

# The effect of quorum sensing signals on nodulation of *Medicago truncatula*

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**By**

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## **Declaration**

This thesis is my own work and does not contain any results that have been generated by people other than myself, except where reference and acknowledgment has been made. These results have not been previously submitted by me for the purpose of obtaining a degree or diploma at any university or tertiary education institution.

Debora Fabiola Véliz Vallejos

October, 2016

*"Our task must be to free ourselves... by widening our circle of compassion to embrace all living creatures and the whole of nature and its beauty."*

Albert Einstein

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## Abstract

N-acyl homoserine lactones (AHLs) act as quorum sensing signals that regulate cell-density dependent behaviours in many gram-negative bacteria, in particular those important for plant-microbe interactions. AHLs can also be recognised by plants, and this may influence their interactions with bacteria. This thesis tested whether the exposure to AHLs affects the nodule-forming, nitrogen-fixing symbiosis between legume hosts and rhizobia. It used the legume, *Medicago truncatula*, and its symbiont, *Sinorhizobium meliloti*, as this model symbiosis has been well characterised on a molecular and cellular basis. In addition, previous studies have characterised the identities and roles of AHLs from *S. meliloti* during nodulation.

First, protocols were established to grow *M. truncatula* plants under conditions conducive to symbiosis and, at the same time, to minimise growth of bacteria growing in and on roots. This was important as bacteria can destroy AHLs and could thus interfere with externally applied AHLs. *M. truncatula* was found to harbour culturable bacteria that were derived from the inside of the seed coat and were recalcitrant to surface sterilisation. A protocol using an antibiotic treatment of the seeds to minimise bacterial growth, and an axenic system growing *M. truncatula* seedlings on large agar plates was chosen for subsequent experiment.

*M. truncatula* seedlings were exposed to a range of synthetic AHLs derived either from its specific symbiont, *S. meliloti*, or from the potential pathogens, *Pseudomonas aeruginosa* and *Agrobacterium vitis*. Increased numbers of nodules formed on root systems treated with the *S. meliloti*-specific AHL, 3-oxo-C<sub>14</sub>-homoserine lactone (HSL), while the other AHLs did not result in significant changes to nodule numbers. The increase in nodule numbers was dependent on AHL concentrations and was repeatedly observed at concentrations of 1 µM and above. No evidence for altered nodule invasion by the rhizobia was found. 3-oxo-C<sub>14</sub>-HSL 'primed' *Medicago* plants before inoculation with rhizobia, indicating that the increase in nodule numbers occurs at early stages and as a direct effect on the plant and not on the rhizobia. Increased nodule numbers following 3-oxo-C<sub>14</sub>-HSL lactone treatment were not under control of autoregulation of nodulation and were still observed in the autoregulation mutant, *sun4* (*super numeric nodules4*). However, increases in nodule numbers by 3-oxo-C<sub>14</sub>-HSL were not found in the ethylene-insensitive *sickle* mutant. Gene expression analysis further suggested that

this AHL affects the expression of ethylene-related genes during nodulation. It was concluded that plant perception of the *S. meliloti*-specific 3-oxo-C<sub>14</sub>-HSL influences nodule numbers in *M. truncatula* via an ethylene-dependent, but autoregulation-independent mechanism.

A comparison of *M. truncatula* with *M. sativa* (alfalfa), *Trifolium repens* (white clover) and *Lotus japonicus* (Lotus) showed that the observed effects of AHLs on nodule numbers, at least at the concentration chosen, were specific to *M. truncatula*, despite *M. sativa* nodulating with the same symbiont. In *M. truncatula*, the effects of AHLs were specific for an increase in nodule numbers, but not lateral root numbers or root length. This result suggests a very specific effect of AHLs on nodulation, possibly via modulation of ethylene-controlled infection, but not on general root developmental processes.

During the investigation of protocols to eliminate bacterial contamination, it was discovered that nodulation phenotypes in response to AHL exposure strongly depended on the presence of plant-associated bacteria. Therefore, the composition and possible role of the *M. truncatula*-associated microbiome was further investigated. High throughput sequencing showed that the antibiotic treatment significantly reduced the presence and composition of the microbiome. Interestingly, application of 3-oxo-C<sub>14</sub>-HSL also significantly altered the microbiome, but this effect was very specific for bacteria belonging to the genus *Pantoea*. Only in the absence, but not in the presence of the majority of the plant microbiome, did *M. truncatula* show increased nodulation in response to 3-oxo-C<sub>14</sub>-HSL, and this was associated with increased expression of early nodulation genes. These results suggest that the bacterial community of *Medicago* affects nodulation responses towards AHLs, particularly towards to 3-oxo-C<sub>14</sub>-HSL, likely by interfering with AHL stability, perception or plant responses.

In summary, this thesis showed that plant perception of AHLs alters symbiosis-specific phenotypes, suggesting that AHLs are not only important to regulate bacterial behaviours during nodulation, but also that the plant has evolved mechanisms to respond to specific AHLs from its symbiont, likely by reducing ethylene signalling or synthesis to enhance the number of nodules.



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## List of abbreviations

AB	antibiotic
ACC	1-aminocyclopropane-1-carboxylate
ACO	1-aminocyclopropane-1-carboxylate oxidase
AHL	acyl homoserine lactone
AI	autoinducer
ANOVA	analysis of variance
AON	autoregulation of nodulation
APX	L-ascorbate peroxidase
AVG	aminoethoxyvinylglycine
B&D	Broughton and Dilworth
BMM	Bergersen's modified medium
CCaMK	calcium/calmodulin-dependent kinase
cDNA	complementary DNA
CHS	chalcone synthase
dag	days after germination
DMSO	dimethyl sulfoxide
dpi	days after inoculation
<i>ENOD</i>	Early nodulin
F media	Fåhraeus media
<i>GADPH</i>	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GH3	auxin-responsive promoter
hai	Hours after inoculation
IFS	isoflavone synthase
ISR	induced systemic resistance
LB	lysogeny broth
LC-ESI-QTOF	liquid chromatography with electrospray ionisation tandem mass
MS/MS	spectrometry
LysM-RLK	lysine motif-receptor-like kinase
$\mu$ M	micromolar
mM	milimolar
MS media	Murashige and Skoog media
<i>NIN</i>	Nodule inception

<i>NSP</i>	GRAS-type nodulation signaling pathway
PAR	photosynthetically active radiation
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
<i>Prp</i>	pathogenesis related protein
<i>PTB</i>	<i>polypyrimidine tract binding protein 1</i>
qRT-PCR	quantitative real-time PCR
QS	quorum sensing
QSSs	quorum sensing signals
R2A	reasoner's 2a
REML	residual maximum likelihood
<i>RIP1</i>	rhizobium-induced peroxidase1
ROS	reactive oxygen species
<i>skl</i>	<i>sickle</i>
<i>sun4</i>	<i>super numeric nodules4</i>
TSA	trypticase soy agar
TY medium	tryptone-yeast extract
<i>wt</i>	<i>wild type</i>
YEB	yeast extract broth



## Chapter 1 General Introduction

### 1.1 Introduction

Higher plants are characterised for being immobile, or rooted organisms. As a consequence they have to adapt to an ever changing environment, not only to survive but also to thrive. Some of the adaptive mechanisms that plants exhibit are mutualistic microbe interactions such as mycorrhiza and bacterial symbiotic associations. These positive interactions allow microorganisms to provide nutrients to the plants and allow the plants to provide protection and a niche for these microorganisms to thrive. A well-known example of a mutualistic symbiosis is the interaction between legumes and soil bacteria generally called “rhizobia”. Rhizobia fix atmospheric nitrogen ( $N_2$ ) into an accessible form to the plant, in a process known as biological nitrogen fixation (BNF). N is one of the macronutrients most highly required to increase crop yield. It has been estimated that annual BNF provides approximately 50 to 70 million metric tons of nitrogen to agricultural systems (Herridge *et al.*, 2008). Unfortunately, excessive inputs of N have caused environmental contamination due to eutrophication of waterways. Costs involved in the production of N fertilisers have risen, which in turn have become an expensive input for crop production. Therefore, legume nitrogen fixation constitutes an important way to boost sustainable agricultural production.

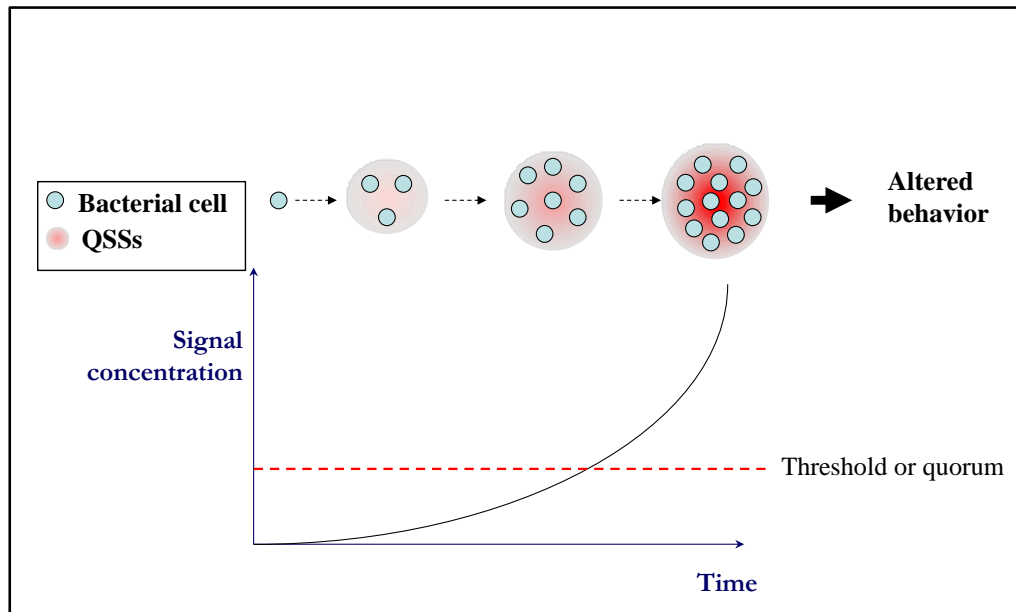
Rhizobia, orchestrate the expression of many bacterial genes important for plant-microbe interactions, including the Rhizobium-legume symbiosis, by a process called quorum sensing (QS). Bacteria produce chemical molecules called autoinducers (AIs). In rhizobia, as in many gram negative bacteria, these autoinducers are acyl homoserine lactones (AHLs). They act as signals that induce a cell to cell communication system in order to modify gene expression in a population-dependent manner (Fuqua *et al.*, 1996; Bassler and Losick, 2006).

This Chapter will present the current research in quorum sensing applied to plant-microbe interactions with emphasis on the Rhizobium-legume symbiosis, particularly looking at nodulation and the plant microbiome.

## 1.2 Quorum sensing: cell to cell communication system

Bacteria have adapted mechanisms to maximize their efficiency to compete in time and space, in order to ensure their multiplication and survival. Some of these mechanisms include motility, synthesis of exoenzymes, exopolysaccharides, surfactants, antibiotic production, biofilm formation, plasmid conjugal transfer and virulence determinant (e.g. Swift *et al.*, 1996; Klein *et al.*, 2009). These activities are regulated by bacterial communication through a phenomenon called quorum sensing (QS), which involves the synthesis, exchange and perception of small diffusible molecules. These molecules regulate transcription activating specific receptors in order to trigger changes in gene expression in a population density-dependent manner (Fuqua *et al.*, 1996; Bassler and Losick, 2006; Choudhary and Schmidt-Dannert, 2010). Thus, bacteria are able to sense their population density as well as other bacterial populations to survive in a constant changing environment.

Neaslon *et al.*, (1970) noticed that in bioluminescent bacteria, luciferase enzyme synthesis was repressed at low population density and activated at high population density. They referred to this phenomenon as autoinduction due to the bacterial cells ability to regulate their luciferase gene expression by recognising their own self-synthesised signal. Later on, Neaslon and Hastings (1979) described for the first time a cell to cell communication system in a bioluminescent symbiotic marine bacterium *Vibrio fischeri*, which produces light only at high population density. However, the term quorum sensing was first mentioned by Fuqua *et al.*, (1994) who described it as the “minimum behavioral unit” in which bacteria take census of their numbers. As the bacterial population grows so does the concentration of small diffusible molecules or quorum sensing signals (QSSs), also referred to as diffusible pheromones or autoinducers. In this sense bacteria are able to count their own numbers by synthesising and detecting the extracellular concentration of QSSs (Bassler and Losick, 2006). When a certain threshold or “quorum” is reached, changes in gene expression are triggered in a coordinated multicellular behaviour (Figure 1.1).



**Figure 1.1** Quorum sensing model. As the bacterial cell population increases over time so does the quorum sensing signals (QSSs) concentration. Modified from Keller and Surette (2006).

Many definitions and classifications of cell to cell communication systems in bacteria have been discussed and proposed according to the type of QS and QS molecules as well as their ecological and evolutionary roles (Winzer *et al.*, 2002; Henke and Bassler, 2004a; Keller and Surette, 2006; Hense and Schuster, 2015). In this study, some of the more commonly used concepts in the quorum sensing field will be defined as following:

- **Quorum sensing:** Cell to cell communication system that allows bacteria to sense their environment to monitor their own cell numbers and their neighbour's cells in order to change their behaviour in a population density manner (Fuqua *et al.*, 1994; Fuqua *et al.*, 2001; Bassler, 2002).
- **Quorum sensing signals:** Chemical molecules or compounds produced by bacteria that possess the following characteristics according to Winzer *et al.*, (2002):
  - Their production occurs during specific stages of growth, under certain physiological conditions, or in response to changes in the environment.
  - They accumulate intracellularly and extracellularly and are recognised by a specific receptor.

- Their accumulation generates a concerted response, once a critical threshold concentration has been reached.
- The cellular response extends beyond physiological changes required to metabolise or detoxify the cell to cell signal molecule.

These criteria help to identify QSSs from other secondary metabolites. For instance, toxic bacterial metabolites or metabolic residues that reach certain concentration may trigger a united stress response in a population (Winzer *et al.*, 2002; Keller and Surette, 2006). In this case, toxic molecules cannot be considered quorum sensing compounds since toxins are not recognised by specific receptors and the bacterial cells only respond to the toxic properties of the molecules *per se* (Winzer *et al.*, 2002).

### **1.2.1 Conventional quorum sensing systems: How do bacteria get socially intimate?**

Conventional quorum sensing systems can be classified in three different categories according to the type of autoinducer and its detection. These are: modified oligopeptides (in Gram positive bacteria), LuxI/R type quorum sensing systems (typically in Gram negative bacteria), and a combination of the two (Figure 1.2) (Henke and Bassler, 2004). In bacteria, gene annotations are in italic lowercase (e.g. *luxI*) and protein annotations in capital letters (e.g. LuxI). In this study, QS system is usually referred to a conventional quorum sensing while QS circuits are the different components inside a defined QS system.

#### **1.2.1.1 Modified oligopeptides**

Quorum sensing in Gram positive bacteria regulates gene expression through secreted modified oligopeptides as autoinducers via a phosphorylation/dephosphorylation cascade (Henke and Bassler, 2004; Miller and Bassler, 2001). The peptide autoinducer signal is synthesised from a peptide signal precursor. This peptide is secreted extracellularly via an ATP-binding cassette (ABC) transporter. When the extracellular peptide concentration reaches a certain threshold, reflecting the cell population density, it is detected by a two component signalling system histidine sensor kinase (Figure 1.2, middle column). Once this transmembrane sensor kinase is autophosphorylated, it transfers the phosphoryl group to the cognate response regulator protein. Thus, the phosphorylated response regulator protein binds to specific target genes activating the transcription of quorum sensing controlled genes (Miller and Bassler, 2001).



	Gram-negative: LuxI/R	Gram-positive: oligopeptide	Hybrid
Circuit organization			
Autoinducer structures	 3-O-C12-HSL/LasI <i>Pseudomonas aeruginosa</i>  C4-HSL/RhlI <i>P. aeruginosa</i>  C8-HSL/Tral <i>Agrobacterium tumefaciens</i>  C6-HSL/LuxI <i>Vibrio fischeri</i>	ERGMT <i>CSF/phrC</i> <i>Bacillus subtilis</i> ADPITRQWGD* <i>comX</i> <i>B. subtilis</i>  YSTCDFIM      GVNACSSLF  INCDFLL      YSTCYFIM AIP I-IV <i>Staphylococcus aureus</i>	 3-OH-C4-HSL/LuxM <i>Vibrio harveyi</i>  AI-2/LuxS <i>V. harveyi</i>  AI-2/LuxS <i>Salmonella typhimurium</i>
Regulated behaviors	<i>P. aeruginosa</i> : Elastase production, rhamnolipid production (virulence factors) <i>V. fischeri</i> : Bioluminescence <i>Erwinia carotovora</i> : Exoenzyme production (virulence), antibiotic production <i>A. tumefaciens</i> : Conjugation of the Ti plasmid <i>Serratia liquefaciens</i> : Pigment production, antibiotic production <i>Sinorhizobium meliloti</i> : Exopolysaccharide synthesis (symbiosis)	<i>B. subtilis</i> : Competence, sporulation <i>S. aureus</i> : Virulence, biofilms <i>Lactococcus lactis</i> : Nisin production <i>Streptococcus pneumoniae</i> : Competence	<i>V. harveyi</i> : Luminescence, type III secretion <i>Vibrio cholerae</i> : Virulence, biofilm production <i>Vibrio anguillarum</i> : Protease production <i>S. typhimurium</i> : Lsr transporter ** <i>Photobacterium luminescens</i> : Antibiotic production ** <i>Clostridium perfringens</i> : Toxin production ** <i>Streptococcus pyogenes</i> : Hemolysin production

**Figure 1.2** Conventional diffusible quorum sensing (QS) in bacteria. The Figure shows the system organization for each conventional QS along with representative microorganisms, their autoinducer structures and the behaviours controlled by them. Abbreviations: Hpt, histidine phosphotransfer protein; RR, response regulator; sensor histidine kinase; P, phosphate. Figure taken from Henke and Bassler, (2004a).

