02 mM
Na_2HPO_4	0.01 mM
Ferric citrate	0.02 mM
NH_4NO_3	1.0 mM
Microelements	
$MnCl_2$	0.27 µM
$CuSO_4$	0.2 µM
$ZnCl_2$	0.05 µM
H_3BO_3	1.6 µM
$Na4_2MoO_4$	0.16 µM
Gelrite	11 g/l
MES	200 mg/ml

Yeast-tryptone medium for *Agr. rhizogenes* cultivation
5 g/l Tryptone; 3 g/l Yeast extract; 1.5 g/l Microagar; 2 mM $CaCl_2$ (has to be added after autoclaving), pH = 7

Media for *A. euteiches* zoospore production:
Corn meal agar medium (CMA - from Sigma Aldrich): 17 g/l
Corn Meal Agar-Yeast extract-ß-sito-sterol-α- Tocopherol Acetat (CMA-HST):
CMA: 17 g/l; Yeast extract: 4 g/l; ß-sitosterol: 0.8 mg/l; α -tocopherol acetat: 100 mg/l
CMA and Yeast-extract were added to 50 mM phosphate buffer (pH 6.8-7). ß-sitosterol and α-tocopherol acetate were added to the suspension, while stirring and heating.
Yeast Extract - Tryptone (HT):
3 % (w/v) yeast extract; 5 % (w/v) tryptone autoclaved with $H_2O_{\,dd}$;
HT is diluted with autoclaved swamps* by 1:4 for final use.
* Swamps water was collected from one pond in Halle, filtered and autoklaved - the vitality of zoospores could not be achieved with tap water.

2.2.2 Axenic cultivation of AM fungal material

For sterile AMF spore propagation, two- partitioned petri dishes were used. One half filled with MSR medium, the other half filled with MSR medium lacking sucrose, so that the roots are preferably growing only on one half of the plate, whereas on the other half pure fungal material is growing. 3-4 pieces a 3-4 cm of carrot roots from a transformed root organ culture were placed on one half of the petri dish (+ sucrose). 4-6 spots of roots were inoculated with a few sterile spores. Plates were sealed with parafilm and stored at 28°C in the dark. To obtain pure spore material, root growth had to be limited to one partition of the plate by cutting overgrowing roots. Pure spore material could be harvested after 6-10 months and was dissolved from the solid MSR medium with 0.01 M citrate buffer.

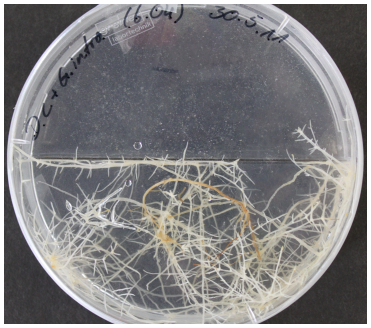

Figure 8: AMF colonized harry roots on two partitioned plates.

Production of non-sterile mycorrhizal inoculum Inoculum was produced using of a leek trap culture. Leek is a good host for mycorrhizal fungus developing large and fine root systems. Leek was cultivated with *R. irregularis* in expanded clay (Blähton, 2-5 mm particle size, Lamstedt, Germany) for at least 6 month in the green house (16 hrs light, 8 h dark at 23 °C/ 18 °C and 50 % relative humidity). After 6 month the substrate is rich in secondary spores and serves as efficient inoculum for further experiments. Inoculated expanded clay was harvested, leek roots were removed and the material was diluted with sterile expanded clay with a ratio of 20:80 (w/w) for further use.

2.2.3 Sterile cultivation of *A. euteiches*

For cultivation of *A. euteiches*, there is one permanent culture, that grows on CMA medium and is subcultured every month on the same type of medium. For zoospore production, one cm^2 from the permanent culture was transferred on a CMA-HST plate (see section 2.2.1) and grown in the dark for 4-5 days. When the entire medium surface was overgrown by the oomycete, 1 cm^2 were cut from the margin of the plate (young hyphae) and transferred to a fresh agar plate (with high edges) and HT solution was added until the gel piece with mycelium was covered. After 48 hrs, the HT solution was poured and the mycelium rinsed with autoclaved tap water. The water was added and incubated for 45 min before being discarded. This procedure was repeated 3 times to eliminate a maximum of sugars and peptides, otherwise zoosporulation might be of poor quality. After rinsing, autoclaved swamps water was added in the petri dish and incubated in the dark for about 28 hours. Then the zoospores were harvested and quantified using the Fuchs-Rosenthal counting chamber. The zoospores were counted and the concentration was calculated with the following formula: $\mathrm{NZ/ml} = \frac{\mathrm{NZ/Square}}{\mathrm{A \times D}} \times 1000$

NZ = Number of Zoospores; A = Area of small square (mm^2); D = Depth of small square (mm)

2.2.4 Cultivation systems for *M. truncatula*

Sterilization and germination of *M. truncatula* seeds Seeds of *M. truncatula* ($\sim$ 50 seeds per tube) were sterilized by shaking them in 1 ml sulphuric acid for 5-8 min (until dark spots became visible on seed coat). Seeds were washed 5 $\times$ in H_2O under sterile conditions and subsequently placed on sterile glass petri dishes with filter paper and 4 ml sterile H_2O. Plates were closed with leukopor, wrapped in aluminum foil and stored for 3 days at 4°C in the dark. One day of incubation in the dark at RT and one day in light at RT followed to obtain seedlings ready to be transferred to the cultivation system.

Aeroponic cultivation The Aeroponic cultivation system is a plant growth system, where plant roots grow in the atmosphere and are regularly sprayed by a nutrient solution. Nutrient solution is filled in a container and is pumped through nuzzles towards the plant roots. The container is closed with styrofoam including openings for the plants. Those openings are filled with rock wool cubes. Seedlings can be transferred into those cubes. The nutrient solution is sprayed in regular intervals (30 min/hr with 4 hrs break during the night). This system assures good oxygen as well as sufficient nutrient supply for the plants. It allows plants to establish a well developed root system and exudates can be easily collected. MSR- medium (see section 2.2.1) without sugar was used to cultivate the plants. 3-4 weeks after seedling transfer, the nutrient solution was exchanged and plants were analyzed after 6 weeks.

Figure 9: *M. truncatula* plants at 8 days (left) or 6 weeks (right) after seedling transfer to the aeroponic system.

***M. truncatula* cultivation on plates with solid MSR medium** 120-mm-square dishes were filled two thirds (8 cm) with MSR medium. On top of the solid medium, 6 seedlings were placed. Plates were sealed with leukopor and the bottom part of each plate was wrapped in aluminum foil for light protection of roots. Plates were placed in a light chamber (16-hrs-light (24°C)/ 8-hrs-dark (20°C)).

***M. truncatula* cultivation on inoculated expanded clay** Seedlings were transferred to pots of 12 cm ∅ with expanded clay containing *R. irregularis* as described in section 2.2.2 and cultivated in the phytochamber under long day conditions (16 hrs light at 26 °C, 8 hrs dark at 20 °C, 40% relative humidity, 150 µmol · m^{-2} · s^{-1} light intensity). Plants were fertilized once in the first 2 weeks and twice per week later on with 10 ml Long Ashton fertilizer (20 % phosphate) (see Paragraph 2.2.1) and were watered twice a week.

2.3 Microscopic works

2.3.1 Ink staining of AMF in root tissue

Roots were incubated in KOH at 70-90°C (depending on the root age and stability) for 10 min. Roots were washed 3 x with H_2O to remove all KOH. An incubation in 2 % acetic acid at RT followed. After this neutralizing step, roots were stained with 5 % black ink (Sheaffer Skrip) in 2 % acetic acid at 90°C for 10 min. Samples were washed one time with H_2O and stored at 4°C until evaluation.

2.3.2 Evaluation of mycorrhization rates via the „Gridline intersection method“

Ink-stained root pieces were spread on a square petri dish with a grid of vertical and horizontal lines. With a binocular at 10 × magnification, mycorrhizal structures were observed. At each point where a root piece intersected a grid line, the presence or absence of AMF structures, such as vesicles or arbuscules was counted. The total of root/ gridline intersects in proportion to the AMF colonized intersects was calculated to estimate the percentage of AMF infected root length [Giovanetti and Mosse, 1980].

2.3.3 Staining with WGA-AlexaFluor488

In order to stain fungal and oomycete structures within root tissue, Wheat Germ Agglutinin (WGA) conjugated to AlexaFluor488 (excitation/emission maxima 495/519 nm; Life Technologies GmbH, Germany) was used. Alexa Fluor; 488 WGA binds to sialic acid and N-acetylglucosamin residues. This property enables the detection of cell wall components of fungi and some oomycetes like *A. euteiches*. For staining, the freshly harvested roots were placed in 50 % EtOH for at least four hours. EtOH was removed and roots were incubated in 20 % KOH for 10 min at 80-90°C (for young roots 80°C is preferable to avoid strong root damage). KOH was removed and samples were washed with H_2O_{dest}. Roots were incubated in 0.1 M HCl for 1-2 hours. Acid was removed and samples were washed with H_2O_{dest}. Samples were then rinsed with 1 X PBS (see: 2.2.1) and a PBS-WGA (0.2 µg WGA-AlexaFluor488 /ml PBS) staining solution was added for at least 6 hours before samples could be analyzed. The stained structures were analyzed with a confocal laser-scanning microscope (LSM 700, Zeiss, Jena, Germany) using the 488 nm laser line for excitation.

2.3.4 Localization of GFP-fused proteins

For subcellular localization of GFP-fused proteins, *N. benthamiana* leaves were infiltrated with the fusion constructs. From infiltrated leaves, discs of about 1 cm^2 were cut out and analyzed with a confocal Laser-Scanning-Microscope LSM 710 (Zeiss) with an Argon laser. For GFP an excitation wavelength of 495-557 nm and an excitation at 485 nm laser line was used.

2.4 Molecular biological methods

2.4.1 Isolation of genomic DNA

100 mg frozen plant material was ground in a Retsch mill at 30 r/s for 2 min and kept frozen permanently with liquid nitrogen. The DNA isolation of plant material was done with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions.

2.4.2 Isolation of Plasmid DNA

For isolation of Plasmid DNA from a bacterial liquid culture the Zyppy^{TM} Plasmid Miniprep Kit (Zymo Research Corporation, 17062 Murphy Ave., Irvine, CA 92614, U.S.A.) was used according to the manufacturer instructions.

2.4.3 Isolation of RNA

For RNA isolation from plant material, the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer instructions. The obtained RNA was quantified with a NanoDrop 1000 (peQLab Biotechnologie GmbH, Erlangen, Germany) and a quality control was done with a QIAxcel system from Qiagen.

2.4.4 Synthesis of cDNA

For cDNA synthesis, 1 µg of RNA in a volume of 10 µl of H^2O was used. 1 µl of a 100 mM Oligo(dT) primer was added, incubated for 5 min at 70°C and quickly cooled down in ice water. 9 µl mastermix including 1 µl Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV), 4 µl M-MLV RT 5x reaction puffer, 4 µl dNTPs (2,5 mM), (Promega, Mannheim, Germany) were added and the following program was applied: 10 min at 40 °C, 50 min at 42 °C, 15 min at 70 °C. The obtained cDNA was diluted 10 $\times$ to serve as template for qRT-PCR.

2.4.5 Agarose Gel Electrophoresis

The separation of PCR products, plasmid DNA or restricted DNA was performed in a 1 % (w/v) agarose gel in 1x TAE buffer (50 x stock solution for TAE electrophoresis buffer: 2 M Tris, 1 M acetic acid ($C_2H_4O_2$: 100 %), 50 mM Na_2EDTA (pH 8.5), add dH_20 to a total volume of 1 L). 1 µl/ 50 ml DNA stain G (SERVA GmbH, Heidelberg, Germany) were added to the heated medium for staining and visualization of the nucleic acid. Before loading the samples on the

gel, a concentration of 2 x loading buffer (10 x Fast Digest Green Buffer, Thermo Scientific) was added to the sample. The gel electrophoresis was conducted at 120 V in 0.5 × TAE buffer. The negative charge of DNA fragments attract those to the anode and a mass dependent separation of the fragments follows. After electrophoresis the gels were analyzed with a gel documentation system (Fusion FX7, Marne-la-Vallée Cedex, France) under UV- excitation. The fragment size was determined with help of a DNA ladder (O´Gene Ruler, 1kb plus, Thermo Scientific).

2.4.6 Polymerase Chain Reaction (PCR)

PCR is a method for targeted amplification of a DNA template strand with the help of two specific oligonucleotides that serve as primer and a heat resistant polymerase [Mullis et al., 1986]. The procedure includes the three steps of DNA denaturation, annealing and elongation that are repeated in cycles (25-40 ×). The PCR reaction was performed with an Eppendorf MasterCycler® Gradient Thermocycler (Eppendorf AG, Hamburg, Germany). For the purpose of genotyping, the GoTaq®- DNA- Polymerase (Bio & Sell e.K., Feucht bei Nürnberg, Germany) was used. The Taq-Polymerase is a very stable polymerase, but also error-prone and therefore not suitable for further use in cloning applications, where the amplified product needs to be a correct copy of the gene of interest. For this purpose a Pfx-Polymerase (AccuPrime™ Pfx SuperMix; Thermo Fisher Scientific) was used that includes a proof-reading function.

GoTaq PCR Reactionmix:	Final volume (20 µl)	AccuPrime™ *Pfx* SuperMix	Final volume (25 µl)
Reactionbuffer BD (10×)	2 µl	AccuPrime *Pfx* SuperMix	22.5 µl
forward Primer (10 µM)	1 µl	forward Primer (2 µM)	1 µl
reverse Primer (10 µM)	1 µl	reverse Primer (2 µM)	1 µl
Template DNA	10-50 ng	Template DNA	10-200 ng
Solution S (10×)	2 µl		
$MgCl_2$ (10×)	2 µl		
dNTP´s (10mM)	200 0.5 µl		
Taq Polymerase	0.5 µl		
H_2O	ad to 20 µl		

Table 2: General scheme of PCR program: The annealing temperature (T_{opt}) was chosen primer - dependent with 3-6°C below the specific melting temperature (T_m) of the primers. The duration of the elongation step was adjusted to the size of the amplified fragment with 1 kb/min.

Process	GoTaq PCR Reactionmix:	AccuPrime™ *Pfx* SuperMix
Initial denaturation	2 min/ 95°C	5 min/ 95°C
35 cycles:		
Denaturation	30 sec/ 95°C	15 sec/ 95°C
Annealing	30 sec/ T_{opt}	30 sec/ T_{opt}
Extension	1min per kb/ 72°C	1min per kb/ 68°C
Final elongation	5 min/ 72°C	
End	∞/ 4°C	∞/ 4°C

2.4.7 Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time polymerase chain reaction is a method for quantification of mRNA in biological samples. A fluorescence signal is emitted by the report dye, that should be proportional to the amplified nucleic acids. The quantification is assessed relative to the transcript amount of a non regulated housekeeping gene. Crucial for quantification is the Ct-value, that indicates the first PCR-cycle reaching a level of fluorescence (threshold) above the background (baseline). As fluorescence dye SYBR® GreenI was used. The binding of SYBR Green to the double strand DNA induces an augmentation of fluorescence intensity, which correlates with the amount of template-DNA. The binding specificity of primers was controlled with a BLAST search on NCBI.The size of amplified fragments was 80-120bp. In the present work qRT-PCR was performed with the CFX Connect^{TM} Real-Time PCR System from BIO-RAD Laboratories, INC. (Munich, Germany). The data evaluation was done with help of the Bio-Rad CFX

Table 3: General scheme for qRT-PCR program: The annealing temperature (T_{opt}) was primer - dependent and was chosen 3-6°C below the specific melting temperature (T_m) of the primers. The duration of the elongation step was adjusted to the size of the amplified fragment with 1 kb/min for reactions with the GoTaq®-Polymerase and 2kb/ min when *Pfu*-DNA-Polymerase was used.

Process	Time [min]	Temp [°C]
Activation of *Taq* DNA-Polymerase	10	95
50 cycles:		
Denaturation	0.5	95
Annealing	1	T_{opt}
Elongation	0.5	72
Melting curve analysis		

Manager. The Ct- values of the target gene (TG) were normalized to the housekeeping genes (Reference gene: RG) with the following formula: $\Delta\mathrm{Ct} = \mathrm{Ct_{TG}} - \mathrm{Ct_{RG}}$. The transcript accumulation of a gene from one sample was determined using the mean of 3 technical replicates. The negative potency of Δ Ct resulted in the actual value of relative gene expression calculated with the following formula: Relative gene expression $= 2^{-\Delta\mathrm{Ct}}$

2.4.8 Affymetrix whole genome array of *M. truncatula*

To analyze the expression pattern of *M. truncatula* genes after different treatments, the Medicago WT Gene 1.1 ST Array Strips from Affymetrix was used. The *Medicago* Genome Array is a 49-format, 11 µm array design. The sequence information for this array was selected from data sources including the TIGR *M. truncatula* gene index, gene predictions from IMGAG, gene predictions from the *S. meliloti* genome, and *M. sativa* EST information made available by TIGR. The array contains 50,902 *M. truncatula* probe sets plus 14 controls: 32,167 *M. truncatula* EST/mRNA-based and chloroplast gene-based probe sets; 18,733 *M. truncatula* IMGAG and phase 2/3 BAC prediction-based probe sets. This equals 48,116 transcripts. The Chips contain up to 26 probes per gene with a single probe length of 25 nucleotides, enabeling an accurate

detection of transcripts. Information such as splice and polyadenylation variants can be distinguished, in contrast to 3'-biased microarrays. The chip contains the hybridization controls: bioB, bioC, bioD from *Escherichia coli* and cre from P1 bacteriophage; Poly-A controls: dap, lys, phe, thr, trp from *Bacillus subtilis* and the housekeeping/control genes beta-actin, GAPDH, glutathione S-transferase, ubiquitin for *M. truncatula* and *M. sativa*.
The RNA was isolated as described in 2.4.3. Quality control was done with the QIAxcel Advanced System from Qiagen according to the manufacturer instructions. With this method the RIN number (RNA Integrity Number) of the RNA could be determined and only samples with a RIN number above 8 were used for further analysis. RNA concentration and purity was analyzed with a NanoDrop 1000 spectrophotometer. The ratio of absorbance readings at 260 and 280 nm (A_{260}/A_{280}) was 2.0-2.2 in all samples.
The Array was performed according to the Affymetrix user manual (see:
http://media.affymetrix.com/support/downloads/manuals/geneatlas_wt_expkit_manual.pdf),
with 500 ng isolated RNA for each biological replicate and four biological replicates per treatment.The obtained data were analyzed with the ArrayStar 5 software (Rockville, USA) and processed with robust multi-array average (RMA). The normalization was done by the quantile mode. The average of the biological replicates for each gene and the fold change (FC) referring to the control was calculated. The significance was determined using the Student's t-test. Parameters for significance were chosen at a FC of at least $\pm$ 2 and a p-value $\leq$ 0.05.

2.4.9 Genotyping of *Tnt1* insertion lines

The Samuel Roberts Noble Foundation´s Medicago *Tnt1* insertion mutant population is the largest collection of DNA-insertion mutants of all legumes. It was established from a starter parental line, containing approximately five copies of the *Tnt1* retroelement transferred into wild-type R108 by *Agrobacterium tumefaciens*-mediated transformation [Pislariu et al., 2012]. To test whether the single plants of the ordered seeds really had the insertion in the gene of interest, a PCR screening using gene-specific primers in combination with *Tnt1* primers, were used to identify *Tnt1* insertions in the genes of interest. To test the presence of the wt allele, primers of the gene of interest depicted as primer 1 and 2 in Fig. 10 A were used to test the presence of the *Tnt1* insertion, the forward primer of the gene of interest (1) was combined with a reverse primer located on the *Tnt1* sequence (3). DNA was isolated from every *Tnt1* insertion plant and two PCR reactions were performed with primer 1+2 and primer 1+3. If both reactions showed a band like in plant 1 of Fig. 10 B, the plant had a heterozygous insertion, if there was only a band for reaction 1+2 (no example shown), there is no insertion in any of the alleles, and if there was a band only in reaction 1+3, it depicts a homozygous insertion of *Tnt1* in both alleles (as shown for plant 2 in Fig. 10 B).

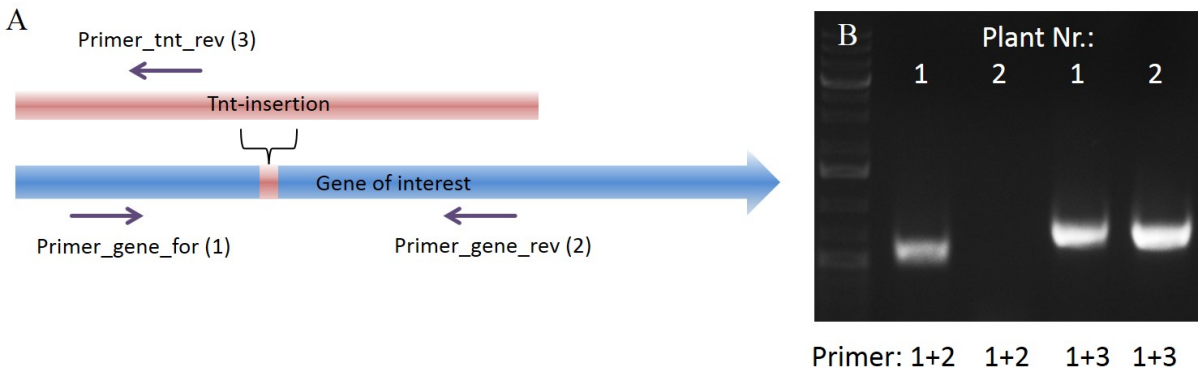

Figure 10: A: Scheme for primer design of *Tnt1* insertion lines; B: Example of a PCR analysis of two different plants with the two primer combinations for wt allele and the *Tnt1* insertion, showing a heterozygous insertion in plant 1 and a homozygous insertion in plant 2.

2.4.10 Cloning techniques

Restriction of DNA DNA restriction was performed with "'fast digest"' Enzymes from Thermo Fisher Scientific. 0.5-1 µg DNA was used for a final reaction volume of 20 µl according to the users manual. To control the DNA restriction, an agarose gel electrophoresis was performed.

Gel extraction of DNA fragments The DNA fragment of interest was separated with gel-electrophoresis and visualized on a UV-light table of the Geldocumentary system BioDocAnalyze (Whatmann Biometra, Göttingen, Germany) at an activation wave length of 360 nm. The fragment of interest was cut with a scalpel and the DNA was extracted from the gel with the MinElute® Gel Extraction Kit from Qiagen according to the users manual.

DNA-fragment purification To clean DNA-fragments gained from a PCR (see section 2.4.6) or restriction reaction (see section 2.4.10), SureClean Kit from Bioline GmbH (Luckenwalde, Germany) was used according to the users manual.

Sequencing All sequencing was done by the company MWG biotech AG (Ebersberg, Germany). Evaluation of the quality of the sequencing file (nucleotide peaks) and the alignment with the original sequence was performed with the software Geneious 6.0.5.

Vector construction with Golden Gate Cloning The Golden Gate Cloning method was described by Engler and Marillonnet (2008). The method relies on the use of type IIs endonucleases, whose recognition sites are distal from their cutting sites. With help of specific primer-overhangs, the target fragment gets flanked by BsaI, on both outer ends of the amplified fragment. The use of type IIs endonucleases allows the combination of restriction and ligation in one single reaction, since after a fragment was ligated once, the restriction site is removed and the ligation is irreversible. In addition, the BsaI sites can be designed in a specific way for

each cleavage site, so that only directional ligation can take place. Thus, over time, all reactions will tend towards the desired assembly product. The overhang sequences themselves must not be palindromic, and the overhang sequences must differ by at least 1 and preferably 2 bps so that the different overhangs are not cross-complementary. The recipient expression vector contains two BsaI restriction sites, complementary with the restriction sites from the entry vector. The entry vector contains a carbenicillin resistance, while the expression vector contains a kanamycin resistance and a LacZ alpha fragment between the two BsaI sites, allowing a double screen for correctly ligated plasmids. For optimal performance of Golden Gate assembly, all used modules should lack any additional BsaI recognition sites, other than those explicitly needed. If in any module there are additional BsaI restriction sites, they need to be removed by targeted nucleotide change (via PCR), without changing the amino acids sequence nor the open reading frame of the gene of interest [Engler et al., 2008].

Gene silencing by RNA interference The method of RNAi uses the mechanism of post transcriptional gene silencing (PTGS), induced by double stranded RNA (dsRNA). The dsRNA is recognized by so called Dicer proteins, that cleave the dsRNA into small fragments of 21-25 bp, so called small interfering RNA (siRNA). Those siRNAs are implemented in RNA-induced silencing complexes (RISC), that are activated after the removal of one strand of the siRNA, to further bind to complementary mRNA, which is then fragmented and thus inactivated. For artificial generation of dsRNA, a vector containing a fragment of the target gene in sense and antisense orientation, separated by a spacer, is needed. When those elements are transformed to the plant and are integrated to the genome and transcribed, double stranded hairpin RNA molecules (hpRNA) are generated, that can be recognized by Dicer proteins and thus lead to PTGS and reduced gene expression [Smith et al., 2000].
In 2003 a binary vector called pRedRoot was constructed in the group of René Geurts [Limpens et al., 2004]. To clone the target gene in sense and antisense direction with spacer in between (Fig. 11) into pRedRoot, several cloning steps were needed. This is the reason why a new vector (pAGH11978) was constructed in our group with the same basic elements, but compatible for the Golden Gate Cloning system (see Fig. 12). In this cloning system the pds intron (phytoene dehydrogenase precursor protein from *Arabidopsis thaliana* (At4g14210)) instead of the above mentioned spacer, was used (recommendation by Sylvestre Marillonnet).

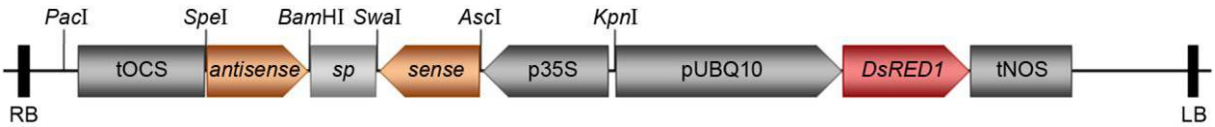

Figure 11: Scheme of the RNAi cassette in the pRedRoot vector, containing the selection marker DsRED. The sense and antisense fragments of the gene of interest with a spacer (sp) in between were ligated into the pRNAi vector between p35S (CaMV 35S-promotor) and the tNOS (Terminator from the nopaline synthase gene from *A. tumefaciens*). In a second cloning step the RNAi construct is cloned in the binary vector pRedRoot. RB: right border, pUBQ10: AtUbiquitin10-Promotor, DsRED1: DsRED aus *D. striata*, LB: left border. [Floß, 2008]

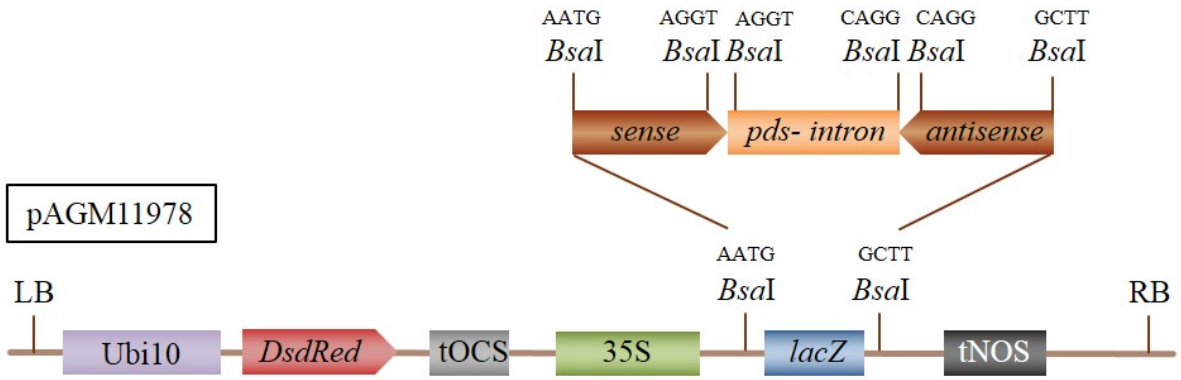

Figure 12: Scheme of the RNAi cassette in pAGM11978 adapted from the pRedRoot vector for the Golden Gate Cloning system. The sense and antisense fragments of the gene of interest and the pds intron were cloned in one cloning step into pAGM11978 between 35S (CaMV 35S-promotor) and tNOS (terminator from the nopaline synthase gene from *A. tumefaciens*). RB: right border, Ubi10: AtUbiquitin10-Promotor, DsRED1: DsRED from *D. striata*, LB: left border.

After construction of a binary vector containing a RNAi construct, the T-DNA was transferred to *M. truncatula* roots by *Agr. rhizogenes* mediated root transformation (section 2.5.5).

2.5 Microbiological Methods

2.5.1 Production of chemically competent *E. coli* cells

In this work "'One Shot® TOP10 Chemically Competent *E. coli*"' cells (Life Technologies GmbH) were used. 2 x 2.5 ml LB-medium was inoculated with cells from a glycerol stock and were shaken at 180 rpm (Shaker, Fluke GmbH, Glottertal, Germany) over night at 37°C. 4 ml of the over night culture was transferred to 400 ml LB medium and shaken at 37°C until an OD_{590} of about 0.375 ($\prec$ 0.4) was reached. The culture was divided in 8 times 50 ml and incubated for 5-10 min on ice. Samples were centrifuged (7 min, 3000 rpm, 4°C) and the pellet was resuspended in 10 ml cold $CaCl_2$- solution (60 mM $CaCl_2$, 15 % glycerol, 10 mM PIPES, pH 7.0, autoclaved). Samples were again centrifuged (5 min, 2500 rpm, 4°C) and pellet was resuspended in 10 ml cold $CaCl_2$- solution. Cells were incubated on ice for 30 min, centrifuged (7 min, 3000 rpm, 4°C) and pellet was solved in 2 ml cold $CaCl_2$-solution. Then cells were aliquoted to 50 µl each in sterile tubes and stored at -80°C.

2.5.2 Transformation of *E. coli* cells

50 µl of cells were thawed on ice, 20 µl of the ligation reaction was added to the vial, mixed gently and incubated for 30 min. The cells were then incubated for exactly 30 sec at 42°C and then placed on ice. 250 µl sterile LB medium were added and shaken for 1 h at 225 rpm. 50-100 µl per transformation were plated on LB agar plates with the corresponding antibiotic (Tab. 1) and X-gal for selection of positive transformants by blue- white screening of constructs, that contain a LacZ selection marker. Plates were cultivated at 37°C over night. White colonies were analyzed by plasmid isolation and sequencing.

2.5.3 Production of chemically competent *Agr. rhizogenes* cells

Agr. rhizogenes cells were cultivated over night in 3 ml LB medium (28°C, 250 rpm). The culture was used to inoculate 200 ml LB-medium, grown at 28°C and 250 rpm until an OD_{600} of 0.5-0.6 was reached. Cells were incubated on ice for 15 min. The cell suspension was partitioned to 4 x 50 ml falcon tubes and centrifuged (all centrifugation steps in this protocol were done as follows: 10 min, 12000 rpm, 4°C). The pellet was washed with 40 ml ice cooled sterile water and resuspended on ice. The suspension was centrifuged again and washed with 20 ml ice cooled sterile water. The suspension was washed again and centrifuged. The pellet was resuspended with 10 ml ice cooled 10 % glycerol. The four fractions were combined and centrifuged. The pellet was solved in 1 ml ice cooled 10 % glycerin and aliquots of 50 µl were transferred to sterile microcentrifuge tubes and stored at -80°C until use.

2.5.4 Transformation of *Agr. rhizogenes* cells

The transformation of *Agr. rhizogenes* was performed by electroporation. Cells were thawed on ice. In parallel electroporation cuvettes (2 mm gap width, Eurogentec, Seraing, Belgium) were cooled on ice. 400-500 ng of plasmid DNA was added to the thawed cells. The cell/ plasmid suspension was pipetted into the cuvette, and inserted into the electroporator (BioRad Laboratories GmbH, Munich, Germany). Immediately after electroporation, 1 ml of LB-medium was added to the cuvette, to flush out the transformed cells and transferred to a falcon tube. The transformed cells were incubated for 3 h at 28°C and 250 rpm. 10 µl of the transformed cells were plated on LB plates with selection antibiotics (Tab. 1) and incubated for 2 days at 28°C.

2.5.5 *M. truncatula* transient root transformation mediated by *Agr. rhizogenes*

To transform roots, the *Agr. rhizogenes* strain Arqua-1 was used. *Agr. rhizogenes* is a root pathogen which elicits adventitious, genetically (Ri T-DNA) transformed roots. A root transformation with this pathogen leads to the development of "composite plants" comprising a transgenic hairy root system attached to a non-transformed shoot [Chabaud et al., 2006]. *Agr. rhizogenes* implements the T-DNA (transfer-DNA is the transferred DNA of the tumor-inducing (Ti) plasmid of Agrobacterium), into the plant cells. The T-DNA containing the transgene of interest in a disarmed binary vector was co-transformed with the resident *Agr. rhizogenes* Ri T-DNA containing the root locus (rol) genes (responsible for root proliferation). The strain Arqua1 is a relatively low virulence *Agr. rhizogenes* strain [Chabaud et al., 2006]. Transformed *Agr. rhizogenes* was cultivated on Yeast-Tryptone (see section 2.2.1) plates with selection antibiotics (Kanamycin and Streptomycin - according to Tab. 1) and treated with 200 µl Acetosyringone (1 mM/mL). Then Agrobacteria were plated and plates were closed with parafilm and incubated in dark at 28°C for 2 days. In parallel *M. truncatula* seeds were sterilized (as described in 2.2.4) and plated on 0.7 % plant agar. The lids of the petri dishes were filled with 1 ml sterile H_2O, closed with Leucopore and kept for 48 h in the dark at 12°C. The seedlings

were freed from the seed coat and 3 mm of the root tip was cut with a scalpel. The cut root of the seedling was then dipped through the bacterial lawn and was directly transferred to square plates containing Fahraeus medium (see section 2.2.1). Only 2/3 of the plates were filled with medium, so that plant shoots had space to develop in the upper section of the plate. 7 plants were transferred on each plate. The sealed plates were cultivated in a climate chamber for 1 week (16 h light period, 90 µM light intensity, 20°C day temperature, 17°C night temperature). Afterwards plants were transferred to a climate chamber (with 24°C day and 20°C night temperature) for 3 weeks. Two weeks after seedling transfer, the plates were watered with 5 ml sterile H_2O.

The root transformation leading to chimeric plants is a transient transformation event and is not inheritable to the next generation. In addition the transformation efficiency is different in every transformation event, due to a different number of integrated T-DNAs of the binary vector in the transformed tissue. Therefore the roots were screened 3-4 weeks after transformation (plants were slowly growing on plates during this time) with help of the dsRed expression, screened on a fluorescence- stereo microscope (LEICA MZIII-Fluoreszenzmikroskop). The red fluorescence coming from the dsRed protein was activated with the wavelength of 583 nm. All roots that did not show strong red fluorescent were removed and the plants were transferred to expanded clay. After 1 week on expanded clay, roots were screened again for red fluorescence, non transformed roots were removed and plants were transferred to the final substrate (expanded clay), if necessary containing inoculum of the corresponding microorganism. During the final harvest, a 1 cm cross section from the now well developed root system was harvested for another transformation efficiency screen and other microscopic analysis, while the top and the bottom part of the roots were frozen immediately for RNA isolation followed by qRT-PCR analysis. This 1 cm of root was classified according to their fluorescence intensity into 5 categories: no, weak, middle, strong and very strong fluorescence. For further analysis, only root systems with a strong or very strong red fluorescence, indicating a high transformation efficiency, were chosen for further analysis. As reference plants for the RNAi analysis, plants were transformed with an empty vector (EV), not containing any target gene sequence, so that any effect induced by the transformation process could be excluded for later data analysis.

2.5.6 Transformation of *N. benthamiana* mediated by *Agr. tumefaciens*

Transformed *Agr. tumefaciens*, carrying the needed construct were plated on LB medium containing Gentamycine and Rifampicin for 2 days at 28°C. Clones were tested for transformation success by colony PCR. Positive transformants were cultivated in 20 ml LB medium at 28°C for 24 h and 180 rpm. The OD_{600} was measured and the needed amount of overnight liquid culture per infiltration was calculated as follows: $\mathrm{OD}_{\mathrm{target}} \times \mathrm{V}_{\mathrm{target}} = \mathrm{OD}_{\mathrm{actual}} \times \mathrm{V}_{\mathrm{culture}}$; with $\mathrm{OD}_{\mathrm{target}} = 0.5$ and $\mathrm{V}_{\mathrm{target}} = 20\mathrm{ml}$

The appropriate amount of overnight culture was centrifuged at 4000 rpm at 4°C for 30 min. The supernatant was removed and the pellet was solved in $1/4$ vol fresh LB medium and then

$1/2$ vol 2 x infiltration medium (2 x infiltration medium: 10% (w/v) sucrose; 20 mM glucose); 8.6 g/l MS- Medium; pH 5.6) were added. H_2O_{dest} was added until V_{target} was reached. The cell suspension was infiltrated on the bottom side of *N. benthamiana* leaves with a 1 ml syringe. Infiltration areas were marked and leaves were analyzed 3-5 days after infiltration.

2.6 Metabolite analysis

2.6.1 Gas chromatography–mass spectrometry (GC-MS) for volatile analysis

Stir bar sorptive extraction (SBSE) devices (GERSTEL-Twisters™, Polydimethylsiloxane phase (PDMS), Gerstel GmbH & Co.KG, Germany) were used to simultaneously collect volatiles emitted from the roots of each treatment. The sampling time was 60 min. The volatiles were analyzed with an Agilent 6890 Series gas chromatograph and an Agilent 5973 Network mass detector controlled by a GC-MSD Chemstation (G 1701EA E.02.02.1431). The gas chromatograph was equipped with a BP5MS column (30 m x 250 µm i.d., 0.25 µm; SGE Analytical Sciences). The GC-MS was operated with the following oven temperature program: 40°C for 3 min, increased of 2°C/min until 60°C, hold for 2 min isothermally, increase of 3°C/min until 180°C, hold for 10 min isothermally. The carrier gas used was helium and was maintained at constant flow rate of (1.2 ml/min) with a Gerstel MPS 2 (Multiple Purpose Sampler) injection system. The cryofocussing program started at -100°C then the temperature increased of 12°C/min to 280°C then, maintained at 280°C for 3 min. Twisters™ desorption was performed with a Gerstel Thermal Desorption Unit (TDU) with the following temperature program (starting temperature, 25°C; increase of 100°C/min until 260°C, held for 4 min at 250°C). The MS analysis was carried out in a full-scan mode, with a scan range of m/z 50-300. The electron impact ionization energy was 70 eV for all measurements. The source temperature was set to 230°C and the quadrupole temperature to 150°C. The raw data from the GC-analysis were deconvoluted and processed by Mass Profiler Professional (MPP; Version 12.1, Agilent Technologies) using the following settings: 70 % abundance/treatment, Oneway ANOVA, p = 0.05 %, fold change $\geq$ 2. Compounds with significant different concentrations between the sample groups were identified by comparing the mass spectra with the Wiley 6.L and Nist 98.L libraries. When possible identification was confirmed by authentic reference compounds. The identity of the following substances was verified with help of standard compounds: Hexanal, Nerolidol, Limonene, ß-Pinene.

2.6.2 Non-targeted metabolite profiling of root exudates with Liquid chromatography–mass spectrometry (LC-MS)

All solutions and solvents used for the sample preparation and analysis were of LC/MS- grade quality (CHROMASOLV; Fluka).

Exudate extraction with SPE columns Root exudates from 2-3 plants were collected in small volumes of water (30 ml). The collected root exudates were directly transferred to C18 columns (Agilent, Bond eluet-C18, 500MG 3ML). Before, the columns were conditioned with 1 ml 100 % MeOH and washed with 1 ml 2 % fumaric acid in H_2O. After enriching the columns with exudates, they were washed with 1 ml H_2O. The exudates were eluted from the column with 1 ml 1 % fumaric acid and were collected in a glass vial (Agilent, Strg vial Kit, 4ml). The eluate was evaporated in a speed vacuum drier. Dried samples were resolved in 200 µl 80 % MeOH by vortexing and 10 min of ultrasonic treatment. The resolved samples were transferred to 1.5 ml Eppendorf tubes and centrifuged for 5 min at 12000 rpm. The supernatant was carefully taken up and filled in LC-MS vials for measurement.

Ultraperformance Liquid Chromatography (UPLC)/ ESI-Quadrupole Time-of-Flight (QTOF) MS Analysis Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with a HSS T3 column (100 mm $\times$ 1 mm, particle size 1,7 µm, Waters) applying the following gradient at a flow rate of 150 µL/min and a column temperature of 40 °C: 0-1 min, isocratic 95 % A (water/formic acid, 99.9/0.1 (v/v)), 5 % B (acetonitrile/formic acid, 99.9/0.1 (v/v)); 1-16 min, linear from 5 to 95 % B; 16-18 min, isocratic 95 % B; 18-20 min, isocratic 5 % B. The injection volume was 2 µL. Eluted compounds were detected at a spectra rate of 3 Hz from m/z 100-1300 using a MicrOTOF-Q-I hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source with a capillary tension of 5 kV in the positiv and 4,5 kV in the negative mode. (Dry gas flow had a rate of 6 l/min at 190 °C). An automatic recalibration against LiHCOO cluster was performed. The raw data analysis was done with the software DataAnalysis (Bruker) V4.0.
The raw data was processed with the XCMS package [Smith et al., 2006]. For feature detection, the XCMS *centWave* [Tautenhahn et al., 2008] algorithm with the following parameters was applied: snthresh = 3, ppm = 20, peakwidth = (5,12). The feature alignment was performed with the standard *group.density* algorithm from XCMS with bw = 3 and mzwid = 0.5.
For MS-MS analysis, the gradient mentioned above was applied with a collision energy of 10 eV and argon used as collision gas. Structure elucidation was only done for signals that were statistically significant. Subtraction of the background signals allowed to reduce the noise significantly. Selected m/z values were extracted as EIC (extracted ion count) in a given Rt (retention time) window. If EIC traces matched perfectly it was assumed that those features belong to the same metabolite forming either adducts or fragments. Known mass differences of adducts and fragments with an error of $\prec$ 5 mDa gave hints about the chemical structure for the

molecule. The vendor specific data analysis software was used to generate sum formula suggestions for fragments and neutral losses to narrow down the number of possible compounds. The mass spectra patterns of hypothesized metabolites were also compared with MassBank entries for additional evidence.

2.7 Different sample processing of root exudates for LC-MS analysis

To test the effect of sample processing on the recovery of exudates after C-18 column purification described in paragraph 2.6.2, a standard solution with ten different substances was prepared including Quercetin, Abscisic acid, Jasmonic acid, Adenosine, Scopoletin, Indole-3-Carboxylic Acid, Glutathione, Ferulic Acid, Indole acetic acid and Phytochelatin. A volume of 200 µl with 50 µM of each substance was prepared. This standard solution was diluted to 30 ml (3 times) and than processed in three different ways:

1. Evaporation of the samples with a rotary evaporator and resolving in 5 ml H_2O dest.
2. Samples were lyophilized and resolved in 5 ml H_2O dest..
3. The complete sample volume was directly applied on the column.
4. Samples were measured without column purification. The 200 µl standard solution was not diluted to 30 ml and not processed, but analyzed directly as positive control.

After column purification, samples were resolved in a volume of 200 µl, which was measured by LC-MS.

The method of direct sample application on the column without evaporation or lyophilization (3) gave the best outcome for most of the substances. Only phytochelatin and ferulic acid had a slightly lower absorption than in the pre-evaporated samples (Fig. 13). For subsequent experiments, samples were directly loaded on the column for LC/MS preparation.

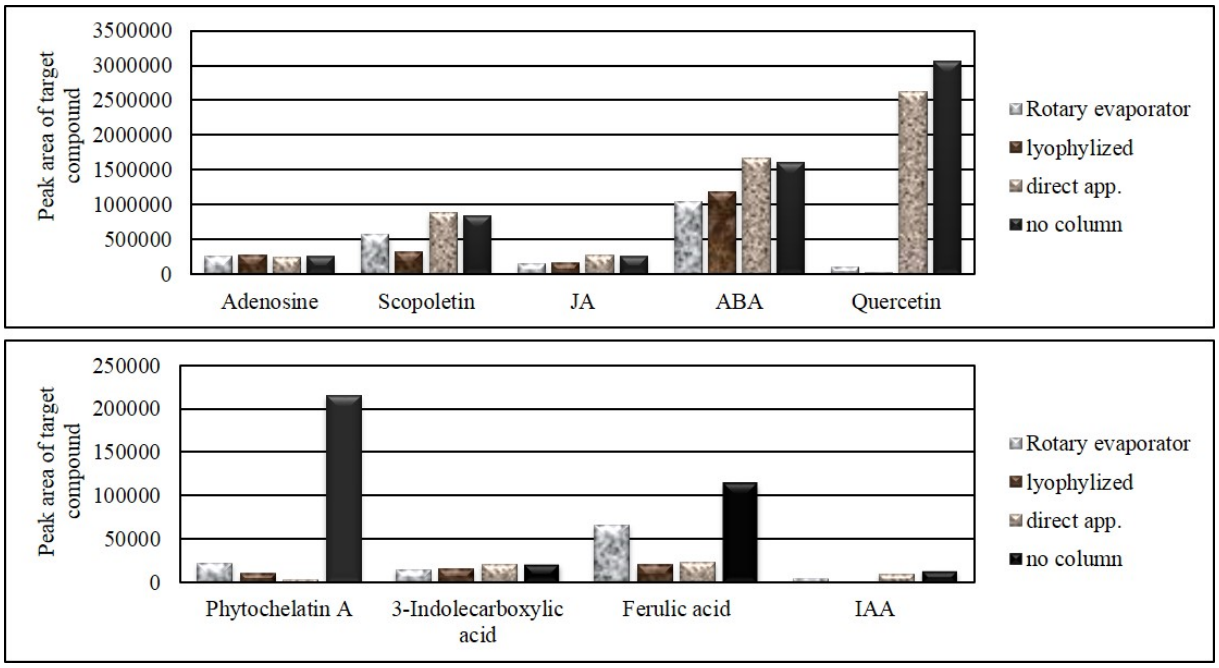

Figure 13: Recovery of ten different substances processed in four ways for LC-MS measurement.