### 1.2.1.2 LuxI/R type quorum sensing systems

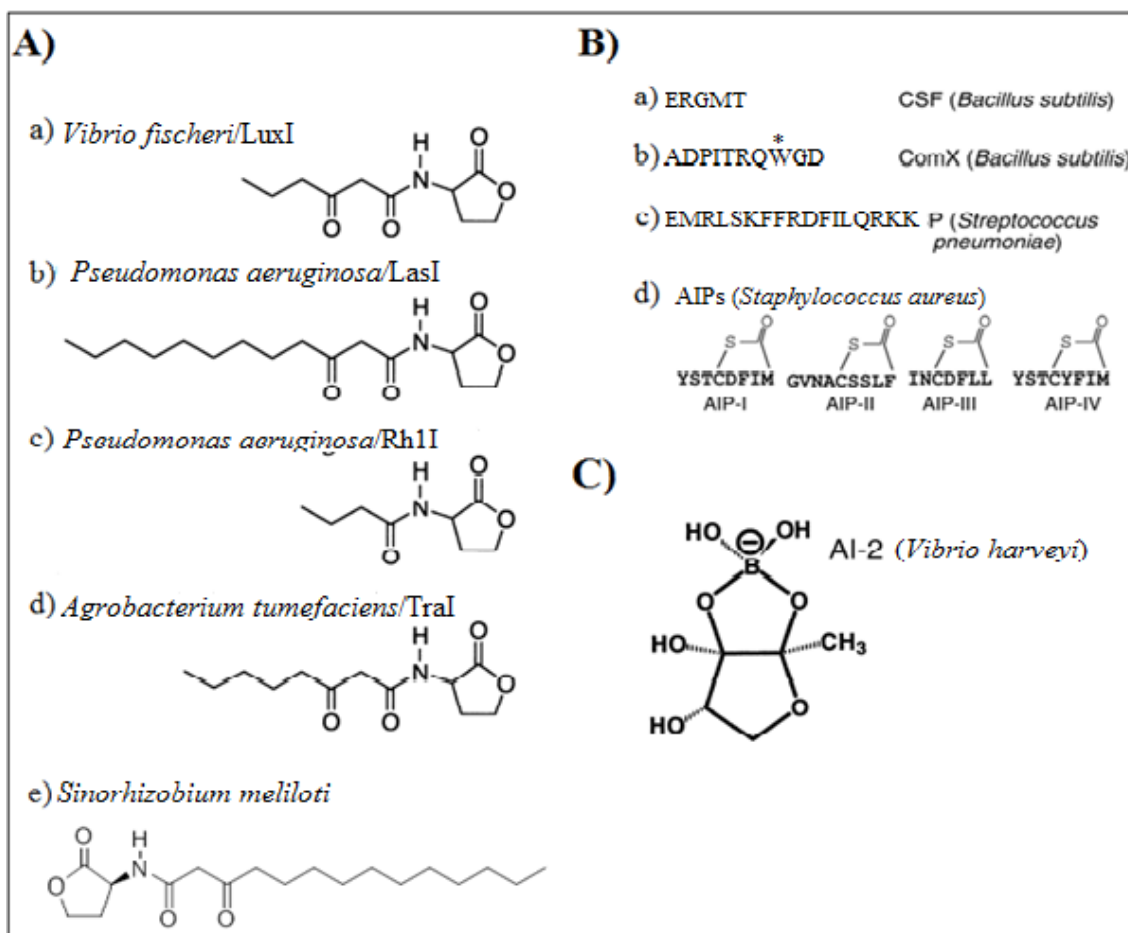
*V. fischeri* was the first bacterium in which quorum sensing was discovered. As a consequence, its QS system, comprising LuxI and LuxR family proteins, became the most extensively studied. The LuxI enzyme catalyses the synthesis of specific acyl homoserine lactones (AHLs). Synthetised AHL molecules freely diffuse out of and into the cell increasing their extra and intracellular concentration proportionally with bacterial cell population within a diffusion-limited environment. When extra- e intra-cellular concentrations of AHLs reach an equilibrium at a certain threshold, AHL molecules are recognised by the LuxR proteins, which subsequently bind different promoter elements, activating downstream transcription of target genes and consequently enhancing RNA polymerase activity (Figure 1.2, first column) (Fuqua *et al.*, 1994; Federle and Bassler 2003; Henke and Bassler, 2004). LuxR-family proteins have two domains: an autoinducer binding domain, located in the amino terminal region and a DNA binding helix-turn-helix (HTH) at the carboxyl terminal motif (Fuqua and Winans, 1994). The biological relevance of QS in *V. fischeri* is explained below.

*V. fischeri* in its free-living state in the seawater, is found at low cell densities (less than  $10^2$  cell/ml) with no luminescence (Fuqua *et al.*, 1996; Fuqua *et al.*, 2001). However, when it colonises the light organs of the sepiolid squid, *Euprymna scolopes*, *V. fischeri* accumulates at high cell densities ( $10^{10}$ - $10^{11}$  cell/ml) (Ruby *et al.*, 1976). Under this high population density, quorum sensing signals, specifically the AHL, N-3-oxohexanoyl-HSL is produced, diffuses and is perceived by the population expressing the luminescence (*lux*) genes (Eberhard *et al.*, 1981; Devine *et al.*, 1989; Dunlap, 1995; Fuqua *et al.*, 1996; Ulitzur, 1995). It is thought that at night the squid needs to be unseen in order to escape from predators and to hunt (Nealson and Hastings, 1979). While this appears to be an obvious advantage to the squid, this hypothesis will require empirical testing in the future. It is then that the light from *V. fischeri*, adjusted to the natural moonlight, makes the perfect camouflage for the squid to hide from its prey in the shallow waters of the ocean. Every morning the squid expels most of the bacteria into the seawater and regrow new bacteria during the day and the cycle is repeated every day (Eberhard *et al.*, 1981; Nealson and Hastings, 1979; Ruby *et al.*, 1976; Ruby *et al.*, 1980; Ruby *et al.*, 1992).

#### 1.2.1.2.1 AHL structures and synthesis

Eberhard *et al.*, (1981) identified the first AHL structure in *V. fischeri* as N-3-oxohexanoyl-HSL (Figure 1.3Aa). Many more AHL structures have been identified in

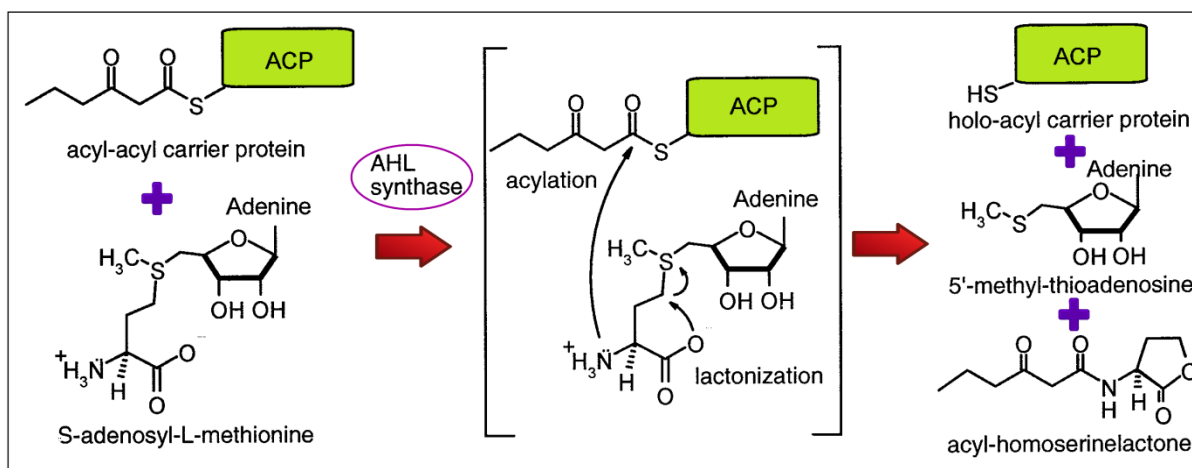
different bacterial species since then (Figure 1.3Ab,c,d,e). Different techniques such as high performance liquid chromatography (HPLC) purification, mass spectrometry and nuclear magnetic resonance (NMR) thin layer chromatography (TLC), ultra-performance liquid chromatography (UPLC), ultra-high-resolution mass spectrometry, and in-situ biosensors have been used to identify them (Shaw *et al.*, 1997; Marketon and González, 2002; Marketon *et al.*, 2002; Teplitski *et al.*, 2003; Fekete *et al.*, 2007).



**Figure 1.3** Some of the QSSs chemical structures from A) Gram negative bacteria a) 3-oxo-C<sub>6</sub>-HSL b) 3-oxo-C<sub>12</sub>-HSL c) C<sub>4</sub>-HSL d) 3-oxo-C<sub>8</sub>-HSL e) 3-Oxo-C<sub>14</sub>-HL. B) Gram positive bacteria CSF: competence and sporulation factor, ComX: Competence pheromone, CSP: Cold shock protein, AIPs: autoinducing peptides C) *V. harveyi*, Furanosyl borate diester. The asterisk above the tryptophan in ComX represents an isoprenyl modification. Figure adapted from Federle and Bassler (2003).

The basic AHL structure is composed of a homoserine lactone ring moiety and an acyl side chain. Different AHLs are distinguished in length, degree of substitution and

saturation of their acyl-side chain (Figure 1.3) (Fuqua *et al.*, 2001). The hydrophobicity of the molecule is a result of the hydrophilic homoserine lactone and the hydrophobic side chain. AHLs, specifically, acyl-side chains, range from 4 to 18 carbons in length (Fuqua *et al.*, 2001; Neumann *et al.*, 2013). Modifications in the oxidation state at the C3 position on the acyl side chain (methyl, ketone, or hydroxyl groups) and the length of the acyl side chain gives specificity to quorum sensing systems (Fuqua *et al.*, 2001; Patel *et al.*, 2013). It has been demonstrated by structural studies of LuxI-type proteins (AHL synthase) that their acyl-binding pocket fits precisely to a specific side chain moiety (Gould *et al.*, 2004). Figure 1.4 shows how the LuxI synthase catalyses the amide bond formation between *S*-adenosyl-L-methionine (SAM), the acyl-acyl carrier protein (ACP) and the acyl SAM intermediate creating the acyl homoserine lactone (Moré *et al.*, 1996; González and Keshavan, 2006).



**Figure 1.4** AHL synthesis reaction. AHL synthases catalyse the acylation of *S*-adenosyl-L-methionine (SAM) by an acyl-acyl carrier protein followed by lactonisation of the methionine moiety. This generates the end product acyl homoserine lactone with the byproducts holo-ACP and 5'-methylthioadenosine. Figure adapted from Watson *et al.*, (2002).

#### 1.2.1.2.2 Some examples of LuxI/R homologs

LuxI/R was thought to be unique to *V. fischeri* and *V. harveyi* (Fuqua *et al.*, 2001). Nonetheless, analogues of this quorum sensing system have been found in over 70 species of Gram negative bacteria (Miller and Bassler, 2001), mostly in proteobacteria (Case *et al.*, 2008; Hense and Schuster, 2015). In other genera, the LuxI/R-like system usually refers to homologues of the *V. fischeri* regulatory systems. Thus, LuxI homologs

are homologous to the LuxI protein in *V. fischeri* and LuxR homologs are homologous to the LuxR protein in *V. fischeri* (Fuqua *et al.*, 2001). Some bacterial species have more than one LuxI homolog, and in that case, a specific AHL combination will be produced. In consequence, each bacterial species recognises their own set of AHL molecules, differentiating their own members from the rest (Federle and Bassler, 2003).

In this conventional system, *luxI* and *luxR* genes are usually located closely in the chromosome. However, sometimes a *luxR* gene is found by its own without *luxI* gene. This is known as an orphan (Fuqua, 2006; Subramoni and Venturi, 2009) or solo LuxR homologs (Case *et al.*, 2008; Patankar and González, 2009; Patel *et al.*, 2013). Even though the LuxR regulatory system is highly conserved in bacteria, substitutions in key amino acids of the AHL-binding domain lead to AHL recognition impairment. This is the case in the solo LuxR homolog OryR in *Xanthomonas oryzae* pv. *oryzae* (Xoo) where OryR cannot bind to AHLs but rather to low molecular weight plant molecule(s) (Fuqua *et al.*, 2001; González *et al.*, 2013). Case *et al.*, (2008) revealed that out of 265 proteobacterial genomes analysed, 68 possess a LuxI/R system, of which 45 genomes lack a complete LuxI/R system (containing a higher ratio LuxRs/LuxIs) and another 45 genomes contained LuxR solos. Fuqua (2006), suggested that an orphan or solo LuxR homolog, QscR (quorum sensing control repressor) from *Pseudomonas aeruginosa* could respond to mono-species as well as multispecies signalling in order to integrate information of mixed bacterial communities.

It is possible to find more than one QS circuit, LuxI/LuxR homologs, in one species. This is the case of *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*. As AHLs from these bacteria were assessed in this work, their QS systems will be reviewed below.

*Pseudomonas aeruginosa* is a gram negative soil bacterium, which can cause plant and animal disease. It possesses at least three QS circuits: Las, Rhl and QscR (Fuqua, 2006). Las/Rhl is composed of LuxI/LuxR homologs, LasI/LasR and RhlI/RhlR, respectively. Las and Rhl affect the expression of 160 to 650 genes, including those involved in virulence factors, biofilm formation, exoenzymes production, nutrient acquisition, motility and signalling pathways molecules such as *Pseudomonas* quinolone signal (PQS), which is critical to its survival (Pesci *et al.*, 1999; Wade *et al.*, 2005; Juhas *et al.*, 2005). The Las and Rhl circuits function in tandem to ensure synchronous timing and thus successful pathogenesis (Miller and Bassler, 2001). LasI catalyses 3-oxo-C<sub>12</sub>-HSL

and RhlI C<sub>4</sub>-HSL synthesis (Pearson *et al.*, 1994, 1995). When a sufficient population density and therefore, 3-oxo-C<sub>12</sub>-HSL concentration, has been reached, LasR, a LuxR homolog, in conjunction with 3-oxo-C<sub>12</sub>-HSL controls the synthesis of virulence factors and also the upregulation of the *rhlR* gene (Ochsner and Reiser, 1995). RhlR binds to C<sub>4</sub>-HSL modulating a diverse range of target genes such as those for siderophores production, involved in iron acquisition, and rhamnolipid biosynthesis, and others involved in virulence, motility, biofilm formation and uptake of hydrophobic substrates (Stintzi *et al.*, 1998; Schuster *et al.*, 2003, 2004). A negative regulator, QscR acts as a repressor of the positive feedback loop, down-regulating genes in the Las, Rhl and QscR system (Fuqua, 2006).

*Agrobacterium vitis* is a well-known plant pathogen, which induces crown gall tumours on grapevine. In order to form the tumours the oncogenic Ti plasmid (pTi), carrying specific virulence genes (*vir*) must be transferred to the host cell nucleus (Lowe *et al.*, 2009). Once a section of the Ti plasmid, T-DNA, is integrated into the plant genome, not only the gall formation begins but also a host specific root necrosis in grapes and a hypersensitive-like response (HR) on non-host plants like tobacco (Hao and Burr, 2006). These pathogenic behaviours are under the control of the cognate *avsI/R* locus. *AvsI* synthesises the long chain AHL molecules and *avsR*, the transcriptional regulator, binds to the AHLs. In addition, *AviR* and *AvhR*, two LuxR solos, are also involved in the regulation of this pathogenesis response (Zheng *et al.*, 2003; Hao *et al.*, 2005). Interestingly, *AvsI* and *AvsR* from *A. vitis* share up to 71% and 38% of identity with *SinI* and *SinR* from *S. meliloti* strain 1021, respectively (Hao and Burr, 2006). In addition, the solo LuxR homologs, *AviR* and *AvhR*, are highly homologous to *ExpR* and two orphan LuxR homologs (SMc00878 and SMc00877) of *S. meliloti* (Zheng *et al.*, 2003; Hao *et al.*, 2005).

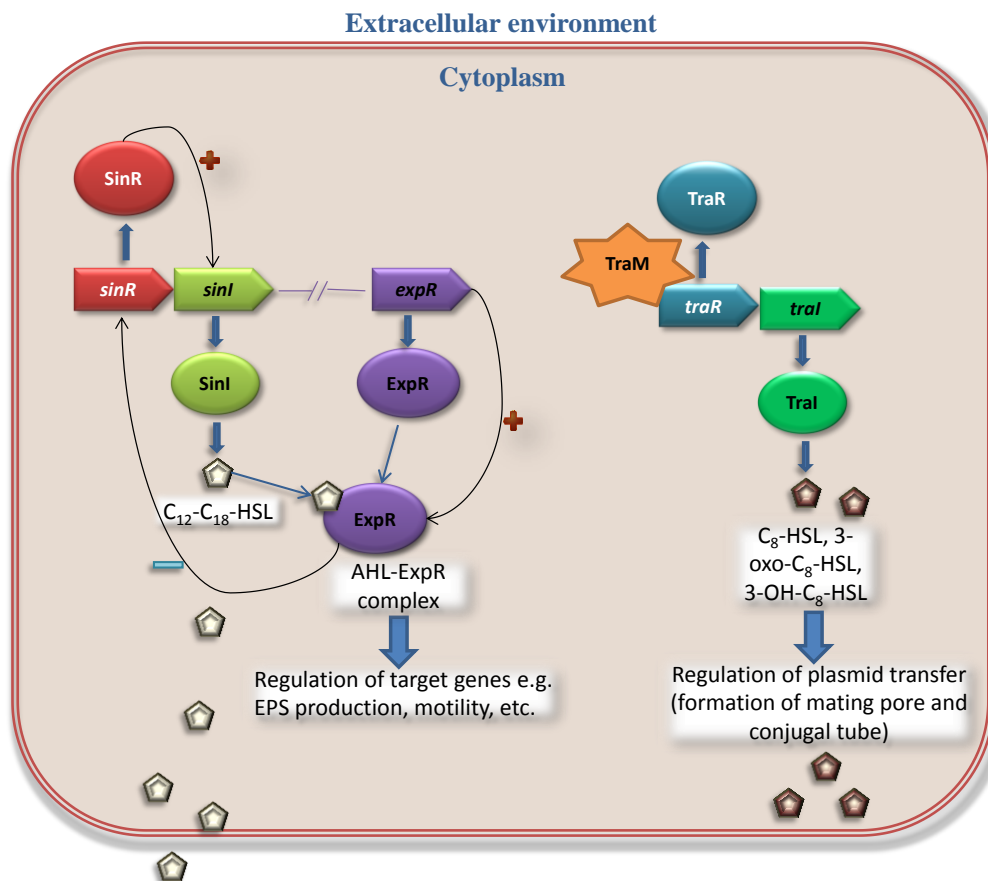
*Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) is a soil alpha proteobacterium gram negative nitrogen-fixing rhizobium, which forms mutualistic symbiosis with legume plants belonging to the genus *Medicago*, *Melilotus* and *Trigonella* (Glazebrook and Walker, 1989; Gurich and González, 2009). To our knowledge, QS in *S. meliloti* has not been completely understood. This QS system is basically composed for the Sin circuit, consisting of *SinR*, a transcriptional regulator and an AHL synthase *SinI*, which catalyses the production of long acyl side chain AHLs ranging from C<sub>12</sub> to C<sub>18</sub>-HSL. In addition, there is a functional orphan LuxR homolog called *ExpR* and the Tra system with *traI*, *traR* and *traM* as in *A. vitis* (Marketon *et al.*, 2002; Marketon and González,

2002; González and Marketon 2003; Charoenpanich *et al.*, 2013). It has also been reported that *S. meliloti* strain 1021 (Rm1021) possesses four additional orphan or solos LuxR homologs encoded by the SMc04032, SMc00878, SMc00877, and SMc00658 loci (Galibert *et al.*, 2001). Only two of them, SMc04032 (NesR) and SMc00878, have a known function. NesR regulates the survival and nodulation efficiency by competition in the rhizosphere and SMc00878 regulates the gene expression of the denitrification pathway (Patankar and González, 2009). Sanchez-Contreras *et al.*, (2007) indicates that two additional putative orphan LuxR homologs, SMc03015 and SMc03016 are also found in Rm1021. However, the functionality of these genes remains unknown. Other components to this QS circuit have been suggested (Hoang *et al.*, 2004). Previously, Marketon and González (2002), suggested that *S. meliloti* had an additional AHL synthase LuxI homolog called Mel system which catalyses the production of short chain AHLs. However, in subsequent studies this was not confirmed (Gao *et al.*, 2005).

The ExpR/Sin quorum sensing circuit is required for most of the free-living and symbiotic cell functions, including the synthesis of the low molecular weight (LMW) fractions of exopolysaccharides succinoglycan (EPSI) and galactoglucan (EPSII), which play an essential symbiotic role in motility, chemotaxis and infection (Pellock *et al.*, 2002; Hoang *et al.*, 2004, 2008; Glenn *et al.*, 2007; Gurich and González, 2009; Nogales *et al.*, 2012), biofilm formation (Wang *et al.*, 2004; Edwards *et al.*, 2009; McIntosh *et al.*, 2008), nodulation and nitrogen fixation (NF) (Loh *et al.*, 2001; Zheng *et al.*, 2006), plasmid transfer (Marketon and González, 2002) and metal transport (Hoang *et al.*, 2004), among others.