3 Results

3.1 Evaluation of 33 *M. truncatula* accessions for mycorrhization capacity

32 accessions of the SARDI core collection based on a world wide *M. truncatula* collection of 346 inbred lines were tested on differential symbiotic activity in regard to AMF. Plants were inoculated with AMF as described in section 2.2.4. Three different harvesting time points (21, 35 and 50 dpi (days post infection)) were tested to identify the stage of symbiotic interaction with the highest differences between *M. truncatula* accessions of the core collection. Three biological replicates per accession and time point were analyzed by quantification of mycorrhization intensity of root tissue. Fungal structures in the roots were stained with ink (see section 2.3.1) and quantified with the method described in section 2.3.2.

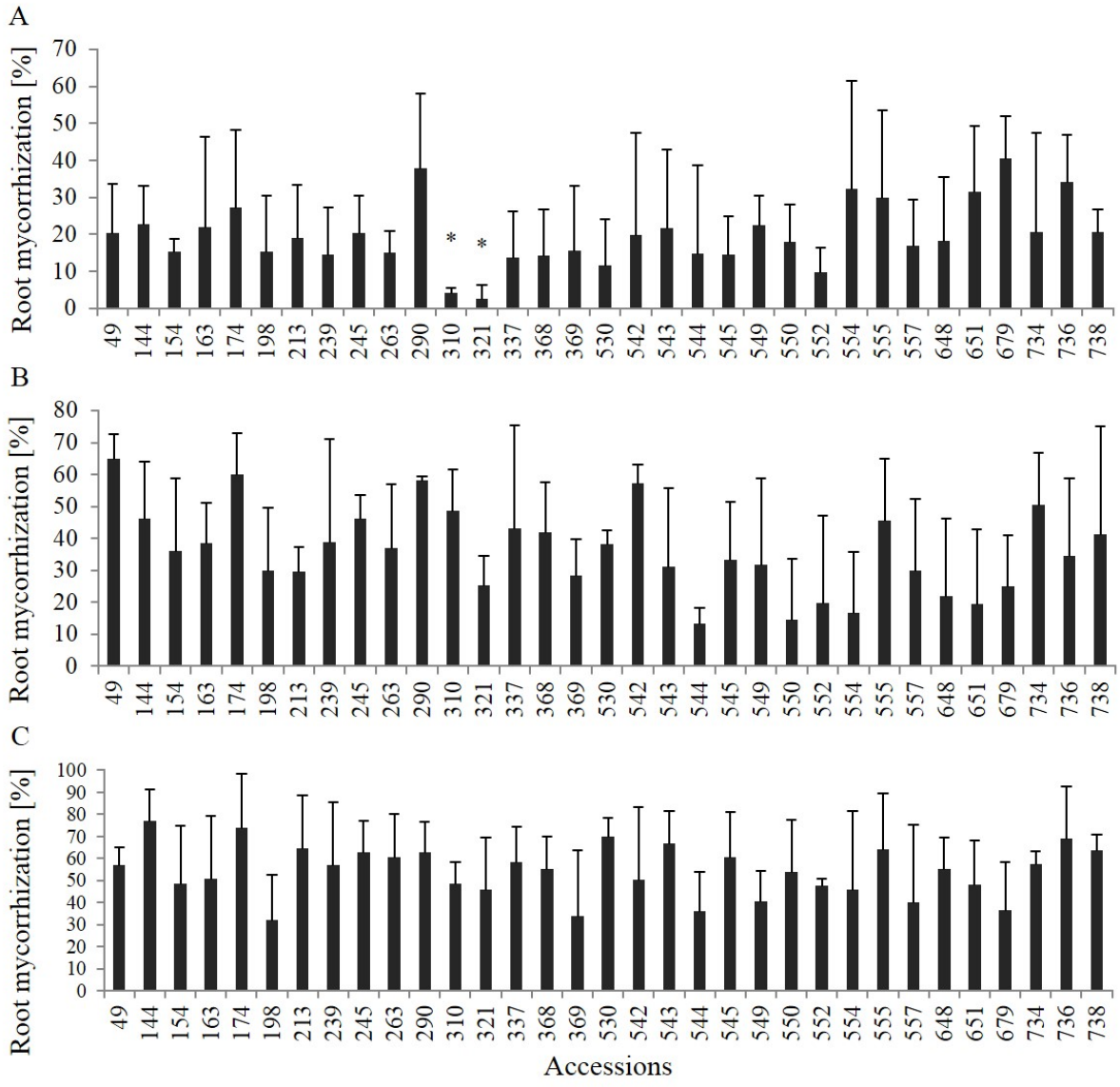

Figure 14: Root mycorrhization of 32 accessions, grown on *G. intraradices* inoculated expanded clay. (A) 21 dpi; (B) 35 dpi; (C) 50 dpi. Bars indicate means $\pm$SD, n $= 3$. Significant differences were calculated between the reference line 738 (A17) and all other ecotypes with a Student's t-test (* p ≤ 0.05)

At 21 dpi, there was a 15 fold difference between the weakest (2.7 %, accession 321) and the strongest (40 %, accession 679) colonization (Fig. 14 A). At 35 dpi, there was a 5 fold difference between the weakest (13 %, accession 544) and the strongest (65 %, accession 49) colonization (Fig. 14 B). At 50 dpi there was only a 2.5 fold difference in colonization between the weakest (32 %, accession 198) and strongest (77 %, accession 144) colonization (Fig. 14 C). Thus the strongest differences in colonization rate could be observed in the earliest time point. Compared to the reference genotype A17 (line 738), only line 310 and 321 showed a significant difference in colonization rate, yet also solely in the earliest time point.

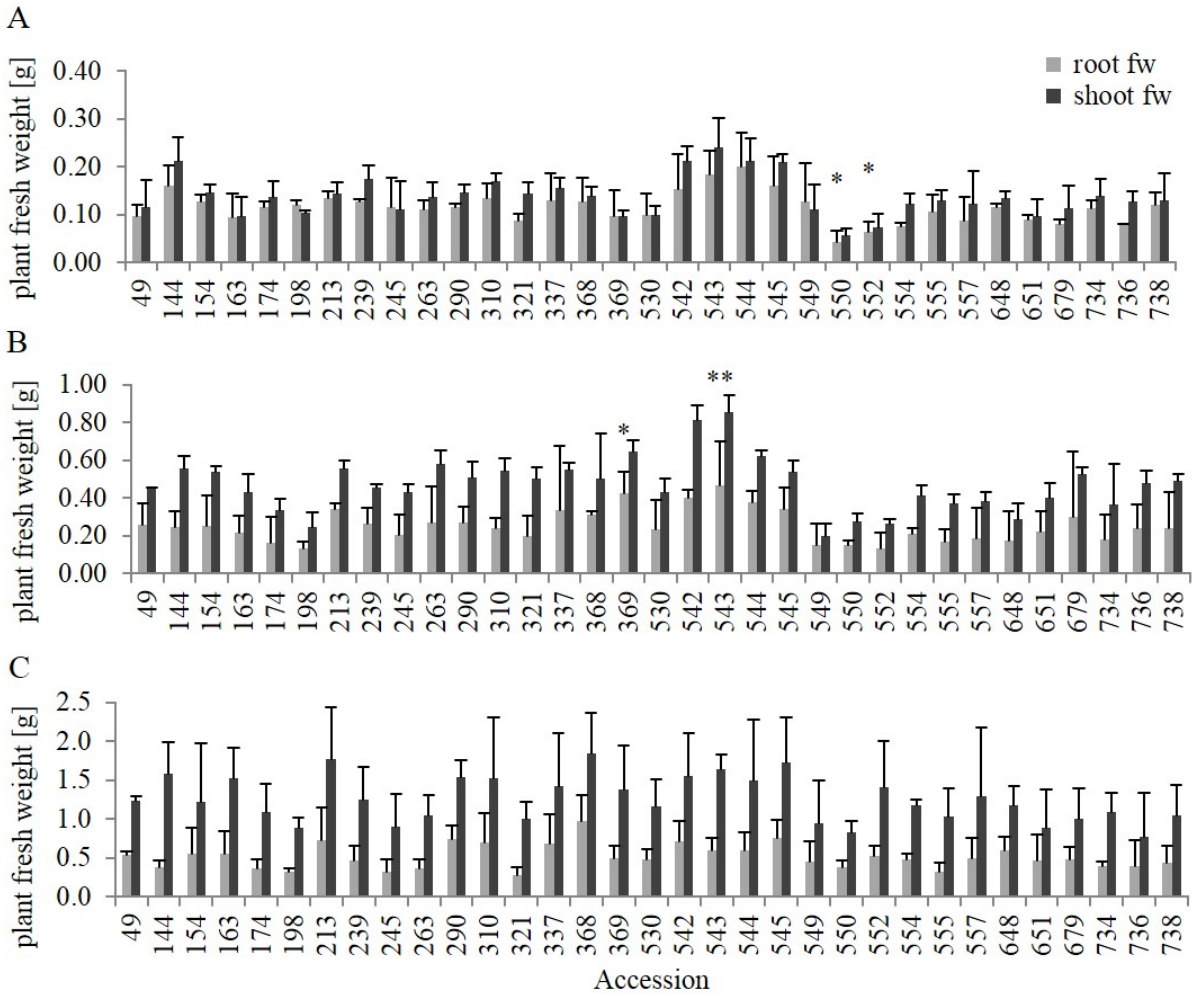

Figure 15: Fresh weight of roots and shoots of 32 accessions, grown on *G. intraradices* inoculated expanded clay. (A) harvested 21 dpi; (B) harvested 35 dpi; (C) harvested 50 dpi. Bars indicate means $\pm$ SD, n = 3 . Significant difference were calculated between the reference line 738 (A17) and all other ecotypes with a Student's t-test (** $p \leq 0.01$, * $p \leq 0.05$).

Fresh weight of roots and shoots were measured to investigate correlations between biomass and mycorrhization rate (Fig. 15). In the first time point, root and shoot biomass within the lines showed a similar level, while the shoot biomass increased stronger then the root biomass towards the later harvesting time points in all ecoptypes. Yet the overall development of shoot and root biomass within one accession compared to the other accessions was rather consistent at the different plant ages. Lines 550 and 552 showed a significantly lower root biomass than

the reference line A17 in the earliest time point. At the second harvesting time point the two lines 369 and 543 showed a higher root fw compared to the reference line. No correlation between mycorrhization rates and fw development could be determined, showing that the ecotype influenced the fw stronger than the stimulating effect on biomass production by AM.
Due to the high standard deviation obtained (Fig. 14), the experiment was repeated. Plants were precultivated for one week on mycorrhiza free expanded clay to select seedlings with a high developmental similarity. Due to this precultivation, the colonization process was faster and plants could be harvested 16 dpi. Seven biological replicates were cultivated to possibly reduce the standard deviation. In this trial, the different ecoptypical mycorrhization patterns observed in the first experiment could not be reproduces. Some lines showed significantly lower and some lines a significantly higher AMF colonization compared to the reference line. Yet the two lines that were found to be significantly differently colonized (310 + 321) did not appear as such in this experiment. (Fig. 16 A). There was again no correlation between mycorrhization intensities and fresh weight (Fig. 16 B). The non-reproducibility of mycorrhization patterns between the

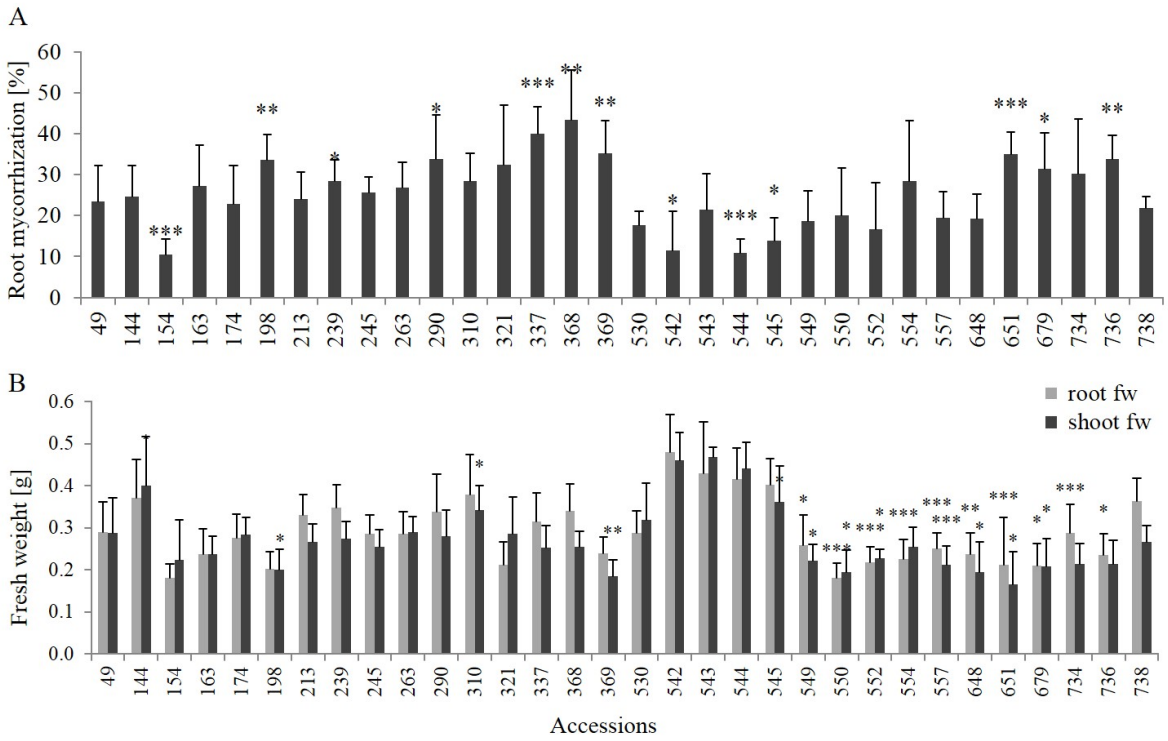

Figure 16: A: Root mycorrhization B: Fresh weight of roots and shoots. The 32 accessions were grown on *G. intraradices* inoculated expanded clay for 16 days. Bars indicate means ± SD, n = 7. Significant difference were calculated between the reference line 738 (A17) and all other ecotypes with a Student's t-test (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$)

two trials led to the hypothesis that the highly effective inoculum of *G. intraradices* might have superimposed the potentially different influences of ecotypes on symbiotic interaction with AMF. Therefore the trial was repeated with a sandy soil from Großbeeren (GB), Germany, with a much lower spore density and higher AMF diversity compared to the inoculum of trap cultures. It was a sandy soil with 1.3 % organic matter, 4 % of clay in the soil dry weight, 15 mg extractable P/ g soil and a pH of 7.6. The earliest time point with good mycorrhization in this

soil was detected at 35 dpi, since the process of mycorrhization was much slower in this natural soil compared to the *G. intraradices* inoculum. The trial on sandy soil (Fig. 17) gave different results compared to the trials on *R. irregularis* inoculum (Fig. 14, 16). The accession 239 had the lowest and accession 543 the highest root colonization of 0.4 % and 18.3 % respectively but compared to the reference line A17 (accession 738), there were no significant differences due to a very high variation between the biological replicates. Due to this variability in colonization between the biological replicates and the fact that the results were different to the first trials, no line with promising outstanding phenotype could be identified and the planned subsequent analysis of root exudates was not done.

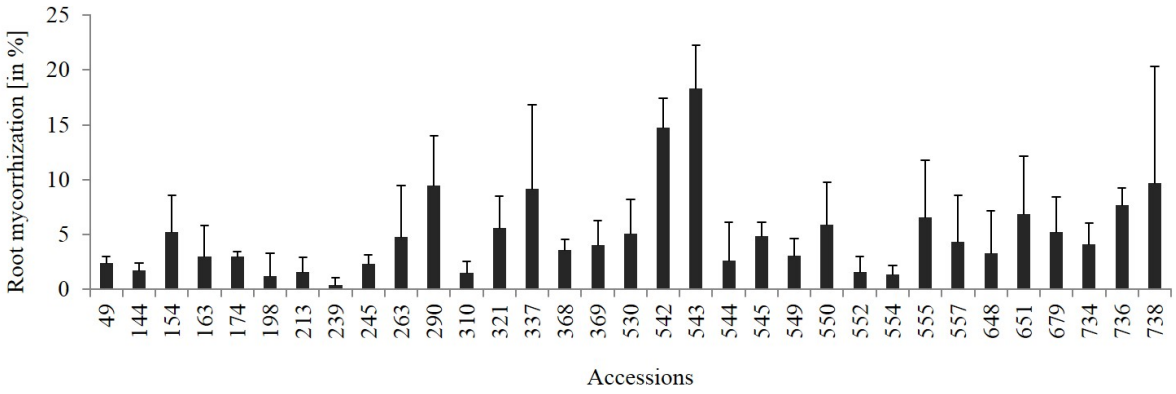

Figure 17: Root mycorrhization of 32 accessions, grown on sandy soil from GB for 35 days. Bars indicate means $\pm$ SD, n = 3. Significant difference were calculated between the reference line 738 (A17) and all other ecotypes with a Student's t-test (* $p \leq 0.05$)

With those highly variable AMF colonization rates in the different *M. truncatula* accessions, it was not promising to further investigate the composition of root exudates, since a clear assignment of any specific metabolites to a mycorrhizal phenotype would not have been possible.
In the publication of Schultz et al (2009), eight lines of the SARDI *M. truncatula* collection were analyzed in a similar manner. They selected different lines of the collection than in this work, yet two lines (530 referred to as F83005 and 736 referred to as DZA045 in Schultz et al.) were overlapping in our studies. Schultz et al. analyzed the root architecture, AMF root colonization, shoot/ root biomass and the plant tissue concentration of diverse nutrients [Schultz et al., 2010]. They could determine significant differences of AMF root colonization between the lines. Line F83005 (530) that they found to have the strongest colonization did not appear as strongly colonized compared to A17 in any of our experiments, independent of the colonization time or the inoculum used. It would be interesting to know if the differences in AFM colonization between the lines could be reproduced in independent experiments. As also observed in the present study, Schultz et al. found no effect of AM colonization on shoot biomass (the root biomass was not clearly determined in their study). In the present study it appeared once more that the process of AMF root colonization is generally variable between individual plants, so that differences between lines will be hard to identify. We suggest that in future, this experiment could be performed assessing the infection phenotype of the different lines with *A. euteiches* and see if infection rates within one line appear more consistent.

3.2 Timing of earliest transcriptional events after AMF contact

As a new project, it was decided to work with the *M. truncatula* reference line A17 and analyze its very early reaction towards different types of microorganisms. *A. euteiches*, a pathogenic oomycete for legumes was selected as counterpart to the symbiotic microorganism of *R. irregularis*. In a first step it should be analyzed when the earliest transcriptional responses of plants towards that microbial contact will be measurable.

3.2.1 Gene expression of known early induced genes

For the preparation of a transcriptome analysis that should elucidate the earliest transcriptional responses of *M. truncatula* roots towards microbial challenge, five early marker genes were selected that are known to be induced shortly after the interaction of *M. truncatula* roots with AMF: *MtENOD11* (AJ297721.1) [Boisson-Dernier et al., 2005], *Cellulose synthase* (*Celsynt*, EC366238), *Expansin related protein 1 precurser* (*MtExplike*, EC366239), *Nodulin-like protein* (*MtNodlike*, EC366236) and *K07C11.4 protein* (*MtK07C11*, EC366248) [Siciliano et al., 2007]. The transcript accumulation was quantified relative to the constitutively expressed reference gene: *M. truncatula Translation elongation factor 1 alpha* (*MtEF1*α). The cultivation system on plate was adapted to the method of Czaja et al., 2012 as described in section 2.2.4

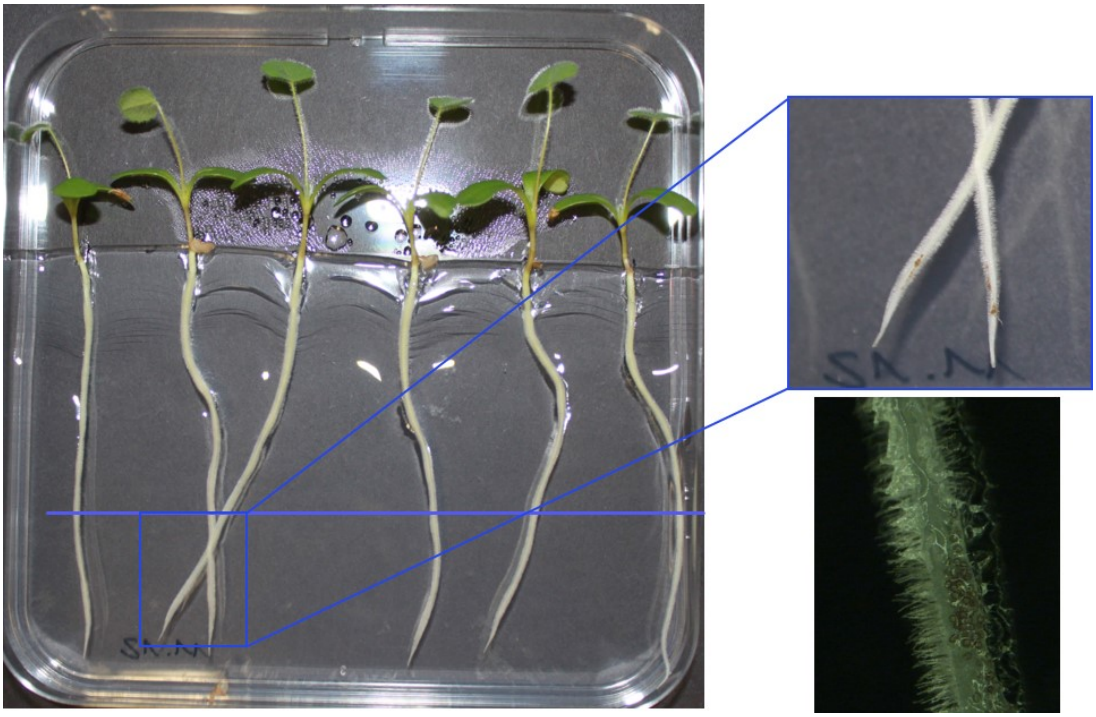

Figure 18: Cultivation and treatment of *M. truncatula* with spores of *R. irregularis* on plates.

30-40 AMF spores were applied on one week old seedlings. Five harvesting time points after inoculation with *R. irregularis* were chosen: 0 (con), 6, 12, 18 and 24 hrs. Spores were applied at different time points, so that the harvesting could take place simultaneously for all samples to exclude diurnal transcriptional changes. For each time point three plates with six plants per plate to be pooled were inoculated by pipetting spores to the bottom two cm of the roots as depicted

(Fig. 18). Inoculated root segments were harvested and frozen in liquid N_2 immediately. RNA was isolated and the transcript accumulation of the five above mentioned genes was quantified with qRT-PCR (Fig. 19). Three of the genes, *MtENOD11*, *MtCelsynt* and *MtExplike* showed a tendency of increasing transcript accumulation over time. *MtNodlike* and *MtK07C11* showed an increase after six hrs, followed by a decrease over time. For further experiments it was an important finding that there was a 1.97-fold change and a 1.43-fold change of transcript accumulation in *MtNodlike* and *MtK07C11* respectively after six hours compared to the control, even though the values were not significantly different. It was suspected genes that were even earlier induced than the selected marker genes. Therefore, the setup for a first test array included the time points 2, 6 and 10 hrs past inoculation.

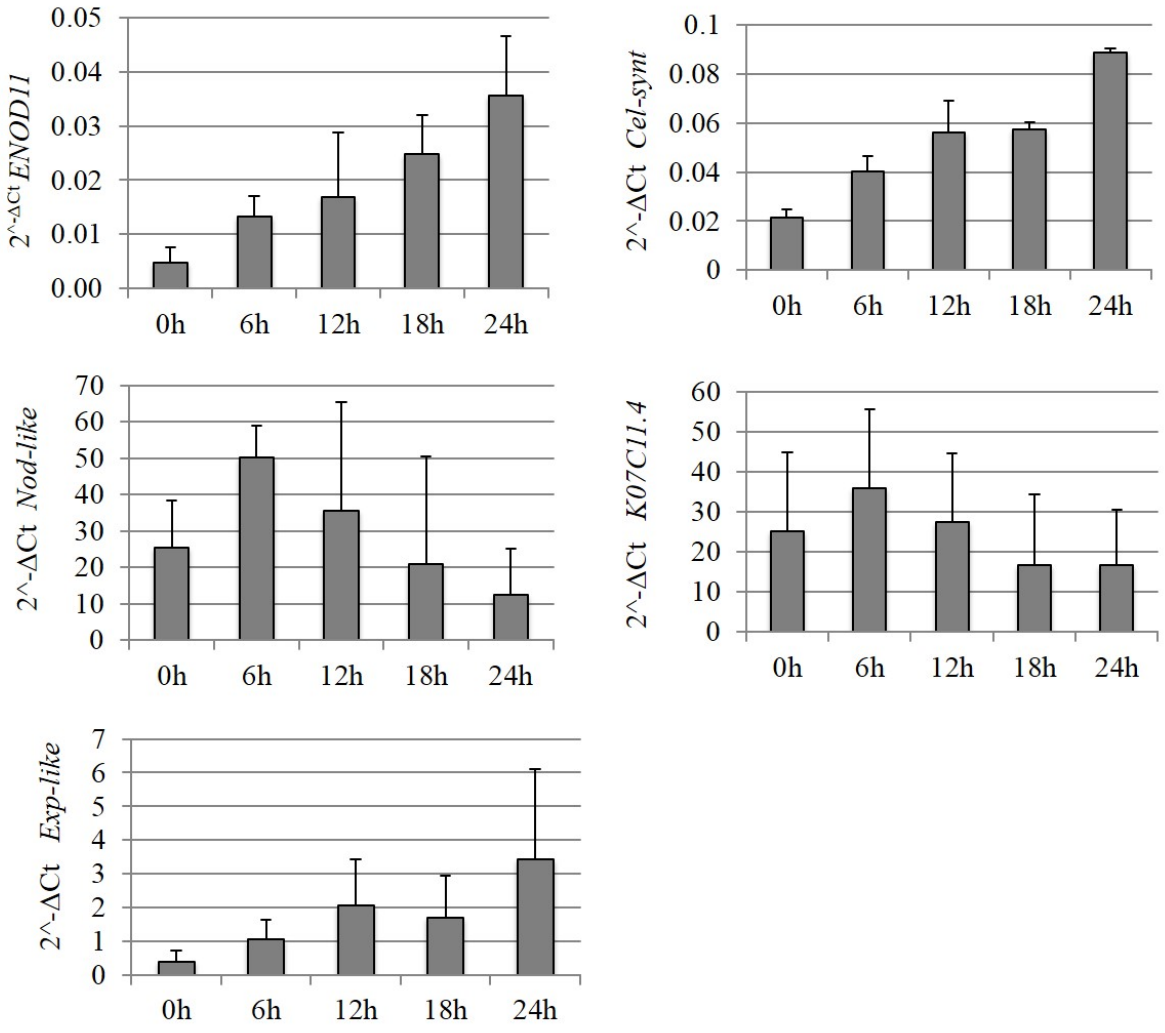

Figure 19: Transcript accumulation of 5 *M. truncatula* marker genes for early response towards AMF contact relative to the reference gene *MtEF1α*, as determined by quantitative qRT-PCR in roots. Plants were grown for one week on MSR medium on plate, treated with *R. irregularis* spores for 0, 6, 12, 18 and 24 hrs. Bars represent mean values ± SD; n = 3, p-values were calculated with a Student´s t-test, for single time points vs. control (0 hrs) (* $p \leq 0.05$).

3.2.2 Test array at 3 different time points after inoculation

To further specify the best suitable time point for a transcriptome analysis, a test microarray was performed. Plants were cultivated and inoculated as described above (Fig. 18). AMF spores were incubated for 0 (con), 2 , 6 and 10 hrs. Six plants were pooled as one sample. The microarray was done with a Medicago GeneChip from Affymetrix. The data shown in Fig. 20 are based on a single biological replicate, for providing an orientation about the strength of transcriptional regulation at different time points. As shown in the venn diagram, 424 genes were differentially regulated 2 hrs after inoculation, 318 and 225 genes were differentially regulated 6 and 10 hrs after inoculation respectively. 80 genes were regulated only after 2 and 6 hrs, 36 genes were

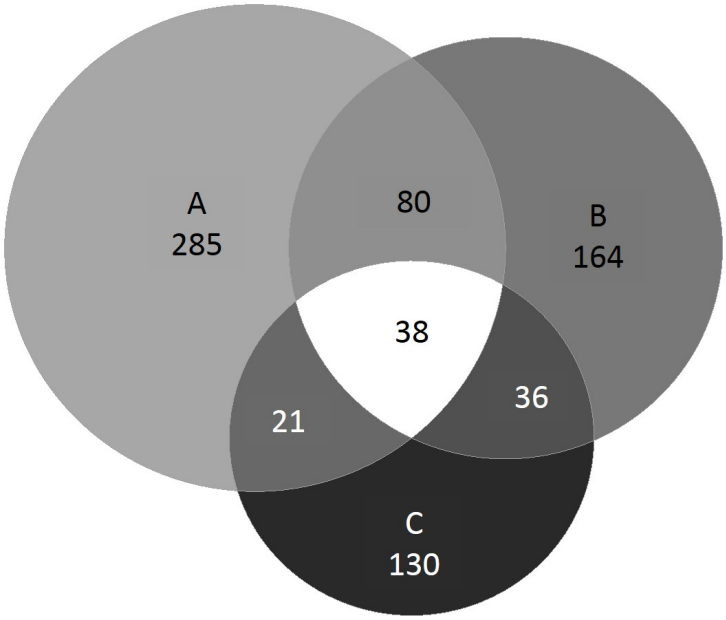

Figure 20: Venn diagram showing number of genes differentially expressed in roots after AMF inoculation at 3 incubation times: A: 2 vs 0 hrs; B: 6 vs 0 hrs; C: 10 vs 0 hrs. Numbers represent the number of genes differentially expressed by ¿ 2fold compared to the control. No replicates were done

regulated only after 6 and 10 hrs and 21 genes were regulated only after 2 and 10 hrs, while 38 genes were regulated at all three time points. Based on these results, further investigation of the early reaction of *M. truncatula* roots on transcriptional level was done at 2 hrs after contact with *R. irregularis* spores or *A. euteiches* zoospores.

3.3 Transcriptional changes 2 hrs after microbial challenge

3.3.1 Microarray analysis

After having selected the most suitable time point to observe the earliest transcriptional responses of the plant roots towards different microbial challenges, a microarray trial with two treatments (30-40 spores of *R. irregularis* per root or about 100 zoospores of *A. euteiches* per root) plus control (mock-treatment with water) for 2 hrs with four replicates each was performed. Plants were cultivated on plates as described in paragraph 2.2.4. Only the bottom 2-3 cm of the roots were treated with spores and harvested for RNA isolation, since the strongest reaction was expected in the youngest tissue. The quality and integrity of the isolated RNA was tested (Fig. 24) and only samples with a RIN number above 8.5 were used for the transcriptome analysis. The microarray was done with the Medicago GeneChips from Affymetrix as described in paragraph 2.4.8.

To identify a differential expression of genes in the treatments versus the control, a robust multichip average normalization based on the Student's t-test was used. Transcripts were defined as being differently regulated with a fold change (FC) of >2 or $<$-2 and a p-value ≤ 0.05.

According to those criteria, 56 transcripts were identified being regulated by the *R. irregularis* treatment, 257 transcripts were regulated by the *A. euteiches* treatment, with 21 genes being regulated by both treatments (Fig. 21). Red numbers indicate the number of transcripts that were up-regulated and blue numbers indicate the number of transcripts that were down-regulated compared to the control. Transcripts that are exclusively regulated by the treatment with *R. irregularis* were mostly up-regulated (34/1), while the treatment with *A. euteiches* resulted in only about twice as many genes up- than down-regulated (159/ 86). Transcripts that were regulated by both treatments included 12 upregulated and 9 down-regulated ones.

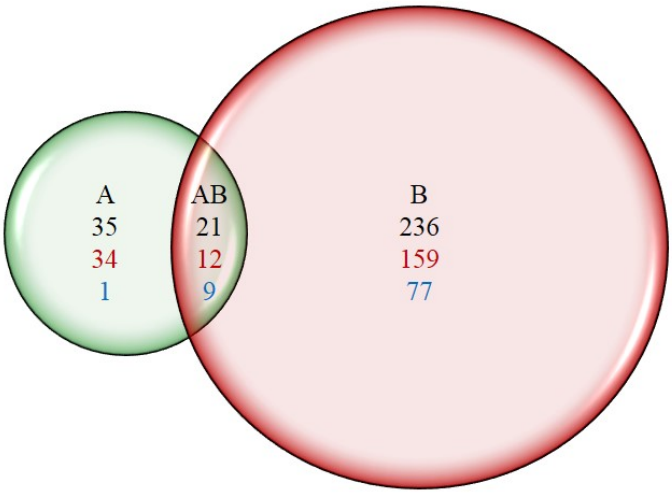

Figure 21: Venn diagram of transcriptional data from *M. truncatula* plants grown on plate and inoculated for 2 hrs with *R. irregularis* (A) or *A. euteiches* (B). Black numbers indicate total count of regulated genes, red numbers indicate up-regulated genes, blue numbers indicate down-regulated genes.

Genes were classified according to their putative functions as shown in Fig. 22.

In the ***R. irregularis*** treated roots (Fig. 22 A) the three most important categories were the ”transcriptional regulation”, ”defense” and ”energy/ sugar metabolism”, with seven transcripts each, being regulated 2 hrs after spore contact. The seven genes of the ”transcriptional regulation” comprise seven transcription factors (TFs), whereof five were up-regulated and the only two pathogen- related transcription factors were down-regulated. The ”defense” category was mainly represented by proteinase inhibitors. Concerning the energy metabolisms, there was an up-regulated glycoside hydrolase and two down-regulated glucosyl transferases. The five ”signaling” genes contain EF-hand calcium-binding proteins, a protein kinase and two proteins, responsible for protein-protein interaction, were all up-regulated after contact to AMF spores. Other genes that were regulated are involved in the protein metabolism, in hormonal regulation and in transport processes.

In the ***A. euteiches*** treated roots (Fig. 22 B), the largest group of transcripts with a known putative function was the group of ”defense related proteins” (32), mainly represented by induced proteinase inhibitors such as kunitz inhibitors. Some proteins involved in the reaction towards oxidative stress and some pathogen related (PR) genes were also present in this group of regulated genes. 23 ”kinases/ phosphatases” were regulated upon *A. euteiches* treatment, probably involved in signaling reaction and regulatory processes. 22 transcripts responsible for ”transcriptional regulation” were found, followed by 18 transcripts related to secondary metabolism including mainly cytochrome P450 E-class genes, that can be involved in biosynthesis of several compounds such as hormones, defensive compounds and fatty acids. 16 transcripts were regulated in the category of ”protein/ lipid-metabolism”. 15 transferases were differentially expressed and 14 transcripts related to transport processes, such as vesicle transport, metal transport, amino acid transport, sulphate and ammonium transport were differentially regulated. Nine transcripts involved in the sugar metabolism or the energy transfer were regulated. Five transcripts, mainly involved in auxin regulation and four transcripts related to signaling were differentially expressed after *A. euteiches* treatment.

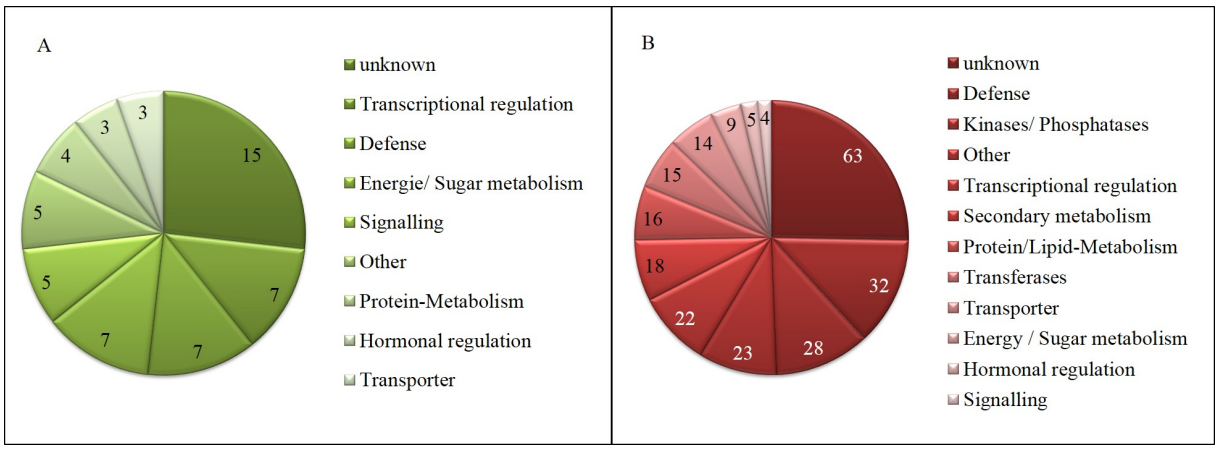

Figure 22: Classification of genes differentially regulated with a FC of >2 or <-2 and p $\leq$ 0.05. A: Genes differentially expressed after treatment with *R. irregularis*; B: Genes differentially expressed after treatment with *A. euteiches*

In Fig. 23 all significantly regulated genes are clustered in 6 groups. Groups A, B and C correspond to the treatment, up-regulated genes are marked in red and down-regulated genes are marked in blue. It is very striking that all genes that are regulated in one direction by one treatment are regulated in a similar direction in the other treatment. There is no gene that is clearly up-regulated in one treatment and down-regulated in the other treatment.

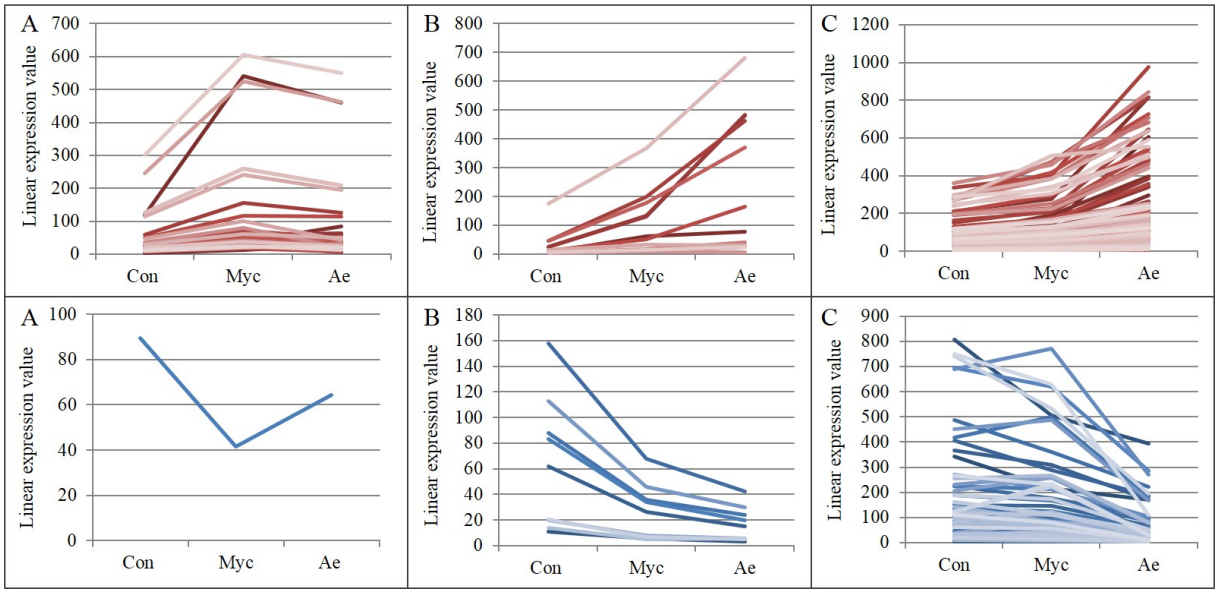

Figure 23: Expression-pattern of regulated genes by microbial treatments for 2 hrs. A: genes significantly regulated by *R. irregularis*; B: genes significantly regulated in both treatments; C: genes significantly regulated by *A. euteiches*. Up-regulated genes are marked in red and down-regulated genes are marked in blue.

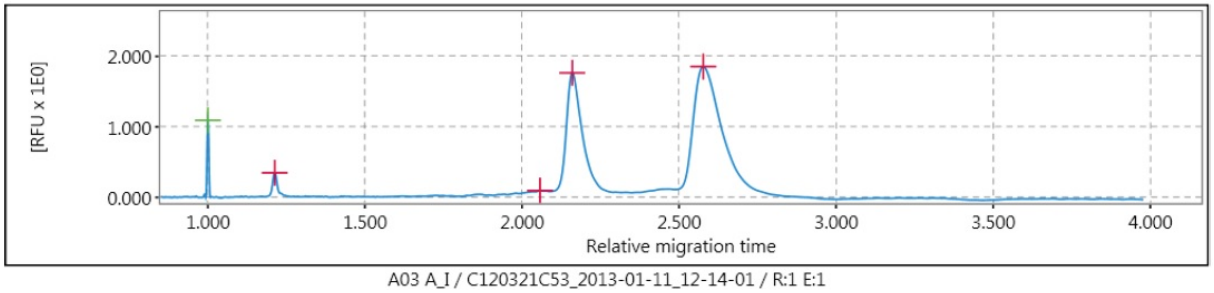

Figure 24: Example of RNA quality control done with the QIAxcel Advanced System of Qiagen of one sample showing a RIN <8.5

3.3.2 Validation of some interesting genes via qRT-PCR

In order to characterize some of the regulated genes, seven of them were selected to be quantified via qRT-PCR. The names and gene identification numbers including their putative function were derived from the NCBI databank (abbreviations are given in square brackets):

M. truncatula Kunitz-type trypsin inhibitor alpha chain	(MTR_6g065460) [*KI*]
M. truncatula AP2 domain-containing transcription factor	(MTR_5g058470) [*PR-TF*]
M. truncatula β*-glucan-binding protein*	(MTR_1g079320) [*ß-GBP*]
M. truncatula (+)-delta-cadinene synthase isozyme C2	(MTR_5g073200) [*TS*]
M. truncatula LysM receptor kinase	(MTR_5g086660) [*LysMI*]
M. truncatula LysM receptor kinase	(MTR_5g086310) [*LysMII*]
M. truncatula Kinase R-like protein	(MTR_7g082510) [*KIN*]

To verify the gene expression, an independent experiment was performed. The same system as for the generation of the array was used, but this time several more time points after spore application were included to see whether the selected genes have a specific early reaction or remain up-regulated within the first 24 hrs. The harvesting time points of 0.5, 2, 6, 10 and 24 hrs after inoculation were selected. The three genes shown in Fig. 25 (*KI*, *PR-TF* and β-*GBP*), had a similar expression pattern in both treatments in the array, like most of the genes observed as shown in Fig. 23. Since we were mainly interested in the differences of the plant root reaction towards the different microorganism, four other genes were selected that had a specific regulation in only one of the treatments, or even showed a tendency of being inversely regulated in the two treatments. For this purpose even genes from the raw data were selected, that were not quite according to the selected statistical parameters. Those four selected genes were *TS*, *LysMI+II* and *KIN* (Fig. 26).

KI was chosen as candidate gene, since the class of kunitz protease inhibitors have been described to play an important role in development and turnover of arbuscules [Rech et al., 2013]. *KI* showed a 5/19 fold higher expression 2 hrs after treatment with the symbiotic or the pathogenic spores/zoospores, respectively. These results could be reproduced in the independent trial with a 6/17 fold higher expression compared to the control 2 hrs after treatment with the symbiotic or the pathogenic spores/zoospores, respectively. The induced expression by *R. irregularis* spores could only be observed after 2 hrs, while the expression returned to a basic level after 6 hrs. In contrast, the treatment with *A. euteiches* zoospores seemed to have a long-lasting effect on the gene expression. After a peak at 2 hrs the *KI* transcript was continuously reduced till the basic level was reached at 24 hrs after inoculation (Fig. 25).

PR-TF was down-regulated in both treatments by 2.4 and 4.2 fold after 2 hrs, respectively. A similar pattern could be observed in the independent trial in all time points (with the exception of the *A. euteiches* treatment after 10 hrs) with *A. euteiches* inducing a stronger down-regulation in most time points compared to the *R. irregularis* treatment and the overall strongest regulation after 2 hrs (Fig. 25).