The expression of the *sinI* gene is under the regulation of the SinR and ExpR proteins (Marketon and González, 2002). At high population density, the production of SinI proteins is upregulated to synthesise long side chain AHLs at its threshold of activation of *sinI* ( $\approx 1$  nM AHL). As a result, the AHL concentrations are increased dramatically. AHLs bind to the ExpR forming the AHL-ExpR complex. This complex regulates a total of 570 genes including the ones responsible for succinoglycan, galactoglucan and flagellum production as well as the Sin circuit SinI/SinR (Charoenpanich *et al.*, 2013; Gurich and González, 2009; Hoang *et al.*, 2004). *sinI* transcription is upregulated by ExpR-AHL complex through *sinR* and *expR*, resulting in a positive feedback loop (Marketon *et al.*, 2003; McIntosh *et al.*, 2009). When the threshold for reduction of *sinR* is reached ( $\approx 40$  nM) *sinR* is repressed, decreasing SinR protein through turnover and dilution of SinR by cell division. In addition, posttranscriptional mechanisms such as

degradation of the AHL synthase transcript *sinI* by RNase E, a bacterial endoribonuclease important for RNA metabolism, has been reported in *S. meliloti*. As a consequence, *sinI* expression is inhibited (Figure 1.5) (McIntosh *et al.*, 2009; Baumgardt *et al.*, 2014). At low AHL concentrations, TraM inactivates TraR until the population density increases when *traI* encodes for the production of 3-oxo-C<sub>8</sub>-HSL, 3-OH-C<sub>8</sub>-HSL and C<sub>8</sub>-HSL activating the response regulator TraR. The Tra system regulates the plasmid transfer through formation of the mating pore and the conjugal tube (Marketon and González, 2002). Interestingly, McIntosh *et al.*, (2009), points out that positive and negative feedback loops in *S. meliloti* are not only affected by AHLs concentration but also by environmental conditions such as phosphate availability in the rhizosphere.



**Figure 1.5** Quorum sensing system of *Sinorhizobium meliloti*. SinI catalyses the production of long side chain AHLs (pentagons). Model based on González and Marqueton, 2003; McIntosh *et al.*, 2009; Charoenpanich *et al.*, 2013). Colourful boxes represent genes that encode ExpR, SinR and SinI proteins (circles). + and – symbols indicate activation and repression of the transcription, respectively. Tra system is borne on a plasmid. Therefore, it is not present in all *S. meliloti* strains.



Exopolysaccharide production and flagellum synthesis are essential for symbiosis (see section 1.3.1). Flagella and EPSII production are required for motility in *S. meliloti*, which in turn, depends on the production of AHLs, particularly C<sub>16</sub>-HSL and 3-oxo-C<sub>16:1</sub>-HSL (Gao *et al.*, 2012). Exopolysaccharides regulation in *S. meliloti* is remarkably complex composed by numerous regulatory circuits that modulate the bacterial response according to several environmental stress factors (Janczarek, 2011). Gurich and González (2009) reported that the inactivation of motility genes by ExpR/Sin QS circuit at high population density is essential for nodule invasion efficiency when *S. meliloti* accumulates in the root. Once the invasion is established ExpR/Sin is repressed (Gurich and González, 2009). Interestingly, in *S. meliloti* strain 1021, *expR* is disrupted due to a native insertion sequence (ISRm 2011-1) which impedes the synthesis of EPSII. Experiments done in this strain, found that a spontaneous excision of this insertion increases EPSII in response to C<sub>16:1</sub>-HSL and 3-oxo-C<sub>16:1</sub>-HSL confirming that Rm1021 is not able to produce EPSII due to this insertion (Pellock *et al.* 2002; Marketon *et al.*, 2003). However, Rm1021 is able to produce EPSI, particularly the low molecular fraction of this polymer essential for symbiosis, establishing a successful colonisation in *M. truncatula* and producing functional nodules (González *et al.*, 1996).

As it has been reviewed, LuxI/R homologs play a crucial role in the regulation of many genes important for competition and survival of gram negative bacteria. LuxI is the population density signal responsible for synthesising the AHL molecules (Fuqua *et al.*, 1994). When the bacterial population increases in numbers, so does the AHL signal concentration. Thus, each AHL binds to the LuxR homolog protein, the population density-dependent transcriptional activator of the DNA (Fuqua *et al.*, 1996; Fuqua *et al.*, 2001). However, LuxI/R is not the only conventional QS system present in bacteria. Others include modified peptides and hybrid QS systems.

### **1.2.1.3 Hybrid quorum sensing systems**

The last conventional QS system is a hybrid between the two conventional QS reviewed above: modified oligopeptides found in gram positive bacteria and LuxI/R found in gram negative bacteria (Henke and Bassler, 2004). Hybrid QS systems were originally identified in *V. harveyi*, which can produce and recognise two autoinducers, AI-1 and AI-2, simultaneously, which is known as coincidence detection (Bassler *et al.*, 1993, 1994). It has been suggested that coincidence detection reduces the background “noise” or trickery from other bacteria (Schauder and Bassler, 2001; Henke and Bassler, 2004).

AI-1 corresponds to an AHL and AI-2 to a furanosyl borate diester. When either AI-1 or AI-2 reach a certain threshold, the signal transduction is activated via a two-component phosphorylation cascade (Figure 1.2, third column). This parallel and “multi-signal” system allows bacteria to communicate with their own (species-specific language) as well as with others (non-species specific language). The AI-2 is synthesised by *luxS*, has also been found in many gram positive and gram negative bacteria regulating inter-species communication. As a result, it has been proposed that AI-2 is a universal signal (Surette *et al.*, 1999; Pereira *et al.*, 2008).

### 1.2.2 Quorum sensing interspecies communication: me and the rest.

Different species of bacteria produce slightly different QSSs. For example, *Pseudomonas* and symbiotic rhizobia produce slightly different versions of AHLs differing in the fatty acyl side chain (Figure 1.3). It seems that different bacterial species have subtle “dialects” that might allow them to differentiate between signals from their own species and signals from other species. This might be extremely helpful as bacteria usually live in complex environments composed of different microbial communities. Not surprisingly, it has been suggested that QS helps bacteria living in these complex ecological environments, eg. rhizosphere and biofilms, to distinguish not only their own but also other species (Fuqua *et al.*, 1996; Case *et al.*, 2008).

With the discovery of AI-2 not only in *V. harveyi* but also in a vast range of gram negative and gram positive bacteria, it was proposed that QS communication existed wide spread among species (Xavier and Bassler, 2003). It is also known that AI-2 regulates many bacterial “behaviours” such as virulence, toxin production; motility, antibiotic production and biofilm formation, among others (See the review Federle and Bassler, 2003). It appears that bacterial “listening ability” is not restricted by their capacity to “talk”. Case *et al.*, (2008), suggest that eavesdropping on other species conversations seems to be predominant among the Proteobacteria. This sophisticated social behavior may give bacteria an advantage to compete and survive in complex diversified bacterial populations (Chandler *et al.*, 2012).

As described before (section 1.2.1.2.2), solo LuxR homologs response regulators and not their cognates LuxI homologs, have been found in abundance in many bacteria (Case *et al.*, 2008). That is also the case in *S. meliloti*, which despite of not producing AI-2, are able to recognise and internalise this molecule from other bacterial species

using its Lsr system (Pereira *et al.*, 2008). Thus, *S. meliloti* ability to eavesdrop other species “conversation” rather than participating actively in the communication provides an advantage during colonisation of a common niche interfering with AI-2 regulated behaviors such as virulence (Pereira *et al.*, 2008). Similar to *S. meliloti*, *P. aeruginosa* does not produce AI-2. However, it has been shown that AI-2 is able to upregulate overlapping subsets of virulence factor promoters (Duan *et al.*, 2003). Eavesdropping on other neighbours might provide important information to *Rhizobium leguminosarum* *bv. viciae* to delay growth through conjugal transfer of the symbiotic plasmid until a quorum has been reached (Wilkinson *et al.*, 2002).

### 1.2.3 Quorum quenching: The war zone.

It has been reported that bacteria are able to disrupt other QS systems by reducing the activity of the AHL receptor or synthesis cognate; affecting the stability and function of the QS signal by removing, inactivating or modifying it (e.g. agonist to antagonist); inhibiting the production of QS signals; producing QS mimic compounds or analogues; producing anti- activator proteins or negative transcriptional regulator homologs and by negative regulation via small RNAs (sRNAs) (Federle and Bassler, 2003; González and Keshavan, 2006; Kalia, 2013).

Removal of the autoinducer from the environment by importing it into the cell through ABC transporters reduces the amount of the signal in the milieu (Federle and Bassler, 2003). In a co-culture with *S. meliloti* and *Erwinia carotovora* (producing AI-2), *S. meliloti* strain Rm1021 was able to remove AI-2 from the extracellular medium (Pereira *et al.*, 2008). Perhaps, masking other species perception that they are in a low population density would give them a better chance to compete for limited energy and nutrient resources.

Moreover, QS inactivation in bacteria is caused by enzymatic degradation of autoinducers. The first such enzyme that was identified and purified was a lactonase from *Bacillus* *sp.* (Dong *et al.*, 2001). Lactonases hydrolyse the lactone ring of the AHL structure, thereby inactivating the molecule. In addition, acylases hydrolyse the AHL amide bond, releasing the homoserine lactone from the fatty acid side chain (Lin *et al.*, 2003). Apart from these enzymes two others have been reported decarboxylases, which hydrolyse the lactone ring and deaminases, which cleave the acyl side chain (reviewed

by Kalia, 2013). These enzymes and derivatives have been found in gram positive as well as gram negative bacteria (Helman and Chernin, 2015).

Volatiles organic compounds from bacteria can also disrupt QS. It has been shown that *P. aeruginosa* and *P. fluorescens*, among other bacteria, produce dimethyl sulfide, which acts as a quorum quencher decreasing both long and short chain AHLs (Chernin *et al.*, 2011). *Streptomyces sp.* produces butyrolactones that act as AHL analogues (Kinoshita *et al.*, 1997). In addition, cyclopeptides and diketopiperazines produced by *Pseudomonas sp.* interfere with QS (Holden *et al.*, 1999).

#### **1.2.4 Interkingdom communication: What happens when bacteria interact with organisms from another kingdom?**

Prokaryote-eukaryote cross talk or interkingdom communication via quorum sensing has been reported with members of fungi, animals and plants (Cugini *et al.*, 2008; Joint *et al.*, 2007; Kalia, 2013). In this study only plant- bacteria interkingdom communications will be discussed, as they form part of the scope of this work.

Bacteria can increase or reduce the performance of plants substantially, in the worst case, they can kill the plant, in the best, they can increase growth by providing extra nutrients, hormones and improved soil structure. Bacteria are efficient colonisers and infecting agents and one of the reasons for this success is their use of AHL signals to coordinate many of their behaviours. Studies have shown that plants are not only able to detect the AHL signals of bacteria, but that they can differentiate between signals from pathogenic and symbiotic bacteria (Gao *et al.*, 2003). Their responses to AHLs depend on the concentration of the signal (Gao *et al.*, 2003; von Rad *et al.*, 2008). In response to AHL signals, *Medicago truncatula* plants have been shown to adjust the accumulation of more than 150 proteins, including defense related proteins, metabolic enzymes, and enzymes of the flavonoid pathway (Mathesius *et al.*, 2003). In addition, AHL signals appear to alter plant development by altering auxin levels in *Arabidopsis* (von Rad *et al.*, 2008). Joseph and Phillips (2003) have shown that QS breakdown products from beneficial root colonising bacteria increase stomatal conductance and transpiration rate in beans (*Phaseolus vulgaris* L.). AHL signals influenced root growth in *Arabidopsis* as well as shoot growth in barley (von Rad *et al.*, 2008; Klein *et al.*, 2009).

Plants can interfere with bacterial QS via reduction of the AHL cognate receptor or synthesis protein and inhibition of AHL molecules by degradation, sequestration and mimicry (Truncado *et al.*, 2015). It has been shown that a number of plant species can react to AHL compounds by producing and exuding so called “QS mimic” compounds - molecules that are perceived as AHL compounds by the bacteria interfering with their QS circuits (Teplitski *et al.*, 2000; recently reviewed by La Sarre and Federle, 2013; Truncado *et al.*, 2015; Koh *et al.*, 2013). QS mimic molecules from plants can inhibit or promote QS-related gene expression in bacteria (Teplitski *et al.*, 2000). This finding suggests that plants have a mechanism to interfere or “quench” bacterial quorum sensing. The production of quorum sensing mimics was enhanced after perception of purified quorum sensing signals by the plant, and differed in response to different structures of quorum sensing signals (Mathesius *et al.*, 2003). Vandeputte *et al.*, (2010), demonstrated that catechin, a flavonoid from the medicinal tree *Combretum albiflorum*, negatively affected *las* and *rhl* gene expression in *P. aeruginosa* PAO1 via interfering with the perception of C<sub>4</sub>-HSL by RhlR resulting in reductions of growth, pyocyanin production and biofilm formation. Further studies demonstrated that naringenin, a flavonone, inhibited the virulence of *P. aeruginosa* decreasing the expression of *las* and *rhl* genes and resulting in reductions of 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL and a defective Rhl-C<sub>4</sub>-HSL complex (Vandeputte *et al.*, 2011). In *E. coli*, naringenin, kaempferol, quercetin and apigenin were found to inhibit biofilm formation. These flavonoids also inhibit biofilm formation and bioluminescence in *V. harveyi*. Also, naringenin suppresses virulence in the latter (Vikram *et al.*, 2010). Interestingly, flavonoids have been linked with legume symbiosis as crucial players to establish nodulation (reviewed by Hassan and Mathesius, 2012). Rajamani *et al.*, (2008), who discovered that riboflavin and its degradation product lumichrome from the algae *Chlamydomonas*, activated LasR response regulator in *Pseudomonas aeruginosa*, which suggests that both compounds act as interkingdom QS signal mimics. Another example is given by Keshavan *et al.*, (2005), suggesting that *Medicago sativa* hinders *S. meliloti* QS by impeding the folding of ExpR.

González *et al.*, (2013), reported that a subfamily of LuxR solo OryR of *Xanthomonas oryzae* pv. *oryzae* (Xoo) is able to recognise low molecular weight plant compounds from rice. However, many of the plant signals interfering with AHL synthesis or perception remain unknown.

## 1.3 The Legume-Rhizobia Symbiosis

Legume plants belonging to the Fabaceae family are the second most important crops in agriculture after cereals crops (Poaceae) like maize, rice and wheat (Smykal *et al.*, 2015). They are a good source of protein, oil and dietary fibre for humans and animals as well as nitrogen, as an amendment for soil (Cook, 1999). One of the reasons for their high protein content is the symbiotic nitrogen fixation with their rhizobia partners. This process consists in the conversion of atmospheric nitrogen (N<sub>2</sub>) into ammonia (NH<sub>3</sub>), which plants are able to utilise. In exchange, plants provide carbon in form of organic acids as a source of energy to rhizobia. The model annual pasture legume *Medicago truncatula* (Gaertn.) (barrel medic or barrel medick or barrel clover) constitutes a great opportunity to study rhizobia-legume interactions due to its features including its small diploid sequenced genome, easy of genetic transformation, self-fertilisation, high seed production and short generation time (Cook, 1999). Moreover, in Australia *M. truncatula* is an important forage crop species cultivated in a lay farming mode (Puckridge and French, 1983).

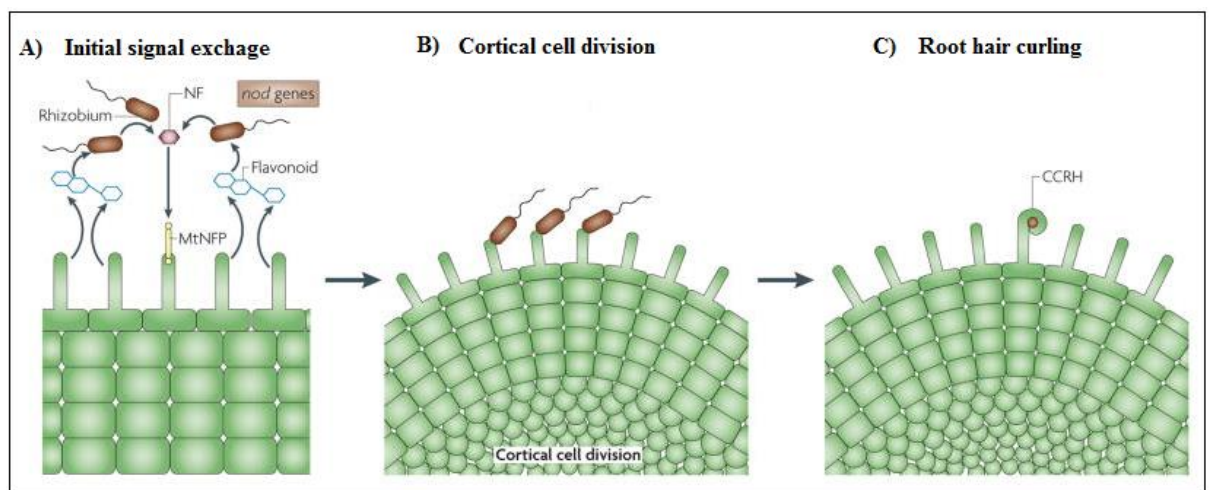
### 1.3.1 Nodulation

The invasion of plant roots by rhizobia is a process that consists of several synchronised steps including the initial signal exchange, initiation of infection, infection thread development, formation of nodule primordia and development of the nodule able to fix atmospheric nitrogen (Gage, 2004; Jones *et al.*, 2007).

The initial signal exchange starts with the release of signal chemicals called flavonoids and betaines that are secreted by host roots into the surrounding rhizosphere in order to attract compatible rhizobia (Gage, 2004). Flavonoids are phenylpropanoid metabolites which act as chemoattractants, regulators of the nodulation (*nod*) genes in rhizobia (Peters *et al.*, 1986; Redmond *et al.*, 1986a). Once rhizobia recognise their partner and reach its root surface, they attach to the roots. Flavonoids bind to rhizobial NodD proteins, which are transcriptional regulators of the LysR family, to induce *nod* gene transcription (Györgypal *et al.*, 1988). In rhizobia, upregulation of *nod* genes leads to the induction of the synthesis and export of lipochitin oligosaccharides (LCOs) or Nod factors (Figure 1.6A). Nod factors consist of a  $\beta$ -1,4-linked *N*-acetyl-D-glucosamine residues, which are species-specific. Bacteria are able to synthesise more than one Nod

factor molecule. Nod factors trigger early nodulation changes in the host such as root hair deformation, membrane depolarization, intracellular calcium oscillations and initiation of cell division in the root cortex which initiates nodule development (Ehrhardt *et al.*, 1996; Felle *et al.*, 1998; Cárdenas *et al.*, 1999; for review see Gage, 2004).

Even though flavonoids usually upregulate *nod* genes in rhizobia, it has been demonstrated that some flavonoids can also downregulate their expression in *S. meliloti* (Zuanazzi *et al.*, 1998). This might be a mechanism by which plants maintain low levels of *Nod* factors to avoid the elicitation of plant defense responses to ensure optimal nodulation (Zuanazzi *et al.*, 1998). Even though Nod factor can be perceived by the plant at extremely low concentrations (1 to 10 pM) (Ehrhardt *et al.*, 1996; Oldroyd *et al.*, 2001), local accumulation of Nod factors on the root hairs it is thought to reach high concentrations (1 to 10 nM) over time activating calcium flux (Shaw and Long, 2003). It has been reported that bradyoxetin, an extracellular quorum-responsive signal molecule produced at high population density by *Bradyrhizobium japonicum* USDA110, indirectly repressed *nod* genes in soybean cultivar Lambert (Loh and Stacey, 2003; Jitacksorn and Sadowsky, 2008). However, this repression was dependent on the environment and the plant cultivar (Jitacksorn and Sadowsky, 2008).



**Figure 1.6** Initial signal exchange between *Medicago truncatula* and *Sinorhizobium meliloti*. A) Flavonoids produced by *Medicago* upregulate *nod* genes in *S. meliloti*. As a result, Nod factors are produced which in turn, are perceived by the plant through a Nod factor receptor (*MtNFP*). B) Attachment of the rhizobia to susceptible root hairs and

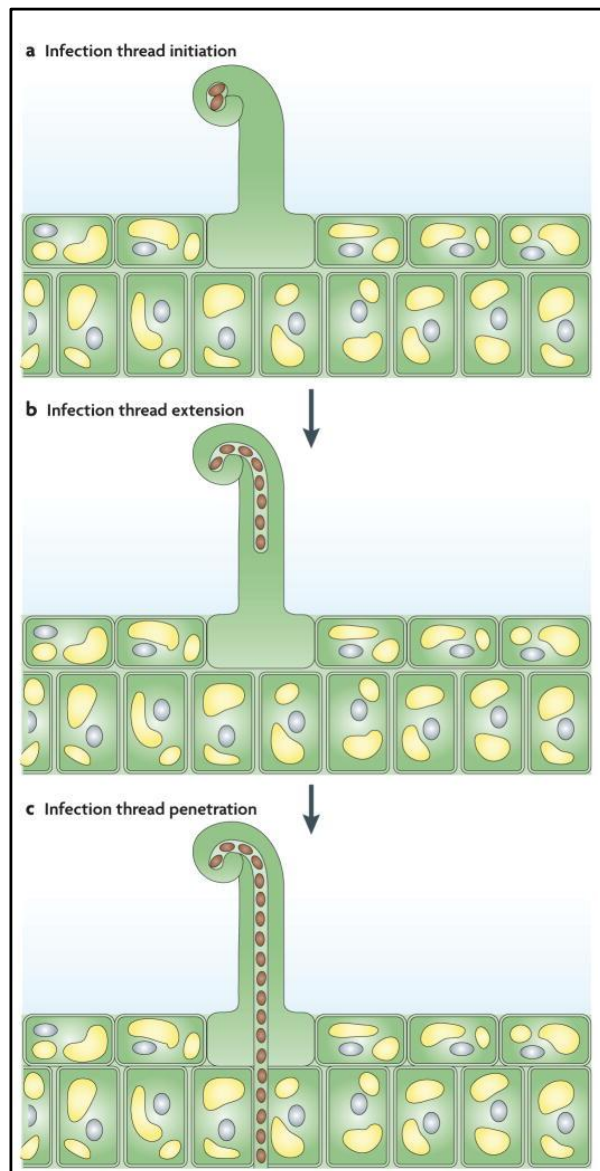
cortical cell division occurring in unison. C) Colonised curled root hair (CCRH). Modified figure from Jones *et al.*, (2007).

The formation of bacterially infected nodules requires a coordinated root development program where two processes occur simultaneously: root hair infection and cortical cell division. The infection of susceptible hair roots starts with rhizobia attaching to young developing root hairs at the root tip zone. At the beginning, they attach loosely via a  $\text{Ca}^{2+}$  dependent step using a protein called rhicadesin. Later, they attach tightly through cellulose fibrils synthesised by the rhizobia (Hirsch, 1992). Nod factors secreted by rhizobia are perceived by receptors on the susceptible root hair inducing membrane depolarization,  $\text{Ca}^{2+}$  influx at the root tip and oscillations in cytosolic calcium ( $\text{Ca}^{2+}$  spiking) (Ehrhardt *et al.*, 1992; 1996). In *M. truncatula* the Nod factor receptor is called *Medicago truncatula* Nod Factor Perception (*MtNFP*) (Figure 6A). In response to Nod factor perception and signal transduction, early nodulation genes (*ENODs*) are induced in epidermal actively growing root hairs (Journet *et al.*, 1994, 2001). However, the importance of Nod factor signalling continues as the invasion extends to the cortex and may also be important when bacteria are released into differentiating the nodule cells (Den Herder *et al.*, 2007; Hadri and Bisseling, 1998).

The infected root hairs deform in several unusual shapes. In the *S. meliloti* and *M. truncatula* symbiosis, they exhibit a typical ‘Shepard’s crook’ shape as rhizobia bind to the root hair, its growth is arrested on one side (Figure 1.6C; 1.7). This deformation can be influenced by plant hormones such as ethylene to regulate the frequency of productive infections, thus, acting as a negative regulator on nodulation (Oldroyd *et al.*, 2001). The root hair curls, trapping the rhizobia inside, followed by progressive invagination of the root hair cell membrane which forms a tubular structure that grows down the root hair to the cortical cells. This structure is known as the infection thread. Rhizobia keep growing and dividing inside the infection thread (Gage, 2004). While this is happening in the root hairs, cortical cells begin to divide (Figure 1.6B). It has been found that rhizobia unable to produce Nod factors and exopolysaccharides (EPS) are impaired to form infection threads successfully (Esseling *et al.*, 2003; Jones *et al.*, 2007). Even though the precise functional role of EPS in symbiosis remains unclear (Kawaharada *et al.*, 2015), EPS in *Medicago* symbiosis were shown to be important for early stages of plant infection, including the attachment of bacteria to the root surface, root hair curling, proper infection thread initiation and extension, bacterial discharge from infection threads, bacteroid development, suppression of plant defence responses



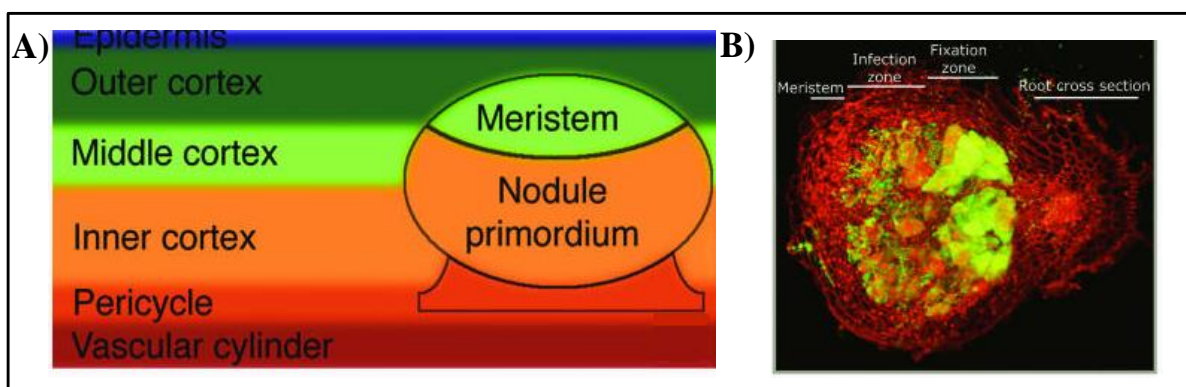
and successful nodulation (Kawaharada *et al.*, 2015; Fraysse *et al.*, 2003; Pellock *et al.*, 2000; Skorupska *et al.*, 2006). However, effective invasions can also occur when synthetic Nod factors and exopolysaccharides are supplied in addition to *S. meliloti* strains (Klein *et al.*, 1988).



**Figure 1.7** Developmental stages of root hair infection in *S. meliloti*. a) Infection threads initiation. b) Infection threads extension. c) Infection threads penetration. Modified figure from Jones *et al.*, (2007).

Cortical cell division initiates nodule formation. There are two main types of nodule structures and nodule differentiation programs, depending on the location of the initial cortical cell divisions. The model legume *M. truncatula* produces indeterminate nodules

as well as other temperate legumes including *Medicago sativa*, *Pisum sativum*, *Vicia faba* and *Trifolium repens*. Indeterminate nodules originate from pericycle and inner cortical cell divisions and are characterised by having an elongated shape and have a persistent meristem at the distal end, which generates new nodule cells that are infected by the rhizobia inside. Indeterminate nodules show a clear gradient of meristematic, infection and fixation zones. The division of the pericycle and inner cortical cells initiates a the nodule primordium. Middle cortical cell divisions create the nodule meristem (Figure 1.8) (Hirsch, 1992; Gage, 2004). On the other hand, determinate nodules found mostly in tropical legumes but also in temperate legumes (e.g. *Lotus japonicus*, *Glycine max*, *Phaseolus vulgaris*) originate from outer cortical cell divisions and tend to have a round shape and lack of the persistent meristem. Therefore, bacterial cells inside the nodule multiply, differentiate and senesce synchronically without the developmental gradient found in indeterminate nodules (Hirsch, 1992; van Spronsen; Mergaert *et al.*, 2006). As this study was done mostly in *M. truncatula*, indeterminate nodulation will be reviewed.



**Figure 1.8** Indeterminate nodule structure A) Representation of an emerging nodule and the root tissues that give rise to different nodule zones. B) Longitudinal section of 10 day-old alfalfa nodule, showing bacteroids (GFP labelled *S. meliloti*) in the different zones of the nodule: meristem, infection zone and fixation zone. At the right it is possible to see the root cross section. Plant tissue is seen counterstained with Propidium iodide (red). Modified figure from Gage, (2004).

While inner cortical cells are dividing, outer cortical cells become polarized and cytoplasmic bridges are formed. The infection threads leave the root hair cells to enter

to the outer cortical cells where they grow through the cytoplasmic bridges towards to the inner cortical cells. When rhizobia reach the inner cortical cells, they are internalised by endocytosis. The unit composed by an individual rhizobia and the surrounding endocytic membrane is called symbiosome (Brewin, 2004). Inside the symbiosome, rhizobia differentiate into their nitrogen fixing form called bacteroids (Jones *et al.*, 2007). Bacteroids are able to fix nitrogen from the atmosphere ( $N_2$ ) via nitrogenase activity. This enzyme reduces  $N_2$  into ammonia ( $NH_3$ ) so plants can use it as a nitrogen source.

### **1.3.2 Control of nodulation**

Nodulation is controlled by abiotic factors such as temperature and nutrient availability. The most extensively studied has been nutrient availability, especially soil nitrogen content (Aranjuelo *et al.*, 2015; Streeter, 1978). It has been shown that plants can control the nodule numbers by different mechanisms including control of plant defence responses, which are partly affected by the gaseous phytohormone ethylene, and a mechanism called autoregulation of nodulation (AON) by the plant host (Gage, 2004).

#### **1.3.2.1 Plant defence responses**

Nodulation is an extremely costly process for the host legume. It has been estimated that the biological cost of nodule establishment, nitrogen fixation and transport for the plant is 12-17 grams of carbon per gram of fixed nitrogen (Crawford *et al.*, 2000). Thus, plants use different mechanisms in response to environmental conditions to regulate nodulation according to their requirements (Mortier *et al.*, 2012). One of these mechanisms involves plant defence responses. Plants are able to defend themselves by controlling invasion of pathogenic microbes by generating a hypersensitive response, which can include the production of reactive oxygen species (ROS) and the modification of the cell wall composition. Membrane depolarization and calcium oscillations, necessary events in the nodulation pathway and root development, have been observed in plant cells responding to pathogenic elicitors such as chitins, oligosaccharides, flagellin and several peptides/peptidoglycans (Deger *et al.*, 2015; see reviews, Vadassery and Oelmüller, 2009; Cheval *et al.*, 2013). There is a growing realisation that plant ROS plays an important role in the establishment and maintenance of nodulation (Gourion *et al.*, 2015). It has been postulated that ROS, in particular hydrogen peroxide, may modulate growth, deformation and root hair curling (Gage, 2004). On the other hand it has been shown that Nod factors are able to reduce the generation of ROS in *M. truncatula* making ROS production transient. Thus, ROS are

linked to the Nod factor signal transduction pathway (Ivashuta *et al.*, 2005; Shaw and Long, 2003a). It has been suggested that the transient production of ROS during nodulation is differentially regulated at different time points (Marino *et al.*, 2009). It has been proposed that hydrogen peroxidase, through a posttranslational modification called sulfenylation, may regulate protein activity, which is important for a successful nodulation (Oger *et al.*, 2012). It has been demonstrated that the *M. truncatula rip1* peroxidase gene, which has sequence motifs with homology to ROS responsive cis elements, has been transcriptionally induced in the presence of Nod factors (Cook *et al.*, 1995; Ramu *et al.*, 1999). In *P. vulgaris*, reductions in ROS production causes a reduction in infection threads formation, density of symbiosomes in the nodule, nodule biomass, nodule numbers and nitrogen fixation (Montiel *et al.*, 2012, Arthikala, *et al.*, 2014). A transcriptome analysis in inoculated *M. truncatula* plants with inhibited ROS production, inactivation of *MtSpk1* gene, which encodes a putative protein kinase and is induced by exogenous hydrogen peroxidase, significantly reduced nodule numbers (Andrio *et al.*, 2013). However, ROS are not the only plant defence responses involved in nodulation. Ethylene biosynthesis has been correlated with plant defence response (Marino *et al.*, 2009).

### 1.3.2.2 Ethylene

Ethylene is a gaseous plant hormone that has been associated with plant growth, development, stress responses and fruit ripening (Yang and Hoffman, 1984). The precursor for the biosynthesis of ethylene is 1-aminocyclopropane-1-carboxylic acid (ACC), which is converted to ethylene by the enzyme ACC synthase. The role of ethylene in nodulation is not yet fully understood. It has been shown that ethylene, which is also involved in plant defence responses, negatively regulates nodule formation, infection thread initiation, root hair deformation, early gene expression, calcium spiking and nodule numbers (Oldroyd *et al.*, 2001). Ethylene acts at early stages in the developmental nodulation pathway presumably at or upstream of calcium spiking and defines sensitivity of the plant to Nod factors (Oldroyd *et al.*, 2001). On the other hand ACC synthase inhibitors such as aminoethoxyvinyl glycine (AVG) have been shown to increase nodulation (Oldroyd *et al.*, 2001; Guinel and Geil, 2002). In addition, an ethylene-insensitive hypernodulation mutant *sickle (skl)* of *M. truncatula* has a mutation in the orthologous gene of *Arabidopsis thaliana* *ETHYLENE-INSENSITIVE2 (EIN2)*, leading to a significant increase in the number of nodules,

which are randomly distributed over the root perimeter of *M. truncatula* (Penmetsa and Cook, 1997; Penmetsa *et al.*, 2008). Besides, Schuegger *et al.*, (2006) found that bacteria producing AHLs in the rhizosphere of tomato induced an ethylene dependent defence response, activating the induced systemic resistance (ISR) against the fungal pathogen *Alternaria alternata*. Apart from ethylene, abscisic acid (ABA) and jasmonic acid (JA) have been reported to have negative effects on nodulation (Mortier *et al.*, 2012).

### 1.3.2.3 Autoregulation of Nodulation (AON) in *M. truncatula*

The numbers of nodules formed on legume roots are typically less than the numbers of initial infections. AON is a mechanism by which the plant closely controls nodule numbers systemically, where successful infections inhibit further cell divisions in other areas of the root (Mortier *et al.*, 2012). This mechanism involves long distance signalling from root to shoot which is converted into a feedback regulatory response. This was demonstrated in a split root study where inoculation of one side of the root inhibits new nodule formations in the other side (Kosslak and Bohlool, 1984). Thus, the AON defines a temporal developmental time window in which roots are susceptible to inoculation with rhizobacteria. Nod factor perception activates AON at different times according to different legume species (Sargent *et al.*, 1987; van Brussel *et al.*, 2002). In *M. truncatula* the super numerary nodules (*sun*) mutant is defective in autoregulation. Thus, *sun* produce nodule numbers excessively in response to inoculation with rhizobia. Because of this, *sun* is called a *super numeric nodules* mutant (Penmetsa *et al.*, 2003). *SUNN* encodes a leucine-rich-repeat receptor-like kinase that acts in the shoot. It most likely perceives a peptide of the CLE family and subsequently transfers a signal to the root to inhibit further nodulation (Mortier *et al.*, 2012; Reid *et al.*, 2011).

The development of nodules and the control of nodule numbers are very similar to the development and regulation of lateral roots. For example, both processes are controlled by environmental factors like N availability (Goh *et al.*, 2015; Jin *et al.*, 2012); both processes require re-initiation of cell division in the root that is controlled by gradients of auxin and cytokinin (Mathesius *et al.*, 2008; Bensmihen *et al.*, 2015) and autoregulation mutants also showed root architecture phenotypes (Jin *et al.*, 2012; Buzas *et al.*, 2007). Therefore, when studying the control of nodule number, it is also of interest to ask to what extent this is nodulation specific, e.g. through control of infection

compared to hormonal effects on root development that could affect both nodulation and root architecture.

## 1.4 The plant microbiome

Agricultural productivity is highly influenced by soil microbes (Philippot *et al.*, 2013; Bakker *et al.*, 2012; Lau and Lennon, 2012). For instance, diverse soil microbial communities associated to *Brassica rapa* improved plant adaptation under drought stress (Lau and Lennon, 2012). Another example is the disease suppressive-soils, which, under favourable conditions for disease development, prevent the outbreak of plant diseases (Bulgarelli *et al.*, 2013). The bacterial root microbiome can play a critical role in disease suppressiveness. This phenomenon is explained by the relative abundance of diverse microbial taxa in the soil more than the presence or quantity of particular taxa (Kent *et al.*, 2002; Berendsen *et al.*, 2012). Therefore, the plant-associated microbiome can confer an adaptive advantage to plants and be used as a powerful tool to improve sustainable agriculture (Bakker *et al.*, 2012; Haney *et al.*, 2015; Goh *et al.*, 2013).

### 1.4.1 Rhizosphere

The rhizosphere, a narrow zone of soil influenced by plant roots, is one of the most dynamic and rich ecosystems on Earth (Tringe *et al.*, 2005). Rhizosphere soil has been found to harbor up to  $10^{11}$  microbial cells per root gram and more than 30.000 bacterial species (Egamberdieva *et al.*, 2008; Mendes *et al.*, 2011). This diverse and complex microbial community harbors a collective genome larger than that of the plant constituting the 'plant's second genome' (Berendsen *et al.*, 2012). Interestingly, this microbial biodiversity is associated with the rhizosphere but not with bulk soil which is known as the 'rhizosphere effect' (Berendsen *et al.*, 2012; Lundberg, *et al.*, 2012). This is a likely result of root exudation, as plant roots exude 5 % to 30 % of the photosynthates into the rhizosphere while the bulk soil, is limited in terms of nutrient acquisition (Marschner, 1995; Bulgarelli *et al.*, 2013). Plants shape their microbiome through their root architecture, root exudates and rhizodeposition. Root exudates may be comprised of low molecular mass compounds including sugars, amino acids and organic acids and heavy molecular mass compounds such as mucilage (Knee *et al.*, 2001; Philippot *et al.*, 2013). Root exudates vary depending on the plant age, soil type, nutrient availability and physiological state of the plant. Rhizodeposition is the secretion

of varied compounds (e.g. flavonoids, antimicrobial compounds) by the rhizodermis and the release of root border cells and root cap cells into the rhizosphere (Bais *et al.*, 2006; Rudrappa *et al.*, 2008; el Zahar Haichar *et al.*, 2008; Bulgarelli *et al.*, 2013; Philippot *et al.*, 2013). Metatranscriptomic analysis in *Arabidopsis* plants revealed that 81 transcripts were significantly expressed at distinct developmental stages, presumably to create a particular microbiome assemblage according to each developmental stage requirements (Chaparro *et al.*, 2014). In addition, in a study conducted under drought stress in a desert farming region in Egypt, the root system of pepper plant (*Capsicum annum* L.) was able to assemble a drought resistance promoting microbial community (Marasco *et al.*, 2012).