A. euteiches contains chitosaccharides, linked to ß-1,6-glucans with a ß-(1,3;1,4)-glucan backbone in their cell wall. Those glucanchitosaccharide fractions of *A. euteiches* are able to induce the expression of defense marker genes in *M. truncatula* seedlings, but yet there is no specific receptor in *M. truncatula* known [Nars et al., 2013]. Therefore, the significantly induced expression (by 1.6-fold) of *ß-GBP* after *A. euteiches* zoospore treatment seemed to be interesting. Due to a later selection of the gene as candidate, material for all time points of the independent trial was scarce and only 2 time points could be analyzed with qRT-PCR. In this independent trial, a very similar expression level compared to the control could be observed after 30 min (increased by 1.9-fold), but not after 2 hrs (Fig. 25).

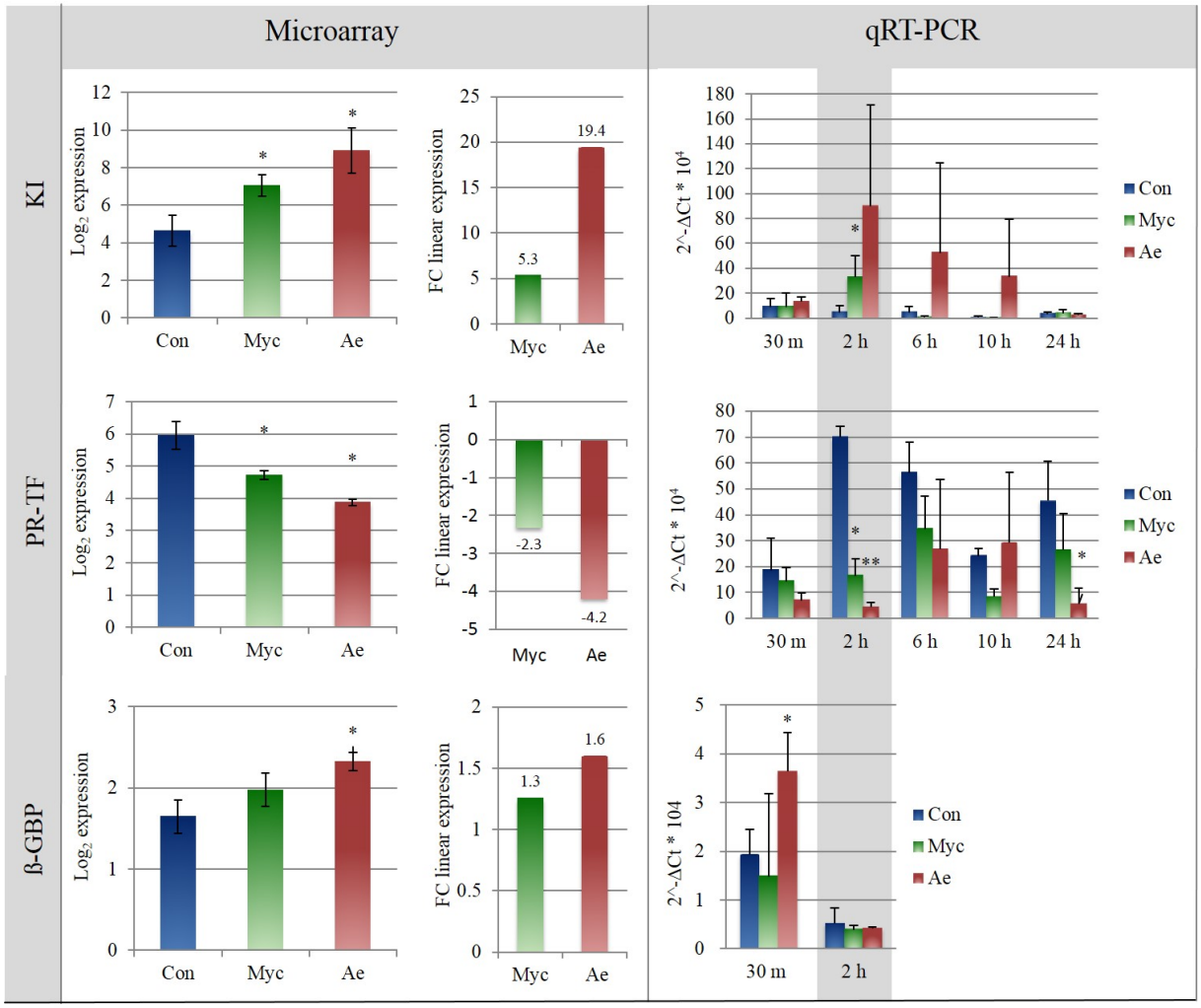

Figure 25: Log_2 expression values of candidate genes according to the transcriptomics (left), the linear fold changes according to the transcriptomics (middle) in *M. truncatula* roots treated with either *R. irregularis* or *A. euteiches* for 2 hrs; Transcript accumulation relative to the reference gene *MtEF1α* generated by qRT-PCR from an independent trial, including 5 time points after inoculation with *R. irregularis* or *A. euteiches* (left). Transcriptomics: n = 4, qRT-PCR: n = 3, Bars represent mean values ± SD; p-values were calculated with a Student´s t-test, for single treatments vs. control (** $p \leq 0.01$, * $p \leq 0.05$).

One of the genes, that showed a very specific response to only one of the treatments (*A. euteiches*) was a *putative ß-cadinene synthase*, which we referred to as *terpene synthase* (*TS*).

Cadinene is a bicyclic sesquiterpene. In *Gossypium arboreum*, a (+)-ß-Cadinene Synthase was described as sesquiterpene cyclase that catalyzes the cyclization of farnesyl diphosphate in the first committed step of the biosynthesis of gossypol, a phytoalexin that defends the plant from bacterial and fungal pathogens [Gennadios et al., 2009]. A (+)-ß-cadinene synthase has not

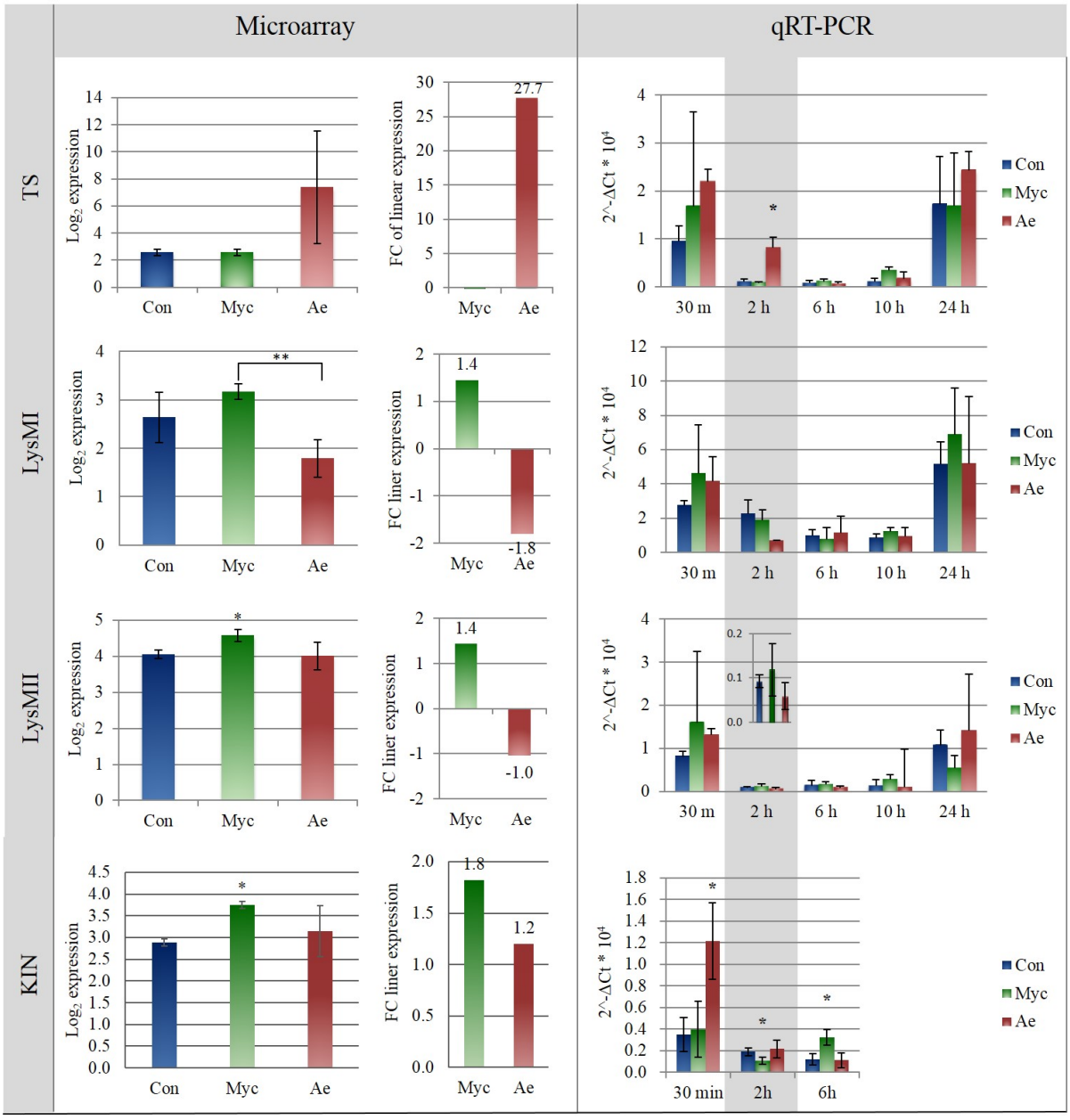

Figure 26: Log_2 expression values of candidate genes according to the transcriptomics (left), the linear fold changes according to the transcriptomics (middle), from *M. truncatula* roots, treated with either *R. irregularis* or *A. euteiches* for 2 hrs; Transcript accumulation relative to the reference gene *MtEF1α* generated by qRT-PCR from an independent trial, including 5 time points after inoculation with *R. irregularis* or *A. euteiches* (left). Transcriptomics: n = 4, qRT-PCR: n = 3, Bars represent mean values ± SD; p-values were calculated with a Student´s t-test, for single treatments vs. control (** $p \leq 0.01$, * $p \leq 0.05$).

been functionally characterized in *M. truncatula* so far. In this transcriptional analysis, a 27 fold induction of the gene 2 hrs after *A. euteiches* treatment could be observed, even though not statistically significant. In the independent trial, a significant up-regulation was found 2 hrs after zoospore treatment (7.2 fold induction), yet this reaction was only temporary and could

not be observed at later time points (Fig. 26).
A special interest was directed to *LysMI/II*. The signaling molecules of AMF are a mixture of sulphated and non-sulphated Myc-LCOs, structurally very similar to the Nod factors produced by rhizobia [Maillet et al., 2011]. While the receptors in *M. truncatula* for the Nod factor perception (MtLyk3/ MtNFP) have already been described [Limpens et al., 2003,Smit et al., 2007], the plant receptors for the perception of Myc-LCOs have not been identified yet. The receptors for the perception of Nod factors are lysin motif containing receptor-like proteins (LysM-RLKs) that lead to the activation of the CSSP (Fig. 7). Due to the high similarity of Myc and Nod-LCOs, a similarity of the receptor domains is assumed. This makes LysM domain containing receptor kinases interesting candidates for further analysis. Two *LysM domain containing kinases* were selected as candidate genes from the transcriptomic analysis. One *LysM domain containing kinase* that we referred to as *LysMI* showed a contrary expression pattern after treatment by the symbiont or the pathogen, what made it interesting to have a closer look, even if the alteration of expression was not statistically significant between the treatments and the control, there was a strong significance when comparing the two treatments directly. A 1.4 fold up-regulation and a 1.8 fold down-regulation after the symbiotic and the pathogenic treatment respectively could be measured. In the independent trial, a 1.7 fold up-regulation 30 min after contact to mycorrhizal spores could be observed, while a 3.3 fold down-regulated by the contact with *A. euteiches* could only be observed after 2 hrs (Fig. 26).
The second selected LysM RLK referred to as *LysMII* had a very similar expression pattern after 2 hrs like *LysMI*, with a significant up-regulation of 1.4 fold after mycorrhizal treatment. The qRT-PCR results showed a 1.3 fold up-regulation and 1.8 fold down-regulation, 2 hrs after myc spore/ *A. euteiches* zoospore treatment, respectively. A similar pattern with a slight up-regulation/ down-regulation can be seen 6 and 10 hrs after the two treatments. The last of the 7 candidate genes was a kinase (KIN), that has an overall similar protein structure like LysMII or also Lyk3 and NFP, with an active kinase domain, a transmembrane domain and a putative receptor domain, which does not contain LysM domains. In the array, *KIN* showed a significant up-regulation of 1.8 fold 2 hrs after mycorrhizal treatment, while no regulation appeared after the *A. euteiches* treatment. In the repetition of the trial, a similar expression pattern was found 6 hrs after inoculation (2.7 fold up-regulated), while 2 hrs after inoculation with *R. irregularis*, the gene expression was even lower compared to the control and 30 min after inoculation the gene was only induced by the pathogen (3.4 fold up-regulated).

3.4 Functional characterization of some candidate genes, early regulated after microbial contact with *R. irregularis* or *A. euteiches*

All selected candidate genes were tried to be analyzed by knock-down or knock-out and/or overexpression of the corresponding genes. *Tnt1* insertion lines were searched in the seed bank of the Samuel Roberts Nobel Foundation to obtain plants that were non-functional in the genes of interest. For those genes where no homozygous *Tnt1* insertion could be found, the method of RNA interference (RNAi) was used, to knock down the function of the genes of interest.

3.4.1 Screen for *Tnt1* insertion lines

For 5 of the candidate genes, *Tnt1* insertions corresponding to the database could be found. In the table below, the name of the insertion is mentioned as well as the sequence identity between the candidate gene and the gene with *Tnt1* insertion according to the Samuel Roberts Nobel Foundation. For all genes there was between 92 and 100 % identity. About 20 seeds per *Tnt1* insertion line were bought. All *Tnt1* insertion lines have several insertions, but not all of those insertions are present in every seed. Therefore, every plant had to be genotyped to control whether the *Tnt1* insertion is actually present in the candidate gene and if this is the case in both (homozygous) or only in one allele (heterozygous). All seeds of all lines were screened and only for *TS* one plant with a homozygous *Tnt1* insertion in the gene of interest was found. For *ß-GBP*, one plant with a heterozygous *Tnt1* insertion was found. The seeds of this plant were collected and the next generation was screened for a homozygous *Tnt1* insertion without success. Meanwhile RNAi constructs were generated for all other genes of interest. Those constructs were used to transiently transform roots (see 3.4.5).

Gene of interest	NF-line-insertion number	Identities
Kunitz inhibitor	NF8638-6	208/209 (99 %)
ß-glucan-bind	NF8493-34	125/127 (98 %)
Terpene synthase	NF10408-18	200/200 (100 %)
LysMI	NF5840-14	122/132 (92 %)
LysMII	NF9719-37	88/88 (100 %)

3.4.2 *Tnt1* insertion for *TS*

The homozygous *Tnt1* insertion line for *TS* was called NF10408. The sequence information given in the databank about line NF10408 including a 200 bp long segment of the gene sequence, located directly behind the *Tnt1* insertion, had a 100 % identity with the candidate gene, MTR_5g073200 according to the NCBI database. At the Noble Foundation, all *Tnt1* insertion lines are generated from the *M. truncatula* cultivar R 108, containing several insertions within one line. NF10408 contains 25 different *Tnt1* insertions. For all further functional analysis of the candidate gene, the knock-out line was compared to the NF10408 plants not containing the

Tnt1 insertion in the gene of interest. In later graphs this control will be referred to as *Tnt1*-BG (NF10408 background). Since *TS* was up-regulated in the array 2 hrs after contact with *A. euteiches*, it should be tested how the knock-out *Tnt1* (*ts*) would react during an infection process of the roots with *A. euteiches*. Thus *ts* plants were inoculated at the age of 2 weeks with about 20.000 zoospores per plant. 10 plants per genotype (ts + *Tnt1*-BG) were treated with zoospores and analyzed 3 weeks after infection.

The phenotype of *ts* and *Tnt1*-BG plants differed strongly in two major aspects (Fig. 27). The shoot biomass was strongly reduced and the plants flowered earlier in the mutant plants compared to the BG plants. The fresh weight of the control plants was about twice as high as the fresh weight of the mutant plants (Fig. 27 A). In *ts* the flowering had started in 75 % of all plants, while the control plants had not started flowering yet (Fig. 27 B).

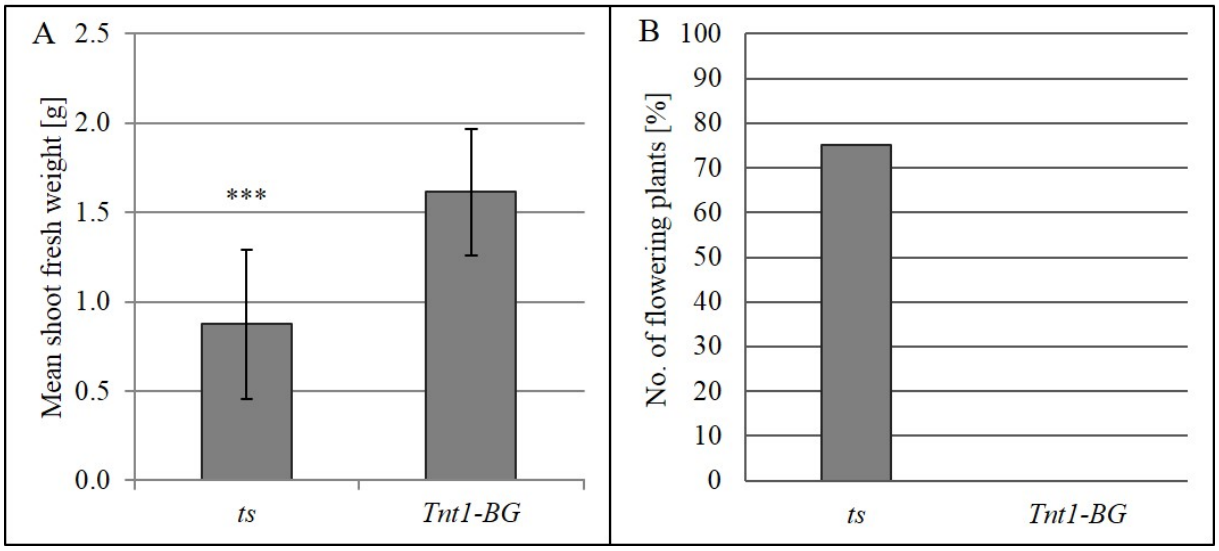

Figure 27: Phenotype of *ts* compared to *Tnt1*-BG plants in 5 week old plants, 3 weeks after inoculation with 20.000 *A. euteiches* zoospores. A: Mean shoot biomass; B: number of flowering plants. Bars indicate means $\pm$SD, n = 10. Significant differences were calculated with a Student's t-test (*** p $\leq$ 0.001, ** p $\leq$ 0.01, * p $\leq$ 0.05)

To test whether this effect was dependent on the presence of the pathogenic stress, plants were grown for 7 weeks without pathogen inoculation. The fresh weight was determined for roots and shoots and again there was a significantly lower fresh weight for both plant parts in *ts* plants, but the phenotypic difference was weaker (Fig. 28). *ts* plants showed about 1.4 fold lower root biomass and 1.7 fold lower shoot biomass compared to *Tnt1*-BG plants. The early flowering could not be reproduced without pathogen treatment (data not shown) and thus seems to be a stress induced response to the *A. euteiches* treatment in lack of the functional TS.

This stress response prompted us to check whether the *ts*- plants had an impaired resistance towards the pathogen. The colonization rate 3 weeks after inoculation with *A. euteiches* was determined in comparison to the control, by quantification of the transcript of an *A. euteiches* marker gene within the root tissue by qRT-PCR (Fig. 29 A). These results showed a roughly 7 fold higher *A. euteiches* marker gene transcript accumulation in *ts*-plants compared to the control plants, even though without statistical significance due to a high variation between the samples. This suggests an impaired plant defense in *ts*-plants.

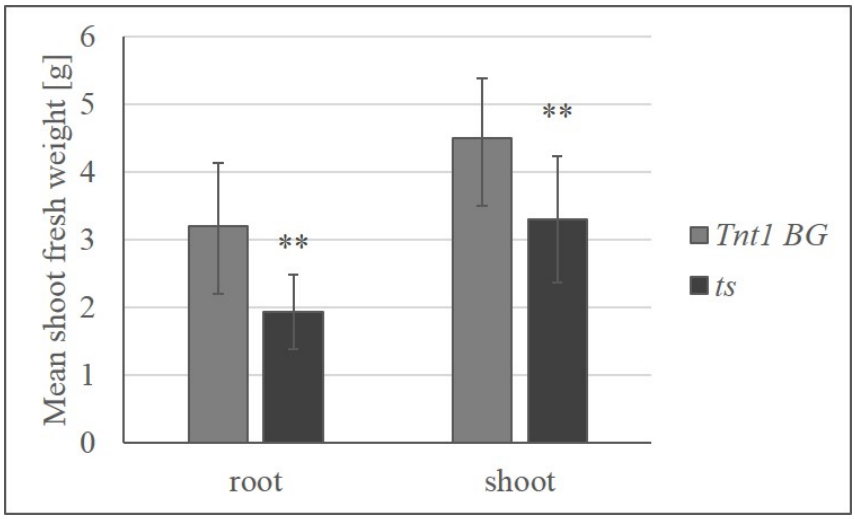

Figure 28: Shoot fresh weight of 7 week old *ts* vs. *Tnt1*-BG plants without treatment. Bars indicate means $\pm$SD, n $= 10$. Significant differences were calculated with a Student's t-test (** p ≤ 0.01, * p ≤ 0.05)

To test whether those effects could also be observed towards the symbiont, *ts* and control plants were inoculated with *R. irregularis* for 3 weeks. Transcript levels of the two colonization marker genes *MtPT4* and *Riß-Tub* were quantified via qRT-PCR (Fig. 29 B+C). No difference between the two genotypes could be observed in regard to mycorrhizal colonization, so TS seems to have a specific function towards the pathogenic organism.

The transcript level of *TS* was also quantified in the *A. euteiches* treated pants. The expression of the gene was 14 fold lower in *ts* compared to *Tnt1*-BG, but a complete absence of the transcript did not occur even though this was expected for a *Tnt1* insertion line (29 D).

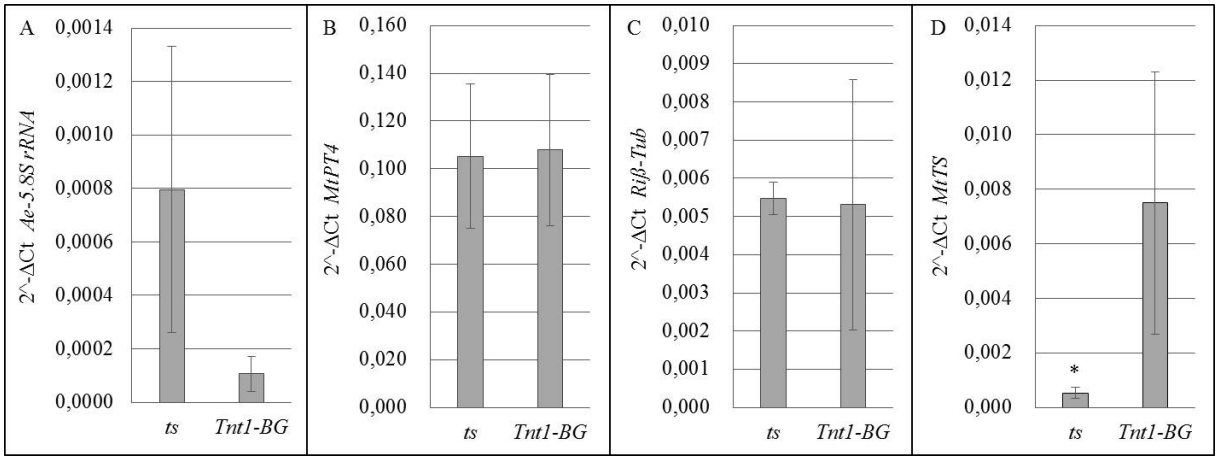

Figure 29: Transcript accumulation of Myc/ Ae marker genes and *MtTS* relative to the reference gene *MtEF1*α in *ts* vs. *Tnt1*-BG, in 5 week old plants, treated with *R. irregularis* for 5 weeks (B+C) or *A. euteiches* for 3 weeks (A+D). A: Relative expression of the *A. euteiches* marker gene *Ae-5.8SrRNA*; B: Relative expression of the mycorrhization marker gene *MtPT4*; C: Relative expression of the mycorrhization marker gene *Ri-ßTub*; D: Relative expression of the gene of interest: *MtTS*. Bars indicate means $\pm$SD, n $= 4$. Significant differences were calculated with a Student's t-test (* p ≤ 0.05)

3.4.3 Functional characterization of putative terpene synthase in *M. truncatula* roots

To functionally characterize the putative TS, all volatiles exuded from *ts* and *Tnt1*-BG plants, after *A. euteiches* treatment were compared. Plants were cultivated on lecatone for six weeks. Then lecatone was removed and plants were transferred to glass vessels (Fig. 30). Volatile absorbing twister were placed in the root system and roots were inoculated with *A. euteiches* zoospores (about 50.000) and incubated for 12 hrs overnight. Twister containing the root volatiles were harvested and measured with GC-MS as described in section 2.6.1. The GC-MS measurement showed a very clear differential result for three volatiles (Fig. 31). Three sesquiterpenes (α-longipinene, copaene and α- himachalene), all having the same chemical formula of $C_{15}H_{24}$ (Fig. 32), were significantly stronger exuded in control plants compared to mutant plants. For GC-MS spectra see Appendix Fig. 68. Based on those results we suggest that TS is a multiproduct sesquiterpene synthase.

Figure 30: Volatile collection of roots after *A. euteiches* treatment.

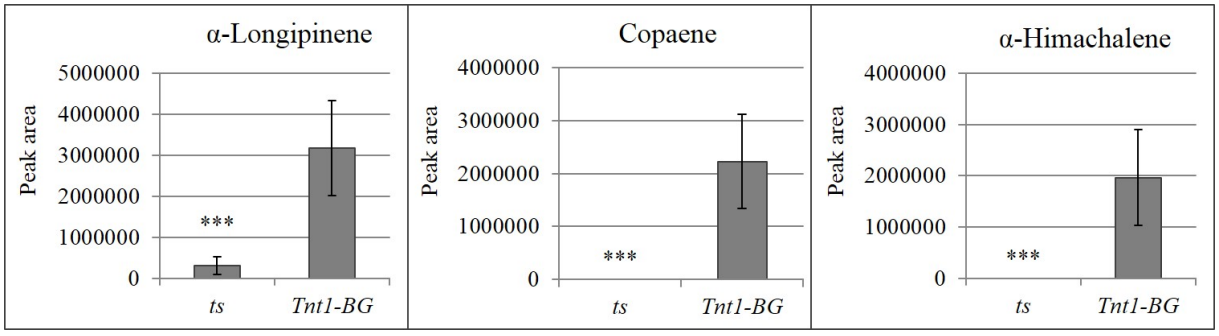

Figure 31: Peak area of volatiles measured with GC-MS, collected from six week old *M. truncatula* roots, 12 h after inoculation with zoospores of *A. euteiches*, Bars indicate means $\pm$SD, n = 10. Significant differences were calculated with a Student's t-test ((*** p $\leq$ 0.001, ** p $\leq$ 0.01, * p $\leq$ 0.05)). .

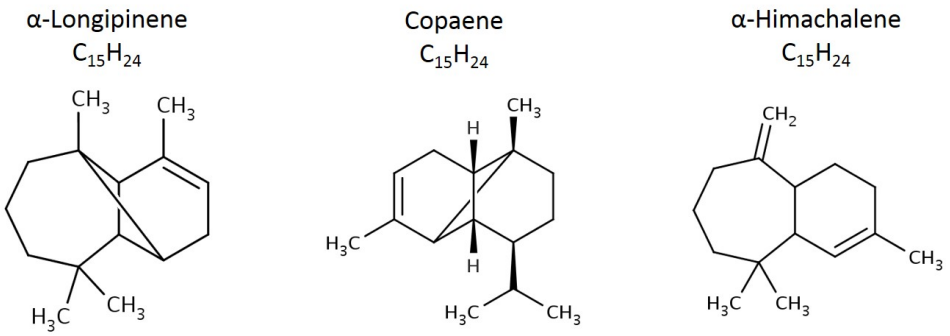

Figure 32: Molecule structure of three sesquiterpenes, synthesized in vivo after *A. euteiches* treatment by the sesquiterpene synthase MTR_5g073200.

3.4.4 *TS*- overexpression

The functional relevance of TS was further analyzed by overexpression to see if an opposing effect to the knock-down could be observed. The *A. euteiches* colonization was analyzed, measuring the gene expression of an *A. euteiches* marker gene 4 weeks after inoculation. As shown in Fig. 33 A, the overexpression of the gene was successful. Yet the induced *TS* gene expression did not affect the colonization of TS-OE plants by *A. euteiches* as can be seen by the expression of the *A. euteiches* colonization marker gene (Fig. 33 B).

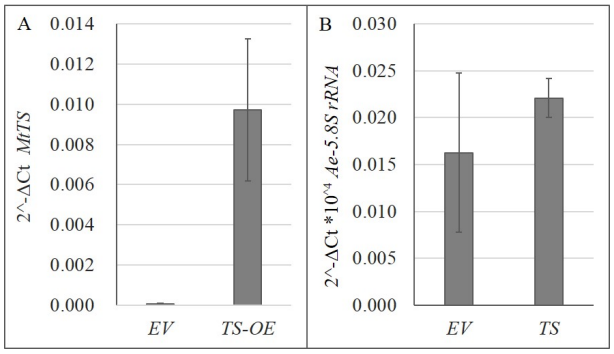

Figure 33: Transcript accumulation of *TS* (A) and a marker gene for *A. euteiches* root colonization (B) relative to the reference gene *MtEF1*α in roots 4 weeks after *A. euteiches* inoculation. Bars indicate means $\pm$SD, n $= 9$. Significant differences were calculated with a Student's t-test (* p ≤ 0.05).

3.4.5 RNAi for some candidate genes

Three of the candidate genes for which neither heterozygous nor homozygous *Tnt1* insertion lines could be found, were selected to be knocked-down by RNAi. For the PCR amplification of the target regions of the selected genes, primers were designed that created an overhang containing a *BsaI* restriction site as well as a 4 bp overhang, complementary to the entry vector at the site of desired ligation. This guaranteed a specificity during the ligation process, so that each fragment could only bind at a certain position in a given orientation (see section 2.4.10). The fragments of each target gene selected for the RNAi constructs were chosen according to the following criteria: 1. Fragments should have the least possible homology to other genes of *M. truncatula*; 2. Fragments should be of a size between 100 - 400 bp; 3. Fragments should not contain introns (to allow the use of genomic DNA as template for the PCR, having a better PCR efficiency than cDNA). The transcripts of one pathogen related TF (*PR-TF*) and two LysM domain containing proteins (*LysMI/ II*) were knocked down by RNAi. *MtPT4* was included in the experiment as control for the RNAi-constructs generated with the Golden Gate Cloning system. Only the *LysMII*-RNAi plants showed a significantly lower target gene expression compared to the empty vector (EV) plants (Fig. 34 B). For *LysMI* and *PR-TF*, there was only a tendency of lower gene expression in RNAi plants. After colonization of RNAi plants with *R. irregularis* for 5 weeks, the colonization rate was determined, based on the two mycorrhization marker genes *MtPT4* and *Riß-Tub*. However none of the RNAi plants showed a significant decrease of the mycorrhization marker genes compared to the control, apart from the control construct with *MtPT4* (Fig. 34 A). Yet it is unclear, whether this missing effect can be explained by the

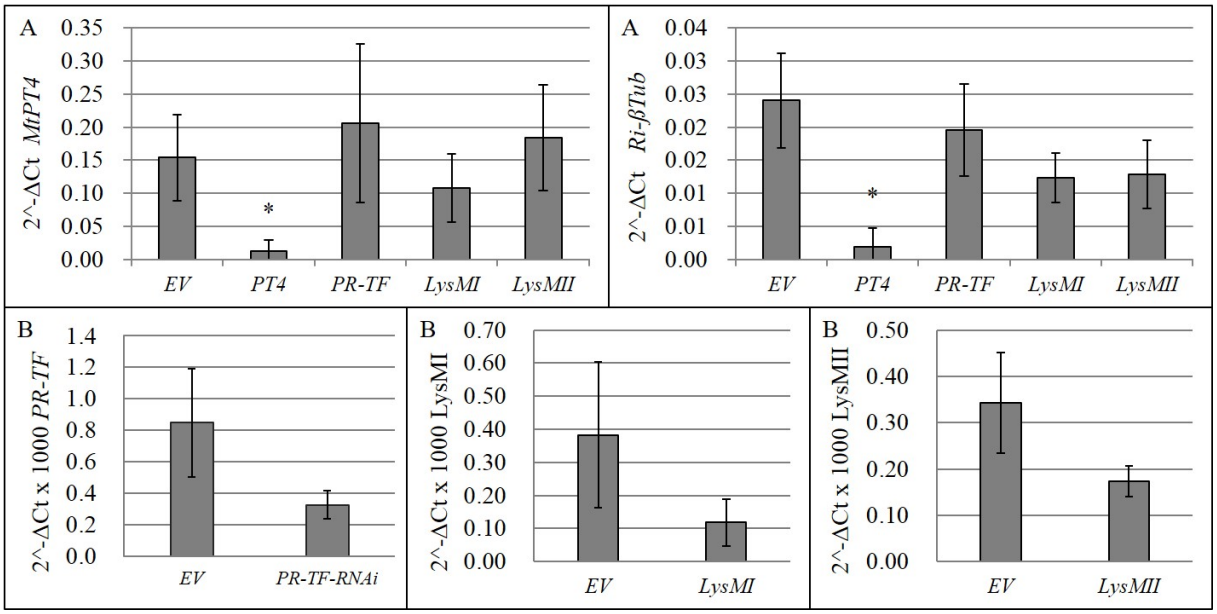

Figure 34: Transcript accumulation relative to the reference gene *MtEF1α* in RNAi roots grown on *R. irregularis* inoculum for 5 weeks of A: the two mycorrhization marker genes *MtPT4* and *Ri-βTub* in plants. B: of RNAi targeted genes *PR-TF*, *LysMI* and *LysMII*. Bars indicate means $\pm$SD, $\mathrm{n} = 5$. Significant differences were calculated with a Student's t-test (* $\mathrm{p} \leq 0.05$)

weak RNAi efficiency or by the fact that those genes were not decisive for the mycorrhization process. The only gene with a trend of correlation between the silencing of the gene and the mycorrhizal colonization, was *LysMI*. The *LysMI* transcript was down-regulated by about 50 %, the mycorrhization marker genes *MtPT4* and *Riß-Tub* were down-regulated by 52 % and 38 % respectively. To be sure that this correlation was substantial, this experiment was repeated with a higher number of transformed plants, so that a higher number of strong fluorescent plants (indicating a good transformation efficiency) could be selected for analysis.

LysMI was further analyzed by a second RNAi approach. Since LysMI does not have a kinase domain, it could only be involved in the recognition of mycorrhizal signaling molecules, in collaboration with a protein containing a kinase domain for the induction of any downstream signaling event. Thus the array data were screened for all kinases and one kinase was found that was specifically up-regulated after AMF spore treatment. The Kinase R-like protein (KIN/ MTR_7g082510) (for the expression values see Fig. 26). The hypothesized interaction between LysMI and KIN would lead to a similar model as described in rice for the two receptor proteins OsCERK1 and OsCEPiP (Fig. 35). The LysM domain containing protein OsCEPiP is the major chitin elicitor–binding protein in rice. It does not contain an intracellular region but appears to be attached to the external side of the plasma-membrane with two LysM motifs in the extracellular portion [Kaku et al., 2006] (LysMI would be a OsCEPiP equivalent). CEBiP is involved in the resistance towards the fungal pathogen *Magnaporthe oryzae* [Kishimoto et al., 2010], and it forms heterooligomers with OsCERK1 after chitin elicitor treatment [Shimizu et al., 2010]. No chitin binding has been shown for OsCERK1, so it was assumed that chitin elicitors could be primarily perceived by CEBiP, followed by signal transduction via the active kinase domain of OsCERK1 [Shimizu et al., 2010] (KIN would be the OsCERK1 equivalent).

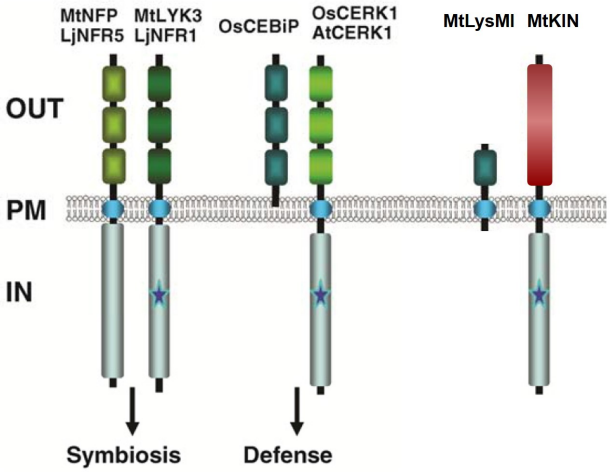

Figure 35: Scheme of the predicted domain structures of chitin receptor proteins. Proteins are shown inserted into the plasma-membrane (PM); the LysM domains (in green) and other receptor domains (red) are extracellular (OUT) and the kinase domains intracellular (IN). Stars in the intracellular domains represent active kinase domains. Modified after [Gough and Cullimore, 2011].

In Fig. 36 the protein structure of the three candidates LysMI, LysMII and the Kinase R-like protein (KIN) is shown. MtLyk3 was used as reference of LysM domain containing proteins since it is already functionally characterized as Nod factor receptor. LysMI is a very short protein of only 18 kDa, including one LysM domain and one transmembrane domain (TM). LysMII has a very similar protein structure as Lyk3 with three LysM domains, one TM and one active kinase domain. The Kinase R-like protein has an extracellular sequence not containing a LysM domain, but also has a TM and an active kinase domain. The Asp-Phe-Gly (DFG) motif located at the beginning of the activation loop is highly conserved among kinases and is important for protein catalysis and as such plays an important role in the regulation of kinase activity [Peng et al., 2013]. This DFG motif can only be found in Lyk3 and KIN.

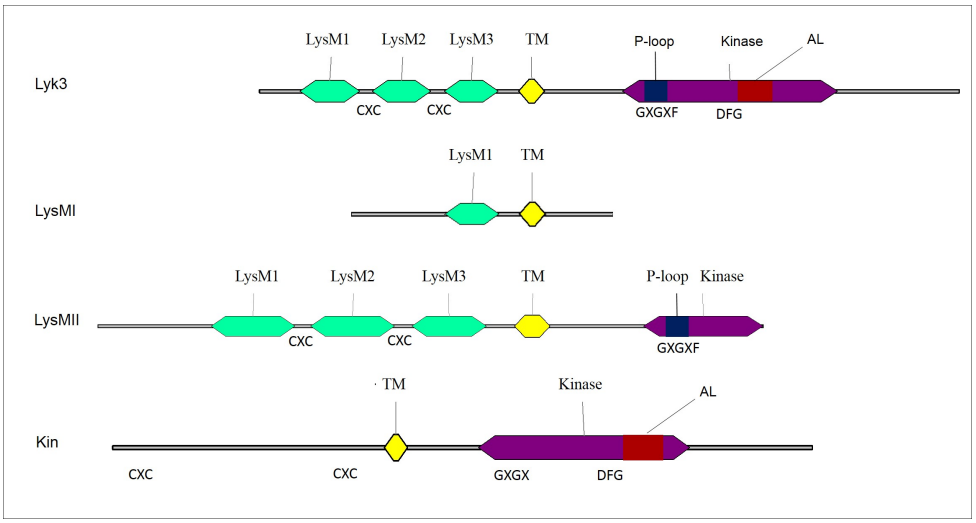

Figure 36: Protein structure of MtLyk3, LysMI/II and KIN, with Lysin motifs (LysM 1-3), transmembrane domains (TM) and the intracellular kinase domain (kinase) containing an activation loop (AL) beginning with a DFG motif.

A knock-down via RNAi and an overexpression of *LysMI* and of the *KIN* in transiently trans-

formed roots was done (Fig. 37). For both genes, not only the knock-down, but also the overexpression worked with good efficiency (Fig. 37 A 3+4 / B 3+4).

The expression of *MtPT4*, a marker for arbuscule abundance, was not affected by an altered *KIN* gene expression (Fig. 37 A1/B1). The marker for overall fungal colonization (*Riß-TUB*), was significantly lower in roots with a lower *KIN* expression and significantly higher in roots with a higher *KIN* expression (Fig. 37 A1/B1). This suggests that *KIN* might play a role in the establishment of mycorrhiza, while later stages, reflected by the *MtPT4* expression were not affected.

The down-regulation or overexpression of *LysMI* did not influence the expression of neither of the mycorrhizal marker genes (Fig. 37 A1/2 and B1/2), so LysMI does not seem to play a major role in the process of mycorrhizal colonization of roots.

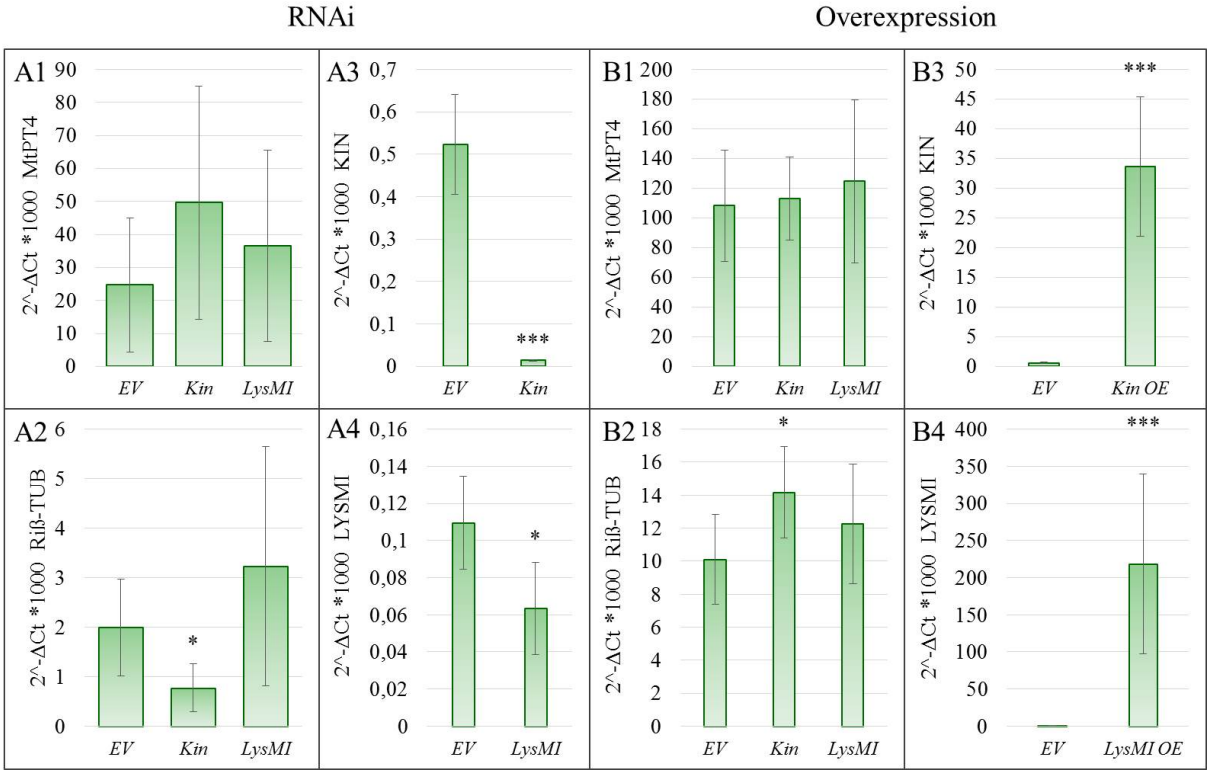

Figure 37: Transcript accumulation relative to the reference gene *MtEF1α* in transiently transformed roots: of the two mycorrhization marker genes *MtPT4* and *Riß-tub* (A 1+2) and RNAi targeted genes *Kin* and *LysMI* (A 3+4) in RNAi roots; of the two mycorrhization marker genes *MtPT4* and *Riß-tub* (B 1+2) and overexpressed genes *Kin* and *LysMI* (B 3+4) in OE roots grown on *R. irregularis* inoculum for four weeks. Bars indicate means $\pm$SD, $\mathrm{n} = 7$. Significant differences were calculated with a Student's t-test (*** $\mathrm{p} \leq 0.001$, ** $\mathrm{p} \leq 0.01$, * $\mathrm{p} \leq 0.05$).