In order to disambiguate terminologies, different concepts will be defined as follows:

- Plant associated bacteria are the set of microbial organisms accompanying the plant
- Microbiome is the set of microbial organisms in a specific niche (Bulgarelli *et al.*, 2013).
- Rhizosphere microbiota is constituted by bacteria, fungi (including mycorrhiza), oomycetes, viruses and archaea that live in the rhizosphere (Philippot *et al.*, 2013).
- Endophytes are the set of microbial organisms inside of plant tissues (Bulgarelli *et al.*, 2013).

Bacterial endophytes along with bacteria from the rhizosphere (rhizobacteria) constitute the largest functional group of the plant growth promoting bacteria (PGPB) (Partida-Martínez and Heil, 2011). Endophytes affect the epigenome and phenotype of plants (Partida-Martínez and Heil, 2011).

#### **1.4.2 Assemblage factors of microbial community in the rhizosphere**

Abiotic and biotic determinants are involved in the assembly of microbial community including plant species, soil type, biotic interactions, climate, plant diversity and agricultural practices (Philippot *et al.*, 2013). In the past, controversy of whether the soil or the plant component was the main determinant in assembly root microbiota was debated. However, in the light of the recent literature, Bulgarelli *et al.*, (2013) proposed a two-step selection process for root microbiota, which includes at first stage a rough microbial differentiation from the bulk soil to the rhizosphere by soil type and rhizodeposition, and a second step that consists in a finer and deeper differentiation

from the rhizosphere into the root microbiota. This dynamic model takes into consideration both soil and plant as cooperative determinants of the root microbiome. In a recent study, plant species, soil chemistry, spatial location and plant genus were found to be sequentially the best statistical predictors of soil microbial communities acting in dependent manner (Burns *et al.*, 2015).

Soil type strongly influences the composition of the plant microbiome (Bulgarelli *et al.*, 2012; Lundberg, *et al.*, 2012). Rhizosphere bacterial communities are more similar to bulk soil communities than to internal root communities (Lundberg *et al.*, 2012). In this context, soil can be considered as a microbial seed bank (Lennon and Jones, 2011). Even agricultural practices such as soil tillage, fertilisation and pesticide application affect the microbial composition in the rhizosphere (Gaiero *et al.*, 2013). On the other hand, plant species and even different genotypes of particular species, growing in the same soil have exhibited distinct microbial communities (reviewed by Berendsen *et al.*, 2012). Plants secrete a myriad of chemical compounds into the rhizosphere including quorum sensing mimic molecules which can interfere with QS related behaviours including virulence factors (section 1.24). Interestingly, AHL-producing bacteria, specifically *Pseudomonas* spp., are found in higher proportion in the rhizosphere than in the bulk soil (Elasri *et al.*, 2001). In a metagenomic approach to assess the root endophytic community of rice growing in field trials, putative functions as well as metabolic process were predicted as prominent features for endophytes to adapt in the root niche. Some of those features included quorum sensing. The authors highlight the potential for endophytes community to improve plant growth and health (Sessitsch *et al.*, 2012).

The rhizosphere is a highly dynamic ecosystem which is subjected to ever constant spatiotemporal changes where microbe-microbe interactions also may explain community differences (Schlaeppli *et al.*, 2013). Cultivars can also affect the composition of the microbial communities in the rhizosphere. Characterisation of the microbial community in the rhizosphere of five potato cultivars indicated that cultivar strongly defined the microbial community structure in the rhizosphere (İnceoğlu *et al.*, 2012).

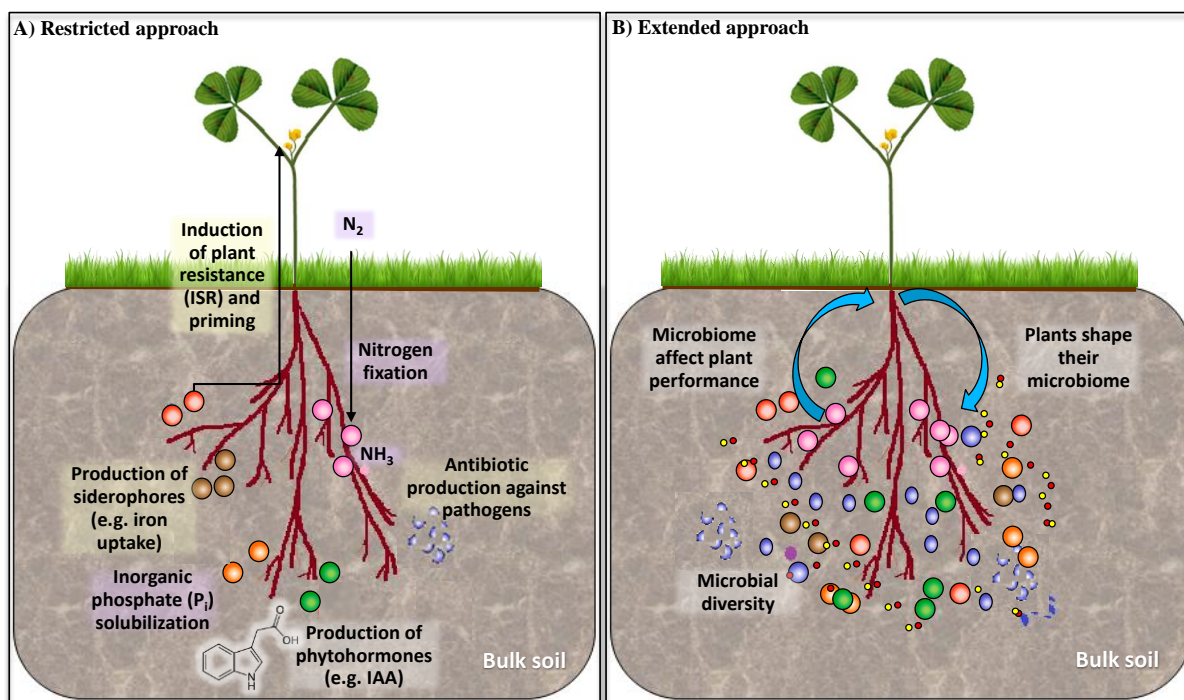
Microorganisms present on or in the seeds can potentially assemble part of the rhizosphere microbiota. Wild and modern maize varieties showed that at least one member of the core seed microbiota was able to colonise the rhizosphere (Johnston-



Monje and Raizada, 2011). This effect is called the ‘maternal effect’ (Philippot *et al.*, 2013). In addition, the same study confirmed that this core seed microbiota was conserved across the different genotypes and that several seed endophytes were able to systemically colonise the plant. This suggests that plants are able to vertically transfer their seed borne microbiota to the next generation.

Taking into consideration these findings, a totally microbe-free plant is an exception to the rule. Plant-microbe interactions phenotype is the result of a complex co-regulation of gene expression between plants and microbes (Partida-Martínez and Heil, 2011). Rosenberg *et al.*, (2007) suggested the term ‘holobiont’ to describe the concept of the eukaryote with its symbionts. Endophytes can be transmitted vertically via seeds or horizontally colonising plant tissues once the plant exists at different developmental stages (Johnson-Monje and Raizada, 2011). Disregarding the fact that plants are surrounded by bacteria and that bacteria can play a crucial role in plant phenotypes, can lead to an overestimation of the contribution of the plant to a given phenotype. Early studies characterising the plant microbiome used to use cultivation as a method to isolate plant associated microbial communities. Culture-dependent studies did not consider non-cultivable microbes and usually they focused on the assessment of a single specific microbe, limiting the comprehension of the microbial community diversity and its effect on the plant performance (Lebeis, 2014). Currently, technologies such as pyrosequencing and Illumina have identified and quantified microbial communities, including diversity of uncultured organisms, in complex systems such as the rhizosphere (Roesch *et al.*, 2007; Bulgarelli *et al.*, 2012; Lebeis, 2014).

Traditional approaches to study plant-microbe interactions used single or specific microbes. For example, *Medicago* plants co-inoculated with *Sinorhizobium medicae* (symbiont) and *P. fluorescens* (rhizobacteria) improved symbiotic efficiency, increasing the rate of nodule initiation, and development as well as nodule number and nitrogen content (Fox *et al.*, 2011). Moreover, in soybean co-inoculations with two endophytic bacteria along with the symbiont, improved nodule number, nodule nitrogenase activity and plant nitrogen content in comparison with single endophytic inoculations. This diversity and evenness in the microbiome improve adaptive plasticity in plants across diverse environments due to a rich community diversity and/or functional redundancy (Figure 1.9) (Bakker *et al.*, 2012).



**Figure 1.9** Schematic representation of different perspectives to study plant associated microorganisms in the rhizosphere. A) The restricted approach refers to the conventional study of plant growth promoting rhizobacteria (PGPR) mechanisms: Biofertilisation (increase of nutrient acquisition) depicted in violet text boxes, Phytostimulation in white boxes and Biocontrol in yellow boxes. B) The extended approach studies the effects of the microbiome on plant performance and vice versa considering biotic and abiotic factors. Abbreviations: ISR, induced systemic resistance,  $P_i$  inorganic phosphate,  $N_2$  atmospheric nitrogen,  $NH_3$  ammonia.

The understanding of QS and how it moderates the interactions between plants and bacteria is in its very early stages. Nonetheless, the realisation of the importance of bacterial communication has been revealed in the continuous increase of publications in this area (Keller and Surette, 2006). A good example of this is exemplified by the web of science (Thomsom Reuters) outcome for the search the key words “quorum sensing”, which is 15,690. While extensive research has focused on studying quorum sensing in bacteria less is known about plant-microbe interactions specifically, legume-rhizobia symbiosis. This is clearly shown by Hartmann *et al.*, (2014) who pointed out a total of 11 studies during 12 years directly addressing the impact of quorum sensing molecules on plant performance or plant-microbe interactions. In particular, it is important to define processes in plants that respond to QS signals and lead to altered plant

performance, which could be of special interest in legume crops due to the opportunity to study symbiotic and pathogenic interactions (Cook, 1999). The model legume *M. truncatula* and its symbiont, *S. meliloti* are an excellent opportunity to study this intricate sophisticated and intimate interaction.

The research of this thesis focuses on the effect of quorum sensing molecules, particularly AHLs, on the *Medicago-Sinorhizobium* symbiosis with particular emphasis on root architecture and nodulation.

The hypothesis of this thesis is that plants respond to QSS by modifying plant growth or development, in particular nodule development.

The questions behind this project are as follows:

- i. Can plants sense QSS and adapt growth and development in response to specific AHLs?
- ii. Does plant perception of quorum sensing signals affect plant nodulation?
- iii. What are the mechanisms involved in the plant response towards quorum sensing signals?
- iv. Do plant associated bacteria influence plant responses to quorum sensing (QS) signals?

The aims for this study are as follows:

- i. Define effects of QS signals from rhizobacteria on growth, development and performance of *Medicago truncatula* cv. *Jemalong* (A17) (Chapter 3).
- ii. Determine whether treatment of *M. truncatula* with QS signals from its symbiont and/or other bacteria alter nodulation (Chapter 4).
- iii. Evaluate the effect of plant associated bacteria in *Medicago* responses to QS signals (Chapter 5).

## Chapter 2 Material and methods

### 2.1 Plant material and growth conditions

#### 2.1.1 *Arabidopsis thaliana*

*Wt Arabidopsis* seeds were surface sterilised in 70% ethanol for two minutes followed by a solution of 5% sodium hypochlorite and Tween20 (5 $\mu$ L/10 mL) for 15 minutes. Then, seeds were rinsed five times with sterile water. Seeds were stratified at 4°C for 48 hours in water in darkness before being placed onto agar plates by pipetting. Plants were germinated and grown on half strength Murashige and Skoog (MS) medium containing 2.154g of MS salts (Duchefa Biochemie B. V., The Netherlands), 10g of sucrose, 0.1g of Myo-Inositol and 0.5g of MES (2-(N-Morpholino)ethanesulfonic acid, 4-Morpholineethanesulfonic acid) and 8g J3 agar (Gelita, Australia) per litre (Murashige and Skoog, 1962). The pH was adjusted to 5.7. Seedlings grew in a controlled growth chamber at 21°C, 24 hours light and 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation (PAR). Plants were grown for seven days and root length was measured from scanned root images using ImageJ 1.44p software (USA). Emerged lateral roots were counted under a stereo microscope (NIKON SMZ745, China).

#### 2.1.2 *Medicago truncatula* (Gaertn.) and other legumes

Seeds of *M. truncatula* wild type (*wt*) Jemalong A17, its mutants *sun4* and *sk1* as well as *Medicago sativa*, *Trifolium repens* and *Lotus japonicus* were scarified with sand paper, surface-sterilised for 10 min in 6% (v/v) sodium hypochlorite, followed by five washes with sterile water, and soaked for 6 hours in a solution containing 200 mg/L amoxicillin and clavulanic acid (Augmentin). For the experiments described in Chapter 3, a number of alternative surface sterilisation protocols were tested as specified in Table 3.2. After this, seeds were rinsed thoroughly with sterile water and placed at 4°C. Seeds were stratified for 48 hours at 4°C and germinated on Fåhræus medium (F) agar plates at 25°C for 16 h in darkness (Fåhræus, 1957) (Table 2.1). Ten seedlings were transferred to square petri dishes (245mmx 245mm x 18mm) (Falcon<sup>®</sup>, USA) containing FM agar (12 g/L J3 agar (Gelita, Australia)) below pH 6 with or without AHLs.

**Table 2.1** Composition of Fåhraeus media

<b>Reagent</b>	<b>Concentration (mM)</b>
<b>Macro elements</b>	
<b>CaCl<sub>2</sub>·2H<sub>2</sub>O</b>	0.9
<b>MgSO<sub>4</sub>·7H<sub>2</sub>O</b>	0.5
<b>KH<sub>2</sub>PO<sub>4</sub></b>	0.7
<b>Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O</b>	0.8
<b>C<sub>6</sub>H<sub>6</sub>FeO<sub>7</sub></b>	0.02
<b>Trace elements</b>	
	<b>Concentration (µM)</b>
<b>H<sub>3</sub>BO<sub>3</sub></b>	46.26
<b>MnSO<sub>4</sub>·4H<sub>2</sub>O</b>	9.11
<b>CuSO<sub>4</sub>·5H<sub>2</sub>O</b>	0.32
<b>ZnSO<sub>4</sub>·7H<sub>2</sub>O</b>	0.77
<b>Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O</b>	0.58
<b>Agar</b>	10 mg/L

All plates were incubated in a growth room in a randomized block design. Plants were grown in a controlled temperature room at 25°C at 120 µmol m<sup>-2</sup> s<sup>-1</sup> of PAR 16h /8h day/night cycle for 21 days after inoculation. *Medicago* seedlings were inoculated with 10 µl *Sinorhizobium meliloti* strain 1021 liquid culture grown in Bergersen's Modified Medium (BMM) (Rolfe *et al.*, 1980) (Table 2.2) to an OD600 of 0.1, three days after transferring *Medicago* seedlings. *T. repens* and *L. japonicus* seedlings were inoculated with *Rhizobium leguminosarum* bv. *trifolii* and *Mesorhizobium loti* three days after seedling transferring, respectively, the same way as described for *Medicago*. *Lotus japonicus* seeds were surface-sterilised following the same protocol as for *M. truncatula* seeds. Subsequently, the seeds were stratified at 4°C and germinated in dark at 21°C for 48-72h. Seedlings were grown on ¼ strength of Broughton and Dilworth medium (B&D) (Table 2.3) in a growth chamber at 28°C with a 16h /8h day/night cycle and 120 µmol m<sup>-2</sup> s<sup>-1</sup> of PAR. B&D medium was adjusted to 6.8 pH with KOH.

**Table 2.2** Composition of Bergensen's Modified Medium to grow *Sinorhizobium meliloti*

Reagent	Amount/Litre
Na <sub>2</sub> PO <sub>4</sub> x 12 H <sub>2</sub> O	360 mg
MgSO <sub>4</sub> x 7 H <sub>2</sub> O	80 mg
FeCl <sub>3</sub>	3 mg
CaCl <sub>2</sub> x 2 H <sub>2</sub> O	40 mg
H <sub>3</sub> BO <sub>3</sub>	3 mg
MnSO <sub>4</sub> x 4 H <sub>2</sub> O	10 mg
ZnSO <sub>4</sub> x 7 H <sub>2</sub> O	7 mg
CuSO <sub>4</sub> x 5 H <sub>2</sub> O	0.25 mg
CoCl <sub>2</sub> x 6 H <sub>2</sub> O	0.25 mg
Na <sub>2</sub> MoO <sub>4</sub> x 2 H <sub>2</sub> O	0.25 mg
Biotin	0.2 mg
Thiamine	2 mg
Na-glutamate	0.5 g
Yeast extract	0.5 g
Mannitol	3 g (solid medium)/10 g (liquid medium)
Agar	15 g (solid medium)

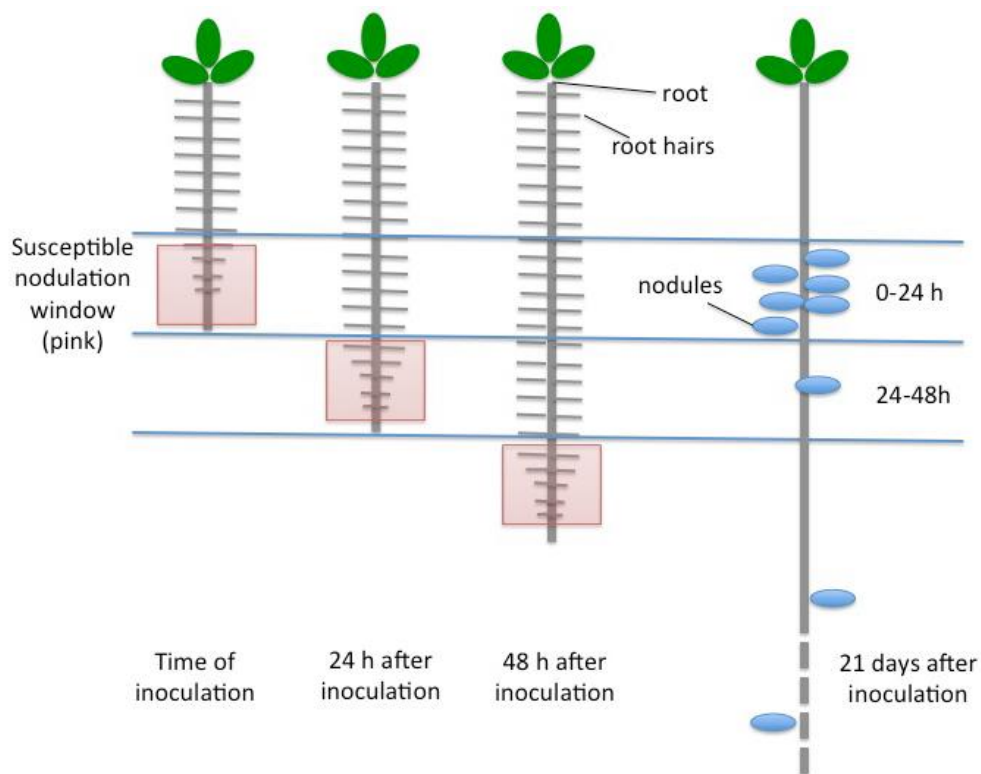
**Table 2.3** Broughton and Dilworth (1971) medium composition.