3.4.6 Subcellular localization of proteins of interest

For TS, LysMI and KIN, a subcellular localization was done by a fusion of the open reading frame to GFP and an expression in *N. benthamiana*, to get a further hint about their functionality. Only the construct with TS (pAGH 12 - see Tab. 5) fused to GFP could be successfully expressed (Fig. 38). Due to our characterization of TS as sesquiterpene synthase, we expected a

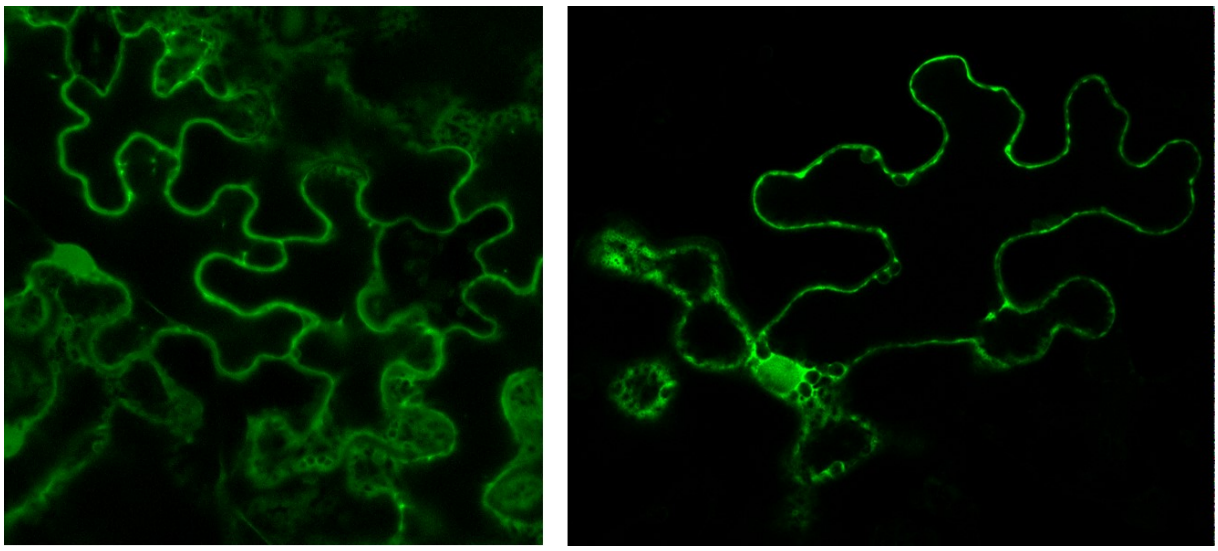

Figure 38: Subcellular localization of GFP-fused TS in transformed *N. benthamiana* leaves.

GFP signal in the cytosol. While the biosynthesis of isoprene, monoterpenes, and diterpenes occurs in the plastid, the sesquiterpenes biosynthesis takes place in the cytosol [Chen et al., 2011]. The GFP signal shown in Fig. 38 suggest a cytosolic localization of TS, yet the interpretation of the pictures is not precise, due to a slight GFP signal in the nucleus, that can not be explained. To confirm the results, a western blot analysis should be performed with the infiltrated tissue to test if the gene of interest fused to GFP was really expressed in the infiltrated *N. benthamiana* leaves.

3.5 Analysis of root exudates and emitted volatiles of aeroponically grown plants

The major aim of this trial was to analyze the plant root exudation short time after microbial contact, yet before a microbial penetration into the root tissue could happen. Root exudates were measured with LC/MS and volatiles with GC/MS. In order to harvest sufficient root exudates for analytical success, plants were cultivated in an Aeroponic system as described in paragraph 2.2.4. Hydroponic systems were tested before without success. The low oxygen supply of the roots in hydroponic culture did not allow the development of large root systems, and the collected exudates were too low concentrated to be measured. In the aeroponic system, plant roots grew without substrate and were sprayed with MSR nutrient solution in regular intervals. This ensured a good nutrient and oxygen supply of the plant roots.
Six weeks after cultivation in the aeroponic system, *M. truncatula* roots were treated with spores of *R. irregularis* or zoospores of *A. euteiches*. The experiment was set up in three different aeroponic containers, each comprising 25 plants. The non-treated plants of one container served as control, the roots of the second container were treated with *R. irregularis* spores and the roots of the third container were treated with zoospores of *A. euteiches* at 8 pm. The *R. irregularis* spores were harvested from axenic carrot root cultures as described in paragraph 2.2.2. One third of the spores of a mature hairy root culture plate was used to inoculate one root system. About 50.000 zoospores of *A. euteiches* were applied per root system (counted as described in paragraph 2.2.3). Before inoculation, volatile absorbing sticks, so called twister, were fixed in each root system (Fig. 39 A). 12 hrs after inoculation (8 am) the twister were collected for GC-MS analysis and the exudates for untargeted LC-MS analysis were collected by washing the roots in 30 ml H_2O_{dest} (Fig. 39 B), whereby three roots were pooled for one sample. Few root segments were stored in 50 % EtOH for later microscopic analysis, while the major part of the roots was frozen immediately in liquid N_2 for RNA isolation.

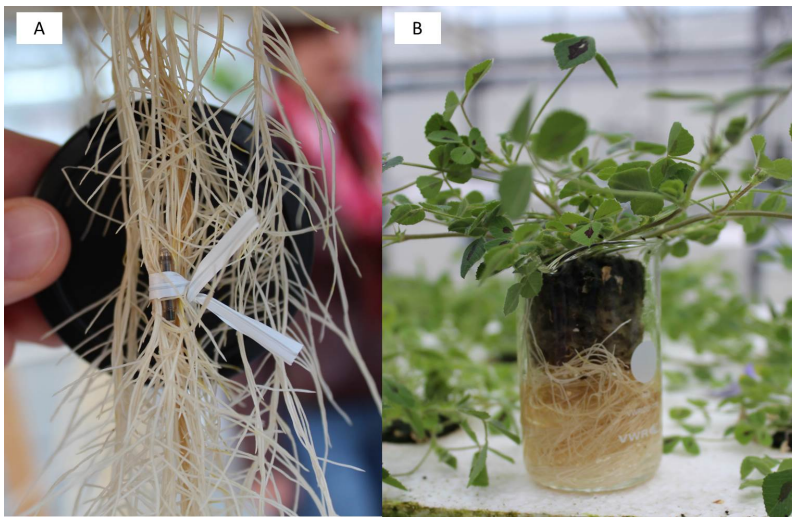

Figure 39: Collection of volatiles/ root exudates 12 hrs after microbial treatment of roots. A: Application of twister for collection of emitted volatiles B: Collection of root exudates by washing roots in 30 ml H_2O_{dest}.

3.5.1 Microscopic sample analysis

In order to be sure that the interaction between the plant roots and the microorganisms was yet in a pre-symbiotic or pre-parasitic state without any root penetration, roots were harvested at the end of the trial (12 hrs after inoculation) and stained with AlexaFluor488 (see section 2.3.3) for visualization of any fungal or fungus-like structures. In the first trial (summer 2013), no colonization of any of the applied microorganisms, nor any microbial contamination was visible. One developmental stage very close to the penetration of the plant tissue was found. A germinated spore of *A. euteiches* with an appressorium on the root surface was detected, but no colonization of the plant roots with any of the two organism was detected 12 hrs after inoculation (Fig. 40 A). The experiment was repeated in summer 2014. A foreign infection was found in that experiment (Fig. 40 B). This circumstance did not allow a good reproducibility of the results, yet the exudates of trial 2 were analyzed.

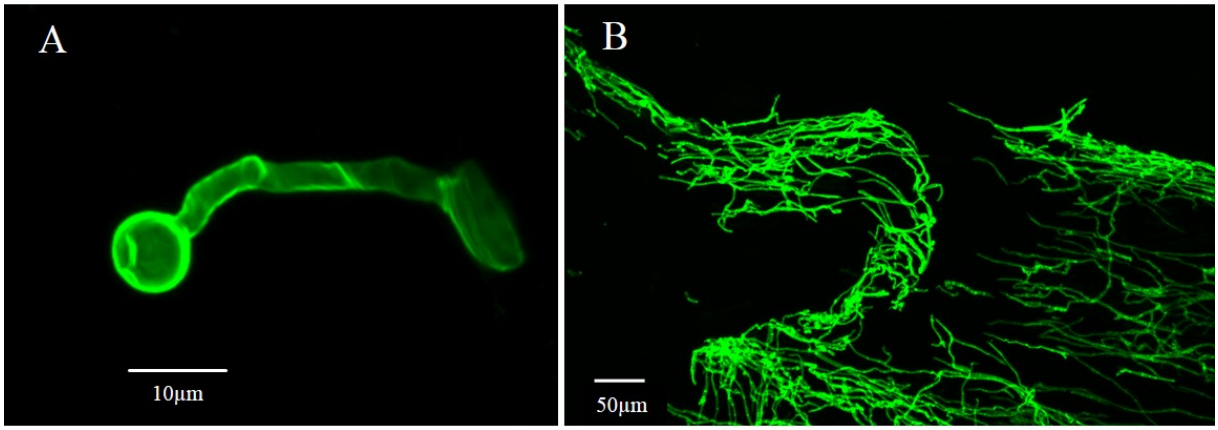

Figure 40: Root samples were stained with AlexaFluor488 after harvest. A: Germinated zoospore with appressorium of *A. euteiches* 12 hrs after application on roots in the first trial (summer 2013). B: Undesired fungal colonization of *M. truncatula* roots, due to a systemic contamination in the second trial (summer 2014).

3.5.2 Root exudates 12 hrs after microbial contact to *M. truncatula* or *A. euteiches* measured by untargeted LC-MS

For the experiment in 2013 only wt plants were used. One container with 25 plants each was used per treatment. 15 plants were selected from each treatment and three plants were pooled per biological replicate leading to a total of five biological replicates. The obtained exudate samples were purified with a C18 column (section 2.6.2) and analyzed by LC-MS in a positive and negative ionisation mode (section 2.6.2). Intensities of measured compounds were very high due to a high concentration of root exudates in the samples.

With the data obtained by untargeted LC-MS measurement, a Principal Component Analysis (PCA) was performed with the ”R” statistic program. Results obtained from the negative ionisation mode are shown in Fig. 41. The first PC explaining 25 % of the features distinguishes between *R. irregularis* treated samples and the rest, while the second PC (11 %) distinguishes between control samples and all treated samples. The data obtained by the positive ionisation mode showed a similar overall picture (data are not shown).

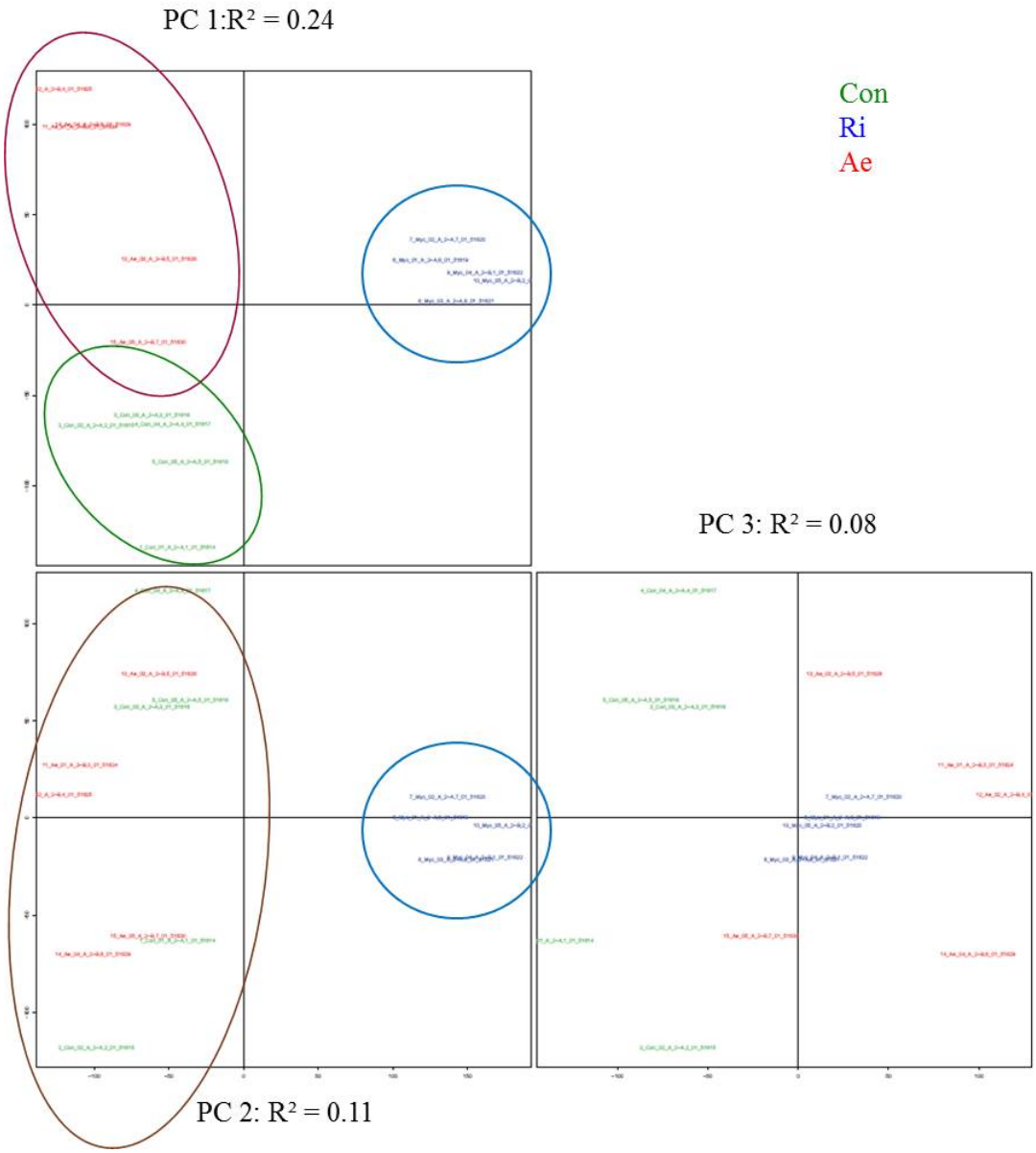

Figure 41: PCA of LC-MS data obtained from root exudates, collected of six week old *M. truncatula* wt plants, 12 hrs after inoculation with spores of *R. irregularis* or zoospores of *A. euteiches* (trial 2013). Oneway ANOVA, $p \leq 0.05$; FC ≥ 2, n = 5.

A stronger influence of the AMF treatment on the root exudates could be observed than after the treatment with *A. euteiches* zoospores. 96 features could be measured in the *R. irregularis* treated samples that refer to significantly stronger exuded substances compared to the control. Only 49 features were significantly stronger exuded in the *A. euteiches* treated roots, while only 7 of the features could be found in both treatments (Fig. 42 A). Also the number of substances that were weaker exuded after microbial challenge was higher after the symbiotic treatment with 37 features, and 29 features after *A. euteiches* treatment, with 8 features being weaker exuded in both treatments compared to the control (Fig. 42 B).

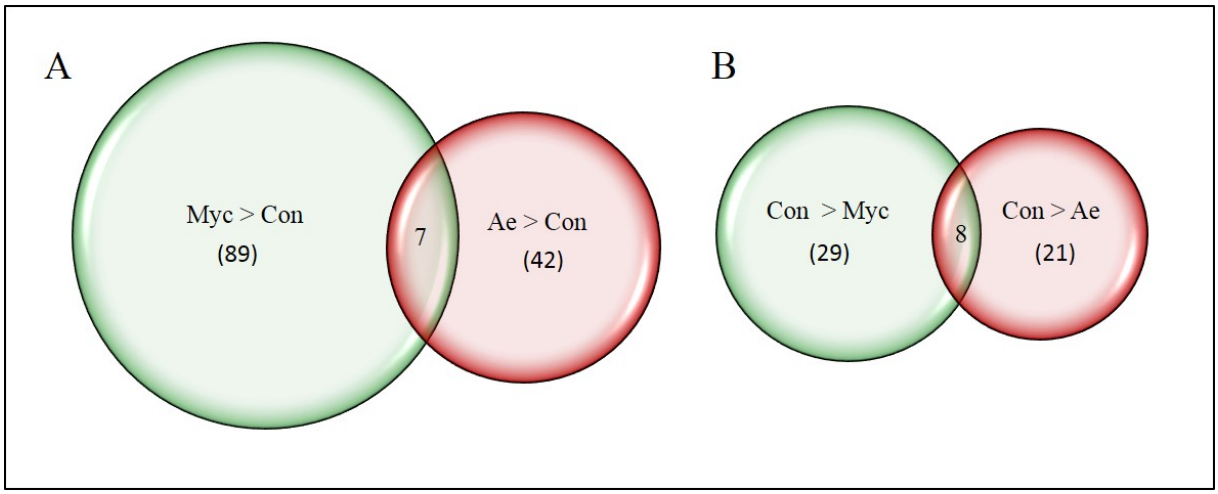

Figure 42: Venn diagram of LC-MS data from root exudates of aeroponically grown six week old wt *M. truncatula* plants, that were inoculated with *R. irregularis* or *A. euteiches* for 12 hrs (trial 2013). A: Exudates that were stronger exuded in the treatment vs. control; B: Exudates that were stronger exuded in the control vs. treatment. Data obtained from the negative and the positive ionisation mode are included.

Independent repetition of the experiment including *dmi3* mutant plants Since the identification of secondary metabolites with the help of tandem MS is a very difficult procedure, and the above mentioned number of features was far too high to be analyzed that way, the complete trial was repeated and only those substances that could also be found in the independent experiment should be analyzed with tandem MS. In this independent trial apart from the microbial treatment, another factor was implemented. A mutant of *DMI3* (Does Not make Infection 3), encoding a Ca^{2+}/calmodulin-dependent protein kinase, that is proposed to function in the Nod/Myc factor signal transduction pathway downstream of calcium spiking (for explanation see Fig. 7), was included as a further genotype. Analysis of exudates from *dmi3* should help to specify exudation patterns after AMF treatment. Substances that are specifically induced by AMF should be missing in *dmi3* since the CSSP is impaired in that mutant.

The experimental setup included three containers as described above, with one treatment per container. Ten wt and 15 *dmi3* plants were planted per container and three plants were pooled per sample, leading to three or four biological replicates for *dmi3* or wt respectively.

The treatment and collection of root exudates and the measurement and data analysis was done like in the first experiment (see section 3.5.2). PCA of those data with PC1 (19 %) and PC2 (11 %) revealed that the treatment of the plants (Con vs Myc vs Ae) had a stronger influence on the root exudate composition than the genotype. All samples with the same treatment cluster close together except two samples of the *A. euteiches* treated wt plants, that appear as outliers in all three PCs. PC3 (8 %) show that the AMF treatment had a stronger influence on the root exudate pattern than the treatment with *A. euteiches* (Fig. 43).

In the second aeroponic trial, an infection with an unknown fungal microorganism appeared in the roots (Fig. 40 B). This unwanted infection could explain, why the overall difference between the control plants and the microbial treated plants was smaller, due to a possible primed state of the plants, that overlapped some part of the reaction induced by the microbial treatment. In

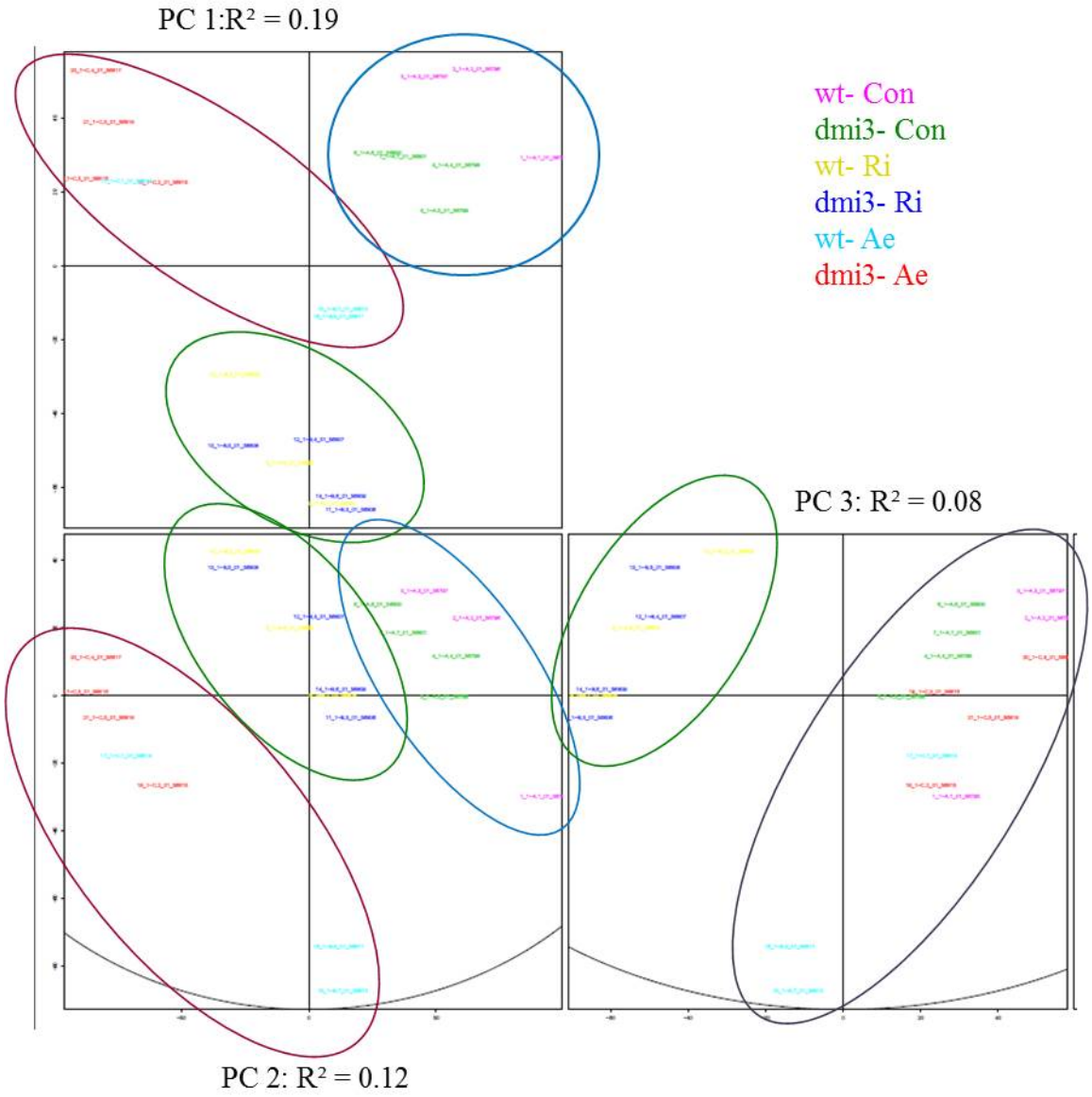

Figure 43: PCA of LC-MS data obtained from root exudates, collected of six week old *M. truncatula* wt and *dmi3* plants, 12 hrs after inoculation with spores of *R. irregularis* or zoospores of *A. euteiches* (trial 2014). Oneway ANOVA, $p \leq 0.05$; FC ≥ 2, n = 3 or 4.

order to keep the data the most comparable to the first experiment, the fold change was reduced to 1.5 (instead of 2). With this statistical parameter, a similar number of features could be observed, that were stronger exuded after the microbial challenge with 40 and 16 features after *R. irregularis* and *A. euteiches* treatment respectively and 6 features present in both treatments (Fig. 44 A). Concerning the number of features that were weaker exuded compared to the control, in the second trial there was a greater regulation by *A. euteiches* with 33 less exuded substances and only 9 less exuded substances in *R. irregularis* treated roots compared to the control, while 6 exudates were reduced in both treatments (Fig. 44 B). In *dmi3* plants the treatment with *A. euteiches* had a very strong effect (Fig. 45). 136 substances stronger and 23 substances weaker exuded in the *A. euteiches* treated vs untreated plants. After *R. irregularis* treatment of *dmi3* 40 substances were stronger and 6 substances weaker exuded compared to

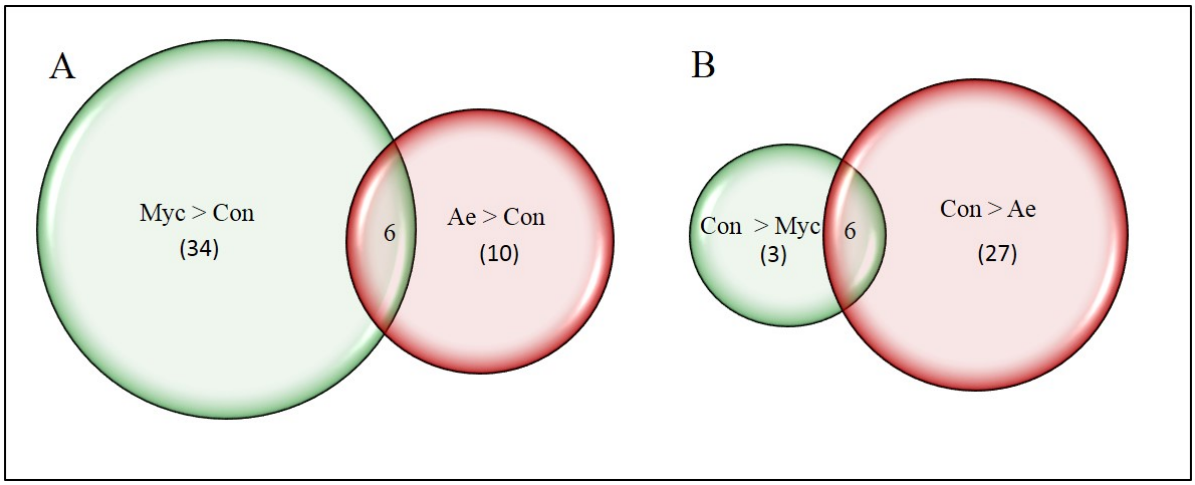

Figure 44: Venn diagram of LC-MS data from root exudates of aeroponically cultivated, six week old plants, that were inoculated with *R. irregularis* spores or *A. euteiches* zoospores for 12 hrs (Second trial). A: Exudates that were stronger exuded in the treatment vs. the control; B: Exudates that were stronger exuded in the control vs. treatment. Data obtained from the negative and the positive ionisation mode are included.

non-treated *dmi3* roots. Comparing Fig. 44 B and Fig. 45 B strong difference especially after the treatment with *A. euteiches* appear between the two genotypes with more substances that are weaker exuded in the wt after pathogen treatment and only few substances being stronger exuded, showing the contrary pattern as in *dmi3*. In contrast to this different reactions of both genotypes after pathogen treatment, the treatment with *R. irregularis* showed a very similar exudate pattern in both genotypes (Fig. 44 A vs Fig. 45 A).

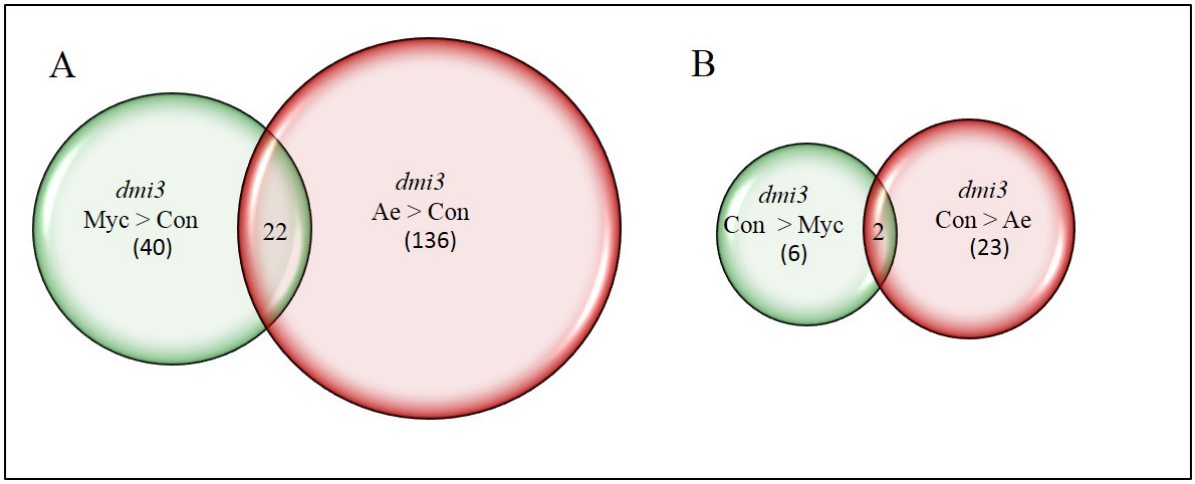

Figure 45: Venn diagram of LC-MS data from root exudates of aeroponically cultivated, six week old *dmi3* plants, that were inoculated with *R. irregularis* spores or *A. euteiches* zoospores for 12 hrs (second trial). A: Exudates that were stronger exuded in the treatment vs. the control; B: Exudates that were stronger exuded in the control vs. treatment. Data obtained from the negative and the positive ionisation mode are included.

Comparing the number of exudates between the genotypes a surprisingly strong effect appeared in *dmi3* after *A. euteiches* treatment with 81 stronger and 5 weaker exuded substances compared to the wt (Fig. 46). Interestingly, the number of regulated substances in the exudates of the control and the *R. irregularis* treated plants is rather similar with only 8 or 12 substances stronger

and 6 or 4 substances weaker exuded in *dmi3* compared to wt respectively.

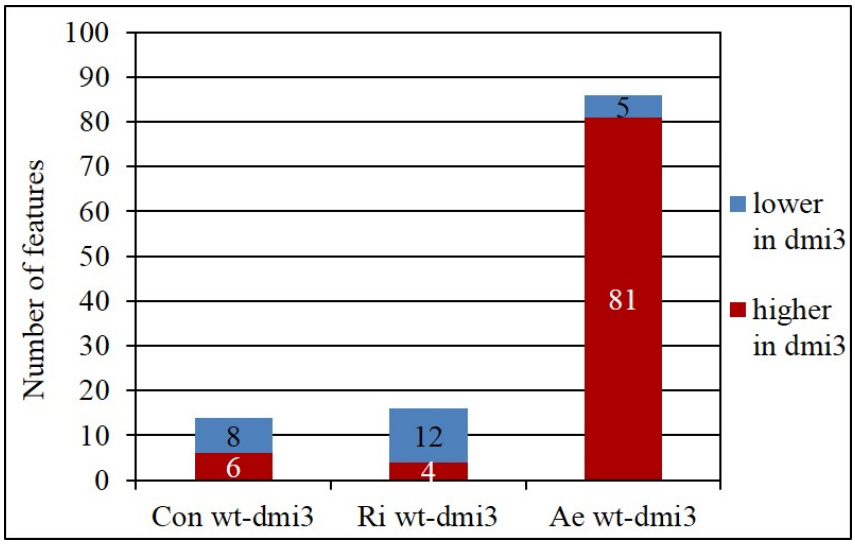

Figure 46: Root exudates of six week old *dmi3* and wt plants that were treated with *R. irregularis* spores or *A. euteiches* zoospores for 12 hrs. Comparison of the two genotypes.

From the experiment done in 2013, several exudates could be likely identified with help of tandem-MS-analysis. All identified flavonoids were stronger exuded by *A. euteiches* treated and weaker exuded by *R. irregularis* treated plants (see Fig. 47). The widespread information about mass spectrometry of flavonoids in plants as well as substantial in-house experience allowed a reliable characterization as flavonoids. Glycosides of naringenin (flavanon), kaempferol (flavonol) and the isoflavonoid medicarpin were found. All glycosylated flavonoids showed a typical glycoside breakdown pattern resulting in a loss of 162,05 (hexose) or 146,07 (deoxyhexose). As aglycons only naringenin, kaempferol and medicarpin were detected. The UV spectra showed a typical shape with two peaks and a shoulder or tail as it was described before [Farag et al., 2007]. MassBank entries of naringeninhexoside and kaempferolhexoside showed a pattern similar to the measured ones and the relative retention times based on a reversed phase column with a standard linear gradient can be well compared with those published by Farag et al. (2007). Two more malonylhexosides at Rt 421s and 432s were found to be significantly altered after infection. The UV shapes correspond to the flavonoid UV trace and the compound at 432s is most likely a methoxy derivative of the compound at 421s. Although the evidences suggest two more flavonoids it was however not possible to assign the signals to a known compound (for more details see Appendix Fig. 67).

The exudation of flavonoids was reduced by *R. irregularis* treatment yet another group of chemically related substances had an opposite exudation pattern (see Fig. 47). These were glycosylated compounds of higher molecular weight than flavonoids with the aglycons having m/z values between 450 and 500. According to literature data, sum formula suggestions and relative retention times these aglycons most likely belong to the class of sapogenins. According to the molecular masses the sapogenins exist in their reduced form as bayogenin (reduced form of medicagenic acid) and hederagenin (reduced form of gypsogenic acid). Other members of this substance class (like soyasapogenols) have not been detected. Attached carbohydrates were

hexoses and deoxyhexoses. The UV spectra of those compounds are unspecific with a peak maximum at 222 nm for all saponins. Unglycosylated bayogenin was also detected in the exudates as well as an unknown compound that is most likely also a saponin. This compound at Rt 553 s has a malonylhexose attached and the sum formula of its aglycon corresponds to a hedaragenin with a loss of water (for more details see Appendix Fig. 67). In contrast the saponins/ sapogenins with two bayogenin glycosides, bayogenin, hederagenin glycoside and one unknown saponin, revealed a significant difference between the two treatments, all having a stronger exudation after AMF spore treatment (Fig. 47).

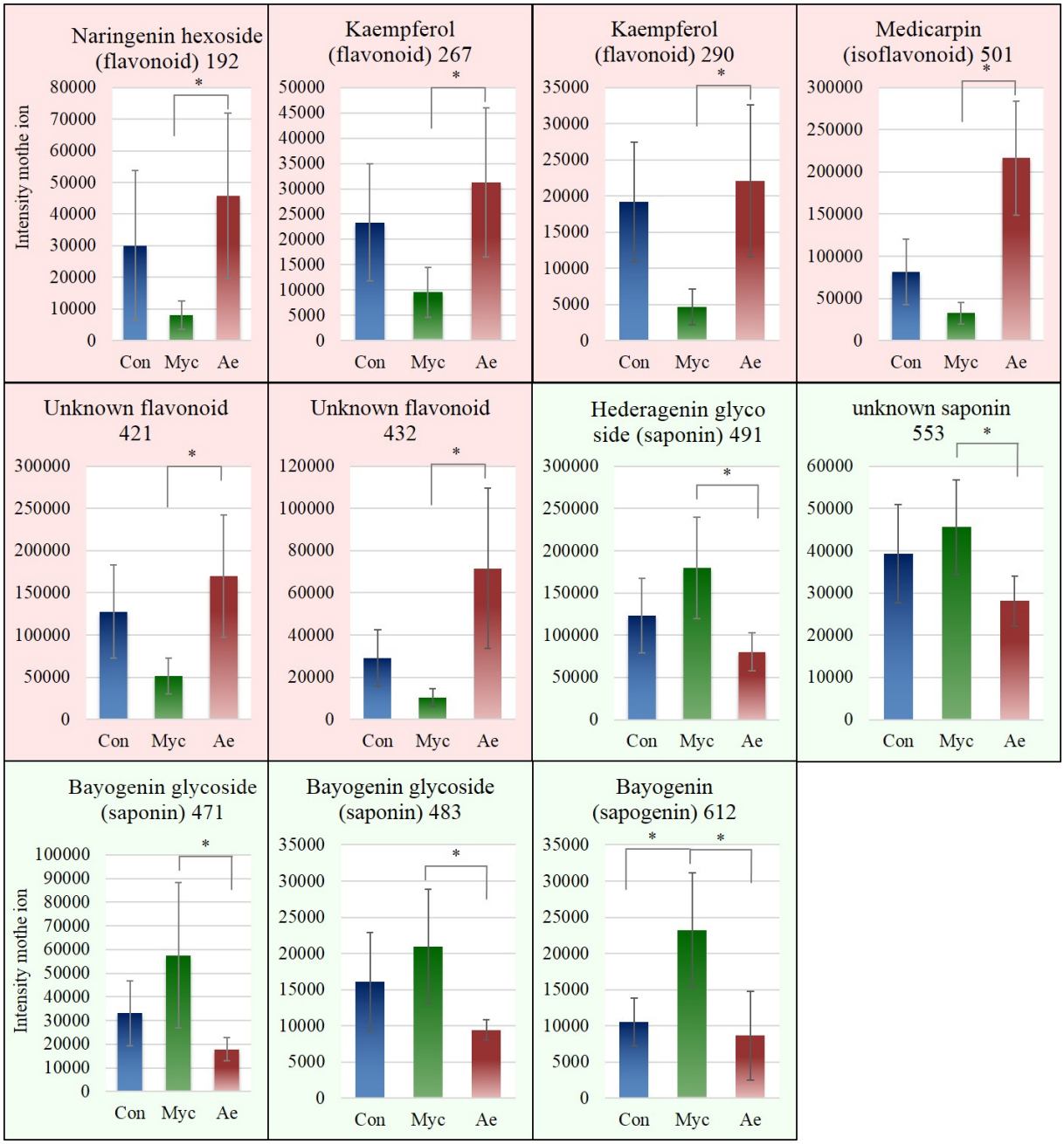

Figure 47: Some identified root exudates of six week old plants treated with *R. irregularis* spores or *A. euteiches* zoospores for 12 hrs. These data were obtained from the first experiment done in 2013. $n = 5$. Significant differences were calculated with a Student's t-test (* $p \leq 0.05$)

The high number of substances found specifically in *dmi3* exudates after *A. euteiches* treatment

could not be identified. Yet the subset of exudates that appeared in *dmi3* after both treatments, was tried to be structurally elucidated. The exudates of *dmi3* plants that were triggered by both microorganisms, predominantly belong to other compound classes than flavonoids or saponins. It can be shown that two metabolites (Rt (retention time) 155s and 335s) are nitrogen containing compounds which strongly differ from treated wt plants, where no substances with heteroatoms were altered. Sum formula suggestions for the smallest fragment at Rt 155s point to a nucleotide derivative or a compound with an amino acid substructure. As it is known that legumes do not synthesize glucosinolates or alkaloids, amino acids and nucleotides are expected to be the dominant heteroatom containing compounds. For the nitrogen containing metabolite at Rt 335s three sum formulas were suggested, but no metabolite could be identified. The metabolite at Rt 419s is identical to the one induced after WT Ae treatment, the others are specific. Mass value and sum formula suggest that metabolites at Rt 452s and 466s and at Rt 494s, 542s and 629s are chemically closely related. The common substructures however remain unidentified (for more details see Appendix Fig. 67).

3.5.3 Volatiles exuded 12 hrs after microbial contact to *M. truncatula* or *A. euteiches* measured by GC-MS

Volatiles exuded of aeroponically grown *M. truncatula* roots after microbial challenge were also analyzed. The collection of volatiles was only successful in the second trial of 2014, so no data from the first trial are available. Directly after the treatment of roots with the two different microorganisms *R. irregularis* and *A. euteiches*, twisters were fixed on the root tissue in order to collect emitted volatiles as depicted in Fig. 39 A. One twister per root was fixed (few roots were left out), leading to a total of 9 biological replicates per treatment in wt and 11 biological replicates per treatment in *dmi3* plants. The absorbed volatiles were measured with GC-MS as described in section 2.6.1.

In the PCA it is very striking to see the negligible effect of the genotype on the root volatile composition compared to the treatment- derived influence (Con vs Myc vs Ae). Component one contributes with 36 % to the sample distribution which is represented by the treatment of the plants with the different microorganisms. Component two contributes only 16 % to the sample distribution, which is reflecting the influence of the genotype (Fig. 48).

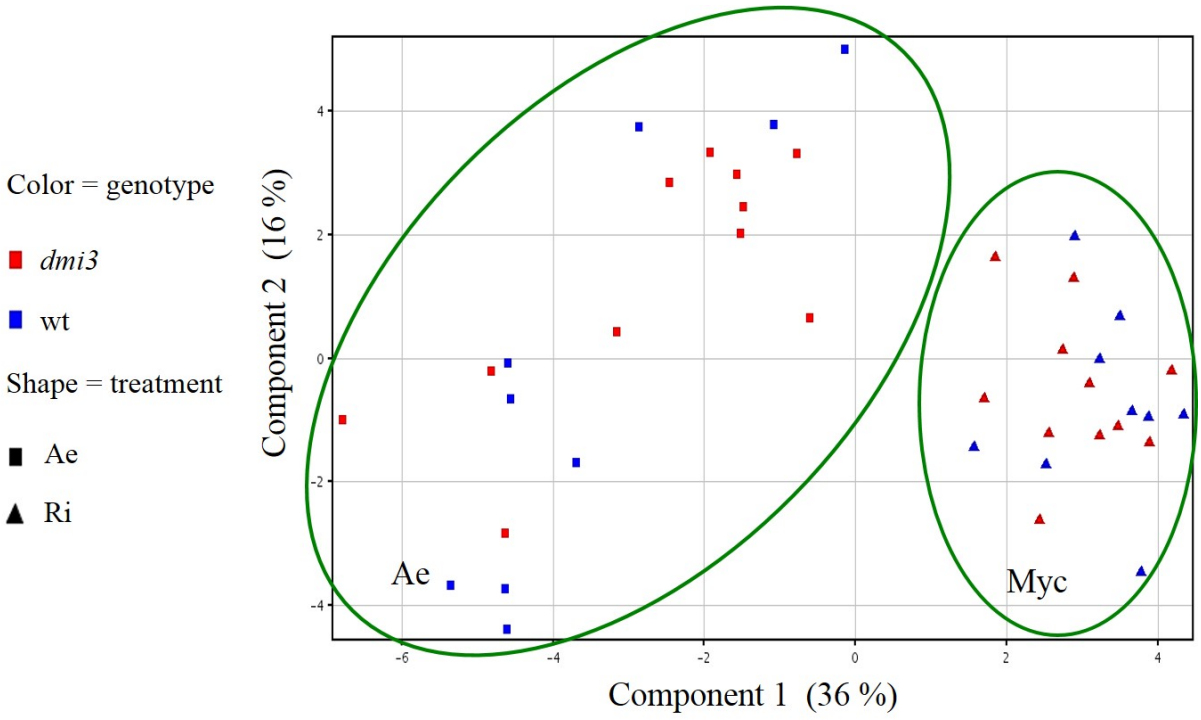

Figure 48: PCA of GC-MS data obtained from exuded *M. truncatula* root volatiles, 12 h after treatment with spores of *R. irregularis* versus treatment with *A. euteiches* zoospores.

In Fig. 49 the comparison of control vs. treatment with *R. irregularis* spores (A) and the comparison of control versus treatment with *A. euteiches* zoospores (B) is shown. Both comparisons reveal a very strong first component with 41 % and 48 %, respectively, which is represented by the treatment of the roots. While there appears some variance within the volatile composition of the control plants (wt and *dmi3*), a very clear clustering of volatiles within the samples of *R. irregularis* or *A. euteiches* treated roots shows up, that is independent of the genotype.

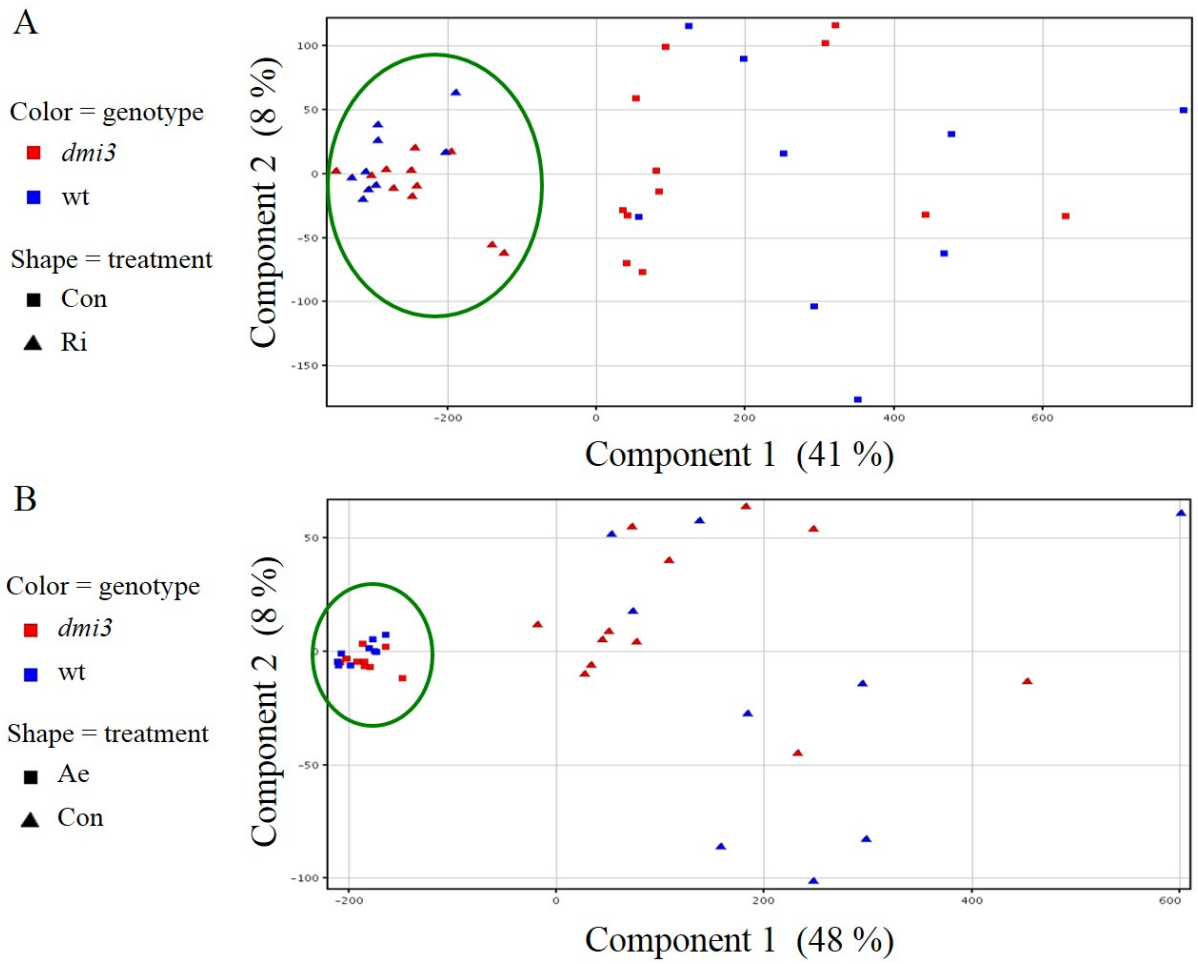

Figure 49: PCA of GC-MS data obtained from exuded *M. truncatula* root volatiles. Comparison of control plants versus plants treated with spores of *R. irregularis* (A) or with zoospores of *A. euteiches* (B) for 12 hrs.

15 volatiles showed significant differences in any of the treatment (Fig. 50). The first three substances belong to the class of azines, a six-membered heterocyclic compound, that contains one or more atoms of nitrogen with a ring structure resembling that of benzene. For all three azines (ethylpyrazine, pyridin and dimethylpyrazine), a stronger exudation appeared only in *dmi3* plants after contact to the pathogen. The exudation of the monoterpene ß-pinene was strongly induced after both treatments, in a *DMI3* independent manner. In contrast, the monoterpene limonene appeared very specifically after the treatment with mycorrhizal spores, while this reaction was not visible in the mutant, indicating a *DMI3* dependent downstream reaction of the limonene biosynthesis. The primary alcohol ethyl-1-hexanol showed accumulation after mycorrhizal treatment only in *dmi3* plants, suggesting a *DMI3* dependent suppression of the synthesis, while the reaction towards the pathogen was induced independent of the genotype. This suggests an overall antimicrobial role of ethyl-1-hexanol, which might be specifically suppressed to promote mycorrhizal interaction. phenylethanone was stronger exuded after both treatments in the wt, whereas the results for *dmi3* plants are not so clear since the control itself showed a higher level compared to the wt. The keton 3-hexanon was released stronger in an unspecific way after both treatments and in both genotypes. The aromatic heterocyclic compound

benzothiazol accumulated to a slightly lower level in the control samples of the *dmi3* plants and a slightly higher level after the pathogenic treatment in the mutant plants. For the two sesquiterpenes nerolidol and viridiflorol and for the nerolidol- epoxyacetate there was a similar pattern of root exudation.

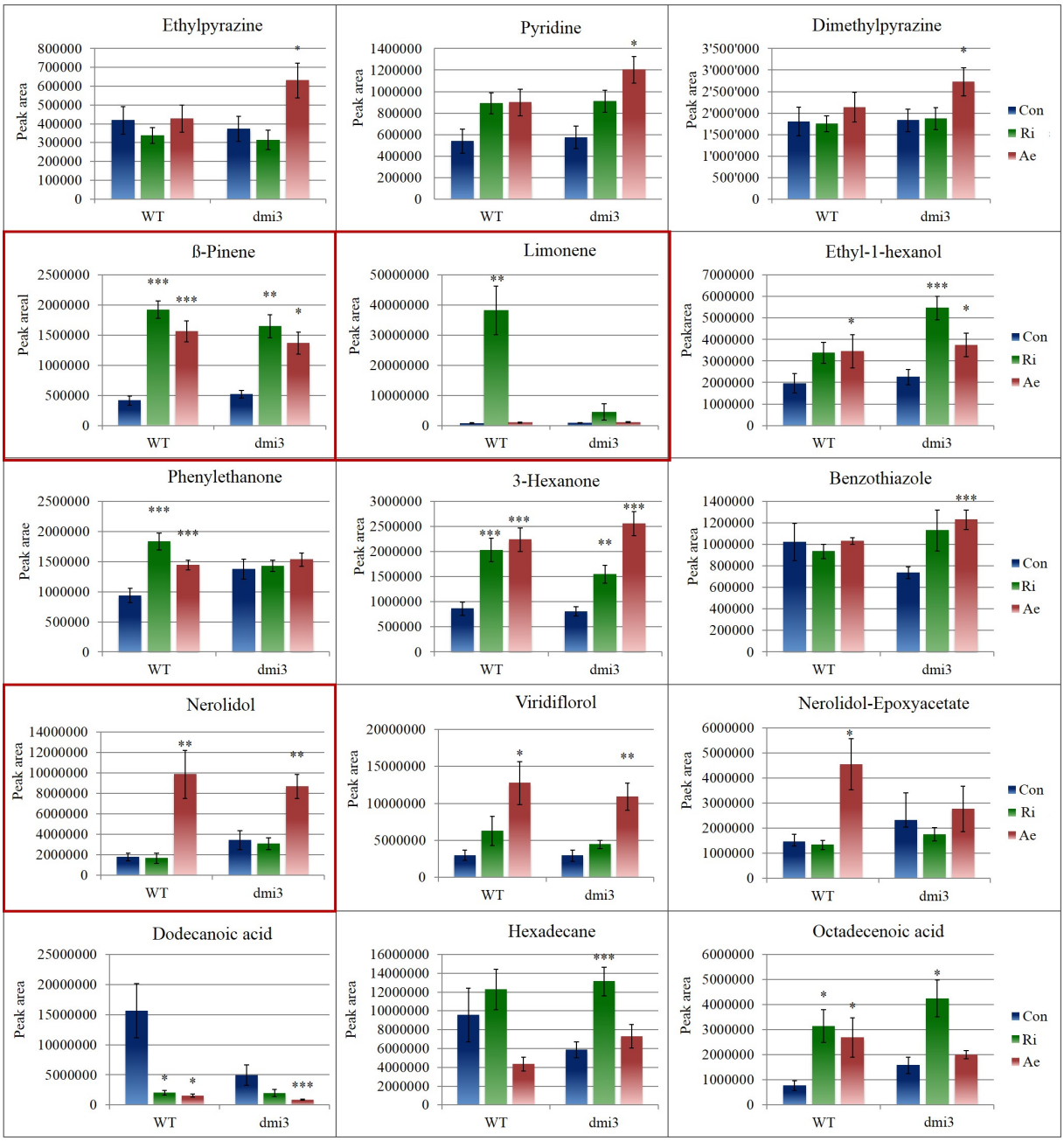

Figure 50: Mean peak area of GC-MS measured volatile compounds. Volatiles were collected for 12 hours, after application of spores of *R. irregularis* or zoospores of *A. euteiches* on roots of *M. truncatula* grown in an Aeroponic system for six weeks. The identity of the substances framed in red was verified using standard compounds. Bars indicate means $\pm$SD, n = 9-12. Significant difference were calculated with a Student's t-test (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$)

They showed an increased exudation after contact to *A. euteiches* zoospores independent of *DMI3*, while there is no regulation by the presence of the symbiotic spores in either genotype. The dodecanoic acid also called lauric acid is a saturated fatty acid. Its exudation was strongly reduced in the wt after contact with both microorganisms. In *dmi3* even the control showed a

lower exudation of lauric acid than the wt roots and this low level was even decreased by the treatment with pathogenic zoospores, indicating a rather weak regulatory influence of *DMI3* on lauric acid towards the two microorganisms. The hexadecane seemed to be induced in the wt under control conditions, while the lack of *DMI3* reduced its exudation. This reduced exudation in *dmi3* was not given anymore when roots were treated with mycorrhizal spores. The octadecanoic acid was increasingly exuded after contact to both microorganisms in the wt. In *dmi3*, its exudation seemed to be even increased after symbiotic stimulation, while the pathogen does not induce a difference compared to the control.

3.5.4 Bioassay to test biological function of limonene

The volatile limonene showed a very specific exudation being induced after the contact with spores of *R. irregularis*. As the high exudation of limonene did not appear in *dmi3* plants, it seems that this reaction is a specific reaction downstream of the CSSP. To better understand the influence of limonene on the colonization process of *M. truncatula* roots with *R. irregularis*, a bioassay was performed. Limonene was applied to plants during the process of colonization. To estimate the limonene concentration that was exuded by plant roots after contact with mycorrhizal spores, a standard of limonene was measured with GC-MS in different concentrations. The peak areas of those concentrations were compared with the peak-area that was measured in the aeroponic trial (Fig. 50). With this comparison it was estimated that a concentration of 0.6 nM corresponded best to the results of the trial. For the bioassay, plants were inoculated with *R. irregularis* inoculum as described in section 2.2.2. Plants were treated with 10 ml 1 nM limonene solution (the concentration was chosen a bit higher than 0.6 nM, to counteract dilution in the substrate) twice a week. Control plants were non-treated. 3 weeks after the inoculation with spores and the treatment with limonene, roots were harvested and the mycorrhizal colonization rate of the roots was determined by quantification of two mycorrhization marker genes *MtPT4* and *Riß-Tub* with q-RT-PCR. No difference in mycorrhizal colonization could

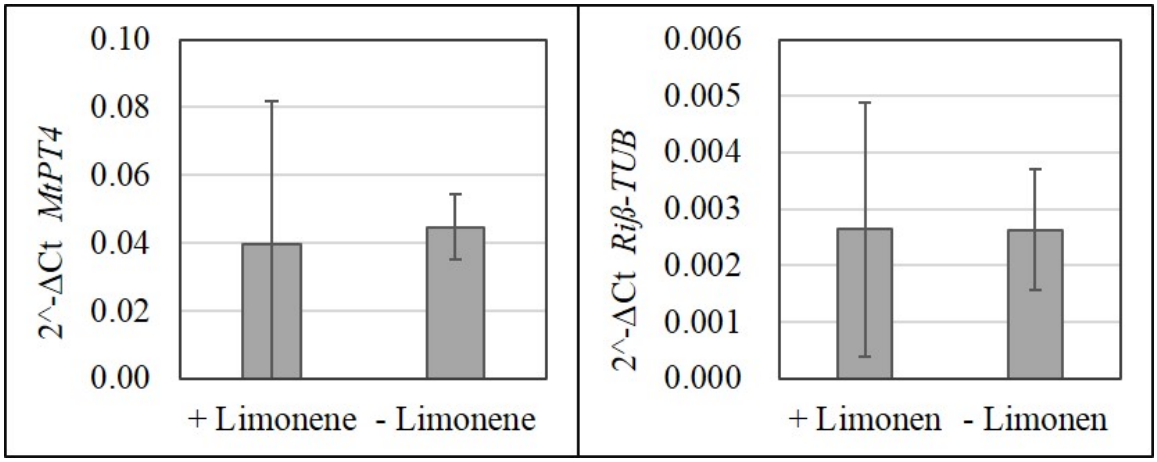

Figure 51: Expression of marker genes for mycorrhizal root colonization in *M. truncatula* after treatment with 1 nM limonene during 3 weeks of mycorrhizal colonization. Bars indicate means $\pm$SD, $n = 9$. Significant differences were calculated with a Student's t-test (* $p \leq 0.05$)

be detected with neither of the genes (Fig. 51). The addition of limonene did not result in a favored condition for root colonization. It remains to be clarified, if a different concentration of limonene- application could have a promoting effect on mycorrhizal colonization, or if the roots have already optimized their endogenous synthesis and exudation of limonene in case of interaction with mycorrhizal fungi.

3.5.5 Bioassay to test biological function of Nerolidol

A second substance with a unique exudation pattern was the volatile nerolidol, a sesquiterpene that showed a very specific exudation induced by the contact with *A. euteiches*. The exudation of nerolidol was induced by the pathogen in both genotypes, though indicating a *DMI3*-independent reaction in the plants. To better understand the effect of nerolidol as potential antimicrobial substance, a bioassay was performed where Nerolidol was applied to the *A. euteiches* zoospores. The zoospores were treated with 5 different concentrations (0 (control), 0.5, 5, 50 or 500 nM) of nerolidol and incubated for 15 min. The number of vital zoospores were counted (see section 2.2.3) and no significant difference in the number of vital zoospores after nerolidol treatment appeared (Fig. 52).

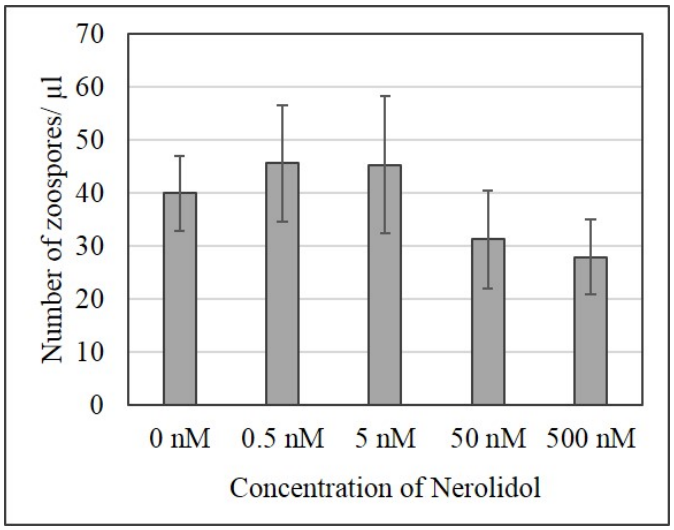

Figure 52: Number of vital *A. euteiches* zoospores 15 min after treatment with different concentrations of nerolidol. Bars indicate means $\pm$SD, n = 3. Significant differences were calculated with a Student's t-test.

3.6 Transcriptional changes 12 hrs after microbial challenge

The roots grown in the aeroponic system that were treated with two different microbial organisms for 12 hrs, were also used to perform a transcriptional analysis. Three roots were pooled to one biological replicate and there were four biological replicates per treatment. The Medicago Gene 1.1.ST Array strip of Affymetrix was used for transcriptome analysis (see section 2.4.8). To identify differentially expressed genes in the treatments versus the control, a robust multichip average normalization based on the Student's t-test was used. Transcripts were defined as being differently regulated, with a fold change (FC) of >2 or $<$-2 and a P-value ≤ 0.05. According to those criteria, 265 transcripts were identified being regulated by the *R. irregularis* treatment, 191 transcripts were regulated by the *A. euteiches* treatment, with 102 genes being regulated by both treatments (Fig. 53). From the 163 genes that were exclusively regulated by the treatment with *R. irregularis*, about two thirds of the genes (106) were down-regulated and about one third of the genes (57) were up-regulated. From the 89 genes that were exclusively regulated by *A. euteiches*, about half of the genes (49) were down-regulated and the other half of the genes (40) were up-regulated. While in the group of genes that were regulated by both treatments, almost all genes were down-regulated (97), while only five genes were up-regulated.

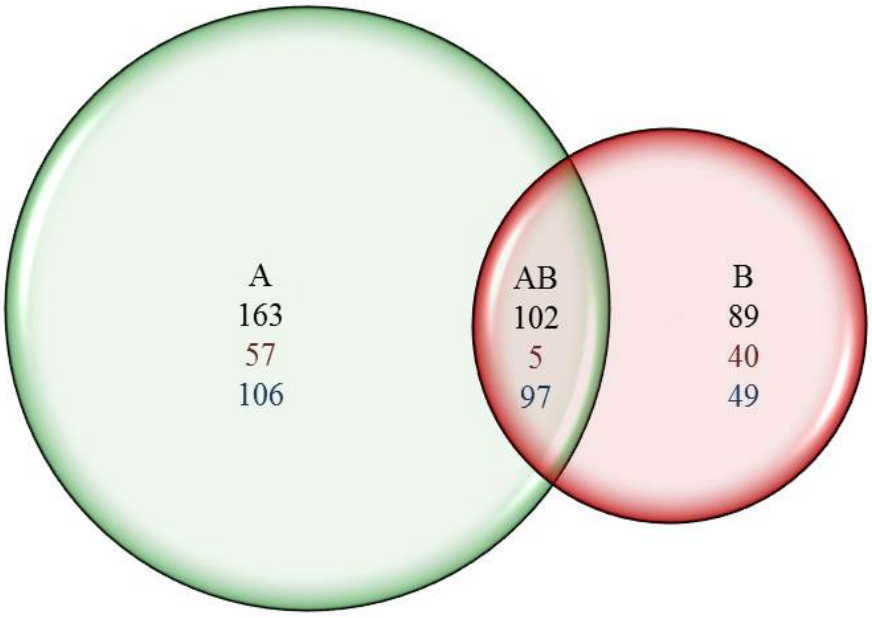

Figure 53: Venn diagram of transcriptome analysis of aeroponically grown *M. truncatula* roots 12 hrs after contact with *R. irregularis* (A) or *A. euteiches* (B). Black numbers indicate total count of regulated genes, red numbers indicate up-regulated genes, blue numbers indicate down-regulated genes.

The classification of regulated genes after ***R. irregularis*** treatment revealed 12 groups (Fig. 54). The largest group of regulated genes (68) contains genes that are not yet identified. 43 genes are involved in transcriptional regulation, including GRAS TFs, pathogenesis-related (PR) TFs, zinc finger containing TFs, homeobox domain containing TFs and basic helix-loop-helix domain containing TFs. 29 genes involved in secondary metabolism were regulated differentially after *R. irregularis* treatment like E-class P450 proteins and terpene synthases. Interestingly also 26 defense related genes were regulated after symbiotic treatment, represented by disease resistance proteins, proteinase inhibitors and leucine-rich repeat domain containing proteins, with some of them being up-regulated and some being down-regulated. 20 different transferases appeared to be regulated including aminotransferases, methyltransferases, glutathione S-transferase and glucosyltransferase. 14 regulated transcripts are involved in the protein or lipid metabolism. From the differentially regulated transcripts after the symbiotic spore treatment, 11 were involved in signaling, mainly represented by calcium-binding EF-hand containing proteins and some TGF (Transforming Growth Factor)-beta receptors, 10 transcripts were involved in transport processes and 8 transcripts were involved in the energy metabolism. Further 7 protein kinases or phosphatases and 5 transcripts involved in redox mechanisms were regulated by the symbiotic treatment.

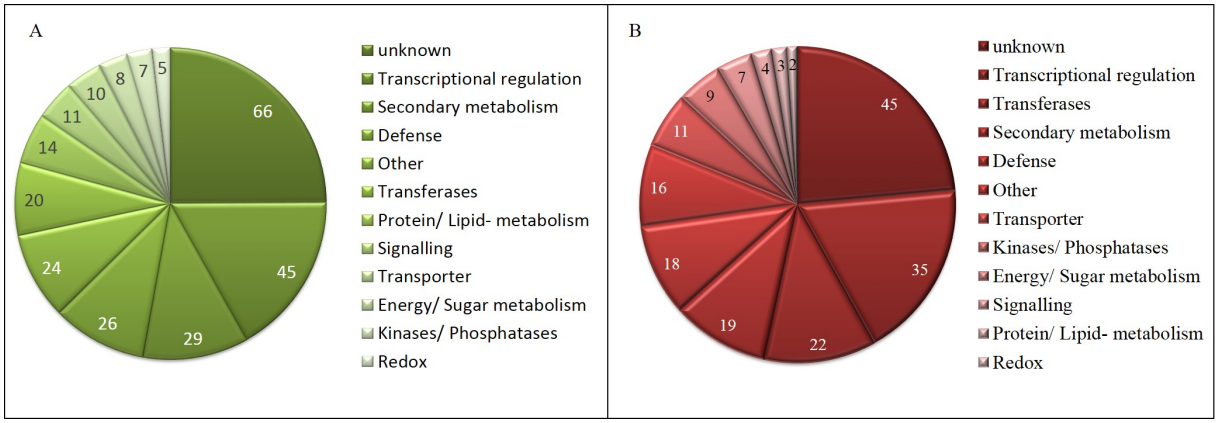

Figure 54: Classification of genes of aeroponically grown roots, 12 hrs after microbial treatment. A: Genes differentially expressed after treatment with *R. irregularis*; B: Genes differentially expressed after treatment with *A. euteiches*

The functional groups of regulated genes after the ***A. euteiches*** treatment showed that the largest group consists of genes of yet unknown function (Fig. 54 B). The second largest group of regulated genes encode proteins involved in transcriptional regulation mainly represented by pathogenesis-related TFs (also called ERF for **E**thylen **R**esponsive **F**actors), but also zinc finger containing TFs, basic helix-loop-helix domain containing TFs and homeobox domain containing TFs were differentially regulated. 22 transferases were differentially induced like polynucleotidyl transferase, glycosyltransferase, methyltransferase, sulfotransferase and glutamine amidotransferase. 19 transcripts were regulated being involved in secondary metabolism like E-class P450 proteins and terpene synthases, but interestingly non of them was exclusively regulated by the pathogen treatment, but they were all regulated by both treatments, mainly

being down-regulated (only one E-class P450 protein was up-regulated by both treatments). 18 defense related transcripts were regulated including disease resistance proteins, proteinase inhibitors and leucine-rich repeat domain containing proteins. Other categories of differentially regulated genes after *A. euteiches* treatment were transporters (11), including K-channel and heavy metal transporters; protein kinases (9), Energy-metabolism (7), Signaling (4), protein/ lipid metabolism (3) and redox- related genes (2). In Fig. 55 all significantly regulated genes are clustered in 6 groups. Groups A, B and C correspond to the treatment, up-regulated genes are marked in red and down-regulated genes are marked in blue. A similar overall trend as in the transcript 2 hrs after microbial treatment appears. Most genes that are regulated in one direction by one treatment, are regulated in a similar direction in the other treatment. There are only very few genes that are significantly up-regulated in one treatment and significantly down-regulated in the other treatment or the other way round. One of those few genes is a putative ankyrine repeat containing protein, that is 1.89 fold up-regulated after mycorrhizal treatment and and 2.22 fold down-regulated after the treatment with *A. euteiches*. There is one putative albumin encoding gene that is down-regulated after AMF treatment by 3.68 and up-regulated after *A. euteiches* treatment by 1.59. All other transcripts that were inversely regulated in the two treatments were of unknown function.

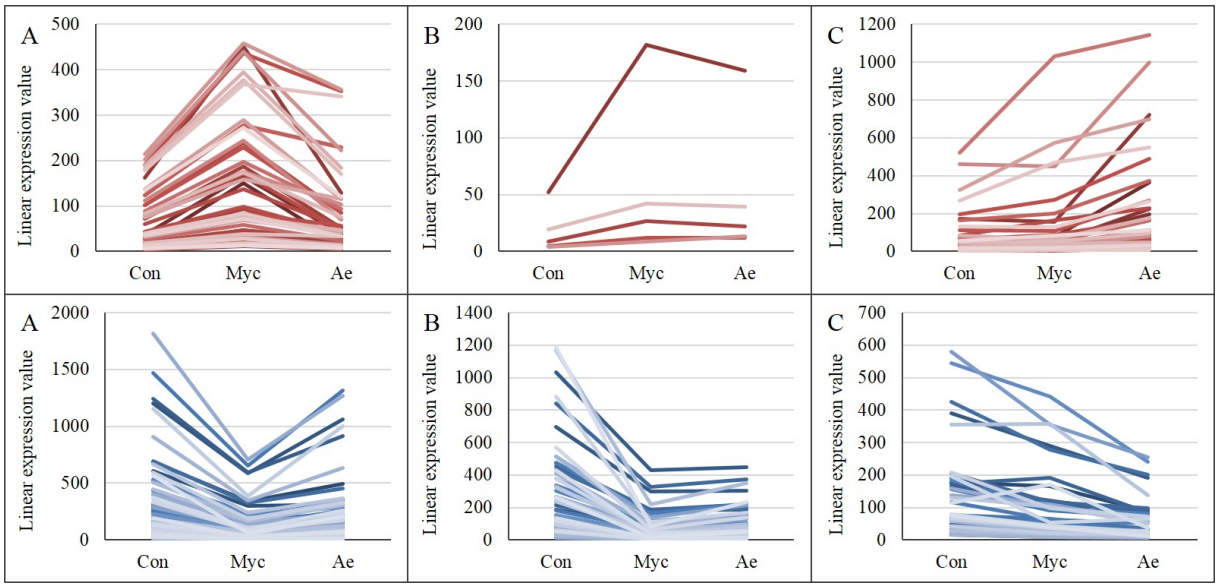

Figure 55: Expression-pattern of regulated genes by microbial treatments for 12 hrs. A: genes significantly regulated by *R. irregularis* treatment; B: genes significantly regulated in both treatments; C: genes significantly regulated by *A. euteiches* treatment. Up-regulated genes are marked in red and down-regulated genes are marked in blue.

The raw data of the two transcriptional studies as well as of the differential reports of the untargeted metabolite analysis will be provided on a CD attached to this thesis.

4 Discussion

In this work the question was addressed how and when plants clearly differentiate the type of organisms they are in contact with. Several publications indicated that the initial stages of AMF root colonization transiently induce plant defense responses, followed by localised suppression at the sites of colonization at later stages [Spanu et al., 1989, Harrison, 1993, Kapulnik et al., 1996, Lambais and Mehdy, 1992, Volpin et al., 1995, David et al., 1998]. Yet all those studies were done several days to weeks after the inoculation of plants with AMF. In this study it should be analyzed how the plant defense mechanisms are activated in very early stages after contact between plants and microorganisms and how the difference of the plant reaction towards a symbiotic or a pathogenic organism would look like. This early reaction was analyzed on transcriptional level 2 and 12 hrs after microbial contact and root exudates were analyzed 12 hrs after contact to *R. irregularis* and *A. euteiches*.

One very general issue of this project to be discussed is the selection of an oomycete pathogen to be compared with AMF. One might suspect, that the plant reaction toward an oomycete is different than towards a fungal organism. So it is suggested to include a fungal root pathogens in future experiments as for example *Rhizoctonia solani*. Yet with its chitin containing cell walls *A. euteiches* might induce an immune response not so different from that induced by fungi. The biotrophy of a pathogen might even have a greater effect on the immune response than the kingdom (*A. euteiches* is a hemibiotrophic, *R. solani* is a necrotrophic pathogen). While SA is the main plant hormone in response to biotrophic pathogens, JA is essential for the immune response against necrotrophic pathogens and herbivores [Pieterse et al., 2012], both leading to different physiological reactions.

4.1 Transcriptomics in *M. truncatula* roots 2 hrs after contact to different microorganisms reveals a mainly quantitative difference in gene expression

A rough estimation about very early transcriptional reactions of the plant induced by contact to AMF spores was done by quantifying the expression of five genes that were described to be involved in the early steps of the AM establishment (*MtENOD11*, *MtCelsynt*, *MtExplike*, *MtNodlike*, and *MtK07C11.4*) at several time points after inoculation. It was found that two of those genes showed the tendency of up-regulation, even 6 hrs after contact to AMF spores (Fig. 19). This gave us the hint that plants are reacting very fast on a transcriptional level, and that there are possibly other transcripts that might be regulated even earlier than 6 hrs after spore contact. Therefore it was decided to have a closer look at the transcript profile of *M. truncatula* 2, 6 and 10 hrs after contact with AMF spores. In this test array it was very stunning to observe that 2 hrs after contact to AMF spores more transcripts were regulated than after 6 hrs or 10 hrs (Fig. 20). From the 2 hrs regulated genes, many transcripts are only shortly regulated and do not appear as differentially regulated after 6 and 10 hrs. This strong early reaction might be assigned to an

unspecific Microbe-Associated Molecular Patterns (MAMP) reaction while a specific reaction to the fungal species of *R. irregularis* might only appear after a process of decoding the Ca-spiking pattern, that allows a species specific identification. The calcium spiking is expected to be induced only minutes after the recognition of the AMF signaling molecule as it was described for *M. truncatula* in contact with Nod-factor [Sieberer et al., 2009], yet the exact time period needed for a downstream reaction to a specific gene activation was not yet elucidated. In order to understand the specificity of the plant reaction 2 hrs after contact to AMF, *A. euteiches*, a well known root pathogen for legumes was implemented in the experiment as comparative element for transcriptomic analysis.

An overall stronger first reaction was induced by the pathogen compared to the symbiont (Fig. 21). Most genes that were significantly up/ or down-regulated by one treatment, were regulated in a similar way by the other treatment. Thus it seems that on the transcriptional level plants react towards different organisms in a general way in the beginning just with different intensities. This observation of similarities in the transcriptional regulation could be explained by the fact that AMF possess similar surface molecules as pathogenic fungi or some oomycetes like chitin and glucans that might act as general elicitors of plant defense response [Boller, 1995]. Yet basic inducible defense mechanisms of the plants including cell wall modifications, enhancement of secondary metabolites and an accumulation of PR proteins [Gianinazzi-Pearson et al., 1996a] are only rarely found in plant-AMF interaction. AMF does not induce major modifications in epidermal or hypodermal cell walls that are in contact with appressorium as this would be induced by a pathogen [Gianinazzi-Pearson et al., 1996b]. The accumulation of secondary metabolites like phenylpropanoid derivates is a common response to pathogen attack [Hahlbrock and Scheel, 1989]. There is some evidence that such defense responses are actively repressed in mycorrhizal roots. In *M. truncatula* roots, transcripts of the *isoflavone reductase*, a gene whose product is a late enzyme in medicarpin biosynthesis, was described to be induced in initial stages of AM symbiosis but subsequently declines as the symbiosis develops [Harrison and Dixon, 1994, Volpin et al., 1995]. This is a hint for the defense suppression mediated by AMF. Concerning PR proteins a similar reaction could be observed. PR-proteins have been grouped to eleven families, many of them acting as hydrolytic enzymes with β-1,3-glucanase or chitinase activities. Those hydrolytic enzymes are powerful inhibitors of fungal growth since they degrade major structural fungal cell wall components [Gianinazzi-Pearson et al., 1996a]. A burst of chitinase activity has been observed during early interaction with AMF, followed by a period where the enzyme activity is repressed to a level below the level in non-colonized roots [Spanu et al., 1989, David et al., 1998, Lambais and Mehdy, 1992].

It was further described that the AMF triggered immune reaction resembles the reaction induced by a biotrophic pathogen [Guimil et al., 2005, Paszkowski, 2006] with a quick but transient increase of endogenous sialicylic acid (SA) followed by the accumulation of defensive compounds (ROS, hydrolytic enzymes, phenylpropanoid derived compounds) [Blilou et al., 1999, Fester and Hause, 2005]. It was assumed that this SA reaction needs to be inhibited by AMF in order to establish successfully in the plant tissue [Jung et al., 2012]. The effector

protein SP7 was proposed to contribute to this inhibition and to allow the biotrophic status of AMF [Kloppholz et al., 2011, Jung et al., 2012]. Those processes are induced after the penetration of the first fungal structures. Thus the observation gained in our experiment that many genes induced by the pathogen are also slightly induced by AMF, indicate that 2 hrs after spore contact the plants are still in a defensive state. The defense response towards the pathogen was however much stronger in this early time point, suggesting that the early MAMP reaction induced by AMF is weaker than the PAMP reaction induced by a pathogen.

4.1.1 Genes differentially regulated in roots 2 hrs after microbial contact

After treatment with *R. irregularis*, seven TFs were differentially regulated. All of them had a very similar expression value by both treatments (symbiont and pathogen) with four TFs being up-regulated and three TFs being down-regulated in both treatments. This indicates an overall similar first transcriptional regulation by both treatments in the plant roots.
Among the defense related transcripts, there were four Kunitz-type proteinase inhibitors (KPIs), that were slightly up-regulated by *R. irregularis*, but 2-3 times higher up-regulated by *A. euteiches*. Three other defense related transcripts were regulated in a similar intensity by both treatments. The expression of KPIs reflects a quantitative reaction of the plant towards different types of organisms. Two transcripts of the category of energy metabolism were glycosytransferases, that were assigned as galactinol synthases in the NCBI database. Galactinol synthase is involved in the biosynthesis of raffinose family oligosaccharides. Among the sugars that are synthesized in a plant, only few are transported in the phloem over a long-distance. Sucrose is the most abundant form of carbon found in the phloem, but also polyols and oligosaccharides of the raffinose family can be found [Lemoine et al., 2013]. Both galactinol synthases are down-regulated after *R. irregularis* and *A. euteiches* treatment. This reduced expression might be in order to control the carbohydrate loss to potential parasitic organisms. This would indicate that AMF is classified as ”parasitic” in the first moments of contact at least in the sense that the sink strength is controlled by the plant. Three genes were found putatively assigned as MTD1 (Methylenetetrahydrofolate dehydrogenase) that belong to the oxidoreductases, acting on the CH-NH group of donors with NAD^+ or $NADP^+$ as acceptor. These genes were stronger up-regulated by the symbiotic than by the pathogenic treatment. Oxidoreductases might be involved in the protection of the plant from oxidative stress, which is often induced by biotic stresses [Salzer et al., 1999]. Most signaling related transcripts are similarly expressed after both treatments apart from one calcium-binding EF-hand which is exclusively expressed after mycorrhizal treatment and one serine/threonine protein kinase which is four times stronger induced by the pathogenic treatment.
The high number of defense related transcripts after pathogenic treatment indicates a stronger virulence of *A. euteiches* derived PAMPs compared to *R. irregularis* derived MAMPs. Such stronger pathogen induced reaction was also reflected by more regulated kinases and phosphatases, TFs and E-class P450 genes that were exclusively regulated by the pathogen. The

biological functions of E-class P450 genes range from the synthesis of lignin, cutin or suberin, to the synthesis or catabolism of hormone or signaling related molecules, the synthesis of pigments and defense compounds, and to the metabolism of xenobiotics [Werck-Reichhart et al., 2002]. Most of those biological functions could be related with a reaction towards *A. euteiches*.

4.1.2 Characterization of selected candidate genes putatively involved in plant-pathogen interaction

***M. truncatula Kunitz-type trypsin inhibitor alpha chain* (MTR_6g065460) [*KI*]:**
KI was selected as interesting candidate gene since some proteases and inhibitors were observed to be involved in controlling plant-microbe interaction. One example has been described for *Zea mays*, where the transcript of the plant protease inhibitor cystatin CC9 is induced by effector proteins of *Ustilago maydis*. Induction of CC9 leads to inhibition of several apoplastic proteases. This effector mediated inhibition suppresses the host immunity and supports the pathogenicity of the *U. maydis* [van der Linde et al., 2012].
Some members of the KPI family have been described to be specifically induced upon AMF colonization. A phylogenetic tree of *M. truncatula* KPIs shows that AM induced KPIs (marked with black dots) cluster together (according to the MtGEA) (Fig. 56). KPI106 an AM induced KPI was shown to interact with the protease serine carboypeptidase (SCP1) and this tandem seems to be essential for the AMF establishment in roots [Rech et al., 2013].

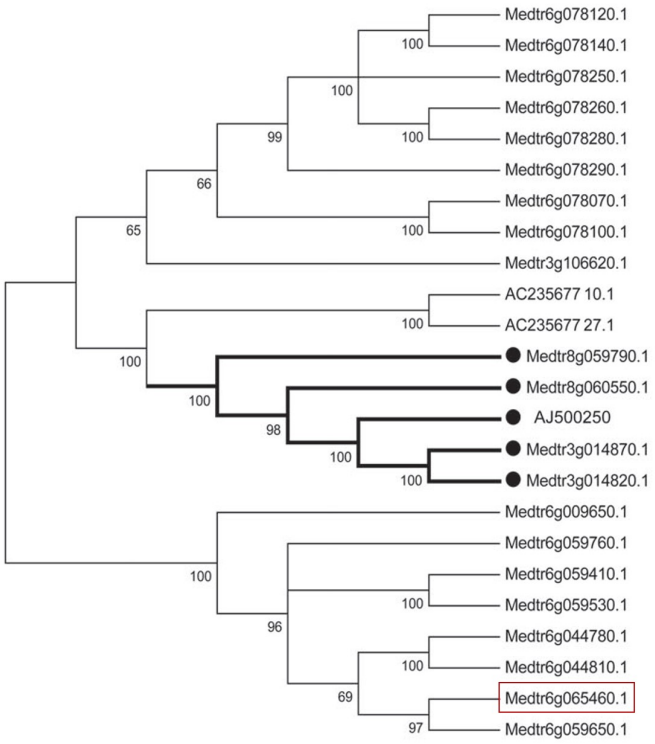

Figure 56: Phylogenetic tree of *M. truncatula* KPIs according to the hapmap 3.5 database. Expression of those KPI genes that were mycorrhiza induced (black dots) according to the MtGEA were found to cluster together. Numbers are bootstrap supported values. [Rech et al., 2013]. The KI found to be regulated by both microorganisms in this work is framed in red.

KI which was found to be induced by both microbial organisms in this study (MTR_6g065460 or Medtr6g044810.1 framed in red in Fig. 56) was not described as AMF induced by Rech et al. (2013). According to the MtGEA, it was rather early induced by different types of biotic and abiotic stresses. In this study the expression of KI was only very shortly induced by AMF while *A. euteiches* caused a strong upregulation of the gene within the first 10 hrs after zoospore contact. This expression pattern is indicating a role of KI related to plant defense. An *A. euteiches* effector triggered induction of KI might be suppressing the host immunity. Recognition of those effectors by plant resistance proteins might explain, why the KI induction only holds for a few hrs after *A. euteiches* treatment and even shorter after *R. irregularis* treatment.

M. truncatula AP2 domain-containing transcription factor* (MTR_5g058470) [*PR-TF]
Since the array done 12 hrs past microbial contact turned out to have many differentially regulated TFs, the discussion of PR-TF will be implemented in the section 4.2.

***M. truncatula* β*-glucan-binding protein* (MTR_1g079320) [β*-GBP*]**
The β*-GBP* was selected as candidate gene since β-glucan is a cell wall component of *A. euteiches*. It should be investigated whether β-GBP that was specifically up-regulated by *A. euteiches* treatment, might be involved in the *A. euteiches* recognition. Even though Oomycetes do not belong to the kingdom of fungi, the order of Leptominales share the feature with fungi, that they contain chitin in their cell wall (not only cellulose like the Peronosporales) [Lin and Aronson, 1970]. It was suggested that the dominant GlcNAc-based cell wall components of *A. euteiches* are chitosaccharides (CO) with either 1,6 lined or 1,4-linked GlcNAc residues [Badreddine et al., 2008]. Those COs might act as PAMP elicitors in plants and specific according PAMP receptors are expected [Boller and Felix, 2009]. Glucan-chitosaccharides induce the expression of defense genes in *M. truncatula* [Nars et al., 2013]. Interestingly it was reported that the *M. truncatula nfp* mutant displays an increased susceptibility towards *A. euteiches* [Rey et al., 2013]. It was also shown that glucan-chitosaccharide fractions of *A. euteiches* cell walls act as defense elicitors in plants and that they can trigger nuclear-associated calcium oscillations independent of the CSSP signal transduction pathway, which was shown by comparison of calcium oscillations in wt, *dmi1*, *dmi2* and *nfp* plants after application of glucan-chitosaccharide fractions [Nars et al., 2013]. Due to the NFP independent activation of defense genes induced by glucan-chitosaccharide fractions, it was assumed that there have to be receptors other than NFP to bind *A. euteiches* glucan-chitosaccharide which might belong to the LysM or glucan-binding protein families [Leclercq et al., 2008, Fliegmann et al., 2011].
One GBP of *Glycine max* was characterized as a hepta-β-glucoside-binding protein which might be part of a yet unknown plasma membrane-associated receptor complex [Mithofer et al., 2000]. For *M. truncatula* a multigenefamily (MtGBP1-5) was described some years later [Leclercq et al., 2008]. *GmGBP* as well as the 5 *MtGBPs* share two highly conserved domains (YNDHHYHLGY and ESTSE) that were assigned to 1.3-β-endonuclease with hydrolase activity [Leclercq et al., 2008] that could not be found in the candidate β-GBP (see alignment

in Appendix Fig. 66). The β-GBP is also shorter compared to the above mentioned proteins with a size of 32 kDa compared to 75 kDa of *MtGBP1*, yet there are some amino acids that are conserved in all aligned sequences (Appendix Fig. 66). In 2013 a huge family of glycoside hydrolises (GH) the so called GH family 81 was described including β-1,3-glucanases from fungi, higher plants and bacteria. An overall protein structure was described including three main domains, referred to as domain A, B and C, where domain A and C are functionally important while domain B is linking domain A and C [Zhou et al., 2013]. The protein alignment of the β-GBP selected in this thesis showed that it only contains domain C. A functional attribute of domain C has not yet been described. In this study a functional characterization of β-GBP could not be performed. Therefore it remains to be elucidated, whether β-GBP is involved in the specific recognition of *A. euteiches* elicitors.

***M. truncatula (+)-delta-cadinene synthase isozyme C2* (MTR_5g073200) [*TS*]**

The expression of *TS* was strongly up-regulated in the array 2 hrs after *A. euteiches* contact. The gene induction appears to be strong but very distinct for an early time-point of the plant-microbe interaction, since at later time-points the transcript was not induced. *ts* plants were more susceptible towards an *A. euteiches* infection, while the colonization with *R. irregularis* was not affected (Fig. 29). It was though interesting to see which metabolites were responsible for this pathogen-protective function in the plant. In frame of this work three sesquiterpenes, α- longipinene, copaene and α- himachalene were found to be synthesized in wt plants induced by contact with *A. euteiches*, that were missing in *ts* plants. Sesquiterpenes are one of the most studied classes of phytoalexins. Phytoalexins act as antimicrobial or antioxidative substances that accumulate rapidly in plant areas of pathogen infection. Yet they are not only toxic to pathogens but also to the plant cells itself. Therefore, the biosynthesis of phytoalexin is not induced until the plant senses the presence of the pathogen and is locally and temporally limited [Paiva, 2000]. The biological function of the three sesquiterpenes was not yet described in literature. Five different derivates of longipinene were found in aerial parts of *Santolina viscosa* and in this context a structural elucidation was performed [Barrero et al., 1994]. α-Copaene was found to be induced in tomato leaves infected by *Botrytis cinerea* [Thelen et al., 2005] and α-himachalene was found as element of the essential oil obtained from leaves of *Anacardium humile* [Winck et al., 2010]. Here the three sesquiterpenes α- longipinene, copaene and α-himachalene were described for the first time as root derived volatiles and a biological role as antimicrobial phytoalexins is proposed. A detailed understanding of its biological function is of major interest due to a possible use for the control of the *A. euteiches* induced root rot disease in agricultural systems. Since a knock-out of *TS* lead to a tendentiously enhanced susceptibility of the plants towards *A. euteiches*, it was presumed that an overexpression of that gene could enhance the resistance of *M. truncatula* towards the pathogen. In fact the *TS*-OE did not affect the infection rate of *M. truncatula* with *A. euteiches* (Fig. 33). This might be explained by the fact that the level of only one enzyme of the sesquiterpene biosynthetic pathway was enhanced and the final quantity of sesquiterpene biosynthesis might have been unaffected.

It is proposed that the functionality of TS, should be further characterized in a yeast expression system to identify further potential products of it. To further investigate the effect of those sesquiterpenes on *A. euteiches*, it is proposed to perform a bioassay, applying the substances on *A. euteiches* zoospores and quantify the effect on the zoospore vitality.