Reagent	Concentration
MgSO <sub>4</sub>	0.25 mM
Fe-citrate	10 µM
CaCl <sub>2</sub>	1 mM
H <sub>3</sub> BO <sub>3</sub>	2 µM
MnSO <sub>4</sub>	1.0 µM
ZnSO <sub>4</sub>	0.5 µM
CuSO <sub>4</sub>	0.2 µM
Co SO <sub>4</sub>	0.1 µM
Na <sub>2</sub> MoO <sub>4</sub>	0.1 µM
KH <sub>2</sub> PO <sub>4</sub>	0.5 mM
K <sub>2</sub> SO <sub>4</sub>	0.25 mM
Agar	10 mg/L

## 2.2 Autoregulation of nodulation (AON)

For the assay of autoregulation of nodulation the position of the root zone susceptible to infection, i.e. the zone of emerging root hairs just behind the root tip (Bhuvaneshwari *et*

*al.*, 1981) was inoculated with *S. meliloti* strain Rm1021. This inoculation zone was marked at the back of the petri dish. The susceptible zone was subsequently marked at 24 and 48 h of inoculation at the back of the petri dish. After 21 days, nodule numbers were counted in the marked windows correlating with the positions of the root zone susceptible to nodulation at the different time points (Figure 2.1).



**Figure 2.1** Schematic representations of autoregulation of nodulation (AON) measurements.

### 2.3 Germination assay

For the germination assay, *wt Medicago* seeds were scarified mechanically with sand paper, surface-sterilised as described in 2.1.2 and placed in a 96 well microtiter plate, one seed per well. A volume of 40  $\mu\text{L}$  of 10  $\mu\text{M}$  AHL solution (Cayman chemicals, USA) or 40  $\mu\text{L}$  of the solvent each AHL was dissolved in (ethyl acetate: acetic acid (1000:1), acetonitrile or dimethyl sulfoxide (DMSO) accordingly), were applied to each well. We used different solvents to dissolve the AHLs. Therefore, the control treatment was respective solvent in which the particular AHL was dissolved. Plates were sealed with Parafilm and covered with aluminium foil to avoid light exposure. Seeds were



stratified for 48 hours at 4°C and subsequently germinated in the individual wells in at 25°C in darkness. The percentage of germination was evaluated at different time points, i.e. 12, 22 and 46 h (Figure 3.11). Results of visually contaminated seeds were discarded.

Seed viability was measured by soaking *Medicago* seeds in a colorless solution of 1% Tetrazolium Chloride (2,3,5 triphenyltetrazolium chloride, Sigma-Aldrich, USA) for two hours. If seeds are viable, the living cells catalyze the formation of formazan, a red precipitate (Kuhn and Jerschel, 1941; Sabnis, 2010). Therefore, seeds that were stained red were counted as viable and the ones un-stained as unviable. Percentage of viability was calculated counting viable seeds among the un-germinated seeds.

## 2.4 Dry biomass

To measure dry biomass of *M. truncatula* plants, plants were harvested at 21 days after inoculation (dai) and separated into root and shoot at the hypocotyl. Three biological repeats with 10 samples each per treatment were introduced into a paper envelope and dried at 60°C for 72h in an incubator (Axyos, Australia). Samples were carefully handled in an air tight plastic box containing silica gel type III (Sigma-Aldrich, USA) to avoid moisture in the samples while they were weighed. Shoot dry biomass, root dry biomass and total dry biomass (shoot and root) were measured on a balance (Mettler Toledo, Switzerland). The biomass of *M. truncatula* nodules was measured by excising all the nodules found per biological repeat, placing them into a previously weighed Eppendorf tube and weighing them on a balance. Fresh nodule biomass per nodule and fresh nodule biomass per plant were estimated by dividing the nodule weight by the nodule numbers and the number of plants, respectively. It was not possible to reliably measure nodule dry weight because the dry biomass of the nodules was too low.

## 2.5 Aminoethoxyvinylglycine (AVG) assay

*Wt* and *sun4* mutant *Medicago* seeds were surface-sterilised and grown as described in section 2.1.2 AVG (Sigma-Aldrich, USA) (10 mM stock in water) was added to Fåhræus medium to a final concentration of 1 µM. Ten seedlings were grown per plate. A total of four plates per treatments were used. Seedlings were grown in a growth

chamber as described before (section 2.1.2). Harvest was done 21 days after inoculation when total nodule numbers were counted.

## 2.6 Screening of plant-associated bacteria

To verify the presence of plant-associated bacteria in the different experiments, aliquots of the sterile distilled water used in the final rinse were plated onto different growth media. To detect bacterial species of slow growth, Reasoner's 2A (R2A) agar media was prepared. In addition, other types of media were prepared to grow different bacteria (Table 2.4). Media was adjusted at a final pH  $7 \pm 0.2$  at 25°C. Plates were incubated at 28°C for three to seven days and then checked for bacterial presence.

**Table 2.4** Composition of diverse media used to grow plant-associated bacteria. All ingredients are listed for 1 liter (L) of medium.

<b>Yeast Extract Broth (YEB)</b>	<b>Trypticase soy agar (TSA)</b>	<b>Lysogeny broth (LB)</b>	<b>R2A</b>
(Ristroph <i>et al.</i> , 1980)	Leavitt <i>et al.</i> , 1955	Bertani (1951)	(Reasoner <i>et al.</i> , 1979)
5g beef extract	15g tryptone		0.5g peptone
1 g yeast extract	5g enzymatic digest of soybean meal	5g yeast extract	0.5 g casamino acids
5g peptone	5g NaCl	10g NaCl	0.5 g yeast extract
5g sucrose	15g Agar J3 Agar (Gelita, Chile)	15g Agar J3 Agar (Gelita, Chile)	0.5 g dextrose
0.5g MgCl <sub>2</sub>			0.5 g soluble starch
15g J3 Agar (Gelita, Chile)			0.3g K <sub>2</sub> HPO <sub>4</sub>
			0.05g MgSO <sub>4</sub> ×7H <sub>2</sub> O
			0.3 g sodium pyruvate
			15g J3 Agar (Gelita, Chile)

## 2.7 Rhizobial growth conditions

*Sinorhizobium meliloti* strain 1021 and *Mesorhizobium loti* were grown overnight at 28 °C in Bergersen's Modified Medium (Rolfe *et al.*, 1980) (Table 2.2) or in TY medium (Beringer, 1974), respectively. TY medium (Vincent, 1970) was prepared using 10g of Bacto-tryptone, 5g of yeast extract and 10g of NaCl for a final volume of one litre. Both

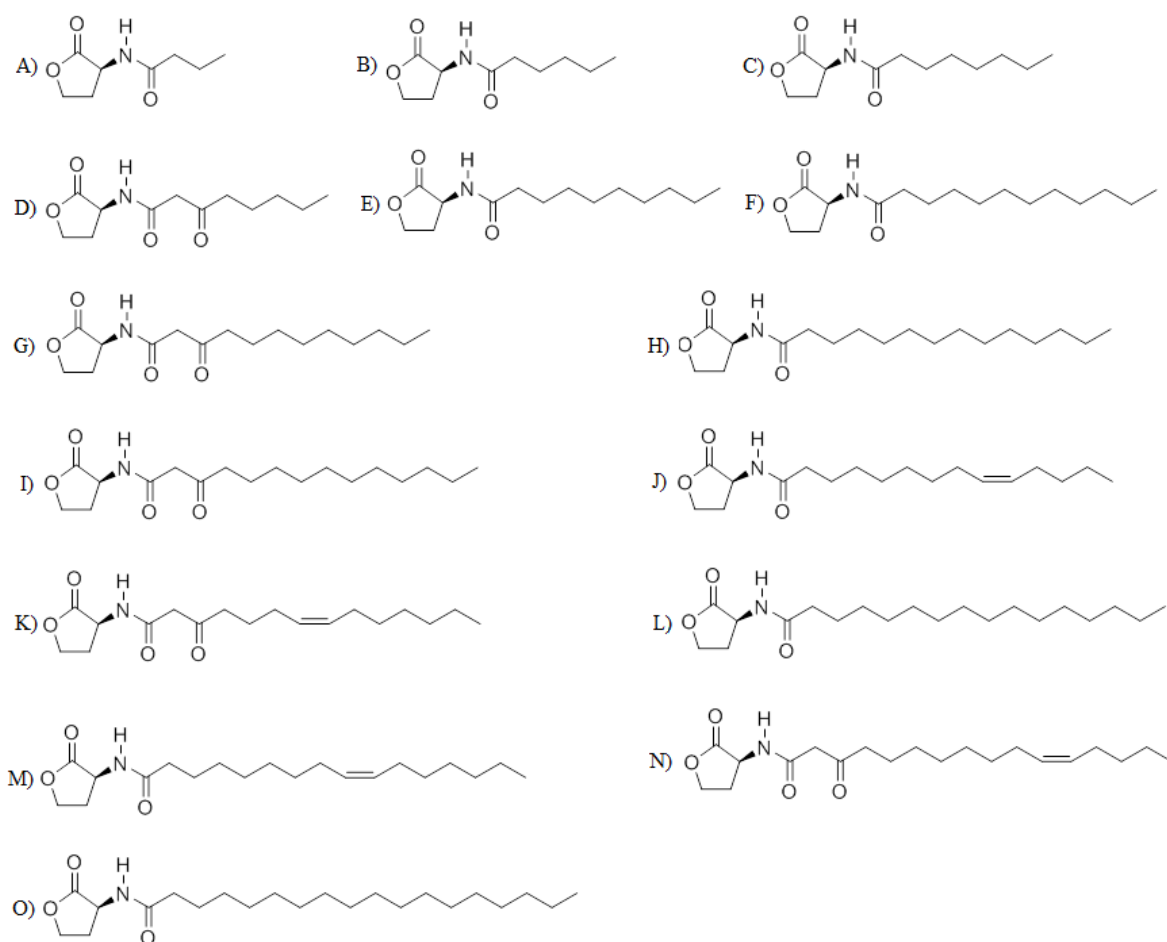
rhizobium species were diluted to OD600 = 0.1 for plant inoculation (Beringer, 1974). *Medicago* was inoculated three days after seed germination and *Lotus* was inoculated one week after seed germination (Dam *et al.*, 2014).

## 2.8 Chemical compounds

The AHLs used (Table 2.5; Figure 2.2) were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA), dissolved in Dimethyl sulfoxide (DMSO, 70mM) and diluted to a final concentration of 1  $\mu$ M or 10  $\mu$ M into the appropriate plant growth medium following autoclaving and cooling of the medium. Solvent diluted to the same concentration as used for AHLs was used as a negative control.

**Table 2.5** Quorum sensing (QS) signal molecules used in the study and organisms known to synthesise them.

QS molecules	Organism	Reference
C <sub>4</sub> -HSL	<i>Pseudomonas aeruginosa</i>	Pearson <i>et al.</i> , (1995)
C <sub>6</sub> -HSL	<i>Sinorhizobium meliloti</i> AK631	Teplitski <i>et al.</i> , (2003)
C <sub>8</sub> -HSL	<i>S. meliloti</i> Rm41	Marketon <i>et al.</i> , (2002), Teplitski <i>et al.</i> , (2003)
3-Oxo-C <sub>8</sub> -HSL	<i>S. meliloti</i> Rm41	Teplitski <i>et al.</i> , (2003)
C <sub>10</sub> -HSL	<i>S. meliloti</i> AK631	Teplitski <i>et al.</i> , (2003)
C <sub>12</sub> -HSL	<i>S. meliloti</i> Rm1021	Marketon <i>et al.</i> , (2002)
3-Oxo-C <sub>12</sub> -HSL	<i>P. aeruginosa</i>	Pearson <i>et al.</i> , (1994)
C <sub>14</sub> -HSL	<i>S. meliloti</i> Rm1021	Teplitski <i>et al.</i> , (2003); Chen <i>et al.</i> , (2003)
3-Oxo-C <sub>14</sub> -HSL	<i>S. meliloti</i> Rm1021	Teplitski <i>et al.</i> , (2003)
3-Oxo-C <sub>14:1-7-cis</sub> (L)-HSL	Synthetic AHL analog	Chhabra <i>et al.</i> , (2003)
C <sub>14:1-9-cis</sub> -(L)-HSL	<i>Agrobacterium vitis</i>	Li <i>et al.</i> , (2005)
C <sub>16</sub> -HSL	<i>S. meliloti</i> Rm41	Teplitski <i>et al.</i> , (2003)
C <sub>16:1-9 cis</sub> -(L)-HSL	<i>S. meliloti</i> Rm1021	Marketon <i>et al.</i> , (2002)
3-Oxo-C <sub>16:1-11cis</sub> -(L)-HSL	<i>A. vitis</i>	Hao and Burr (2006)
C <sub>18</sub> -HSL	<i>S. meliloti</i> Rm1021	Marketon <i>et al.</i> , (2002)



**Figure 2.2** Chemical structures of the AHLs used in this study **A)** C<sub>4</sub>-HSL, **B)** C<sub>6</sub>-HL, **C)** C<sub>8</sub>-HSL, **D)** 3-Oxo-C<sub>8</sub>-HL, **E)** C<sub>10</sub>-HL, **F)** C<sub>12</sub>-HSL, **G)** 3-Oxo-C<sub>12</sub>-HSL, **H)** C<sub>14</sub>-HL, **I)** 3-Oxo-C<sub>14</sub>-HL, **J)** C<sub>14</sub>:1-9-cis-(L)-HSL, **K)** 3-oxo-C<sub>14</sub>:1-7cis-(L)-HSL, **L)** C<sub>16</sub>-HSL, **M)** C<sub>16</sub>:1-9 cis-(L)-HSL, **N)** 3-Oxo-C<sub>16</sub>:1-11 cis-(L)-HSL, **O)** C<sub>18</sub>-HSL.

## 2.9 Microscopy

Three day-old *M. truncatula* seedlings were inoculated with the green fluorescent protein (GFP)-expressing *S. meliloti* strain pHC60-GFP (Cheng and Walker, 1998). At 21 days after inoculation, a 0.5 mm long root segment containing nodules was excised from each plant and embedded in 3% agarose. These blocks were sectioned at 100 µm thickness on a vibratome (1000 plus, Vibratome Company, St Louis, MO, USA) and sections arranged in order on a microscope slide. In order to standardise the measurements of the nodule area and infection zone in all the samples, segments with the biggest diameter corresponding to the middle section of each nodule were selected and assessed. The preparations were examined immediately under a Leica

Microsystems DM5500 B microscope equipped with epifluorescence detection (Leica, Wezlar, Germany). Two images were taken per sample: One after excitation at 365 nm to visualize the flavonoids visible in the root cortex tissue and the other after excitation at 470 nm to visualize the GFP-fluorescence inside the infected nodule zone. These images were overlapped, and the ‘total nodule area’ and the area of the ‘infection zone’ (as indicated in Figure 4.5) were measured and analysed using Leica LAS 4.4 software (Leica, Wezlar, Germany). The ‘remaining nodule area’ was calculated by subtracting the ‘infection zone’ from the ‘total nodule area’.

## 2.10 Quantification of flavonoids in *M. truncatula* roots

The flavonoid content of roots was determined by LC-MS/MS according to Farag *et al.*, (2007) with modifications as specified below. Flavonoids were extracted from wild type *M. truncatula* roots four days after exposure to AHLs (or solvent control) and 24 hours after inoculation with *S. meliloti* or a mock treatment (bacterial growth medium). For each treatment, a 2 cm long root segment from the root tip upwards (encompassing the root zone susceptible to infection with rhizobia) was excised and immediately frozen in liquid nitrogen. For each treatment, 15 root segments were pooled, and five replicates of 15 roots each were independently collected and analysed. Frozen root tissue was ground in a TissueLyser LT (Qiagen, Hilden, Germany). To each sample, 20 ng of luteolin was added as an internal standard, as luteolin was not detected in *M. truncatula* roots. Flavonoids were extracted with 1 mL of 80% methanol for 14 h at 4°C on a rotator in the dark and centrifuged at 10000 rpm for 30 min in at 4°C. The supernatants were dried in a Speedvac centrifuge for approximately 60 min. The pellet was resuspended in 45% methanol for analysis.

Flavonoids were separated on an Agilent 6530 Accurate Mass LC-MS Q-TOF (Agilent Technologies, Santa Clara, USA). The samples were run in ESI (electrospray ionization) mode in the Jetstream interface in the negative mode and injected (7 µl) onto an Ascentis® Express 2.7 µm C18 2.1 × 50 mm column (Supelco/Sigma Aldrich, St. Louis, MO, USA). Solvent A consisted of 0.1 % aqueous formic acid and solvent B consisted of 90 % acetonitrile containing 0.1 % formic acid. The elution of the flavonoids was carried out with a linear gradient from 10-50 % solvent B from 0-8 min, 50-70 % solvent B from 8-12 min (then hold from 12-20 min), 70-10 % solvent B from 20-21 min (then hold from 21-30 min) at a flow rate of 200 µl min<sup>-1</sup>. The mode used by

the instrument to operate was in extended dynamic mode over a range of  $m/z$  50-1000 using targeted collision induced dissociation (CID;  $N_2$  collision gas supplied at 18 psi) MS/MS. Naringenin, quercetin and morin were analysed comparing their respective flavonoid standards from Sigma Chemicals. The mass spectra for biochanin A, medicarpin, daidzein, formononetin, liquiritigenin, isoliquiritigenin and chrysoeriol in the samples were compared to the MassBank database (Horai *et al.*, 2010). The data analysis was done using Agilent Mass Hunter Workstation Software Qualitative Analysis version B.05.00 (2011).

## **2.11 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Two centimetres root tip samples were harvested from five *wt M. truncatula* seedlings for each biological replicate. These biological replicates were used for each treatment. Samples were finely ground in mortars using pestles and liquid nitrogen. Approximately 60 mg of finely ground tissue were transferred to a new Eppendorf tube for RNA extraction using the Spectrum plant Total RNA kit (Sigma, USA). The extracted and purified RNA was quantified on a NanoDrop ND1000 UV/Vis spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized from equal amounts of RNA for all treatments using the Superscript III First Strand cDNA synthesis kit (Life Technologies, USA). The final cDNA was diluted five times in miliQ sterile water.

A total of 5  $\mu$ L reaction volume was prepared as shown in Table 2.6. The primer dilution was prepared by taking 5  $\mu$ L of the reverse and forward primers into 90  $\mu$ L of MQ sterile water to a final concentration of 5  $\mu$ M. Out of this solution, 0.5  $\mu$ L were used per reaction. cDNA and Fast SYBRGreen (Applied Biosystems, USA) were mixed to compose a master mix, of which 4.5  $\mu$ L were used per reaction. The qRT-PCR primers used for assessing the expression of plant defence response genes and nodulation genes are listed in Table 2.7.

**Table 2.6** Reagents used per qRT-PCR reaction.

<b>Elements and reagents</b>	<b>Volume per reaction (<math>\mu\text{L}</math>)</b>
Reverse primer (5 $\mu\text{M}$ )	0.25
Forward primer (5 $\mu\text{M}$ )	0.25
cDNA	2
Fast SYBRGreen PCR	2.5
Total volume per reaction ( $\mu\text{L}$ )	5





**Table 2.7** Primers used to measure expression of plant defence and nodulation genes in *wild type (wt) M. truncatula (A17)*.