4.1.3 Characterization of candidate genes putatively involved in plant-AMF interaction

***M. truncatula LysM receptor kinase* (MTR_5g086660) [*LysMI*]** and
***M. truncatula LysM receptor kinase* (MTR_5g086310) [*LysMII*]**
The two LysM domain containing proteins were selected as candidate genes, since a role in the perception of Myc LCOs was hypothesized. Both genes were only slightly induced by the contact with *R. irregularis* spores. Despite the non-significance of the induction, the functionality of the genes was further analyzed. This was decided due to the high relevance of the identification of receptors for mycorrhizal signaling molecules. A low induced gene expression might be explained by a constitutive expression of receptors at the plant plasma membrane, so that the rhizosphere can be permanently screened for the presence of microbial signals. Another reason might be a very localized regulation of gene expression only at sites of direct plant-microbial contact. Measuring the gene expression of the complete root tissue would thus lead to a dilution effect. In Arrighi et al. (2006) the expression of the receptor kinase *NFP* was analyzed in *M. truncatula* at different stages after inoculation with *Sinorhizobium meliloti* (0, 1, 2 and 3 dpi) visualizing the gene expression by a promoter-GUS fusions. They could show that without inoculation the gene was expressed at potential sites of colonization which are the root hairs in young lateral roots. Later on, the expression changed spatially to areas where nodules were developed. This study indicates that the expression of receptors might be spatiotemporally controlled in dependence of the infection process.
In *M. truncatula* a LysM-RLK family of 16 members is known, that was divided into three subfamilies (I-III) according to their kinase domains (Fig. 57). Those 16 family members com-

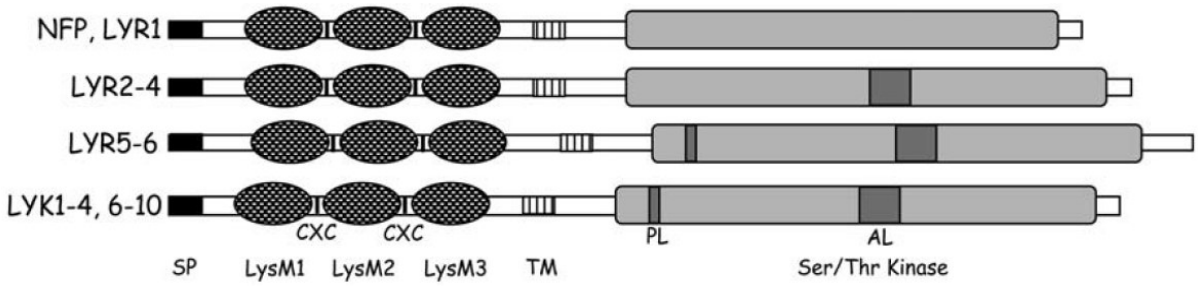

Figure 57: Scheme of the domain structure of 16 *M. truncatula* LysM-RLKs. SP: Signal peptide; LysM1, LysM2, and LysM3: three LysM domains; CXC: Cys-X-Cys motif; TM: transmembrane segment; PL: P-loop; AL: activation loop; Ser-Thr kinase: predicted intracellular kinase domain [Arrighi et al., 2006].

prise NFP, a LYK cluster (LYK1-10) and a LYR cluster (LYK related genes: LYR1-6). All *M. truncatula* LysM-RLK proteins are predicted to have an N-terminal signal peptide (SP), three LysM domains in the extracellular regions that are separated by two highly conserved Cys-X-

Cys motifs, a transmembrane-spanning domain and a Ser-Thr kinase domain in the cytoplasmic part of the protein [Arrighi et al., 2006]. The phylogenetic tree of all LysM-RLKs from *M. truncatula*, *L. japonicus*, *A. thaliana* and *Oryza sativa*, based on their kinase domains, led to the classification of three main clades, that differ in the number of exons per gene, with a LysM-I clade having 10-12 exons, and LysM-II and LysM-III clade having one and two exons, respectively [Arrighi et al., 2006]. Clade I included LYK 1-10, clade II included NFP and LYR 1-4 and clade III included only LYR 5 and 6 (Fig. 58). A phylogenetic tree based on the protein

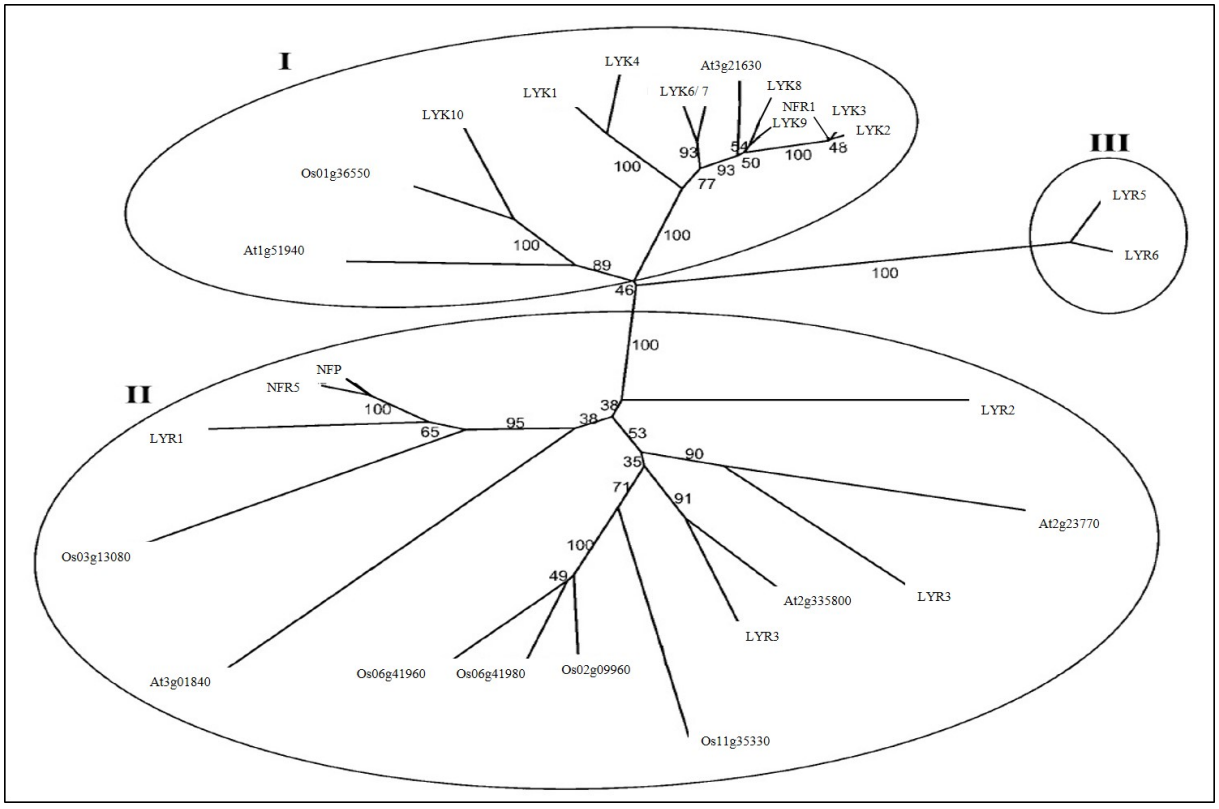

Figure 58: Phylogenetic tree of kinase domains of LysM-RLK proteins of *M. truncatula* (16), *O. sativa* (6), *A. thaliana* (5) and *L. japonicus* (NFR1/ NFR5) with bootstrap values [Arrighi et al., 2006].

alignments of only the *M. truncatula* LysM RLKs (missing LYK8 and LYR2 that could not be found in the NCBI database, but including the candidates LysMI and LysMII) showed a similar structure to the tree shown by Arrighi et al. (2006), which was based on the kinase domains. In the phylogenetic tree based on the complete protein structures, two main clades were found, where one clade included NFP, LYK 10 and LYR 1-6, while the second clade included LYK 1-4/ 6-9 and the two candidate proteins LysMI and LysMII (Fig. 59).

According to the results of this study, a role of LysMII for the plant-AMF interaction could not be confirmed. Nevertheless it should be commented on the identity of the gene. It is suggested that *LysMII* (MTR_5g086310) is the full length sequence of the above mentioned *LYK2*. The overlapping sequences of *LysMII* with *MtLYK2*, partial sequence (Locus: AY372420) have an identity of 99% according to the NCBI databank. This less than 1% of difference is represented by 2 bp out of 2615 bp, which might be a mistake within the database. This is also reflected by the extreme vicinity of the two protein sequences in the phylogenetic tree (Fig. 59).

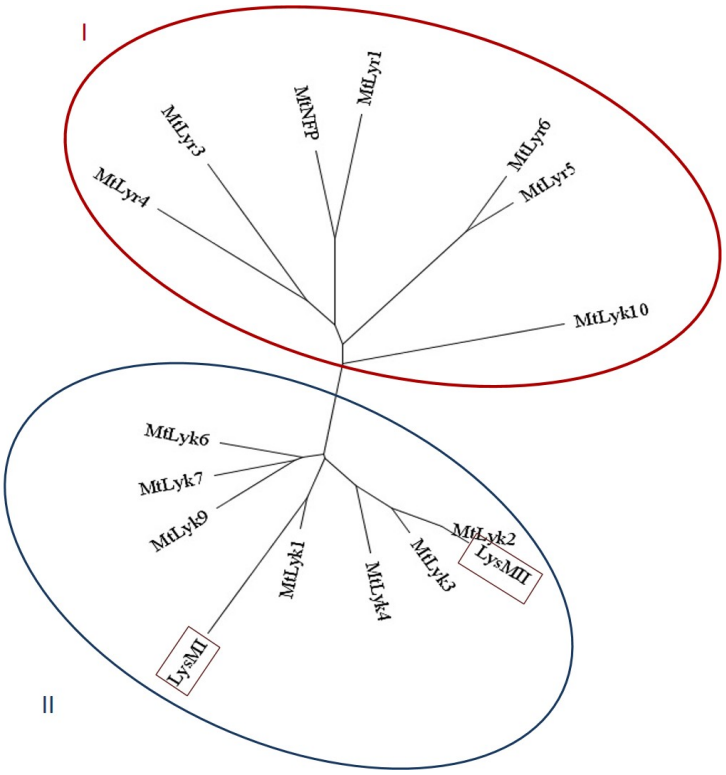

Figure 59: Phylogenetic tree of 16 LysM-RLK proteins of *M. truncatula*, divided into two groups labeled I and II.

Unfortunately also a role of LysMI in the mycorrhizal recognition could not be confirmed based on the obtained data from this study.

***M. truncatula Kinase R-like protein* (MTR_7g082510) [*KIN*]**

In contrast to the two proteins LysMI/II, KIN showed a slight but significant role for AMF root colonization. The AMF marker gene *Ri-β-tub*, which indicates the overall fungal root colonization, was lower in RNAi-plants and higher in OE-plants. KIN has a transmembrane domain, an active kinase domain and an extracellular domain, lacking any LysM domains and it was slightly induced by *R. irregularis* treatment, but also strongly induced 30 min after *A. euteiches* treatment. Based on those facts we hypothesize that KIN might be a receptor, which recognizes molecules other than LCOs (those are very likely to be recognized by LysM-domain containing receptors) and might be involved in a more unspecific recognition of MAMPs, that are present in both applied microorganisms.

Camps et al. (2015) published that the known signaling compound of AMF, the Myc LCOs activate three different signaling pathways in *M. truncatula*. MtDMI3 decodes the calcium signaling induced by Myc-LCOs and activates different TFs, including the MtNSP (Nodulation Signaling Pathway), which regulates again a high number of genes. The assumption of the existence of different signaling pathways was suggested by a transcriptome analysis performed in *M. truncatula* wt , *dmi3* and *nsp1* plants after treatment with Myc-LCOs. They identified genes that were regulated in a DMI3- and NSP1-dependent; DMI3-dependent and NSP1-independent; and DMI3- and NSP1-independent way [Camps et al., 2015]. We suggest to employ those mu-

tants to differentiate the signaling pathway in which KIN is involved. The gene expression could be quantified by qRT-PCR, 2 hrs after contact with spores (or exudates of spores) in mutants and wt roots, and the dependence of gene expression on DMI3 or NSP1 could be traced. If a DMI3 dependent but NSP1 independent expression could be found, it would be interesting to identify the TF that might regulate the kinase and thus belong to a NSP1 independent signaling pathway which is not yet characterized. One question that could be answered with this trial is if the kinase is involved in the perception of some fungal signals or if it is involved in DMI3 downstream responses.

4.1.4 Concluding remarks on transcriptomics 2 hrs after microbial contact

The transcriptomic analysis in *M. truncatula* roots after contact to different types of microorganisms after 2 hrs complements an important peace within the puzzle about early microbe induced reactions of plants. It could be observed, that the overall reaction towards AMF resembled the reaction towards a pathogen, yet the AMF induced reaction had a lower intensity. Having a closer look at the transcriptional regulation it appears that already at this early stage some genes are specifically induced, so it is suggested that the plant already distinguishes the two microorganisms to a certain level. It should be considered that in this experimental setup (plate-assay), plants were not grown in a natural environment and also the fact that plants were only exposed to two distinct microorganism would never appear under natural conditions and surely influences the specific transcriptional reaction.
To set those results in context to the root exudates, transcriptome analysis done with plants in a similar system and developmental stage as was used for the root exudate analysis (aeroponics) is of high relevance. The developmental stage of the plants cultivated on plate was far from an adult plant with highly developed root system. Therefore the transcriptomics were also done with the roots from the aeroponic experiment (12 hrs treatment), which will be discussed in the next section.

4.2 Transcriptomics 12 hrs past microbial contact to *M. truncatula* roots reveal an important role of TFs for a microbial-specific plant reaction

This transcriptional analysis was performed on plants cultivated in an aeroponic system for 6 weeks. Compared to the array done with seedlings treated for 2 hrs, the plants in this experiment were in a different developmental stage and roots were treated with microorganisms for 12 hrs. One major difference that appeared compared to the 2 hrs transcriptomics was the higher number of differentially regulated genes by the treatment with *R. irregularis* than after the treatment with *A. euteiches* (Fig. 53). One recurring observation was that only few genes were inversely regulated by both treatments while most transcripts were regulated in the same direction by both treatments with different intensities (Fig. 55).

Regarding the early AMF induced transcriptional regulation in plants, there was one interesting study by Gallou et al. (2011). The transcript of potato at three different stages of AMF colonization were analyzed: pre-stage (1 dpi), early stage (3 dpi) and late stage (12 dpi). They found that in the pre-stage there were more regulated genes (658) than in the early stage (381), while most genes were regulated under full colonization in the late stage (865). The fact that at 1 dpi there was a stronger transcriptional response than at 3 dpi is in accordance with our results, that showed the strongest transcriptional reaction in the earliest tested time-point (Fig. 20).

The transcriptomics 12 hrs past microbial contact also showed many differentially regulated TFs, most of them specifically regulated by only *R. irregularis* or *A. euteiches*.

Two WRKY TFs were specifically up-regulated by AMF treatment. In Gallou et al. (2011) it is hypothesized that WRKY TFs might be involved in the expression of salicylic acid (SA)-dependent defense responses. It seems that during early stages of the interaction with AMF, the plant reacts by activating some defense responses, similar to that induced by a biotrophic pathogen. These AMF induced responses are suppressed in later stages of the colonization. AMF induces a quick but transient increase of endogenous SA which is followed by an accumulation of defensive compounds, such as ROS and phenylpropanoids [Jung et al., 2012]. These findings are in agreement with the fact that some defense related transcripts accumulated in this experiment like disease resistance proteins and proteinase inhibitors. In the study of Gallou et al. many WRKY TFs (9) were found to be induced in the pre-stage of the colonization. It has been reported that numerous members of the multigene family of WRKY TFs are involved in the transcriptional response associated with a plant immune response [Gallou et al., 2012]. According to those data a role of the two WRKY TFs in an AM induced plant defense response is possible.

Two GRAS TFs were induced by *R. irregularis*. One of them being classified as DELLA, was up-regulated, while the other one was down-regulated by AMF. In context with the AM symbiosis, two DELLA TFs called *MtDELLA1* and *MtDELLA2* were shown to be required for successful arbuscule development. GA (gibberellic acid) is a negative regulator of arbuscule formation and the two above mentioned DELLA proteins negatively regulate the GA response. This was shown in *M. truncatula* roots [Floss et al., 2013] as well as in pea roots [Foo et al.,

2013]. DELLAs were also found to serve as mediators for the crosstalk of various phytohormones, eg. for the fine-tuning between GA and JA signaling [Hou et al., 2010]. In *Arabidopsis*, DELLA proteins also act as negative regulators of GA signaling that act immediately downstream of the GA receptor [Eckardt, 2007]. The upregulation of one DELLA protein 12 hrs after AMF contact indicates a very early control of GA signaling induced by AMF. GRAS proteins exert important roles in very diverse processes such as signal transduction, meristem maintenance and development. [Bolle, 2004]. This also supports that those two early AMF regulated GRAS TFs might be involved in signaling processes or hormonal regulation.

The so called ERFs (Ethylene-responsive element binding factors) were the most abundant group of TFs regulated in this approach (12). 8 ERFs were specifically up-regulated by pathogen treatment and 4 ERFs were downregulated by both microbial organisms in a similar way. The PR-TF (ERF) that was found to be regulated 2 hrs after microbial contact was down-regulated by both treatments. ERFs contain a highly conserved DNA binding domain called ERF domain. In *A. thaliana*, ERFs were described to respond to extracellular signals and bind to GCC-boxes in the DNA. Some defense-related genes that are induced by ethylene were found to contain a GCC box (AGCCGCC). Thus ERFs seem to be a mediator between a stress-induced ethylene reaction and a downstream response to it [Fujimoto et al., 2000]. Sakuma et al. (2002) has classified ERF-AP2 domain containing proteins from *A. thaliana* into 5 different subfamilies based on similarities of the protein sequence in their DNA-binding domain: AP2 subfamily, RAV subfamily, DREB subfamily and the ERF subfamily (Fig. 60) [Sakuma et al., 2002].

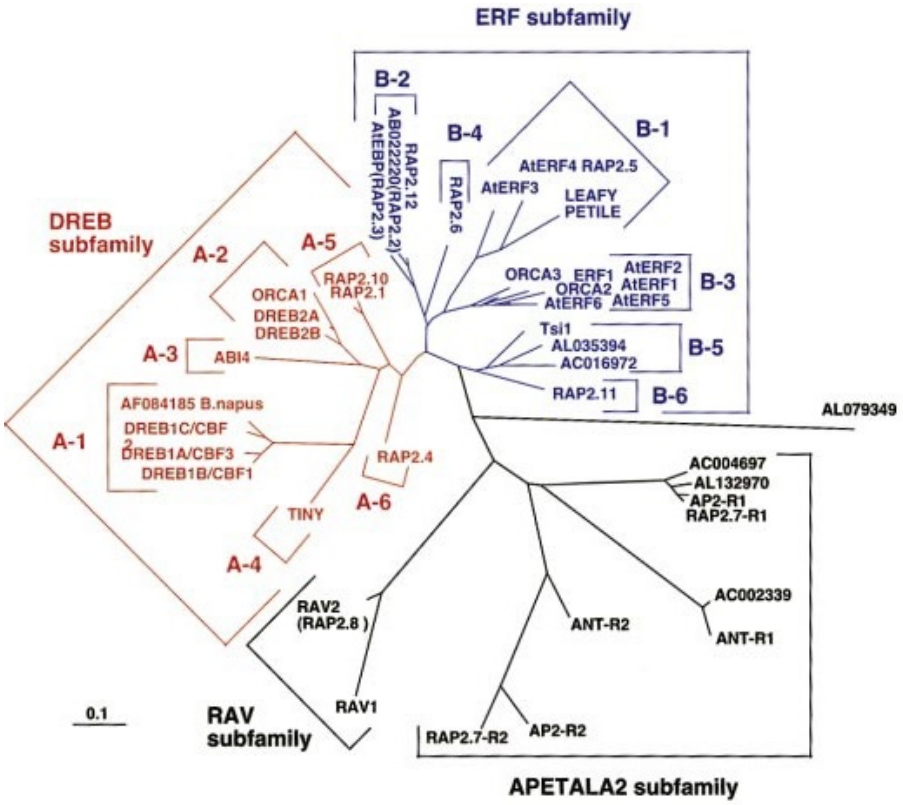

Figure 60: Phylogenic tree of ERF-related proteins clustering to 5 subfamilies after Sakuma et al., 2002.

According to Sakuma et al. (2002), there are 145 genes encoding ERF proteins in *A. thaliana*. 121 of those proteins contain the conserved WLG motif in the middle of the ERF domain, that can be found in all 13 above mentioned ERFs that were transcriptionally regulated by pathogen contact. In Fig. 61 a blast of the ERF domain amino acid sequence of the 13 regulated ERFs is shown, with the highly conserved WLG motif framed in red. Further it was suggested by Sakuma et al. (2002) that the amino acids of position 14 and 19 may play a decisive role in the recognition of DNA-binding sequences (also framed in red in Fig 61).

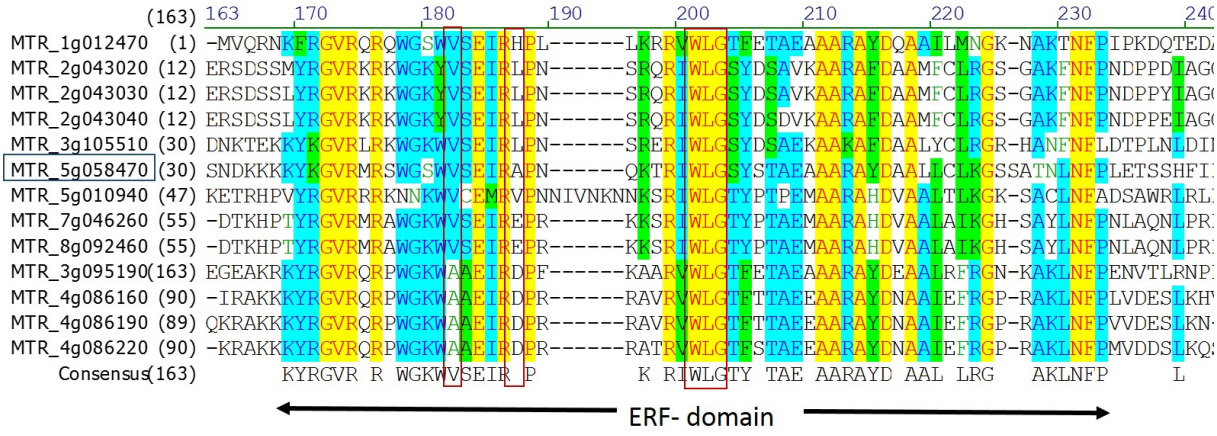

Figure 61: Blast of the ERF domain amino acid sequence of 12 transcriptionally regulated ERFs 12 hrs after *A. euteiches* contact and one ERF TF transcriptionally regulated 2 hrs after *A. euteiches* contact (framed in blue). Framed in red are amino acid position 14, 19 and WLG motif.

According to this classification, 4 ERFs belong to the ERF subfamily with alanine at position 14 and aspartic acid at position 19, while 9 ERFs belong to the DREB subfamily with a valine at position 14 and glutamic acid, histidin, leucine, alanin or valine at position 19 (Fig. 61). The ERF domain amino acid sequences of the 13 ERFs cluster into two main groups (Fig. 62). One group

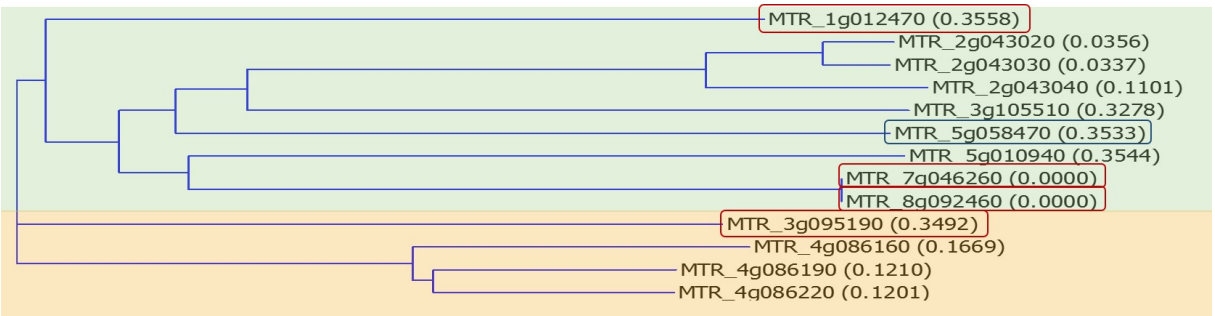

Figure 62: Phylogenic tree of amino acid sequence of the ERF domain of 13 ERFs whose transcript were differentially regulated 12 hrs (or 2 hrs - framed in blue) after contact with *A. euteiches*. One group (highlighted in green) corresponds to the DREB subfamily and the other group (highlighted in orange) corresponds to the ERF subfamily. The genes being repressed by the contact with both microorganisms are framed in red.

(highlighted in green) corresponds to the DREB subfamily and the other group (highlighted in orange) corresponds to the ERF subfamily. The genes being repressed by the contact with both microorganisms (framed in red), appear in different places within the phylogenetic tree, yet 3 of them belong to the DREB subfamily. The PR-TF regulated 2 hrs after contact is framed in

blue. Sakuma et al. (2002) states that the proteins from the ERF subfamily are involved in the ethylene signal transduction induced by biotic stress while DREB proteins were rather related to abiotic stresses like cold, drought and salt. The results of this transcriptomics suggest that also proteins of the DREB subfamily of ERFs respond to the presence of a pathogenic organism but it remains open if this might happen in an ethylene independent manner.
A subset of the huge family of ERFs had been identified as active repressors. The responsible domain is the so called ERF-associated amphiphilic repression (EAR) motif, which contains a consensus sequence of (L/F)DLN(L/F)(x)P [Kagale et al., 2010]. This sequence was not found in any of the 12 identified ERFs of this array so they were not classified as repressors.

One suggestion for the future work on TF would be to identify target genes of *e.g.* the WRKY TFs and the ERFs that were regulated specifically by the different treatments. As a first step for the target identification a successful knockdown or overexpression should be done for the TFs to later on analyze transcriptional differences induced by it. One specific challenge for this is that many of the TFs (especially the ones with big families) have very similar nucleotide sequences and thus it is hard to find specific RNAi target regions to silence the transcript of only one specific TF. It is also questionable if it makes sense to target only one single TF, since similar TFs might be able to take over their tasks, thus no major difference in downstream gene regulation would be visible. This would justify targeting a group of TFs with high sequences homology to increase the chance to get an impaired gene expression of genes, targeted by this group of TF, yet the exact functionality of each single TF would stay veiled. If knockdown or overexpression is successful, the target genes can be identified with the microarray technology, to identify the downstream regulation of TFs. Yet a microarray does not give information about whether targets are regulated directly by the TF through binding to regulatory sequences or whether regulation is indirect, through the activation of intermediate genes. To get a more detailed information, methods such as chromatin immunoprecipitation (ChIP) and Dam methylase identification (DamID), should be applied to reveal where in the genome the transcription factor is bound [Taverner et al., 2004].

4.3 *M. truncatula* root exudates and volatiles emitted 12 hrs after microbial contact contain specific secondary metabolites depending on the microorganism

It has been suggested in previous studies that secondary metabolites of root exudates are critical for the specialized associations between plants and different species of rhizosphere microorganisms. Plants are dependent on the interaction with the soil microbial community and a supposedly plant species specific chemical communication takes place [Broeckling et al., 2008]. Yet there is no clear picture on the range of secondary metabolites exuded by *M. truncatula* as reaction to a first contact with different types of microorganisms like symbiotic or pathogenic ones. The experiment of this study gives a first insight about the composition of secondary metabolites in root exudates of *M. truncatula* 12 hrs after treatment with the symbiont *R. irregularis* or the pathogen *A. euteiches*. Not many studies have analyzed the secondary metabolites of *M. truncatula* root exudates so far. First of all this is due to the challenge of collecting pure root exudates that are not contaminated with soil born substances that would impair the delicate method of untargeted LC-MS analysis. This challenge could be nicely solved by using an aeroponic system to cultivate the roots, that permitted the development of a high root biomass and thus high root exudation and an easy exudate collection by simply washing the exudates from the roots. By cultivating plants in the aeroponic system, roots are lacking a natural environment that implies different physicochemical properties of soils that might also influence root exudate composition. This could not be taken into account in this experiment. A second major challenge is the huge amount of different secondary metabolites potentially synthesized by plants. This highly complicates the interpretation of chromatograms and the structure elucidation based on databank information. Moreover it appears that root exudates of *M. truncatula* differ highly from exudates of other plants as for example *Arabidopsis thaliana*, which even hampered the identification work. Yet the groundwork of some groups on root metabolites of *M. truncatula* was supportive for the identification of some root exudates. One study analyzed triterpene saponins from *M. sativa* and *M. truncatula* and identified a large number of saponins from *M. truncatula*. They also showed that *M. truncatula* roots contain a much more complex mixture of saponins than *M. sativa* especially due to a greater diversity in conjugation of saponins. This greater diversity of conjugates may play a role for storage or even signaling and plant defense [Huhman and Sumner, 2002]. In a later study the triterpene saponins of *M. truncatula* within different tissues (root/ leaf/ seeds) were qualitatively profiled and quantified. It was found that roots contained the highest number of saponins followed by leaf and seeds, respectively [Huhman et al., 2005]. In root tissue and cell cultures of *M. truncatula* 35 polyphenols were identified, including isoflavones, flavones, flavanones, aurones and chalcone [Farag et al., 2007].

4.3.1 Root exudates identified by untargeted LC-MS

To analyze the root exudates, two independent aeroponic experiments were performed, one in July/ August 2013 and one in July/ August 2014. In the first experiment only *M. truncatula* wt plants treated with the different microorganisms were analyzed. In the second experiment *dmi3* plants were added to compare them to wt plants. One finding that appeared in both years was that on the level of root exudates, plants reacted stronger to the symbiotic contact with *R. irregularis* than to the pathogen *A. euteiches* (Fig. 42, 44). This was correlating with the number of regulated genes, that was higher after the symbiotic treatment than after pathogen treatment in the same experiment (Fig. 53). Yet in the transcriptomics done with seedlings that were inoculated with the two different microorganisms, the number of regulated genes was much higher in the *A. euteiches* treated plants than in *R. irregularis* treated plants. To clarify if this pattern was also related to the developmental stage of the plants in the different experiments, it is proposed to perform an aeroponic experiment with only 2 hrs of incubation time after spore/ zoospore inoculation and see if the transcriptional regulation in the adult plants would be similar to the transcriptional regulation in the seedling stage. The higher number of root exudates that was specifically exuded after *R. irregularis* treatment was also reflected by the sample distribution in both PCAs, where *R. irregularis* treated samples showed the clearest distance to all other samples (Fig. 41, 43). So far we can not explain why the AMF treatment induces a higher number of root exudates.

Some exudates of the trial of 2013, could be identified via MS-MS, even though a control using standard substances has not been performed. Interestingly all flavonoid related metabolites were stronger exuded after the contact with the pathogen and weaker exuded after the symbiotic treatment. Here the so far known role of flavonoids in root exudates of *M. truncatula* should be discussed (Fig. 63).

In legumes, flavonoids have a diverse functional spectrum. They are synthesized through the pheynylpropanoid or acetate-malonate metabolic pathway which is well described in different plant species. Interestingly compared to legumes, *Arabidopsis* lacks some enzymes (chalcone reductase and some isoflavone synthase enzymes) and can therefore not produce the subset of isoflavonoids [Weston and Mathesius, 2013]. Diverse flavonoids are typically produced in the roots and stored as glycosydes or aglycones and are released by either root exudation, tissue decomposition or leaching [Rao, 1990]. Flavonoids are involved in protecting the plants against different abiotic stresses and can be involved in the acquisition of different nutrients like N, P and Fe by changing the chemical properties of the soil or by acting as chelators [Gholami et al., 2014]. Regarding beneficial plant-microbe interactions it was described that flavonoids play a major role in stimulating the symbiotic interaction with rhizobia or AMF. The recognition of the Nod factor by plants induces up to 30 *nod* gene- induced flavonoids, that were isolated from nine legume genera [Cooper, 2007, Cesco et al., 2012, Gholami et al., 2014]. Of those flavonoids, luteolin and apigenin act as chemo-attractants, guiding rhizobia to the root surface [Cooper, 2007, Weston and Mathesius, 2013].

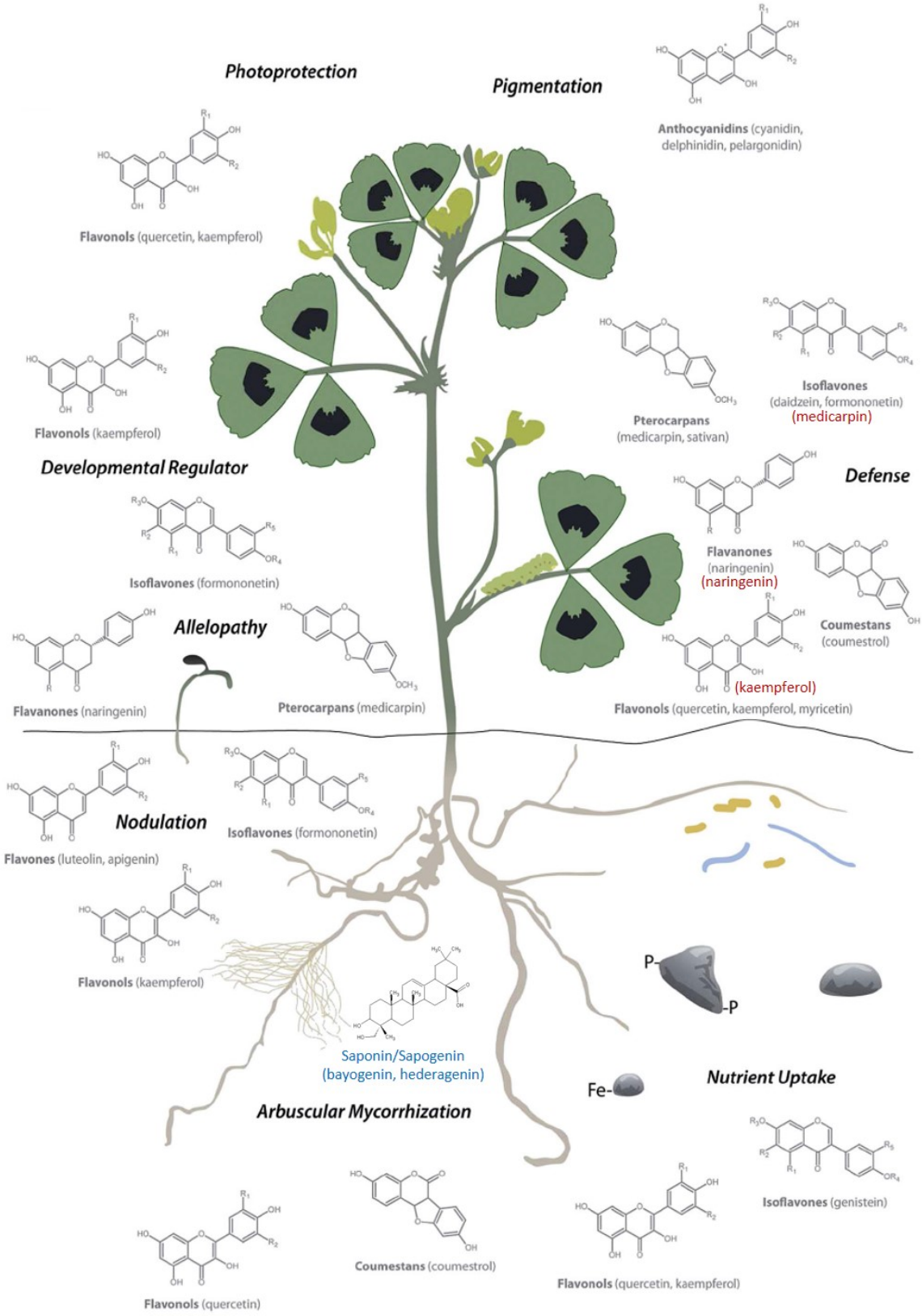

Figure 63: In planta biological functions of different secondary metabolites with some representative compounds, depicted on a *Medicago* plant. Metabolites found in this work to be exuded after *A. euteiches* contact (red) and after *R. irregularis* contact (blue). Modified after [Gholami et al., 2014].

For the symbiotic interaction with AMF, flavonoids play an important role inducing AMF spore germination, hyphal growth/ branching and root colonization [Hassan and Mathesius, 2012]. Hyperoside, the 3-O-galactoside of quercetin exuded by roots of *Medicago sativa* (*M. sativa*) seems to stimulate the spore germination of two different *Glomus* species [Catford et al., 2006]. Within the class of isoflavonoids, coumestrol which is closely related to medicarpin was identified as stimulator for hyphal growth of AMF in *M. truncatula* [Morandi et al., 2009]. Medicarpin itself was described to accumulate in *M. truncatula* colonized roots by *G. versiforme* [Harrison and Dixon, 1994], but a supportive role of medicarpin for AMF has not been confirmed yet. So far medicarpin has rather been characterized as phytoalexin being toxic to a number of fungal plant pathogens *e.g.* it was shown to protect *M. sativa* from the pathogenic fungi *Rhizoctonia solani* [Naoumkina et al., 2010]. This would be in line with the results of this work, since here a pathogen-induced exudation of medicarpin in roots treated with *A. euteiches* was found, and a rather lower exudation after AMF treatment (Fig. 47).
The flavanone naringenine interferes with the production of *Pseudomonas aeruginosa* derived virulence factors that are controlled by quorum sensing (QS) [Vandeputte et al., 2011]. In this study naringenin was higher exuded after the pathogen treatment and thus a similar role as described for the interaction with *Pseudomonas aeruginosa* might account for the interaction with *A. euteiches*. The flavonol kaempferol that was slightly higher exuded by plants inoculated with *A. euteiches* in this experiment, has so far been described as repellants for root nematodes [Weston and Mathesius, 2013], but according to the results of this work, it might also be involved in the defense towards other pathogens.
All putative sapogenins were found to be stronger exuded after AMF treatment and lower exuded after pathogen treatment (Fig. 47). Sapogenins are the basic molecules of saponins and represent the aglycone (non-saccharide) portions of the natural products family of saponins [Gholami et al., 2014]. Some observations about saponins suggest their involvement in plant defense mechanisms: they are elicited by jasmonates, which are known to trigger plant defense responses [Suzuki et al., 2005, Naoumkina et al., 2010] and are also increased in plant tissues that were exposed to herbivore attacks [Agrelli et al., 2003]. Furthermore a biological activity against phytopathogenic fungi, bacteria, insects and nematodes suggests their involvement in plant defense [D' Addabbo et al., 2011, Carelli et al., 2015]. In this experiment three exudates of the class of sapogenins were found to be specifically stronger exuded after symbiotic spore contact. This observation is very interesting regarding a report about a nodule- promoting effect of saponins in *M. truncatula* [Confalonieri et al., 2009]. *AsOXA1*, a gene of *Aster sedifolius* encoding β-amyrin synthase was expressed in *M. truncatula*. The β-amyrin synthase is a key enzyme in the biosynthesis of sapogenins and finally triterpene saponins (see Fig. 64). The expression of *AsOXA1* lead to a significantly increased production of bayogenin, hederagenin, soyasapogenol E and 2β-hydroxyoleanolic acid in roots and this was accompanied by an increases nodulation of the root tissue [Confalonieri et al., 2009]. This indicates a role of saponins beyond antimicrobial functions, which was also suggested earlier by a group that analyzed β-amyrin synthase genes from pea and *M. truncatula* (PSY and MtAMY1) and found that

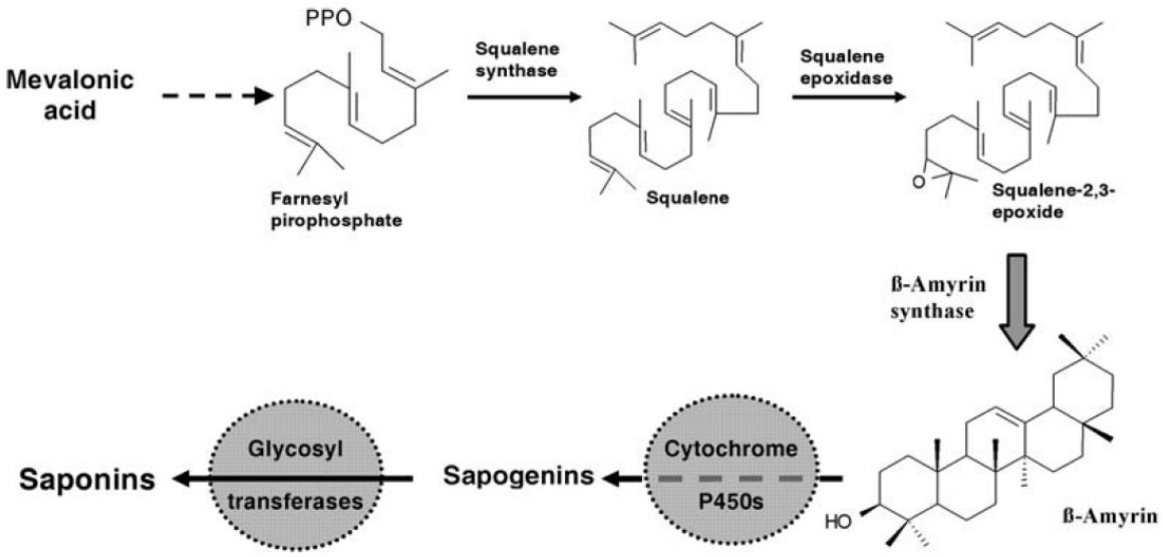

Figure 64: Biosynthetic pathway of sapogenins/ triterpene saponins in *M. truncatula* [Confalonieri et al., 2009].

their expression is induced in nodules [Iturbe-Ormaetxe et al., 2003]. Apparently sapogenins play a role for symbiotic interactions and it is recommended to have a closer look at the detailed effect of sapogenins on AM. This could be done with a bioassay, analyzing the effect of the single sapogenins on AM fungal development, like hyphal growth and branching. Plants with a silenced β-amyrin synthase encoding gene should be analyzed for AM development within roots. So it is suggested that certain saponins might not act as phytoalexins but be even stimulating for the symbiotic interaction.

A very surprising result appeared in the experiment of 2014, where *dmi3* plants were included. The fact that *dmi3* plants are impaired in the CSSP led to the expectation that the root exudate composition after *R. irregularis* treatment (which induces the CSSP in wt plants) would be strongly influenced by the lack of the functional DMI3. Indeed, only few substances were found that were differentially exuded in *dmi3* compared to the control after mycorrhizal treatment with only 12 substances reduced and 4 substances stronger exuded. This suggests that the CSSP does not highly influence the plant reaction regarding the exudation pattern. It seems that the CSSP-induced reaction is rather limited to changes in cellular reorganization, so that the AMF can enter the root tissue. In contrast the treatment with the pathogen *A. euteiches* lead to a highly different root exudation between wt and *dmi3*. It is speculated that a PAMP from *A. euteiches* might also activate the DMI3 but lead to a reaction that is independent of the CSSP. This reaction might be responsible for plant defense reactions involving the exudation of antimicrobial substances.

4.3.2 Volatiles

In the present study not only exudates but also volatiles were analyzed that were emitted by plant roots 12 hrs after microbial contact. The collection of volatiles and its analysis with GC-MS was only done in the second trial of 2014.

VOCs exuded by roots have been described to be involved in plant-microbe interactions in different ways. One aspect of VOCs is the involvement in antimicrobial and antioxidant activity [Candan et al., 2003, Cimanga et al., 2002, Dorman and Deans, 2000] as well as in plant defense against herbivores [Arimura et al., 2008,Dicke et al., 2009]. On the other hand, volatiles especially monoterpenes can be attractants as they represent a potential carbon source for microbial rhizosphere organisms. Some organisms such as *Pseudomonas fluorescence* or *Alcaligenes xylosoxidans* have been able to feed on monoterpenes as unique carbon source [Kleinheinz et al., 1999].

Two different monoterpenes were specifically influenced by the microbial contact in this experiment: β-pinene and limonene. A potential antimicrobial effect of both was shown in a study that analyzed the antibacterial activity of essential oils from different herbs. Those substances were assayed against ten different human pathogenic bacteria. Linalyl acetate and limonene showed the lowest antibacterial activity among the components tested, followed by α-pinene, β-pinene [Sokovic et al., 2010].

β-Pinene-exudation is strongly induced by both treatments in both genotypes and seems to be a general answer of plants to biotic stimuli. So it is suggested that β-pinene might act as signaling substance for within and inter-plant communication, to activate priming. VOCs were formerly described to act as within-plant signaling compounds [Heil and Silva Bueno, 2007] and an induced priming by VOCs of neighboring plants were found especially after herbivore attack but might also hold true for other microbial challenges [Dudareva et al., 2004].

The monoterpene limonene appeared to be specifically exuded after AMF treatment in a DMI3 dependent manner. This suggests that limonene might be an attractant for AMF. Yet a bioassay where limonene was applied on roots during the AMF colonization process did not show an increased colonization level of limonene-treated vs. non-treated plants (Fig. 51). The detailed effect of limonene on the AMF phenotype such as hyphal growth or branching after limonene application is advised to be analyzed in further experiments.

The two sesquiterpenes nerolidol and viridiflorol were specifically induced by the pathogen in a DMI3 independent manner (Fig. 50). At a first sight this would suggest an antimicrobial effect of those sesquiterpenes. Yet the bioassay done with nerolidol did not indicate any direct effect on the vitality of *A. euteiches* zoospores (Fig. 52). So far nerolidol was only described as being emitted by plants under herbivore attack [Degenhardt and Gershenzon, 2000], but an antimicrobial effect has not yet been described. Since a direct impact on the microbial vitality was not observed, which would have been expected from a phytoalexin, it is proposed that the mode of action of nerolidol is rather indirect like for example a disturbance of quorum sensing within species communication. QS is a microbial communication tool that is used by bacte-

ria to coordinate processes like biofilm formation and population growth. QS has also been discovered in fungi. In *Candida albicans* filamentation is controlled by the sesquiterpene alcohol farnesol and aromatic alcohol tyrosol was found to control growth, morphogenesis and biofilm formation [Albuquerque and Casadevall, 2012]. Even a cross-kingdom communication between oomycetes and bacteria was described [Kong et al., 2010].
It can be concluded that plants have a distinct reaction towards different types of microorganism on the level of root emitted volatiles already at very early stages of plant microbe-interactions.

4.4 Model about early interaction of *M. truncatula* with different types of microorganisms and outlook

Summing up the insights gained in this study and combining it with already published data, a very hypothetical model about a possible strategy of plants reacting towards a symbiotic partner in comparison to the reaction towards a pathogen should be drawn here: The early transcriptional regulation in roots seems to combine a defense response with a specific response towards the symbiont. Regarding the composition of secondary metabolites found in root exudates, only few substances were induced by both treatments while a subset of secondary metabolites were found to be very specifically exuded after AMF contact. This in one sense broad and in another sense specific reaction of plants towards different organisms could be provided by a combination of different receptors at the plasma membrane. Receptors for Myc-LCOs and potential other specific AMF signaling compounds that lead to the CSSP activation as well as receptors that recognize a general fungal presence (like chitin receptors) and induce a PAMP induced defense response, might exist. Biologically this would make sense, since a mimic of AMF derived signaling molecules could be used by pathogens to overcome immune reactions and facilitate a tissue penetration. Keeping the defense responses active, a first colonization is permitted by the activation of CSSP. At the moment of fungal tissue penetration, a further critical discrimination might happen, induced by AMF specific effectors as for example SP7 [Kloppholz et al., 2011]. This would than inhibit plant defense in early stages of root colonization so that the full development of the AM symbiosis can take place as was formerly described.
To further elucidate processes induced by microbial stimuli, the transcriptome should be analyzed in mature plants at different early time points after microbial contact. The implementation of mutants like *dmi3* to better understand the role of the CSSP and a possible co-involvement of DMI3 in decoding pathogen-induced signaling processes is recommended. It is also advised to repeat the root exudate analysis especially of *dmi3* and to analyze if the strongly *A. euteiches* induced response of root exudates can be reproduced. In this study only few secondary metabolites detected in root exudates could be identified, missing out the numerous metabolites that were specifically emitted by *dmi3* plants after *A. euteiches* treatment. More effort should be done to identify more *M. truncatula* derived root exudates and the use of an aeroponic plant cultivation system is highly recommended for exudate collection of future studies.

5 Summary

Plants are permanently confronted with the rhizosphere microbial community and are deeply impacted by it. For agricultural plant production it is of major importance to understand mechanisms that induce resistance towards pathogens or that support the interaction with beneficial organisms. To better understand the first steps of recognition and interaction of plants towards its close environment, the very early reaction of the model plant *M. truncatula* towards the symbiont *R. irregularis* or the pathogen *A. euteiches* was analyzed. This was done by analyzing the transcriptome and the secondary metabolites of root exudates at 2/ 12 and 12 hrs after microbial contact, respectively. Transcriptional differences were captured with the help of microarray analysis. Untargeted LC-MS served as method to analyze secondary metabolites of root exudates and VOC were measured with GC-MS.

The transcriptomic data revealed that many genes were regulated in a similar way after treatment with the symbiont or the pathogen, while the intensity of the regulated gene expression differed clearly. Regarding the number of significantly regulated genes, *A. euteiches* treatment had a much stronger impact than *R. irregularis* treatment. In order to get an understanding about the biological function of some selected candidate genes, RNAi-lines/ OE-lines were generated or *Tnt1* insertion lines were ordered and their colonization phenotype was evaluated. With the homozygous *Tnt1* insertion line for the candidate gene *TS* an important role of TS for the plant resistance towards *A. euteiches* could be confirmed. Furthermore, the three sesquiterpenes α-longipinene, copaene and α- himachalene were found to be emitted from *A. euteiches* treated roots in a TS-dependent manner, suggesting an antimicrobial function. The candidate gene *KIN* was shown to slightly influence the root colonization by *R. irregularis* yet a detailed characterization about the functionality of KIN could not be done in frame of this work.

For analysis of roots exudates, plants were cultivated in an aeroponic system, which allowed an easy root exudate collection. Untargeted LC-MS analysis revealed major differences between exudate patterns of roots in contact with *R. irregularis* or *A. euteiches*. *R. irregularis* treatment had a stronger impact on root exudation than *A. euteiches* treatment, while both treatments had a much stronger impact on root exudation than the genotype, including wt and *dmi3*. One major finding of the LC-MS measurements was that metabolites from the class of flavonoids were stronger exuded after *A. euteiches* treatment, while metabolites from the class of sapogenins were stronger exuded after *R. irregularis* treatment. The most surprising result was the very strong impact of *A. euteiches* on root exudates of *dmi3* plants with about five times more specifically exuded metabolites than after *R. irregularis* treatment.

VOC analysis also revealed a much stronger effect of the treatment than the genotype on emitted VOC composition. VOCs could be found that were emitted in a very treatment specific manner, like for example the three sesquiterpenes nerolidol, viridiflorol and nerolidol-epoxyacetate, that were only emitted after *A. euteiches* contact, while the monoterpene limonene was specifically emitted after *R. irregularis* contact. Few VOCs were found to be exuded in a DMI3 dependent manner while most of them were exuded independent of the genotype.

6 Zusammenfassung

Pflanzen unterliegen dem ständigen Einfluß der mikrobiellen Gemeinschaft der Rhizosphäre. Für die landwirtschaftliche Produktion ist ein gutes Verständnis über pflanzliche Mechanismen für Toleranz bzw. Resistenz gegenüber Pathogenen oder für die verbesserte Interaktion mit Symbionten sehr wichtig. Um die ersten Schritte der Erkennung und Interaktion von Pflanzen mit ihrer Umwelt besser zu verstehen wurde in dieser Studie die frühe Antwort der Modellpflanze *M. truncatula* auf Kontakt mit dem symbiontischen Pilz *R. irregularis* oder dem Pathogen *A. euteiches* analysiert. Der Ansatz hierfür war zum einen eine transkriptionelle Analyse der Pflanzenwurzeln 2 bzw. 12 Stunden nach mikrobiellem Kontakt mit Hilfe der Mikroarray Technologie. Zum anderen wurden die exudierten Sekundärmetabolite bzw. Volatile von *M. truncatula* Wurzeln 12 Stunden nach mikrobieller Behandlung mit LC/ GC-MS analysiert.
Die Transkriptomdaten zeigten, dass viele Gene durch beide Mikroorganismen auf ähnliche Weise reguliert wurden, jedoch mit deutlichen Unterschieden in der Intensität der Regulation. Die *A. euteiches*- Behandlung beeinflußte die Regulation von deutlich mehr Genen als die Behandlung mit *R. irregularis*. Um die biologische Funktion einiger ausgewählter Kandidatengene besser zu verstehen, wurden RNAi/ Überexpressionslinien generiert bzw. *Tnt1*-Insertionslinien bestellt. Mit Hilfe der *Tnt1*-Insertionslinie für das Kandidatengen *TS* konnte eine wichtige Rolle von TS für die pflanzliche Resistenz gegenüber *A. euteiches* nachgewiesen werden. Zudem konnte gezeigt werden, dass die drei Sesquiterpene α- Longipinene, Copaene and α- Himachalene nach Kontakt mit *A. euteiches* in einer TS- abhängigen Weise von Wurzeln exudiert wurden. Des weiteren konnte ein Einfluß des Kandidatengens *KIN* auf die Kolonisierungsrate der Wurzeln mit *R. irregularis* gezeigt werden, eine detaillierte funktionelle Charakterisierung konnte im Rahmen dieser Arbeit jedoch nicht erfolgen.
Zur Analyse der Wurzelexsudate wurden Pflanzen in einem aeroponischen System angezogen, welches eine einfache Sammlung von Exsudaten ermöglichte. Die LC-MS Analyse zeigte, dass die Zusammensetzung von Wurzelexsudaten sich nach beiden Behandlungen deutlich unterschied wobei der symbiontische Kontakt einen stärkeren Einfluß ausübte als der Kontakt mit dem Pathogen. Insgesamt hatten beide Behandlungen einen deutlich stärkeren Effekt auf das Exudationsmuster als der Pflanzengenotyp (wt vs. *dmi3*). Alle Exsudate, die aus der Klasse der Flavonoide identifiziert werden konnten, zeigten verstärkte Exudation nach Kontakt mit *A. euteiches*. Hingegen wurden alle Exsudate aus der Klasse der Sapogenine stärker nach der Behandung mit *R. irregularis* exudiert. Erstaunlich war der dominante Einfluß von *A. euteiches* auf die Wurzelexudation in *dmi3* Pflanzen, mit etwa fünf mal soviel spezifisch exudierten Metaboliten wie nach der *R. irregularis* Behandlung. Die Analyse von VOCs ergab ebenfalls einen deutlich stärkeren Einfluß der Behandlung auf die Exudatzusammensetzung als des Genotyps. Einige VOCs wurden Behandlungs-spezifisch exudiert wie z.B. die drei Sesquiterpene Nerolidol, Viridiflorol und Nerolidol- Epoxyacetat, deren Exudation durch *A. euteiches* stimuliert wurden. Dahingegen wurde das Monoterpen Limonen spezifisch nach *R. irregularis* Kontakt freigesetzt.

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7 Appendix

Figure 65: Stock solutions for MSR Medium (Modified Strullu Romand Medium)

Solution 1: Macroelements (1 l)	**Amount [g]**	**Final concentration [µM]**
$MgSO_4*7H_2O$	73.9	2998
KNO_3	7.6	752
KCl	6.5	872
KH_2PO_4	0.41	30
Solution 2: Calcium nitrate (1 l)		
$Ca(NO_3)*4H_2O$	35.9	2062
Solution 3: Vitamins (500 ml)		
Calcium panthotenate	0.09	1.89
Biotin	0.0001	0.004
Nicotinic acid	0.1	8.12
Pyridoxine	0.09	5.32
Thiamine	0.1	2.96
Cyanocobalamine	0.04	0.29
Solution 4: NaFeEDTA (500 ml)		
NaFeEDTA		0.16
Solution 5: Microelements (500 ml)		
a) $MnSO_4*4H_2O$	1.225	81.13
b) $ZnSO_4*7H_2O$	0.14	4.87
c) H_3BO_3	0.925	149.6
d) $CuSO_4*5H_2O$	1.1	44
e) $Na_2MoO_4*2H_2O$	0.12	4.96
f) $(NH_4)6Mo_7O_{24}*4H_2O$	1.7	13.75
Mix solution a, b and c. Add 5 ml of solution d, 1 ml of e and 1 ml of f. Adjust vol to 500 ml.		

Table 4: Sequences of oligonucleotides for PCR analysis. The specific use is indicated above each group of primers. The melting temperature (T_m) is based on the calculation of the OligoCalc software (salt-adjusted values) and might differ slightly from other calculations. The annealing temperature was chosen 3-6°C below the specific T_m of the primers. All primer sequences were controlled for their specificity with the NCBI database. Potential hairpin formation or potential self-annealing sites of the oligonucleotides were tested with OligoCalc and were avoided if possible.

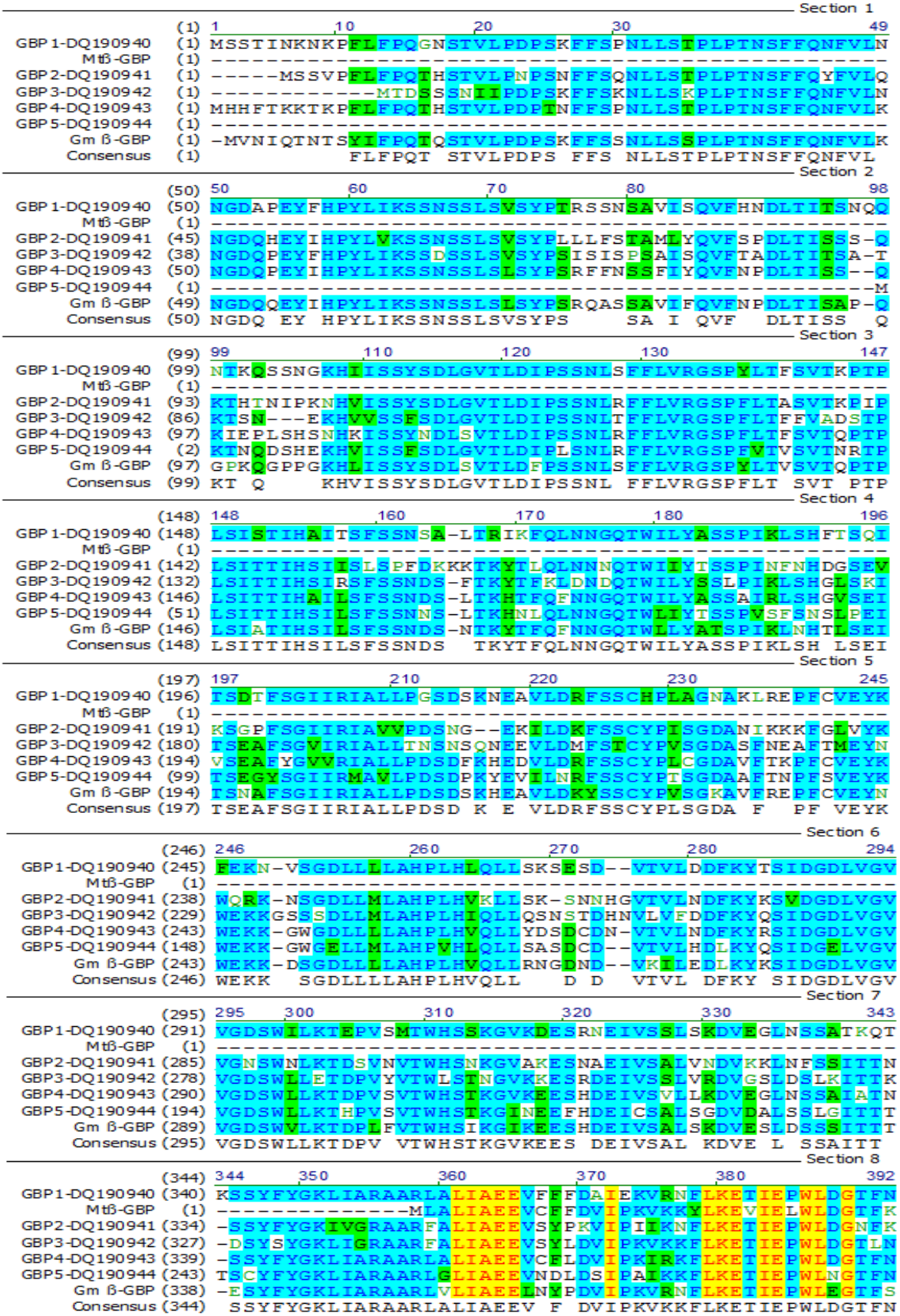
Primer Name	**Sequence**	**T_m [°C]**
Reference gene (qRT-PCR)		
MtEF1α-fw	5´-AGAAGGAAGCTGCTGAGATGAAC-3´	62.9
MtEF1α-rev	5´-TGACTGTGCAGTAGTACTTGGTG-3´	62.9
Marker genes for colonization rate of *R. irregularis* and *A. euteiches* (qRT-PCR)		
MtPt4-fw	5'-ACAAATTTGATAGGATTCTTTTGCACGT-3'	62.7
MtPt4-rev	5'-TCACATCTTCTCAGTTCTTGAGTC-3'	62
Ri-ßTub-fw	5'-CCAACTTATGGCGATCTCAACA-3'	60.1
Ri-ßTub-rev	5'-AAGACGTGGAAAAGGCACCA-3'	58.4
Ae-5.8S rRNA-fw	5'-TGTCTAGGCTCGCACATCGA-3'	60.5
Ae-5.8S rRNA-rev	5'-AGTGCAATATGCGTTCAACGTTT-3'	59.2
Genes to test early induced response to Myc-treatment (qRT-PCR)		
MtENOD11-fw	5'-TCTCCATCCCACAATATGCC-3'	58.4
MtENOD-rev	5'-GGAGGCTTGTAAATTGGAGG-3'	58.4
MtCelsynt-fw	5'-ATGGTATGAAGGAGGCTTGG-3'	58.4
MtCelsynt-rev	5'-AAAGTAAGCCTCAGGACACC-3'	58.4
MtExplike-fw	5'-TTGACTATGCTAACCCACCC-3'	58.4
MtExplike-rev	5'-GTATTGCGAACCTTGACTCC-3'	58.4
MtNodlike-fw	5'-AGTGGAACCACTGAGCCAAC-3'	60.5
MtNodlike-rev	5'-AGAAACTAGCCCAACCACCA-3'	58.4
MtK07C11.4-fw	5'-CATTCGATCCGATCCAAACC-3'	58.4
MtK07C11.4-rev	5'-ACTGGTGACGTTGACTTTGG-3'	58.4
Candidate genes selected from transcriptomics (qRT-PCR)		
CS-fw	5'-CTCTAGGGAAGCTTCAGTTC-3	58.4
CS-rev	5'-CTGTTGGCCTAAGACATTGC-3'	58.4
Kunitz-inh-fw	5'-CCATCAATCTTTGGAGCAGC-3'	58.4
Kunitz-inh-rev	5'-TATCATGGCCTGCTTCTAGC-3'	58.4
PR-TF-fw	5'-CCAAAGCCAATGCAACAACA-3'	56.4
PR-TF-rev	5'-CCCAACTTCTCATTCTTACTCC-3'	60.1
ß-Gluc-Bind-fw	5'-CACATGGAACGAATCAGACG-3'	58.4
ß-Gluc-Bind-rev	5'-CATCACCATAAGCCATTCCC-3'	58.4
LysMI-fw	5'-TTCCTGTGGAGAAAGACTGG-3'	58.4
LysMI-rev	5'-CAACTCAGCATCCATCTTGG-3'	58.4
LysMII-fw	5'-CAACACAGTTATGGAGTGGC-3'	58.4
LysMII-rev	5'-AAATGGCTTCTTGCAACGCC-3'	58.4
Kinase-R-fw	5'-CCCAACCTCAAGTTCAAAGC-3'	58.4
Kinase-R-rev	5'-ACGGTTACTCCAAATCCAGC-3'	58.4

Primer name	**Sequence**
Primer for RNAi constructs with Golden Gate Cloning	
29-LysMI-s-for	5'-tt ggtctc a aatg GGAGTTTAGCTACTGTTATCGG-3'
30-LysMI-s-rev	5'-tt ggtctc a acct CGATATTTCTCAACTTTGGGGGC-3'
31-LysMI-as-for	5'-tt ggtctc a cagg CGATATTTCTCAACTTTGGGGGC-3'
32-LysMI-as-rev	5'-tt ggtctc a aagc GGAGTTTAGCTACTGTTATCGG-3'
33-LysMII-s-for	5'-tt ggtctc a aatg GGTGTGATGTAGCCTTAGCT-3'
34-LysMII-s-rev	5'-tt ggtctc a acct GAAATCTGGCTGTTCCCACA-3'
35-LysMII-as-for	5'-tt ggtctc a cagg GAAATCTGGCTGTTCCCACA-3'
36-LysMII-as-rev	5'-tt ggtctc a aagc GGTGTGATGTAGCCTTAGCT-3'
37-CS-s-for	5'-tt ggtctc a aatg TGTTGATGTTGAGGGGATG-3'
10-CS-s-rev	5'-tt ggtctc a acct AGTTCTTGTGGAGAGCTTGC-3'
11-CS-as-for	5'-tt ggtctc a cagg AGTTCTTGTGGAGAGCTTGC-3'
38-CS-as-rev	5'-tt ggtctc a aagc TGTTGATGTTGAGGGGATG-3'
WRKY-s-for	5'-tt ggtctc a aatg ATGGAGGAGGATTTGGACAG-3'
WRKY-s-rev	5'-tt ggtctc a acct TTGTGGATCTCTTCCTTGGG-3'
WRKY-as-for	5'-tt ggtctc a cagg TTGTGGATCTCTTCCTTGGG-3'
WRKY-as-rev	5'-tt ggtctc a aagc ATGGAGGAGGATTTGGACAG-3'
21-PR-TF-s-for	5'-tt ggtctc a aatg AAGCTGCTGCAAGAGCCTAT-3'
22-PR-TF-s-rev	5'-tt ggtctc a acct GAGGATGATGAAGTTGGTGG-3'
23-PR-TF-as-for	5'-tt ggtctc a cagg GAGGATGATGAAGTTGGTGG-3'
24-PR-TF-as-rev	5'-tt ggtctc a aagc AAGCTGCTGCAAGAGCCTAT-3'
45-ß-Glucan-bind-s-for	5'-tt ggtctc a aatg ACGAATCAGACGGGATGTAG-3'
46-ß-Glucan-bind-s-rev	5'-tt ggtctc a acct GACCACAAAACTCCCATGATCC-3'
47-ß-Glucan-bind-as-for	5'-tt ggtctc a cagg GACCACAAAACTCCCATGATCC-3'
48-ß-Glucan-bind-as-rev	5'-tt ggtctc a aagc ACGAATCAGACGGGATGTAG-3'
41-MtPT4-s-fw	5'-tt ggtctc a aatg TTACCCTCTTTGGTCTAGCG-3'
42-MtPT4-s-rev	5'-tt ggtctc a acct CGAAGAAGAATGTTAGCCCG-3'
43-MtPT4-as-fw	5'-tt ggtctc a cagg CGAAGAAGAATGTTAGCCCG-3'
44-MtPT4-as-rev	5'-tt ggtctc a aagc TTACCCTCTTTGGTCTAGCG-3'
49-pAGM11978-sense	5'-TTCATTTGGAGAGGACACGC-3'
50-pAGM11978-antisense	5'-CAAGACCGGCAACAGGATTC-3'

Table 5: Description of plasmids. ORF: Open reading frame, Spec: Spectinomycin, Kan: Kanamycin

Construct name	Entry vector	Construct description	Resistance
pAGH1	pICH41308	LysMI complete ORF for OE Level	Spec
pAGH3	pICH41308	TS ORF for OE	Spec
pAGH4	pAGM1287	TS ORF for OE without stop codon for GFP fusion	Spec
pAGH5	pICH41308	KIN ORF for OE	Spec
pAGH11	pAGH11978	pAGH11978 + pAGH3 (TS-OE)	Kan
pAGH12	pAGH11978	pAGH11978 + pAGH4 (TS-OE) + pAGT279 (GFP)	Kan
pAGH17	pAGH11978	pAGM11978 + LysMI s + as + pds intron	Kan
pAGH18	pAGH11978	pAGM11978 + LysMII s + as + pds intron	Kan
pAGH19	pAGH11978	pAGM11978 + TS s + as + pds intron	Kan
pAGH22	pAGH11978	pAGM11978 + PR-TF s + as + pds intron	Kan
pAGH24	pAGH11978	pAGM11978 + PT4 s + as + pds intron	Kan

Figure 66: Alignment of amino acid sequence of one GBP of *Glycine max* (GmGBP), the five formerly described genes of the *M. truncatula* multigenefamily (MtGBP1-5) and the candidate gene MtβGBP.



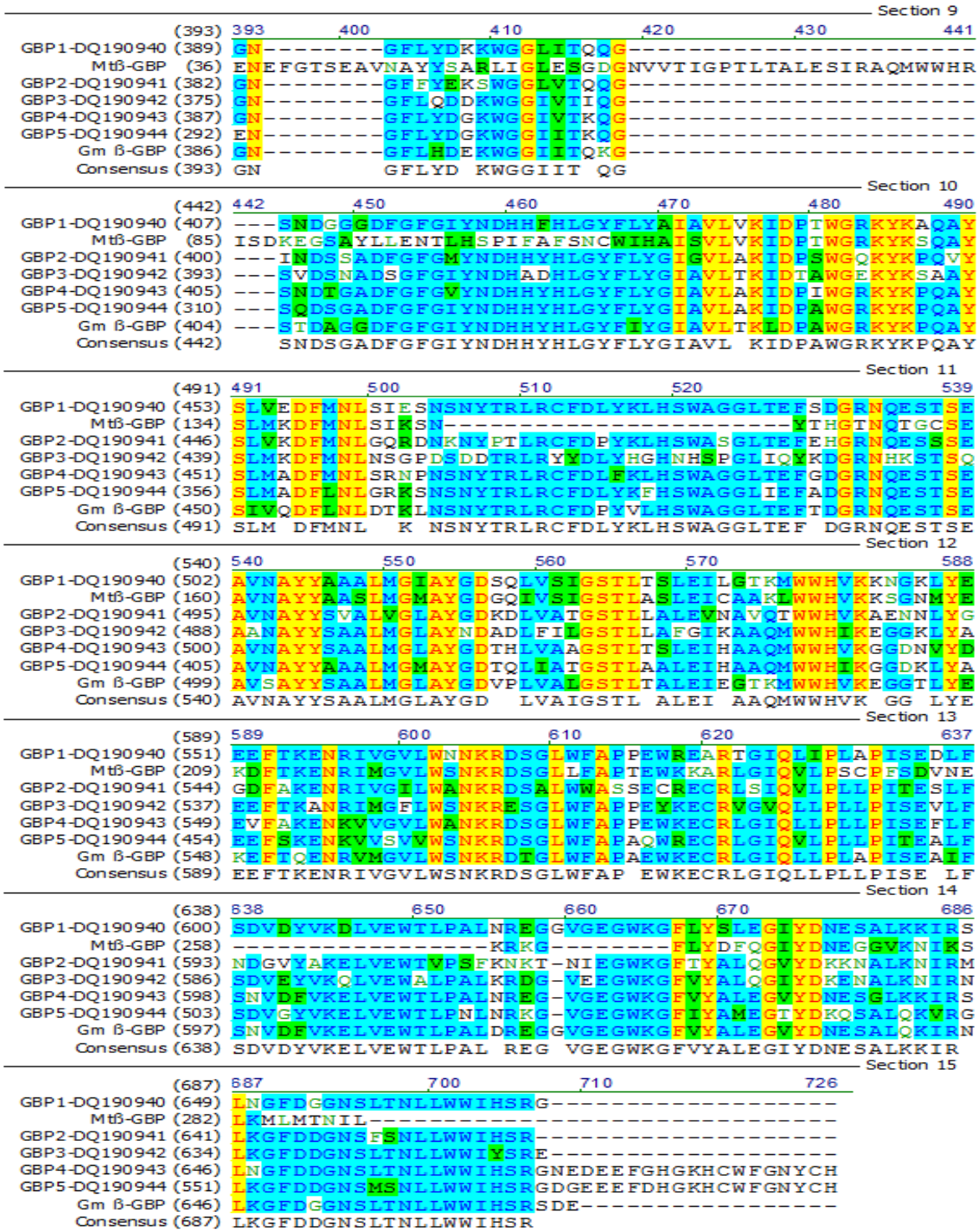
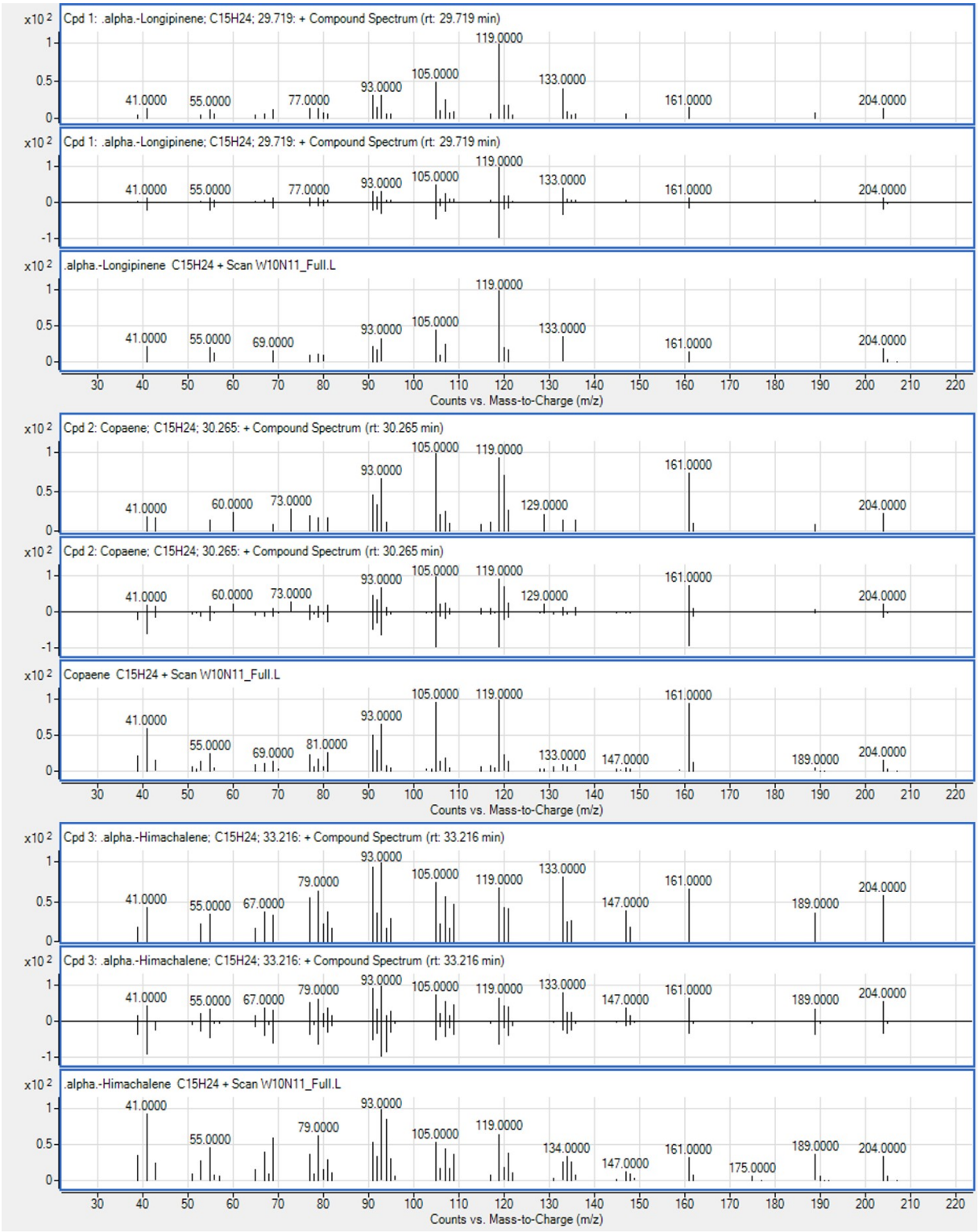

Section 9
(393) 393 400 410 420 430 441
GBP1-DQ190940 (389) GN--------GFLYDKKWGGLITQQG----------
Mtß-GBP (36) ENEFGTSEAVNAYYSARLIGLESGDGNVVTIGPTLTALESIRAQMWWHR
GBP2-DQ190941 (382) GN--------GFFYEKSWGGLVTQQG----------
GBP3-DQ190942 (375) GN--------GFLQDDKWGGIVTIQG----------
GBP4-DQ190943 (387) GN--------GFLYDGKWGGIVTKQG----------
GBP5-DQ190944 (292) EN--------GFLYDGKWGGIITKQG----------
Gm ß-GBP (386) GN--------GFLHDEKWGGIITQKG----------
Consensus (393) GN GFLYD KWGGIIT QG
Section 10
(442) 442 450 460 470 480 490
GBP1-DQ190940 (407) ---SNDGGGDFGFGIYNDHHFHLGYFLYAIAVLVKIDPTWGRKYKAQAY
Mtß-GBP (85) ISDKEGSAYLLENTLHSPIFAFSNCWIHAISVLVKIDPTWGRKYKSQAY
GBP2-DQ190941 (400) ---INDSSADFGFGMYNDHHYHLGYFLYGIGVLAKIDPSWGQKYKPQVY
GBP3-DQ190942 (393) ---SVDSNADSGFGIYNDHADHLGYFLYGIAVLTKIDTAWGEKYKSAAY
GBP4-DQ190943 (405) ---SNDTGADFGFGVYNDHHYHLGYFLYGIAVLAKIDPIWGRKYKPQAY
GBP5-DQ190944 (310) ---SQDSGADFGFGIYNDHHYHLGYFLYGIAVLAKIDPAWGRKYKPQAY
Gm ß-GBP (404) ---STDAGGDFGFGIYNDHHYHLGYFIYGIAVLTKLDPAWGRKYKPQAY
Consensus (442) SNDSGADFGFGIYNDHHYHLGYFLYGIAVL KIDPAWGRKYKPQAY
Section 11
(491) 491 500 510 520 539
GBP1-DQ190940 (453) SLVEDFMNLSIESNSNYTRLRCFDLYKLHSWAGGLTEFSDGRNQESTSE
Mtß-GBP (134) SLMKDFMNLSIKSN----------YTHGTNQTGCSE
GBP2-DQ190941 (446) SLVKDFMNLGQRDNKNYPTLRCFDPYKLHSWASGLTEFEHGRNQESSSE
GBP3-DQ190942 (439) SLMKDFMNLNSGPDSDDTRLRYYDLYHGHNHSPGLIQYKDGRNHKSTSQ
GBP4-DQ190943 (451) SLMADFMNLSRNPNSNYTRLRCFDLFKLHSWAGGLTEFGDGRNQESTSE
GBP5-DQ190944 (356) SLMADFLNLGRKSNSNYTRLRCFDLYKFHSWAGGLIEFADGRNQESTSE
Gm ß-GBP (450) SIVQDFLNLDTKLNSNYTRLRCFDPYVLHSWAGGLTEFTDGRNQESTSE
Consensus (491) SLM DFMNL K NSNYTRLRCFDLYKLHSWAGGLTEF DGRNQESTSE
Section 12
(540) 540 550 560 570 588
GBP1-DQ190940 (502) AVNAYYAAALMGIAYGDSQLVSIGSTLTSLEILGTKMWWHVKKNGKLYE
Mtß-GBP (160) AVNAYYAASLMGMAYGDGQIVSIGSTLASLEICAAKLWWHVKKSGNMYE
GBP2-DQ190941 (495) AVNAYYSVALVGLAYGDKDLVATGSTLLALEVNAVQTWWHVKAENNLYG
GBP3-DQ190942 (488) AANAYYSAALMGLAYNDADLFILGSTLLAFGIKAAQMWWHIKEGGKLYA
GBP4-DQ190943 (500) AVNAYYSAALMGLAYGDTHLVAAGSTLTSLEIHAAQMWWHVKGGDNVYD
GBP5-DQ190944 (405) AVNAYYAAALMGMAYGDTQLIATGSTLAALEIHAAQMWWHIKGGDKLYA
Gm ß-GBP (499) AVSAYYSAALMGLAYGDVPLVALGSTLTALEIEGTKMWWHVKEGGTLYE
Consensus (540) AVNAYYSAALMGLAYGD LVAIGSTL ALEI AAQMWWHVK GG LYE
Section 13
(589) 589 600 610 620 637
GBP1-DQ190940 (551) EEFTKENRIVGVLWNNKRDSGLWFAPPEWREARTGIQLIPLAPISEDLF
Mtß-GBP (209) KDFTKENRIMGVLWSNKRDSGLLFAPTEWKKARLGIQVLPSCPFSDVNE
GBP2-DQ190941 (544) GDFAKENRIVGILWANKRDSALWWASSECRECRLSIQVLPLLPITESLF
GBP3-DQ190942 (537) EEFTKANRIMGFLWSNKRESGLWFAPPEYKECRVGVQLLPLLPISEVLF
GBP4-DQ190943 (549) EVFAKENKVVGVLWANKRDSGLWFAPPEWKECRLGIQLLPLLPISEFLF
GBP5-DQ190944 (454) EEFSKENKVVSVVWSNKRDSGLWFAPAQWRECRLGIQVLPLLPITEALF
Gm ß-GBP (548) KEFTQENRVMGVLWSNKRDTGLWFAPAEWKECRLGIQLLPLAPISEAIF
Consensus (589) EEFTKENRIVGVLWSNKRDSGLWFAP EWKECRLGIQLLPLLPISE LF
Section 14
(638) 638 650 660 670 686
GBP1-DQ190940 (600) SDVDYVKDLVEWTLPALNREGGVGEGWKGFLYSLEGIYDNESALKKIRS
Mtß-GBP (258) ----------KRKG---------FLYDFQGIYDNEGGVKNIKS
GBP2-DQ190941 (593) NDGVYAKELVEWTVPSFKNKT-NIEGWKGFTYALQGVYDKKNALKNIRM
GBP3-DQ190942 (586) SDVEYVKQLVEWALPALKRDG-VEEGWKGFVYALQGIYDKENALKNIRN
GBP4-DQ190943 (598) SNVDFVKELVEWTLPALNREG-VGEGWKGFVYALEGVYDNESGLKKIRS
GBP5-DQ190944 (503) SDVGYVKELVEWTLPNLNRKG-VGEGWKGFIYAMEGTYDKQSALQKVRG
Gm ß-GBP (597) SNVDFVKELVEWTLPALDREGGVGEGWKGFVYALEGVYDNESALQKIRN
Consensus (638) SDVDYVKELVEWTLPAL REG VGEGWKGFVYALEGIYDNESALKKIR
Section 15
(687) 687 700 710 726
GBP1-DQ190940 (649) LNGFDGGNSLTNLLWWIHSRG----------
Mtß-GBP (282) LKMLMTNIL----------
GBP2-DQ190941 (641) LKGFDDGNSFSNLLWWIHSR----------
GBP3-DQ190942 (634) LKGFDDGNSLTNLLWWIYSRE----------
GBP4-DQ190943 (646) LNGFDDGNSLTNLLWWIHSRGNEDEEFGHGKHCWFGNYCH
GBP5-DQ190944 (551) LKGFDDGNSMSNLLWWIHSRGDGEEEFDHGKHCWFGNYCH
Gm ß-GBP (646) LKGFDGGNSLTNLLWWIHSRSDE----------
Consensus (687) LKGFDDGNSLTNLLWWIHSR

Figure 67: Features obtained by LC-MS that were selected for structure elucidation. Some of them could be putatively identified, for others only sum formulas (SF) could be proposed. Column six indicates UV spectrum with maximum absorption in nm; s: UV-spectra shoulder.

Retention time	Features found	Annotation	proposed SF	rank / proposals / accuracy	UV λmax	compound class	putative compound	Massbank record
Ae > Myc								
193	457.1131	M+Na	C21H22NaO10	1 / 5 / 10 ppm	220; 284	Flavonoid	Naringeninhexoside	PR020069
	435.1328	M+H				Flavanon		
	417.1222	M+H - H2O						
	433.1148	M-H	C21H21O10	2 / 5 / 10 ppm				
235	457.1138	M+Na	C21H22NaO10	2 / 6 / 10 ppm	224; 283; 325s	Flavonoid	Naringeninhexoside	PR020069
	435.1315	M+H				Flavanon		
	273.0779	M+H - Hex	C15H13O5	1 / 2 / 15 ppm				
	255.0672	273 - H2O						
	227.0728	255 - CO		1 / 1 / 15 ppm				
267	595.1720	M+H	C27H31O15	2 / many / 10 ppm	220; 266; 327s	Flavonoid	Kaempferol [Hex-doHex]	TY000226
	449.1132	M+H - doHex				Flavonol		
	287.0578	M+H - doHexHex	C15H11O6	1 / 2 / 10 ppm			Kaempferol	
290	617.1522	M+Na			223; 266	Flavonoid		
	595.1697	M+H	C27H31O15	2 / many / 10 ppm		Flavonol	Kaempferol [Hex-doHex]	TY000226
	449.1112	M+H - doHex	C21H21O11	2 / 6 / 10 ppm				
	287.0566	M+H - doHexHex	C15H11O6	1 / 2 / 10 ppm			Kaempferol	
	661.1466	M-H + NaHCOO						
	593.1547	M-H	C27H29O15	1 / many / 10 ppm				
421	557.1649	M+Na					Unknown	
	535.1832	M+H	C26H31O12					
	287.1282	M+H - Malonylhexoside	C17H19O4	1 / 3 / 10 ppm	222; 270s			
432	587.1770	M+Na			223; 275; 310s		Unknown	
	565.1944	M+H	C27H33O13					
	317.1398	M+H - Malonylhexoside	C18H21O5	1 / 3 / 15 ppm				
501	293.0804	M+Na			225; 285	Isoflavonoid	Medicarpin	
	271.0976	M+H	C16H15O4	1 / 3 / 15 ppm		Pterocarpan		
	243.1039	M+H - CO						
	137.0612	271 - C8H6O2	C8H9O2	1 / 1 / 15 ppm				
	123.0468	271 - C9H8O2						
Ae > Myc								
471	981.5121	M+Na	C48H79O19		222	Sapogenin	Bayogenin [doHex-Hex-Hex]	
	959.5276	M+H				Triterpenoid		
	797.4744	M+H - Hex						
	651.4109	797 - doHex						
	635.4222	797 - Hex						
	489.3559	635 - doHex	C30H49O5	2 / 2 / 10 ppm				
	471.3377	489 - H2O						
483	835.4476	M+Na			222	Sapogenin	Bayogenin [Hex-Hex]	
	813.4707	M+H	C42H69O15	2 / 4 / 10 ppm (no N,S)		Triterpenoid		
	651.4143	M+H - Hex						
	489.3550	651 -Hex	C30H49O5	2 / 2 / 10 ppm				
	471.4355	489 - H2O	C30H47O4	2 / 4 / 10 ppm				
491	965.5135	M+Na			222	Sapogenin	Hederagenin [doHex-Hex-Hex]	
	943.5315	M+H	C48H79O18	1 / 4 / 10 ppm (no N,S)		Triterpenoid		
	781.4787	M+H - Hex						
	619.4247	781 - Hex						
	601.4128	619 - H2O						
	455.3521	601 - doHex	C30H47O3	1 / 3 / 10 ppm				
553	743.4009	M+Na	C39H60NaO12	3 / many / 10 ppm	222		Unknown	
	721.4191	M+H		1 / 2 / 10 ppm (no N,S)				
	703.4069	M+H - H2O						
	455.3526	703 - Malonylhexoside	C30H47O3	1 / 2 / 10 ppm				
	437.3426	455 - H2O						
612	511.3385	M+Na	C30H48NaO5	2 / 3 / 10 ppm	222	Sapogenin	Bayogenin	
	489.3343	M+H				Triterpenoid		
	471.3486	M+H - H2O	C30H47O4	2 / 3 / 10 ppm				
dmi3 Myc+ dmi3 Ae > dmi3 Con								
155	320.0775	M+Na			217; 259s		Unknown	
	298.0967	M+H						
	136.0613	M+H - C9H6O3	C5H6N5	1 / 2 / 20 ppm		Nucleotid		
			C4H10NO4	2 / 2 / 20 ppm		modified amino acid		
335	376.1720	M+Na	C19H23N5NaO2		221; 258s		Unknown	
			C22H27NaOS					
			C19H31NNaOS2					
	354.1902	M+H						
	308.1839	M+H - H2O - CO						
	290.1745	308 - H2O		4 suggestions				
407	385.0725	M+Na			222		Unknown	
	363.0901	M+H						
	319.1009	M+H - CO2						
	275.1057	319 - CO2						
	213.1464							
419		see Rt 421					Unknown	
452	701.3492	M+Na			222		Unknown	
	679.3720	M-H						
	517.3159	M+H - Hex		8 suggestions				
466	787.3505	M+Na			222		Unknown	
	765.3648	M+H						
	517.3129	M+H - Malonylhexoside		9 suggestions				
494	339.1204			9 suggestions	222		Unknown	
542	339.1205	M+Na	C18H20NaO5	1 / 6 / 10 ppm	222		Unknown	
	317.1469	M+H						
	299.1353	M+H - H2O						
	271.1343	299 - CO	C17H19O3	1 / 2 / 15 ppm				
	165.0556	271 - C8H10 ?						
	121.0649	165 - CO2	C8H9O	1 / 1 / 20 ppm				
629	339.1015	M+K						
	323.1250	M+Na	C18H20NaO4	2 / 3 / 10 ppm	222		Unknown	
	301.1433	M+H	C18H21O4	1 / 2 / 10 ppm				
	283.1352	M+H - H2O	C18H19O3	2 / 4 / 15 ppm				
	121.0633	283 - C10H10O2	C8H9O	1 / 1 / 20 ppm				

Figure 68: GC-MS spectra of three sesquiterpenes α- longipinene, copaene and α- himachalene including measured spectrum (top), differential spectrum (middle) and database spectrum (bottom).