<b>Name</b>	<b>Sequence</b>	<b>TC</b>	<b>Reference</b>
Chalcone synthase_F	5' CGCTGTCACATTTTCGTGG 3'	Medtr5g007717	Hassan (2015)
Chalcone synthase_R	5' AACACACCCCATTCAGTCC 3'	Medtr5g007717	Hassan (2015)
Peroxidase_F	5' GGTCTTCTTCAAACGGACCA 3'	Medtr 7g093370	Hassan (2015)
Peroxidase_R	5' TGGACTGAACAAAGGCTTCA 3'	Medtr 7g093370	Hassan (2015)
Auxin responsive GH3 product_F	5' TGGCACGTCCAGTTCTAACA 3'	Medtr2g081860	Hassan (2015)
Auxin responsive GH3 product_R	5' AGGCCACAAAGCATCTGAGT 3'	Medtr2g081860	Hassan (2015)
LysM domain containing receptor like kinase 3_F	5' TGGGCATGCTACTGGTAGTG 3'	Medtr 5g086130	Hassan (2015)
LysM domain containing receptor like kinase 3_R	5' ACAGCTCCAAATCCACCTTG 3'	Medtr 5g086130	Hassan (2015)
Glutathione S-transferase_F	5' AAGAGCAAGAAGCTGCCAAG 3'	Medtr2g070180	Hassan (2015)
Glutathione S-transferase_R	5' TGATGTTGCCAAAGGTCTCA 3'	Medtr2g070180	Hassan (2015)
Type A response regulator_F	5' GAATTGCATGTGCTTGCTGT 3'	Medtr4g106590	Hassan (2015)
Type A response regulator_R	5' CGCTGTTCTCTCCATCCAAT 3'	Medtr4g106590	Hassan (2015)
Chitinase class III_F	5' CATGCAACACTGGTCTCTTCA 3'	Medtr6g023340	Hassan (2015)
Chitinase class III_R	5' GCAATTGGGTGGGTTACAAT 3'	Medtr6g023340	Hassan (2015)
Pathogenesis related protein 5-1 (Prp)_F	5' GGTGGCGGTAAGCAACTAAA 3'	Medtr8g096900	Hassan (2015)

Pathogenesis related protein 5-1 (Prp)_R	5' GTTCCCTGAACCGTCAAAGT 3'	Medtr8g096900	Hassan (2015)
Isoflavone synthase_F	5' GATCGACGGGTATGTGGTTC 3'	Medtr4g088195	Hassan (2015)
Isoflavone synthase_R	5' CTGCTTCACCTTCACCAACA 3'	Medtr4g088195	Hassan (2015)
<i>MtNSP1_F</i>	5' GCGATTTCGCCACTGGATTC 3'	Medtr 8g020840	Untergasser <i>et al.</i> , (2012)
<i>MtNSP1_R</i>	5' CAGCCTCGCCTTCCATCATT 3'	Medtr 8g020840	Untergasser <i>et al.</i> , (2012)
<i>MtNSP2_F</i>	5' GGCCTAGAATTGCAGGCTCGT 3'	Medtr3g07271	Untergasser <i>et al.</i> , (2012)
<i>MtNSP2_R</i>	5' TTGCAAAGCTCACCGGA ACTC 3'	Medtr3g07271	Untergasser <i>et al.</i> , (2012)
<i>MtNIN_F</i>	5' GGGAGAAAGTCCGGGGACAA 3'	Medtr5g099060	Untergasser <i>et al.</i> , (2012)
<i>MtNIN_R</i>	5' GACACACACCGATGCTCTTTGC 3'	Medtr5g099060	Untergasser <i>et al.</i> , (2012)
<i>ENOD11_Fa</i>	5' CTTGTTCTCTAGGGCTTGC 3'	contig_77244_1	This study
<i>ENOD11_Ra</i>	5' AATTGGATTGGAGGCATATTG 3'	contig_77244_1	This study
<i>ENOD11_Fb</i>	5' AAAGCCACCACTTCCTAAACCG 3'	contig_77244_1	This study
<i>ENOD11_Rb</i>	5' TTAATTGGTCGCTTACGCGATGG 3'	contig_77244_1	This study
<i>ENOD11_Fc</i>	5' TATGGTAACCAGCCTCCACCTAGC 3'	contig_77244_1	This study
<i>ENOD11_Rc</i>	5' GCATTGGTAAACCTTGTTGCTTGC 3'	contig_77244_1	This study
<i>ENOD40_Fa</i>	5' TCCTAAACAGTTTGCTTTGTGC 3'	TC94515	This study
<i>ENOD40_Ra</i>	5' GCTTAATTATTGCTTCTGCTACTCA 3'	TC94515	This study
<i>ENOD40_Fb</i>	5' GCCTGTGTTTGTGCTTCGTA 3'	TC94515	This study
<i>ENOD40_Rb</i>	5' TGGCAATTTCTCAAAGGAAGA 3'	TC94515	This study
<i>ACO_Fa</i>	5' AGCCATGGGAAGATCAGTTG 3'	Medtr3g083370	This study
<i>ACO_Ra</i>	5' GTCCTTCCAATCTCATCGTAT 3'	Medtr3g083370	This study
<i>ACO_Fb</i>	5' TCTTTGAGCTGGTGAATCATGGC 3'	Medtr3g083370	This study
<i>ACO_Rb</i>	5' TGGTTAACCTCTCCACAGTGTCC 3'	Medtr3g083370	This study
<i>ACO_Fc</i>	5' TTCTTTGAGCTGGTGAATCATGGC 3'	Medtr3g083370	This study
<i>ACO_Rc</i>	5' TGGTTAACCTCTCCACAGTGTCC 3'	Medtr3g083370	This study

<i>ASP_Fa</i>	5' GACCAGGACATCGTTGCTTT 3'	Medtr4g061140	This study
<i>ASP_Ra</i>	5' TCAAATCCAGAACGCTCCTT 3'	Medtr4g061140	This study
<i>ASP_Fb</i>	5' CCAGAAGGCTGTTGAGAAGG 3'	Medtr4g061140	This study
<i>ASP_Rb</i>	5' AACGGAGCATAAGAGGAGCA 3'	Medtr4g061140	This study
<i>RIP1_Fa</i>	5' GTTTGCCCTCAAGCATTACC 3'	Medtr5g074860	Hassan (2015)
<i>RIP1_Ra</i>	5' GCAGGACTGATCCATCACAA 3'	Medtr5g074860	Hassan (2015)
<i>RIP1_Fb</i>	5' GCTAGATGATACCCCAAATTTCA 3'	Medtr5g074860	This study
<i>RIP1_Rb</i>	5' CCACAGAAAATCCTCTGATTGA 3'	Medtr5g074860	This study
GADPH_F	5' TGCCTACCGTCGATGTTTCAGT 3'	MtC00030_GC	Kakar <i>et al.</i> , (2008)
GADPH_R	5' TTGCCCTCTGATTCCTCCTTG 3'	MtC00030_GC	Kakar <i>et al.</i> , (2008)
PTB_F	5' CGCCTTGTCAGCATTGATGTC 3'	Medtr3g090960	Kakar <i>et al.</i> , (2008)
PTB_R	5' TGAACCAGTGCCTGGAATCCT 3'	Medtr3g090960	Kakar <i>et al.</i> , (2008)
PPRrep-F	5' GGAAAACCTGGAGGATGCACGTA 3'	Medtr6g079920	Kakar <i>et al.</i> , (2008)
PPRrep-R	5' ACAAGCCCTCGACACAAAACC 3'	Medtr6g079920	Kakar <i>et al.</i> , (2008)
MtUBQ_F	5' GCAGATAGACACGCTGGGA 3'	Medtr8g027610	Kakar <i>et al.</i> , (2008)
MtUBQ_R	5' AACTCTTGGGCAGGCAATAA 3'	Medtr8g027610	Kakar <i>et al.</i> , (2008)
ERN1_F	5' TGCATGCCTTCTTCGAGGTTTCG 3'	Medtr7g085810.1	This study
ERN1_R	5' TCCTGGAAGCAAGAGGAGAATCC 3'	Medtr7g085810.1	This study
contig_70694F	5' ACGAGAACGATCCTGAAGAGATGC 3'	contig_70694_1	This study
contig_70694R	5' TGTTGAGGTTGTTAGCGTCGTTG 3'	contig_70694_1	This study

F: Forward primer; R: Reverse primer.



In order to assess the expression of nodulation genes in inoculated and uninoculated *wt Medicago* in the presence of AHLs with or without antibiotics, primer pairs were designed indicated as ‘this study’ in Table 2.7. The design of qRT-PCR primers was done using Primer3Plus (Untergasser *et al.*, 2007), Quantprime (Arvidsson *et al.*, 2008) and ProbeFinder software in the Universal Probe Library Assay Design Center (Roche Applied Science, Germany). Different parameters were taken into consideration such as melting temperature (T<sub>m</sub>) between 58°C and 61°C, primer size no more than 23 base pairs, PCR amplicon lengths of 100–150 base pairs (bp) and limited self-complimentary. The specificity of the transcripts were tested on *wt M. truncatula* cDNA dilutions (1/10, 1/100 and 1/1000). Housekeeping gene primers were also tested as internal control genes (Table 2.8) (Kakar *et al.*, 2008). For the presented results, *GADPH* (Glyceraldehyde 3-phosphate dehydrogenase) was chosen as the most stable expressed housekeeping gene. (The amplification was done on ABI 7900HT Sequence Detection System (Applied Biosystems, USA). The cycle program used was as follows: stage 1: 95°C, 20 sec; stage 2 ×40 times: 95 °C for 1 sec, 60°C for 20 sec, Melt curve×1: 95 °C for 15 sec, 60°C for 1 min, 95 °C for 15 sec. Taking into consideration primer efficiency, melting curve and standard deviation and error, 14 primers were chosen (Table 2.8). The primers not listed in Table 2.8 had already been tested by Hassan (2015).

**Table 2.8** Primer efficiency. Chosen primers are underlined.

<b>Primer</b>	<b>n</b>	<b>mean Efficiency</b>	<b>St Dev</b>	<b>SEM</b>
<u>MtNSP1</u>	6	1.932	0.032	0.013
<u>GADPH</u>	8	1.927	0.048	0.017
<u>MtNSP2</u>	8	1.906	0.059	0.021
PTB	9	1.885	0.135	0.045
<u>MtNIN</u>	9	1.929	0.069	0.023
PPRrep	6	1.933	0.019	0.008
<u>ENOD11a</u>	9	1.914	0.036	0.012
MtUBQ	4	1.921	0.032	0.016
ENOD11b	5	1.917	0.049	0.022
ENOD11c	8	1.937	0.056	0.020
ENOD12	7	1.824	0.114	0.043
<u>ENOD40a</u>	8	1.926	0.035	0.012
ENOD40b	9	1.895	0.026	0.009
<u>ACO_a</u>	9	1.929	0.020	0.007
<u>ACO_b</u>	9	1.916	0.085	0.028
<u>ACO_c</u>	7	1.941	0.033	0.013
ASPa	9	1.875	0.029	0.010

<u>ASPb</u>	8	1.925	0.029	0.010
<u>SH</u>	8	1.925	0.032	0.011
<u>RIP1b</u>	8	1.882	0.070	0.025
<u>ERN1</u>	53	1.959	0.048	0.007
<u>contig_70694_1</u>	63	1.959	0.048	0.006

The qRT-PCR data analysis was done according to Ramakers *et al.*, (2003) using the software LinRegPCR version 2014.2 (Ruijter *et al.* 2009). Statistical analysis was carried out with GraphPad Prism version 5.02 (2008) using One-way ANOVA on the three biological replicates (three technical replicates for each biological replicate were done).

## 2.12 Nitrogen fixation

The nitrogenase activity in plants was estimated by the acetylene reduction assay (ARA) (Turner and Gibson, 1980). 21 days old *wt Medicago* plants growing on Fåhræus medium treated with 3-oxo-C<sub>14</sub>-HSL at 1 and 10 µM and solvent-treated plants (controls) were used for the ARA assay. Roots from five plants were excised and placed in an air tight glass vessel sealed with suba seals. Three biological repeats were used per treatment. Acetylene gas (C<sub>2</sub>H<sub>2</sub>) was injected at 10% (v/v) and samples were incubated for 1h at 30 °C. Gas samples, extracted with a syringe, were analysed using a gas chromatograph Shimaduzu GC-9AM. Nitrogenase activity was calculated from peak areas relative to acetylene and ethylene standards.

## 2.13 Bacterial isolations

*Medicago* roots, derived from seeds treated with and without antibiotics, were weighed in 1.5 ml Eppendorf tubes on a balance (Mettler Toledo, Switzerland). These roots were ground with sterile sand using a sterile mortar and pestle. One ml of sterile phosphate-buffered saline (PBS) (Tble 2.9) was added to the sample and the bacteria were extracted by vortexing for 15sec. Serial dilutions were done with sterile miliQ water followed by plating on LB and/or R2A agar. Plates were incubated in the dark at 28°C for three or seven days. The most dominant strains were chosen for DNA sequencing by Sanger Sequencing in The Australian Genome Research Facility Ltd. (AGRF), Australia.

**Table 2.9** Composition of phosphate-buffered saline.

Salt	Concentration (mmol/L)	Concentration (g/L)
NaCl	137	8
KCl	2.7	0.2
Na <sub>2</sub> HPO <sub>4</sub>	10	1.42
KH <sub>2</sub> PO <sub>4</sub>	1.8	0.24

## 2.14 Next-generation high-throughput 454 pyrosequencing

DNA was extracted from *Medicago* root samples treated with or without antibiotics exposed to 3-oxo-C<sub>14</sub>-HSL or to the solvent (control) with DNeasy® Plant Mini Kit (QIAGEN®, USA) following the manufacturer's instructions. In order to evaluate which primer pair could differentiate the plant 16S DNA from the bacterial 16S DNA, three primer combinations were tested. The primer pairs were: 799F-1193R, 799F-1525R, 799F-1394R (Table 2.10). The PCR was conducted with Platinum®Taq DNA Polymerase (Invitrogen) and run in a C1000 Touch™ Thermal Cycler (Biorad). Conditions were as follows: 95°C, 3:00 min; [95°C, 0:30 sec; 55°C, 0:30 sec; 72°C, 1:00 min×35 cycles; 72°C, 5:00 min; 12°C indefinitely. To visualise the amplicons, DNA was separated in 1.2% agarose gels at 100 volts for ~ 30-45 minutes.

For bacterial community analysis purpose, the bacterial 16S rRNA genes were amplified with the 799F-1193R primer set in the same condition as above, and the visualised bands were purified from the gel with QIAquick gel extraction kit (QIAGEN®, USA). These purified bands were sent to Molecular Research DNA Molecular Research LP (Shallowater, Texas, USA) for 16S pyrosequencing. Barcoded amplicon sequencing processes were performed by Molecular Research DNA under the trademark service (bTEFAP®) described by Dowd *et al.*, (2008). Samples were sequenced utilising Roche 454 FLX titanium instruments and reagents, following manufacturer's guidelines. For the sequencing data processing initially the barcodes and primers were removed from the sequences. Short sequences (< 200bp), sequences with ambiguous base calls and sequences with homopolymer runs exceeding 6bp were discarded. Sequences were denoised and Operational taxonomic units (OTUs) were defined at 97% similarity followed by removal of singleton sequences and chimeras (Edgar 2010, Dowd *et al.* 2008a, Eren *et al.* 2011, Dowd *et al.* 2008b, Capone *et al.*

2011, Swanson *et al.* 2011). Final OTUs were taxonomically assigned using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov), DeSantis *et al.* 2006, <http://rdp.cme.msu.edu>) and each OTU count was converted to percentage for subsequence statistical analysis.

**Table 2.10** Sequences of primer pairs tested to amplify 16S DNA.

Primer	Sequence	Reference
799F	5'- AAC MGG ATT AGA TAC CCK G-3'	Chelius and Triplett (2001)
1193R	5'- ACG TCA TCC CCA CCT TCC-3'	Bulgarelli <i>et al.</i> (2012)
1525R	5'-AAGGAGGTGWTCARCC-3'	Calvo-Bado <i>et al.</i> , (2011)
1394R	5'-ACGGGCGGTGTGRTC-3'	Liu <i>et al.</i> , (1997)

## 2.15 Rarefaction and Principal components analysis (PCA)

We estimated rarefaction curves for each sample individually using a rarefaction calculator software Analytic Rarefaction 1.3 freely available at <http://strata.uga.edu/software/index.html> (Holland, 2003). Repeats were averaged per treatment. Eigenvalues for PCA were calculated by Past software (Paleontological Statistics) version 3.10 (Hammer, 2001).

## 2.16 Statistical analyses

Statistical analyses were done with GraphPad version 3.06, RStudio version 0.98.501 and Genstat 15th Edition. ANOVA and Student's t-tests were performed at  $p < 0.05$  level. Statistical analysis of taxonomical and functional bacterial profiles were done with STAMP software (Donovan Parks and Robert Beiko 2.1.3) with post-hoc Tukey Kramer with multiple corrections Benjamini-Hochberg FDR ( $p < 0.05$ ) Effect size (Eta-Squared). The effect size quantifies the extent of the significant difference between the samples within the ANOVA test.



## Chapter 3 Optimisation of growth conditions to test the effects of AHLs on plant phenotypes

### 3.1 Abstract

Bacteria synthesise AHLs and interfere with AHL signaling. Therefore, the importance of having a bacteria-free growth method to test the effect of AHLs on plant phenotype is crucial. This Chapter explores the optimisation of a surface seed sterilisation protocol as well as growth methods to evaluate the effect of AHLs on plant phenotype, particularly *Medicago truncatula* nodulation and root architecture. Initially, we sterilised *Medicago* seeds with a wide range of surface seed sterilisation methods without success. We discovered that after germination, seed-borne bacteria colonised plant surfaces. Thus, we optimised the seed sterilisation protocol by adding an antibiotic treatment. Furthermore, we conducted several experiments using different methods to grow *Medicago* in closed and open systems. We found that growing plants in plates was the best method to test AHLs on plants. All further experiments were carried out growing plants in plates. *Medicago* plants showed a nodulation-specific response to AHLs, whereas root architecture showed very little response to AHLs in *M. truncatula*. This was in contrast to strong responses in root architecture in *A. thaliana*. This suggests that legumes might respond differently to AHLs than non-legumes and that AHLs specifically modulate nodulation responses in legumes.

### 3.2 Introduction

Root systems play a crucial role in plant survival, health and productivity, especially at early stages of plant growth and development (Nicola, 1998). Root length, lateral root density and root biomass have been widely used as root parameters associated with plant growth, stress and health (Ortíz-Castro *et al.*, 2008; Zhan *et al.*, 2015; Zhan and Lynch, 2015). Previous studies have shown that different AHLs can reduce or increase the root length in *Arabidopsis* plants (von Rad *et al.*, 2008). One possible mechanism by which AHLs appear to alter plant development is by influencing auxin and cytokinin signaling in *Arabidopsis* shoot and root tissues (von Rad *et al.*, 2008). Auxin responses were also activated by some AHLs in *M. truncatula* roots, although it has not been

tested whether this is associated with altered root architecture (Mathesius *et al.*, 2003). Joseph and Phillips (2003) have shown that AHL breakdown products from beneficial root colonising bacteria increase stomatal conductance and transpiration rate in beans (*Phaseolus vulgaris* L.). AHL signals also influenced shoot growth in barley (Klein *et al.*, 2009). In most cases, it has been shown that the effect of QS on plant phenotypes is structure-and concentration-dependent. However, as yet there is little understanding about how plants “use” the ability to sense bacterial soil populations, and whether it gives them an advantage in their interaction with bacteria. The main aim of this Chapter was to develop a system to study the effects of rhizobial AHLs on *M. truncatula* to answer whether *Medicago* would specifically recognize AHLs from its symbiont and alter responses that are symbiosis-specific.

Taking into consideration that roots sense abiotic and biotic factors in the soil, including AHLs, we were interested to test their effects on root phenotypes of the model legume *M. truncatula*. As *M. truncatula* establishes a mutualistic symbiosis with *Sinorhizobium meliloti* we particularly explored nodulation phenotype in response to AHLs. As a method to validate our results in *Medicago* we also tested the effect of AHLs on the non-legume model plant *Arabidopsis thaliana* (L.) Heynh. Furthermore, we analysed the possible effect of AHLs on *Medicago* seed germination as this process and early seedling developmental stages are critical for the establishment of a plant when they are the most vulnerable to biotic and abiotic stresses.