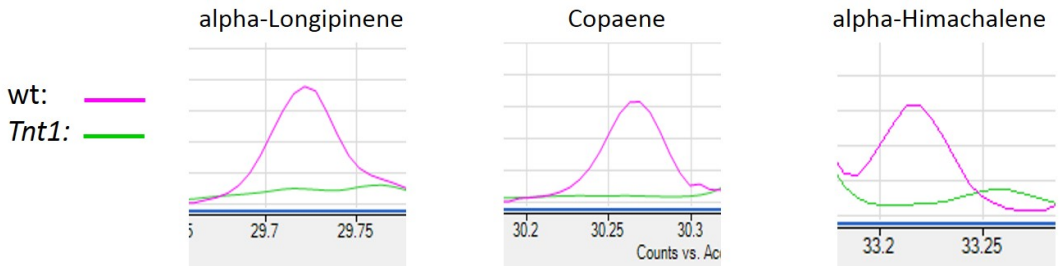
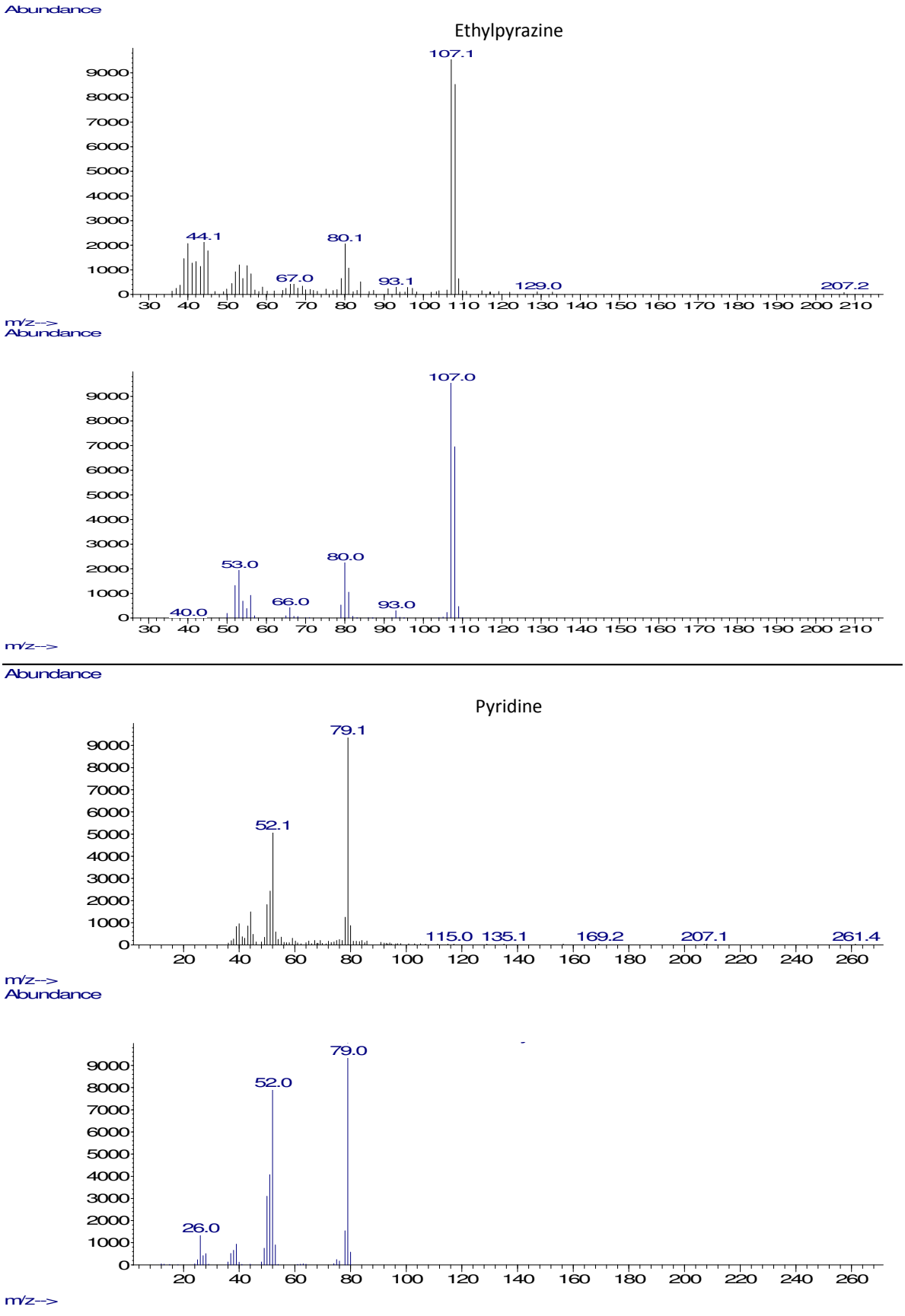
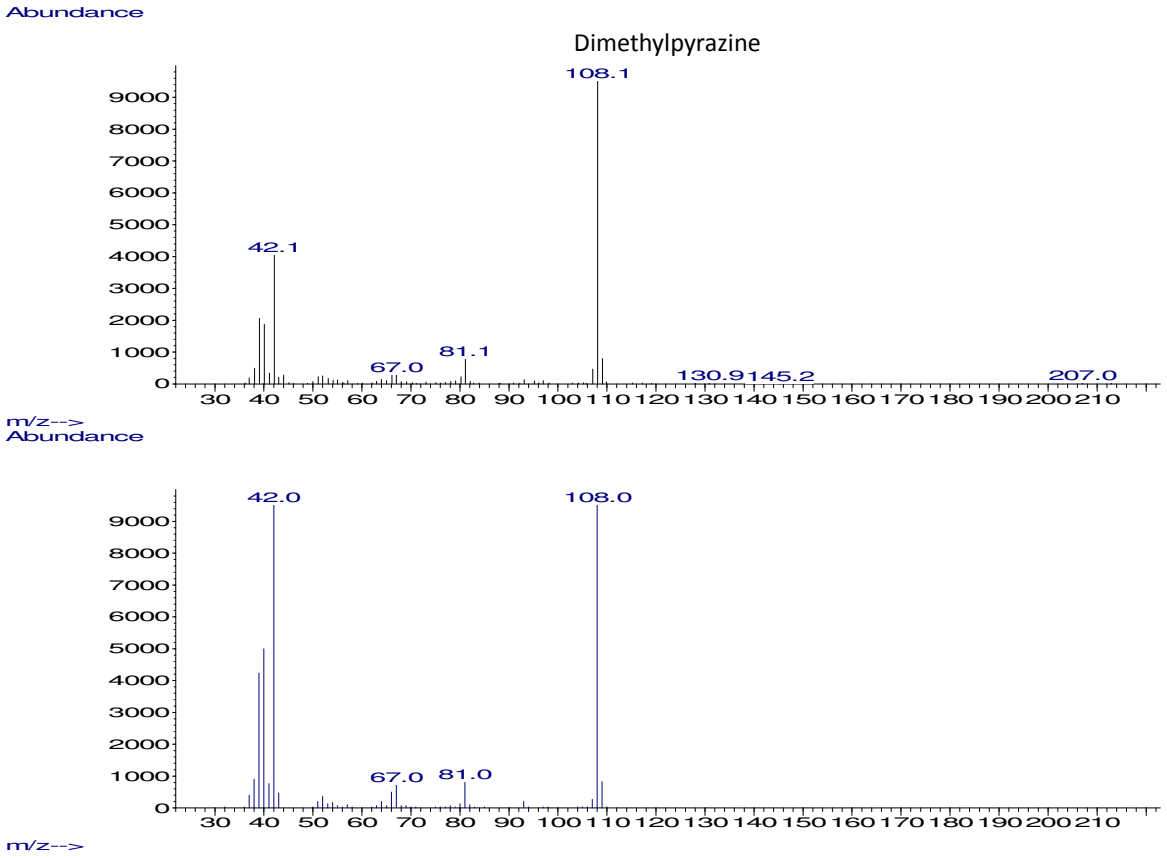
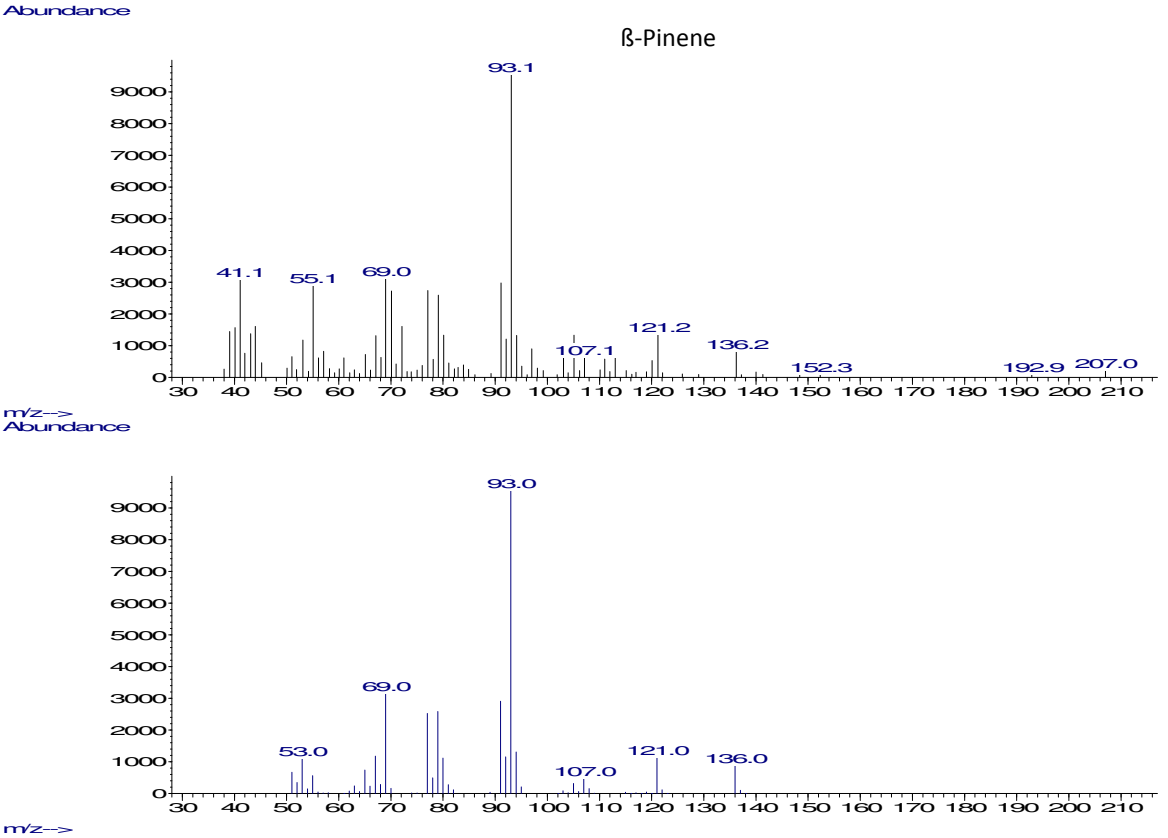
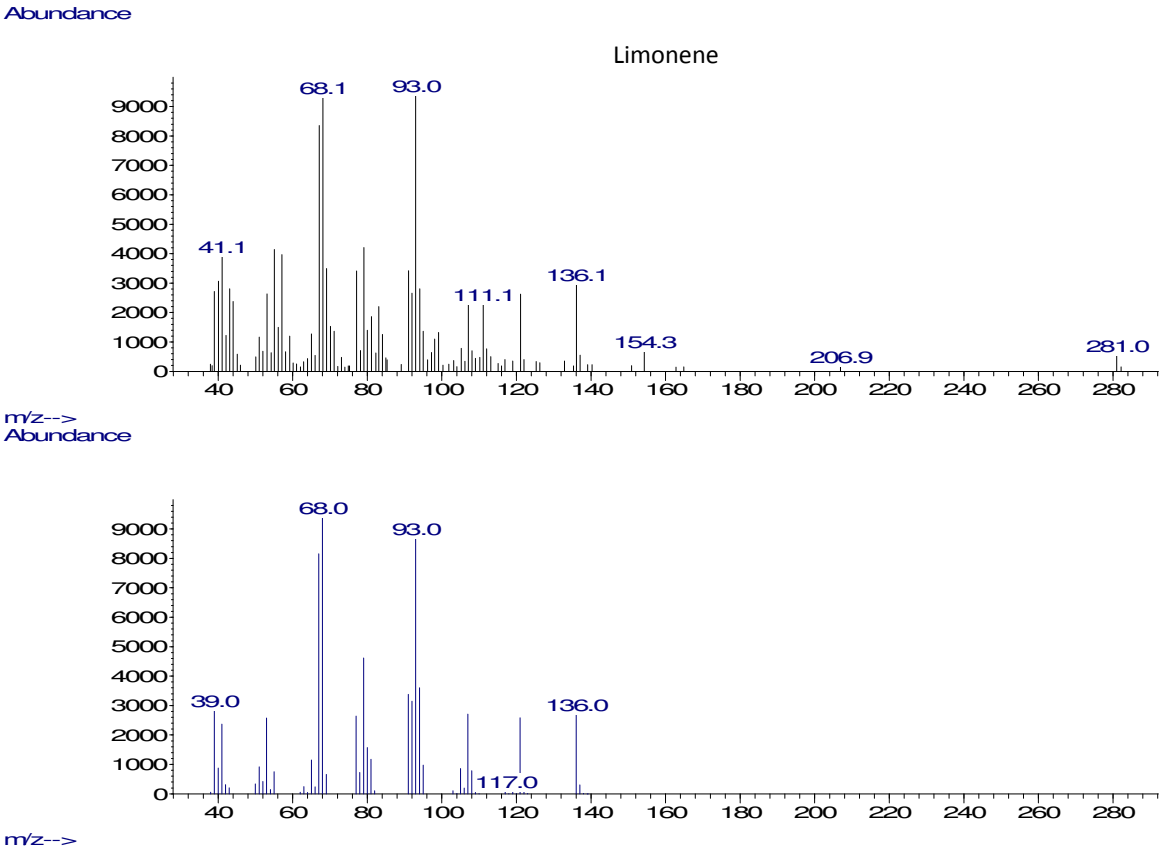


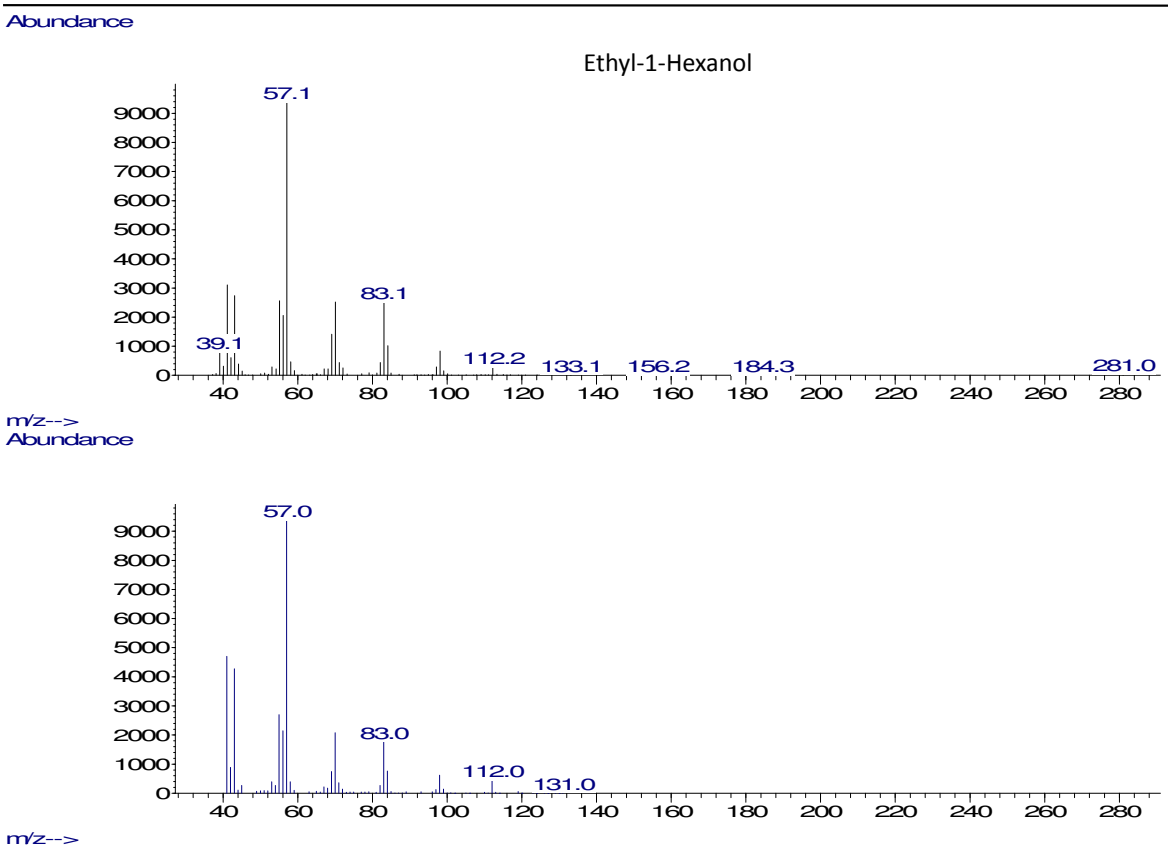
Figure 69: GC-MS spectra of differentially exuded VOCs, with measured spectra (top) in comparison with data-bank spectra (bottom).

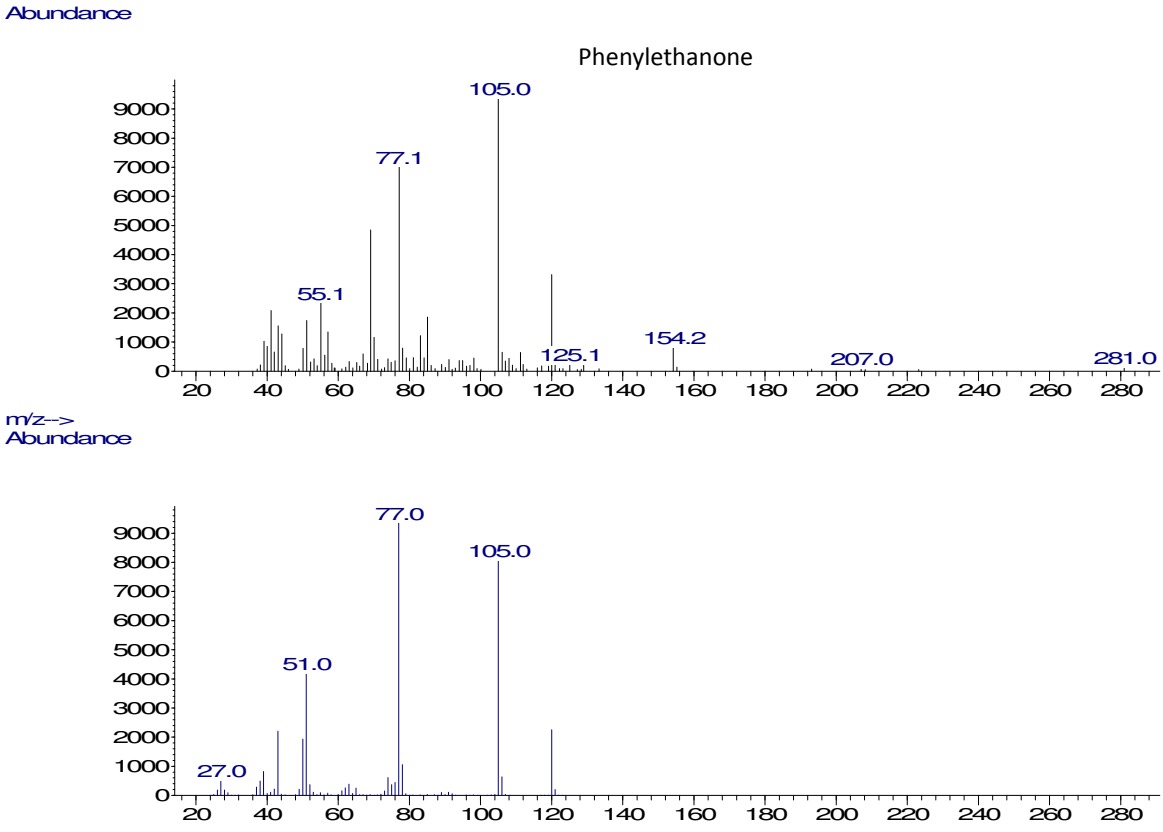



Abundance
Dimethylpyrazine
108.1
42.1
67.0
81.1
130.9145.2
207.0
m/z-->
Abundance
42.0
108.0
67.0
81.0
m/z-->


Abundance
ß-Pinene
93.1
41.1
55.1
69.0
107.1
121.2
136.2
152.3
192.9
207.0
m/z-->
Abundance
93.0
69.0
53.0
107.0
121.0
136.0
m/z-->


Abundance
Limonene
68.1
93.0
41.1
111.1
136.1
154.3
206.9
281.0
m/z-->
Abundance
68.0
93.0
39.0
136.0
117.0
m/z-->


Abundance
Ethyl-1-Hexanol
57.1
83.1
39.1
112.2
133.1
156.2
184.3
281.0
m/z-->
Abundance
57.0
83.0
112.0
131.0
m/z-->


Abundance
Phenylethanone
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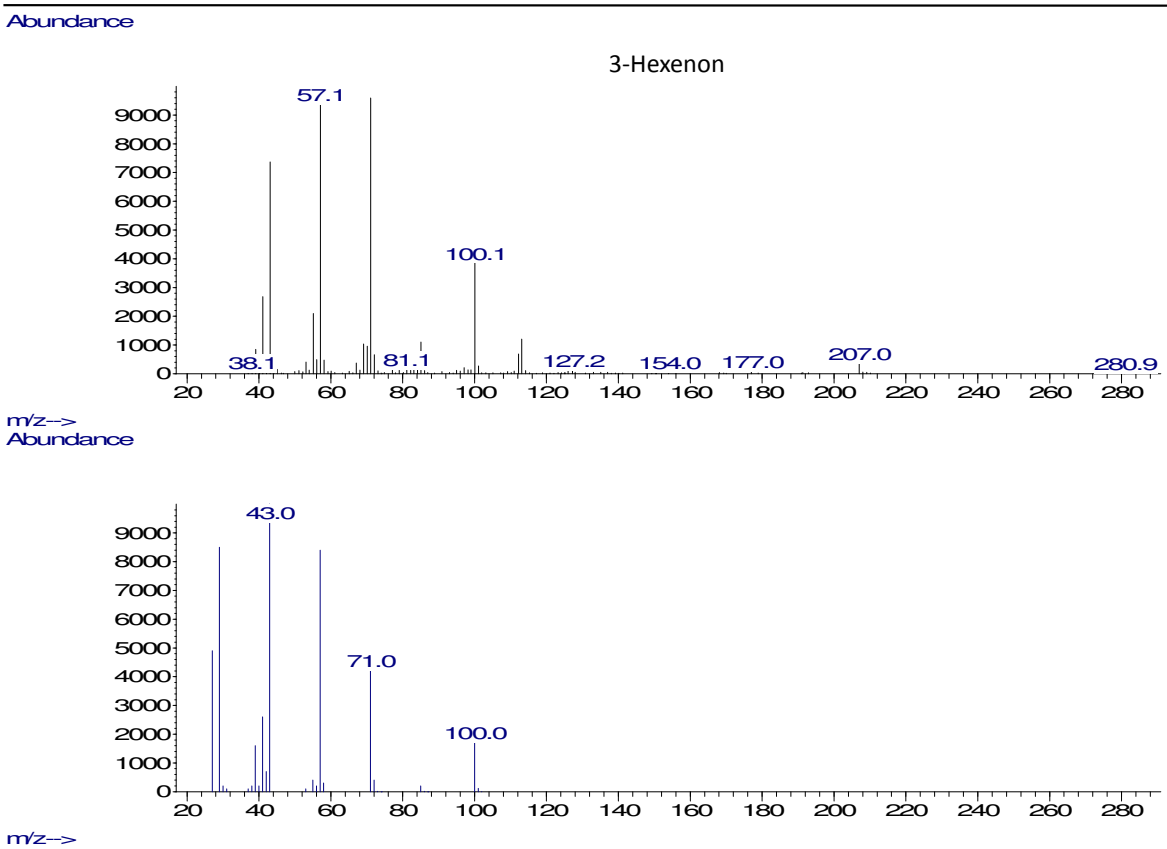
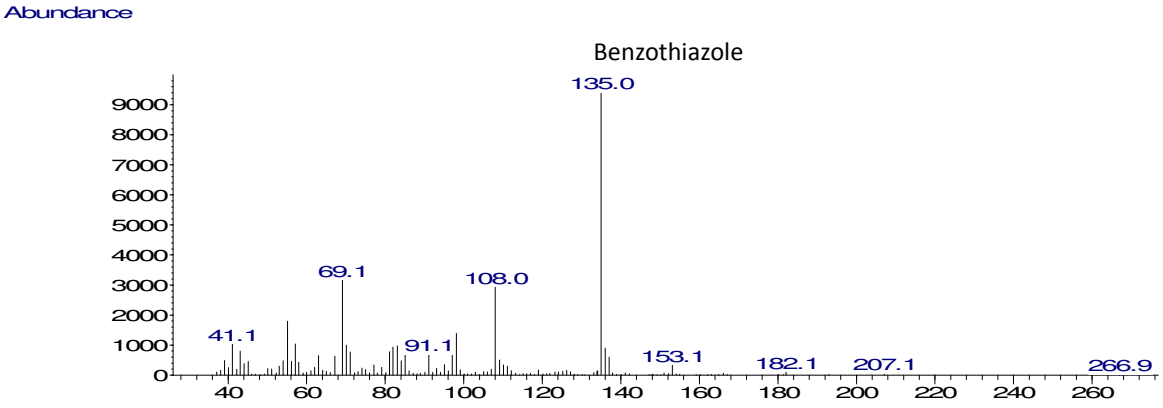
77.1
55.1
125.1
154.2
207.0
281.0
m/z-->
Abundance
77.0
105.0
51.0
27.0
m/z-->


Abundance
3-Hexenon
57.1
100.1
38.1
81.1
127.2
154.0
177.0
207.0
280.9
m/z-->
Abundance
43.0
71.0
100.0
m/z-->


Abundance
Benzothiazole
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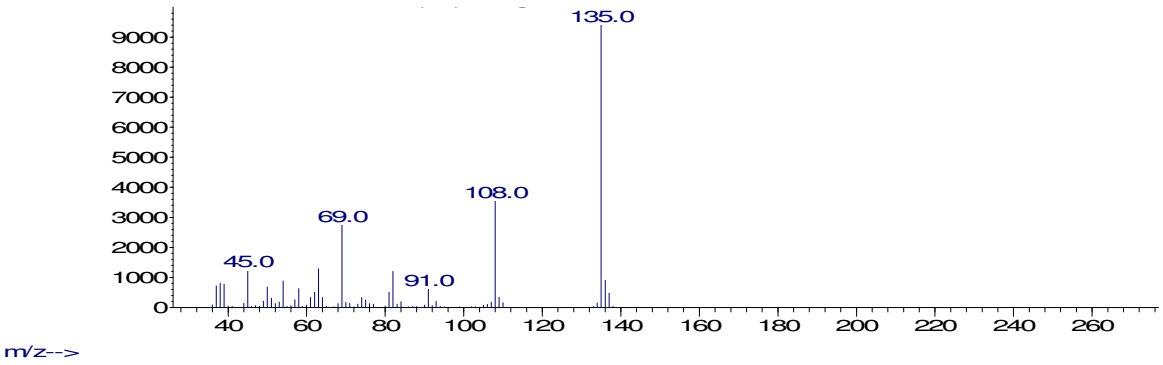
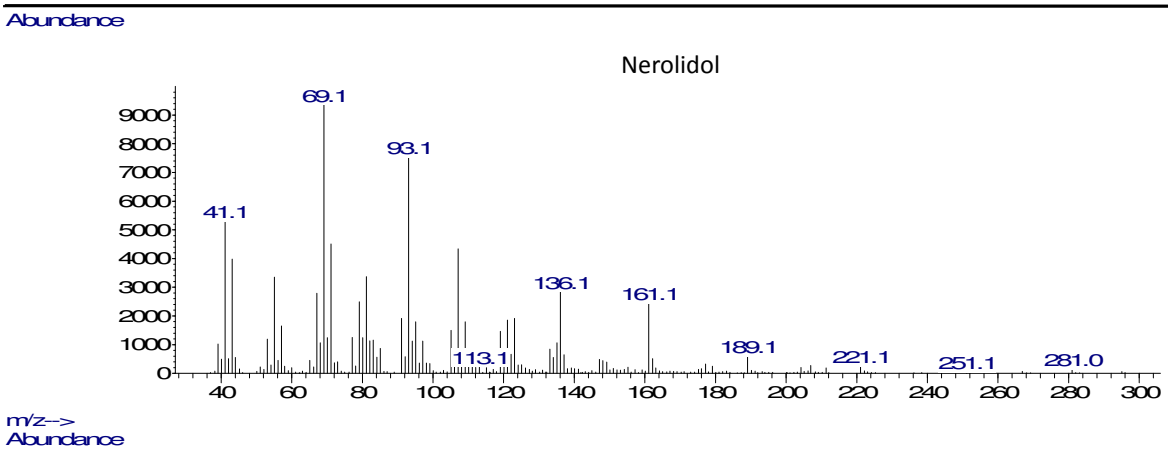
69.1
108.0
41.1
91.1
153.1
182.1
207.1
266.9
m/z-->


Abundance
135.0
108.0
69.0
45.0
91.0
m/z-->


Abundance
Nerolidol
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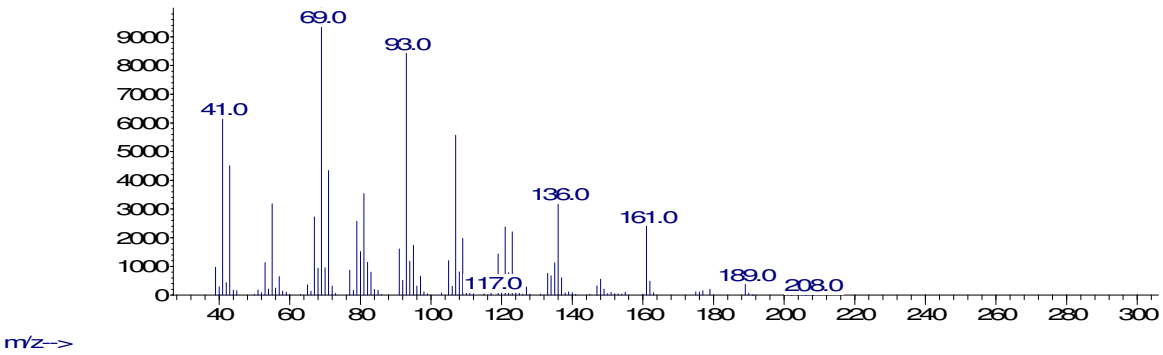
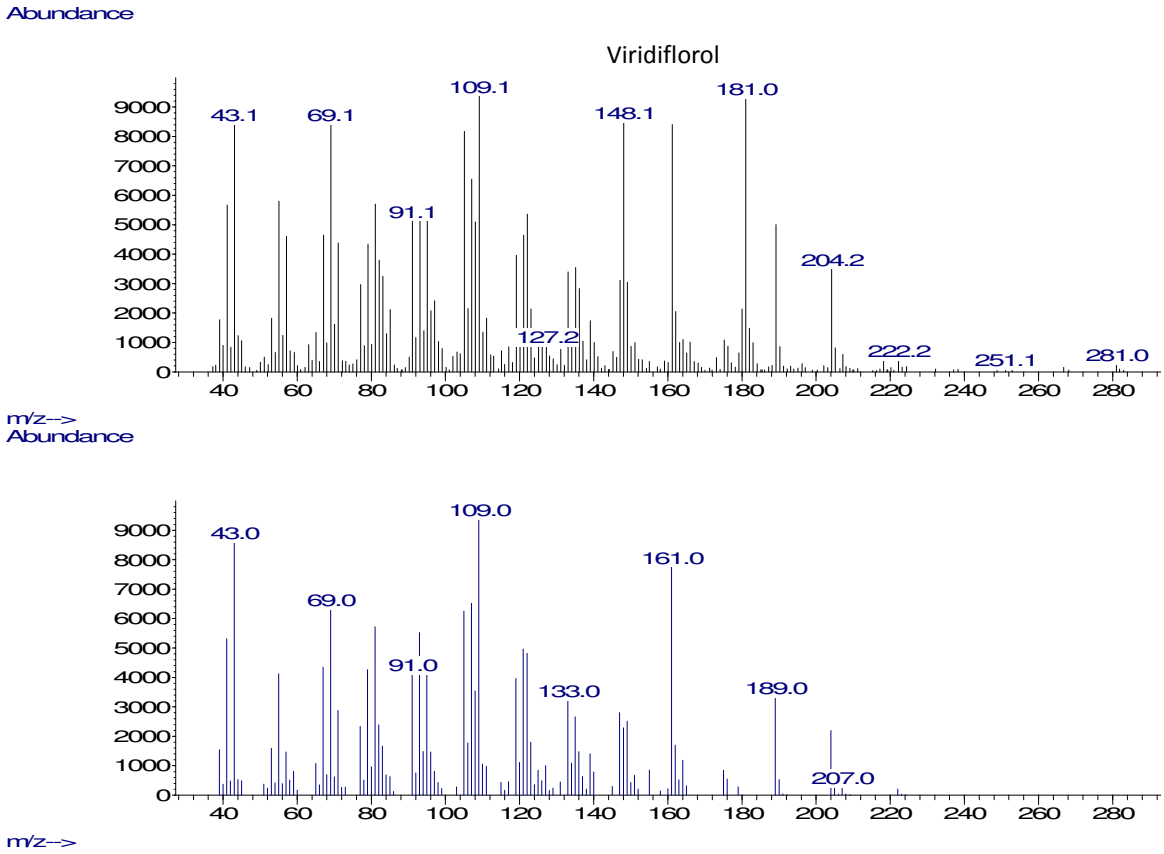
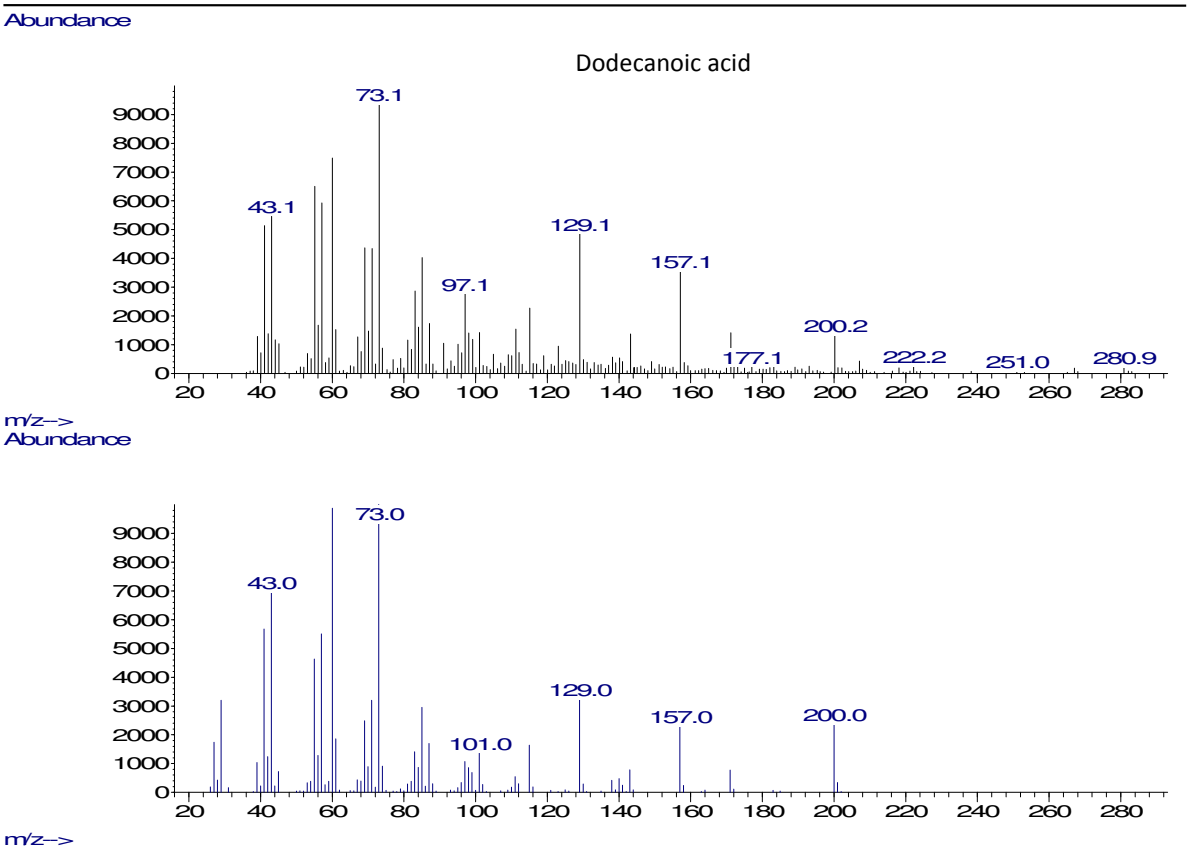
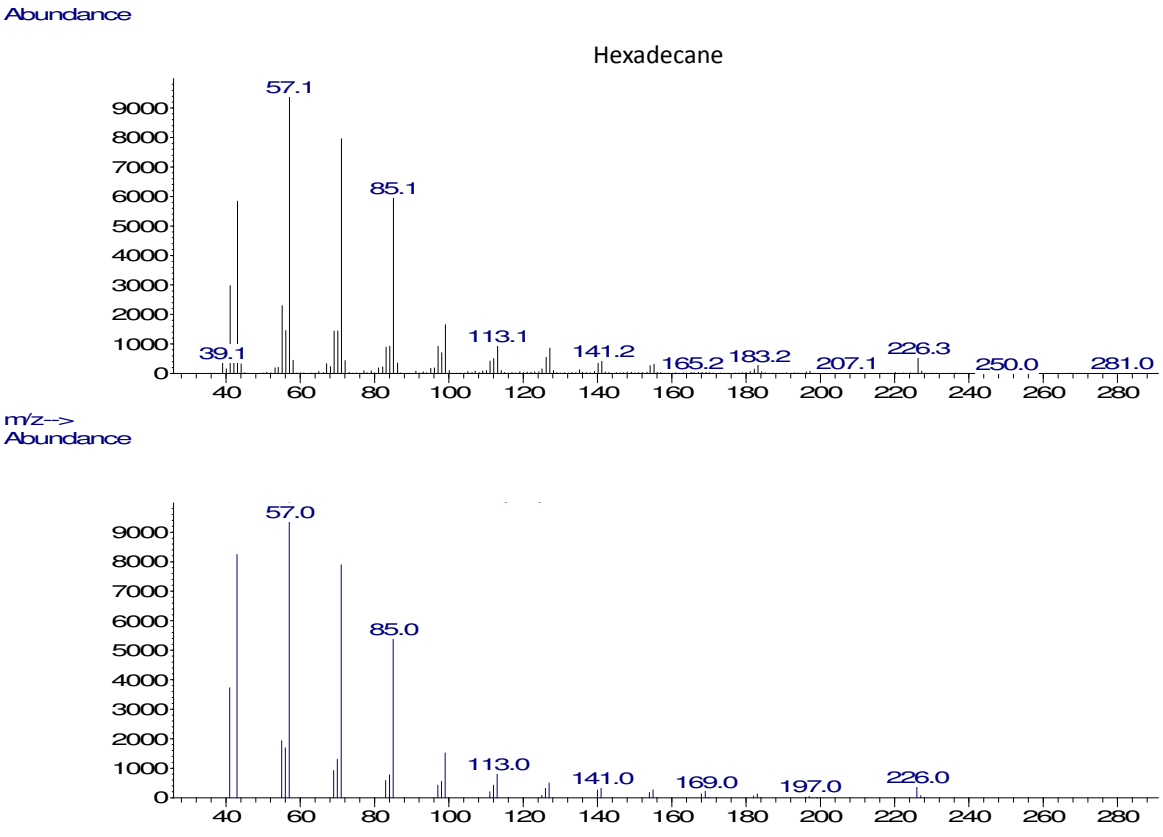
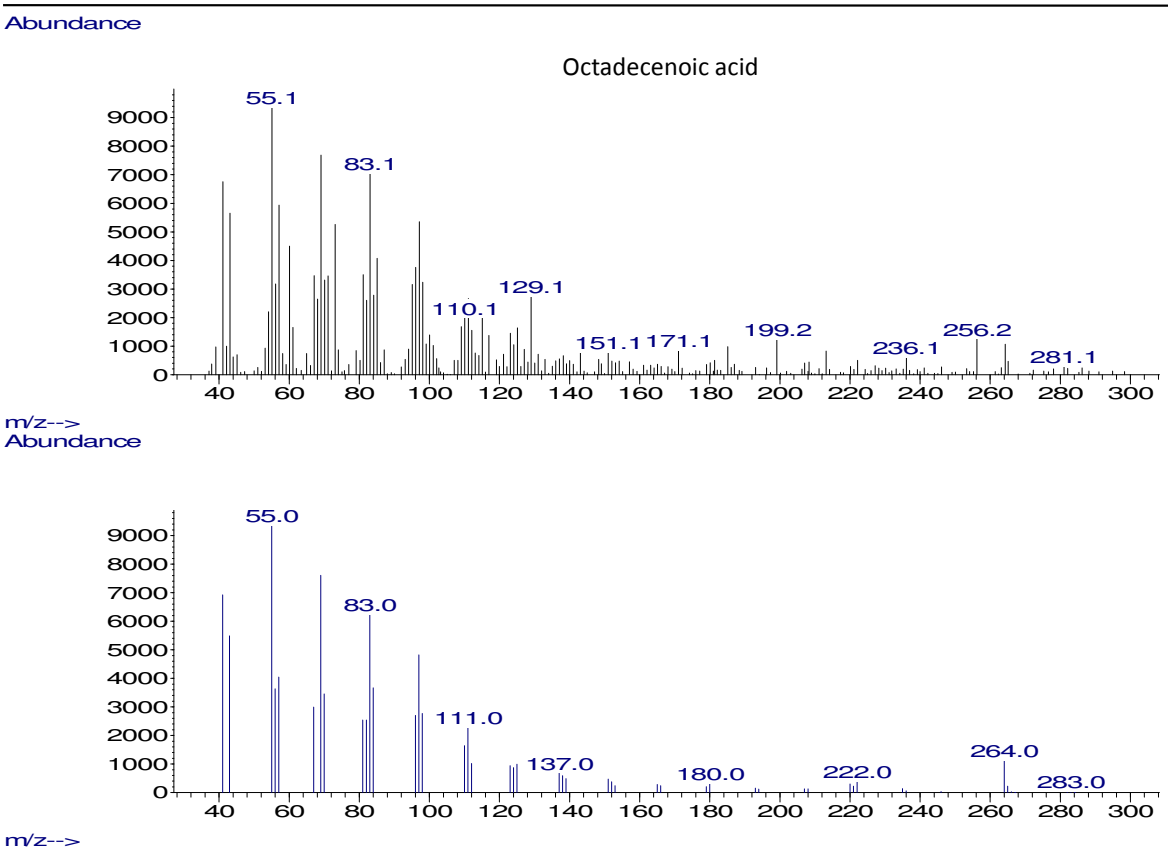
93.1
41.1
136.1
161.1
113.1
189.1
221.1
251.1
281.0
m/z-->


Abundance
69.0
93.0
41.0
136.0
161.0
117.0
189.0
208.0
m/z-->


Abundance
Viridiflorol
43.1
69.1
109.1
91.1
148.1
181.0
204.2
127.2
222.2
251.1
281.0
m/z-->
Abundance
43.0
109.0
69.0
91.0
161.0
133.0
189.0
207.0
m/z-->


Abundance
Dodecanoic acid
73.1
43.1
129.1
157.1
97.1
200.2
177.1
222.2
251.0
280.9
m/z-->
Abundance
73.0
43.0
129.0
157.0
200.0
101.0
m/z-->


Abundance
Hexadecane
57.1
85.1
39.1
113.1
141.2
165.2
183.2
207.1
226.3
250.0
281.0
m/z-->
Abundance
57.0
85.0
113.0
141.0
169.0
197.0
226.0
m/z-->


Abundance
Octadecenoic acid
55.1
83.1
129.1
110.1
151.1
171.1
199.2
236.1
256.2
281.1
m/z-->
Abundance
55.0
83.0
111.0
137.0
180.0
222.0
264.0
283.0
m/z-->

8 Acknowledgments

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9 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende wissenschaftliche Arbeit selbstständig und ohne fremde Hilfe angefertigt habe. Ich erkläre weiterhin, dass ich keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und die den Werken wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.
Mit dieser Arbeit bewerbe ich mich erstmals um die Erlangung des Doktorgrades. Diese Arbeit wurde an keiner anderen Fakultät oder Universität zur Begutachtung eingereicht.

Halle (Saale), den 26. Oktober 2015

Dorothée Klemann

Curriculum Vitae

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PERSONAL DETAILS

Born	27th May 1983, in 87435 Kempten, Germany
Citizenship	German, Suisse
Marital status	single
Gender	female

EDUCATION

Since 2011	Doctoral studies at the Leibniz- Institute for Plant Biochemistry in Halle (Saale) in the group of Prof. Bettina Hause: Jasmonate function & Mycorrhiza with the topic "Early responses of *Medicago truncatula* roots after contact with the symbiont *Rhizophagus irregularis* or the pathogen *Aphanomyces euteiches*"
2007 - 2010	Master studies of agricultural sciences, University of Hohenheim; Degree: M. Sc.; Specialization: Plant nutrition; The master thesis with the topic: „Genetic diversity of arbuscular mycorrhizal fungi (AMF) and the influence of AMF on salt tolerance in *Medicago truncatula*" was performed at the Leibniz Institute for Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany.
2004 - 2007	Bachelor studies of agricultural sciences, University of Hohenheim; Degree: B. Sc.; Topic Bachelor thesis: „Analysis of the understory vegetation in different land use systems on the island of Leyte, Philippines"
1993 – 2003	Hilda-Gymnasium Pforzheim, Degree: „Abitur"

INTERNATIONAL EXPERIENCES

02.2012	Consultant in the Baha´i gardens in Haifa, Israel, for introduction of mycorrhizal fungi as organic fertilizer.
11.2007-02.2008	Internship in the quality department of SanLucar Fruits S.L., Puzol, Valencia, Spain.
08.-10.2006	Research on biodiversity in different land use systems (Agroforestry vs. monoculture), Leyte State University, Baybay, Leyte, Philippines.
08.-10.2005	Internship on an organic farm in Minzac, Bordeaux, France
11.2003-03-2004	Language stay in Guatemala, Central America
12.1999-03.2000	Language stay in New Zealand

SCIENTIFIC ACTIVITIES

2015	05	Talk: "Early responses of *Medicago truncatula* roots after symbiotic or pathogenic interaction" at the conference "Plant-Environment Interaction" in Heidelberg, Germany
2014	04	Talk: "Early response of *Medicago truncatula* roots after contact to a symbiont or a pathogen" at the "International Students Conference on Microbial Communication", Jena, Germany
2013	04	Talk: "Early response of *Medicago truncatula* roots after contact to a symbiont or a pathogen" at the "Plant Science Students Conference" in Halle (Saale), Germany
2012	09	Talk: "Early response of *Medicago truncatula* roots in contact with *Rhizophagus irregularis*" at the "Third annual joint meeting on Plant Microbe interactions", Burgundy Regional Council, Dijon, France
2012	05	Poster presentation at the "28th New Phytologist Symposium – Functions and ecology of the plant microbiome", Rhodes, Greece
2011	09	Poster presentation at the „Botanikertagung 2011- The interdisciplinary Congress for Plant Sciences", Berlin, Germany

LANGUAGE SKILLS

German (mother tongue)

English (fluent)

French (good)

Spanish (basic knowledge)

METHODOLOGICAL SKILLS

General:	Microsoft Office, LaTEX, VekotNTI, handling of genome databases
Molecular biological methods:	Cloning techniques (Golden Gate), PCR, qRT-PCR, LC/GC-MS, transient plant transformation
Others:	Diverse plant cultivation methods (Aeroponics, Hydroponics)

OTHER

- Voluntary social activities with youth
- Co-founder of the association: „Food Revitalization & Eco-Gastronomic Society of Hohenheim" („FRESH"), which won a price from the UNESCO for its engagement in sustainable development.

INTERESTS AND HOBBIES

Classical singing

Travelling

Gardening/ Bee-keeping