Some of the questions we wanted to answer were: What type of growth system is the most suitable to test plant responses to AHLs? Do AHLs affect nodulation, root architecture and seed germination in *M. truncatula*? Does *Medicago* respond specifically to different AHLs? This Chapter pursued to answer the previous questions presenting the development and optimisation of different plant growth and seed surface sterilisation protocols in order to test the effect of AHLs on plant phenotype.

## 3.3 Results

### 3.3.1 The effect of AHLs on *Medicago truncatula* under glasshouse conditions

Initially we assessed the possible effect of AHLs on *wild type* (*wt*) *M. truncatula* plants under glasshouse conditions. In order to develop a system to study AHL-plant responses in soil *M. truncatula* plants were grown in pots at the Australian National University (35°18'29"S 149°07'28"E) in the greenhouse facility in Canberra, Australia. The pot

size and shape (70 mm (W) x 70 mm (L) x 200 mm (H)) allowed the young root system to expand in depth without an early restriction. A pilot assay of four treatments was established to evaluate the effect of a particular AHL applied to either shoot or root (Table 3.1). An important factor to take into consideration when testing AHLs on plants is AHL degradation. C<sub>4</sub>-HSL was chosen due to its short acyl side chain which makes it more rapidly degraded by lactonolysis (ring opening) than molecules with longer chains as it has been found that lactonolysis in C<sub>4</sub>-HSL increased with increased pH but decreased as the N- acyl side chain was lengthened (Yates *et al.*, 2002). Therefore, if this short acyl side chain AHL show a significant response under glasshouse conditions that would mean that this open system would be suitable to test AHL responses on plant phenotype. A concentration of 0.1 µM AHL was chosen to evaluate the effect of low AHL concentrations on plants under glasshouse conditions, a concentration that was shown to result in proteome changes in *M. truncatula* before (Mathesius *et al.*, 2003).

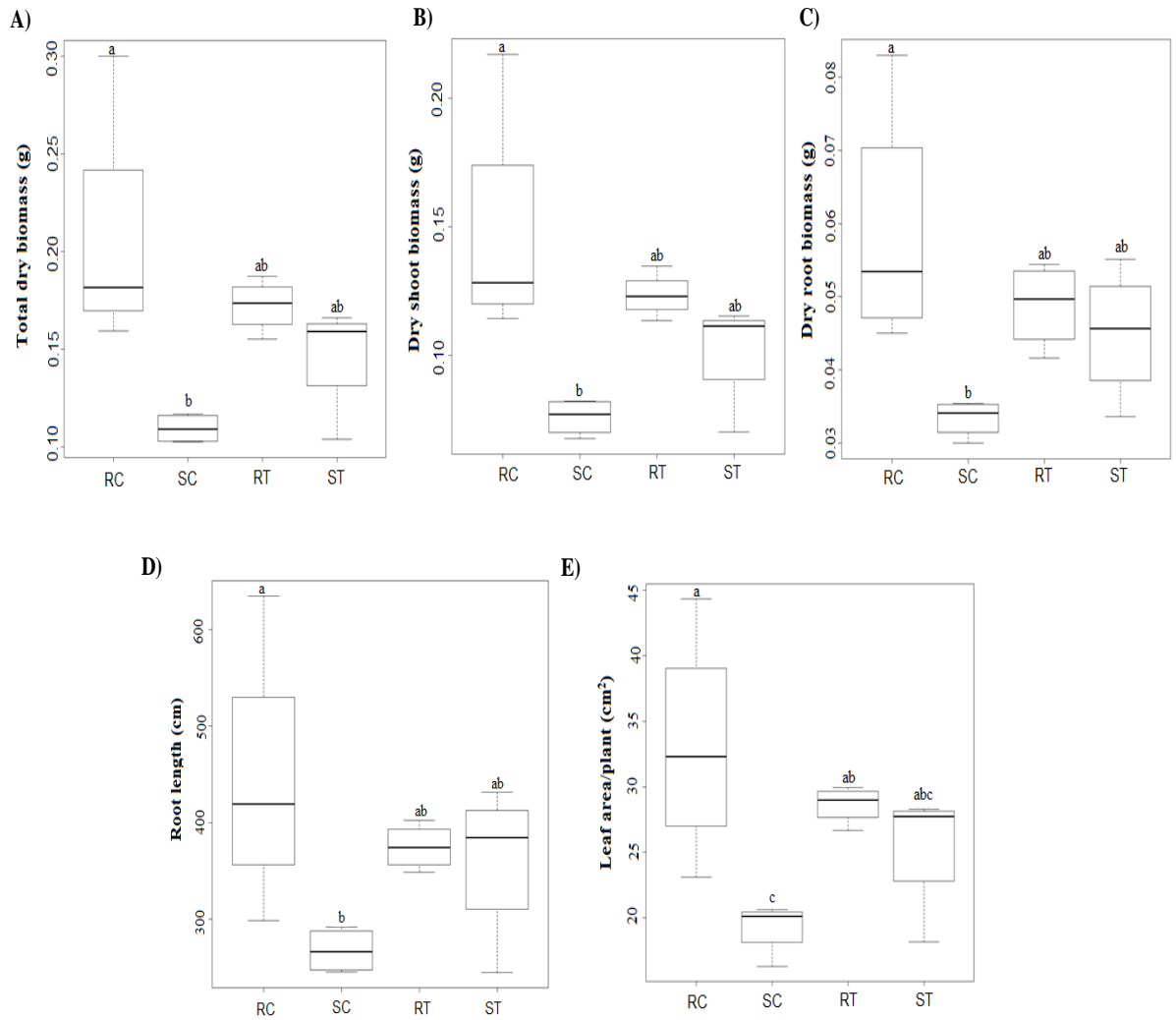
**Table 3.1** Treatments used in the glasshouse experiment.

Treatment code	Description
RC	Root control (solvent applied to the roots)
SC	Shoot control (solvent applied to the shoot)
RT	Root treatment (C <sub>4</sub> -HSL applied to the roots)
ST	Shoot treatment (C <sub>4</sub> -HSL applied to the shoots)

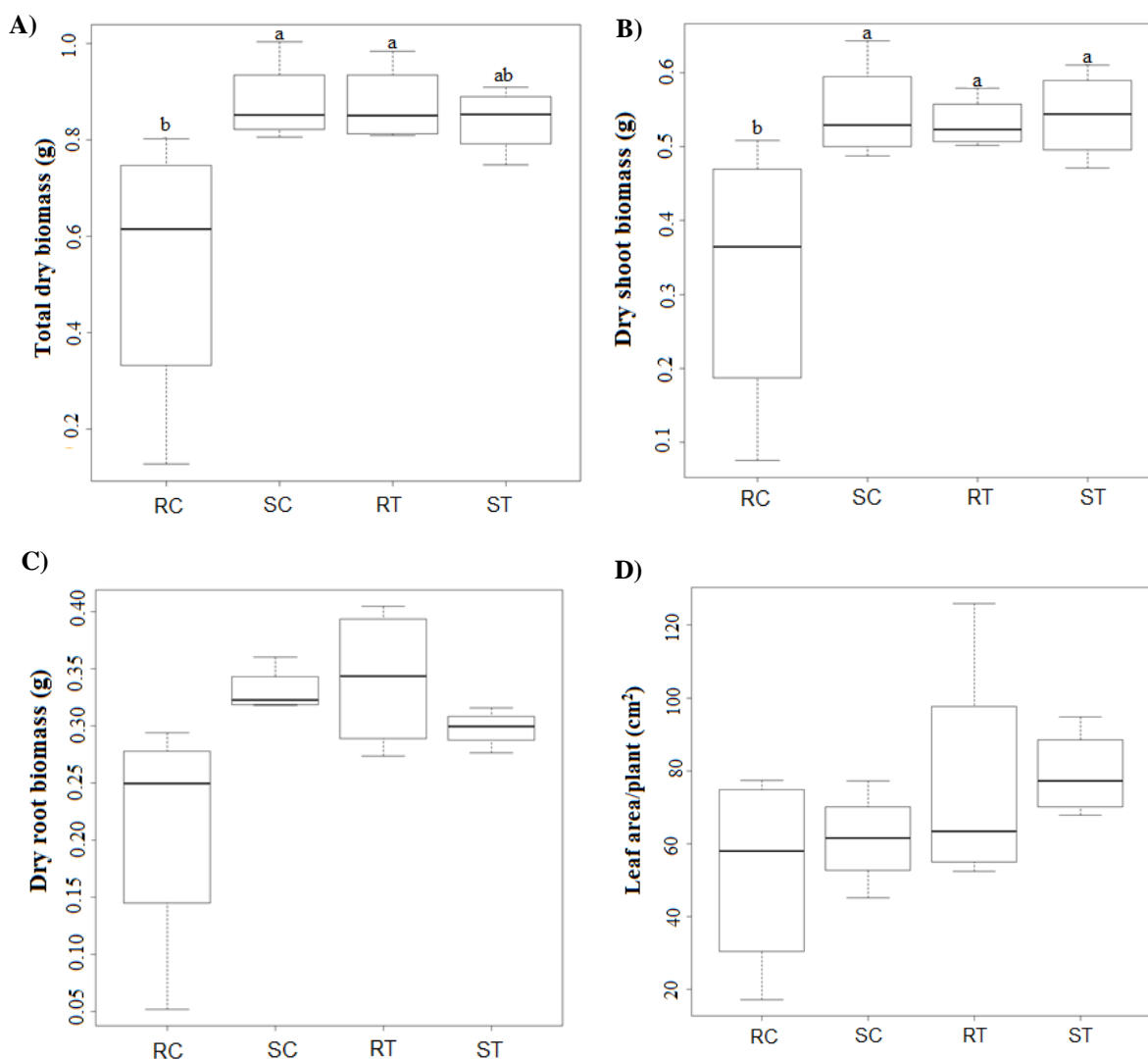
At 18 days after transferring seedlings to pots *Medicago* plants were treated on either shoot or root with C<sub>4</sub>-HSL. Shoot application was done by spraying the leaves and covering the pot with a plastic sheet in a transparent plastic box to avoid cross application (Figure 3.7E middle and right pictures). Root treatment was done by pouring 250 mL of the solution to the perlite. Controls containing solvent only were treated in the same way. Plants were treated three times per week for three weeks. Then half the plants were harvested. The rest was grown for another 22 days with reduced frequency of the C<sub>4</sub>-HSL application, once per week. Watering was done twice per week or when plants needed. Each pot contained one plant, four replicates per treatment. Plants were grown during the summer of 2012, under day/night temperatures of 25/20 °C, under natural light. The statistical design used a randomised complete block design.

Physiological parameters such as shoot and root biomass, total biomass, leaf area per plant and total root length were evaluated. To measure biomass, plants were divided

into roots and shoot. Roots were washed with abundant water to clean them from residual perlite. Both shoots and roots were wrapped with wet tissue contained in plastic bags in a box and transported to the laboratory for further processing. Leaves were scanned and leaf area was calculated by ImageJ 1.44 p software. Roots were scanned and root length calculating using WinRhizo pro Arabidopsis 2009 software (Regent instruments Inc.). All the samples were put inside envelopes to dry out for 72 h at 60<sup>0</sup>C. In the first harvest, shoot, root and total dry biomass, root length and leaf area of AHL treated plants did not show significant differences in comparison with the control. However, shoot and root control were significantly different ( $p<0.05$ ; Figure 3.1). For the second harvest there was no difference in regard to dry root biomass and leaf area (Figure 3.2C, D). Root control application of AHL had a significant effect on shoot and total dry biomass. It was not possible to determine root length at this stage as the root system was too big to be measured.



**Figure 3.1** Phenotypic responses of wild type *M. truncatula* plants at 38 days post-seedling transferring. A) Total dry biomass (g); B) Dry shoot biomass (g); C) Dry root biomass (g); D) Root length (cm); E) Leaf area per plant (cm<sup>2</sup>). RC: Root control, SC: Shoot control, RT: Root treatment, ST: Shoot treatment. Different letters indicate statistical difference between groups ( $p < 0.05$ ; One way ANOVA with Tukey post-test,  $n = 4$ ).



**Figure 3.2** Phenotypic responses of wild type *M. truncatula* plants at 65 days post-seedling transferring. A) Total dry biomass (g); B) Dry shoot biomass (g); C) Dry root biomass (g); D) Leaf area per plant (cm<sup>2</sup>). RC: Root control, SC: Shoot control, RT: Root treatment, ST: Shoot treatment. Different letters indicate statistical difference between groups ( $p < 0.05$ ; One way ANOVA with Tukey post-test,  $n = 4$ ).

Because of the large variability observed in these experiments, compared to the very small effects of AHLs on plant growth, we did not pursue the use of this glasshouse system. One factor that could have contributed to this variability is the presence of bacteria in this non-sterile system. These bacteria could have interfered with AHL breakdown (Dong *et al.*, 2001; Lin *et al.*, 2003). In addition, the large differences between shoot and root controls made this growth system difficult to work with.

### 3.3.2 A growth system to test AHLs on *Medicago truncatula*

We proceeded to assess different growth methods for *Medicago* under controlled conditions. Magenta jars, pouches and plates were assessed as a potential growth system searching for desirable characteristics such as axenic conditions, long term plant growth, accessible symbiont-inoculation, AHL application and an easy and fast screening for root phenotypes. All commercially synthesised AHL compounds (Table 2.5, Chapter 2) were of analytical grade stored at -20°C. The compounds were dissolved in ethyl acetate: acetic acid (1000:1), acetonitrile or dimethyl sulfoxide (DMSO) depending on their solubility (Table 3.2). The stock solution was diluted further with the same solvent to reach the final concentration desired in the media.

**Table 3.2** Solvents tested to dissolve synthetic AHLs.

Solvent	AHLs
Ethyl acetate: acetic acid (1000:1)	C <sub>4</sub> -HSL
	C <sub>6</sub> -HSL
	C <sub>8</sub> -HSL
	3-Oxo-C <sub>8</sub> -HSL
	C <sub>10</sub> -HSL
	C <sub>12</sub> -HSL
	3-Oxo-C <sub>12</sub> -HSL
	C <sub>14</sub> -HSL
	3-Oxo-C <sub>14</sub> -HSL
	C <sub>14:1</sub> -9-cis-(L)-HSL
	C <sub>16</sub> -HSL
	C <sub>16:1</sub> -9 cis-(L)-HSL
Acetonitrile	3-Oxo-C <sub>14:1</sub> -7-cis (L)-HSL
	3-Oxo-C <sub>16:1</sub> -11 cis-(L)-HSL
Dimethyl sulfoxide (DMSO)	C <sub>18</sub> -HSL

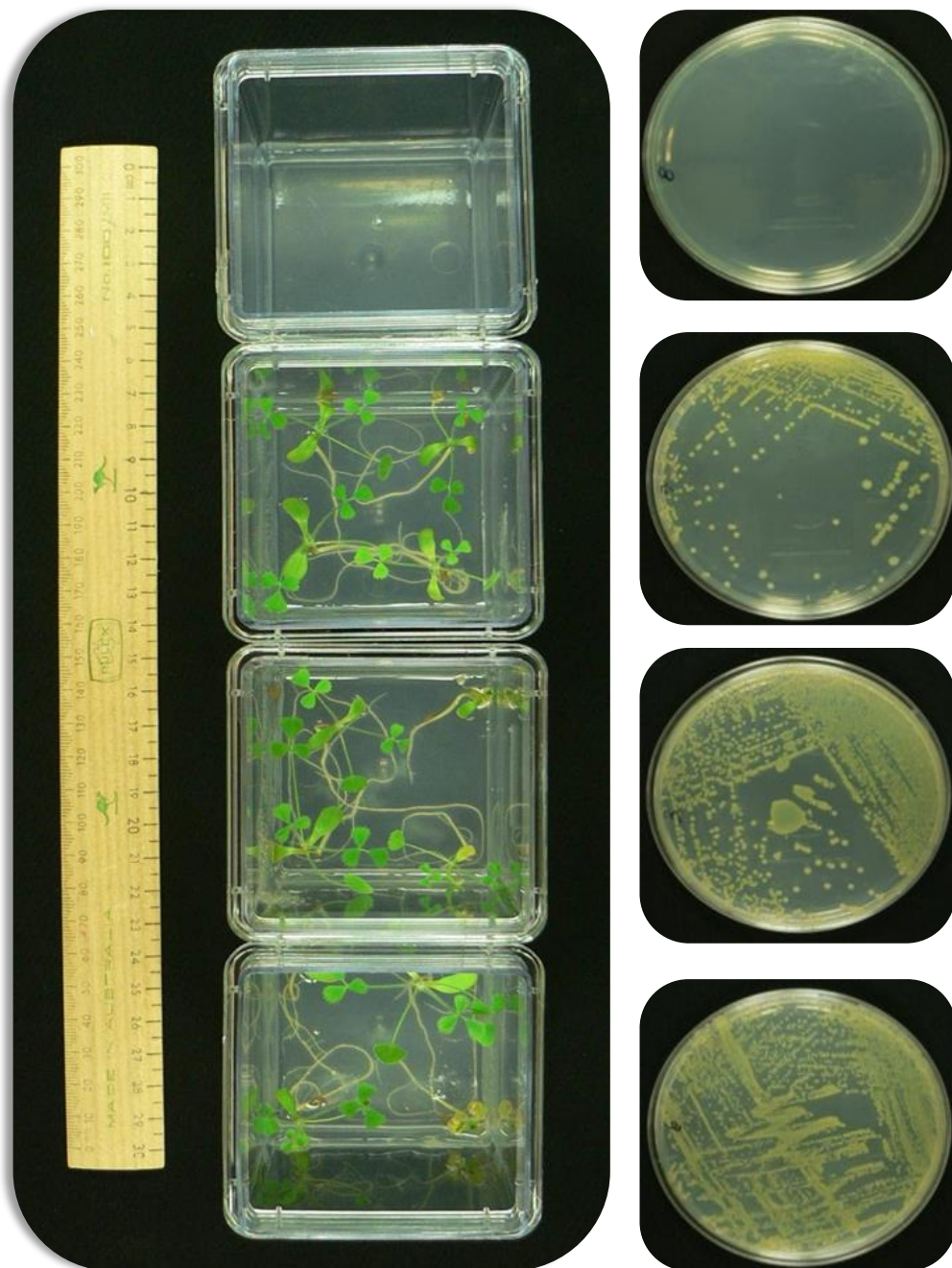
#### 3.3.2.1 Seed sterilisation

To test if *M. truncatula* seedlings respond phenotypically to AHLs we needed to ensure that the seedlings did not have any bacterial contamination as they could interfere with the AHL molecules applied. To test for the presence of contaminating bacteria, we grew plants on nutrient agar. When growing *wt M. truncatula* (A17) seedlings on Fåhraeus (F) media agar plates, we often observed bacterial contamination around the seedlings. In order to investigate if the bacteria were coming from the environment, the media or the seed, we grew *Medicago* seedlings in air tight magenta jars using only media as the

control treatment. Several types of media including Lysogeny broth (LB), Trypticase soy agar (TSA), Yeast Extract Broth (YEB) and Reasoner's 2A (R2A) were used to isolate bacteria. We chose LB medium as it was an easy and fast way to determine bacterial contamination visually.

We discovered that the contamination came from the seeds and not from the environment or the media as no bacteria could be grown from the control treatment (only media) (Figure 3.3). Several surface seed sterilisation protocols were tested in order to eliminate bacteria which could interfere with AHLs (protocols without symbol, Table 3.3). As these protocols were unsuccessful in eliminating surface contamination, we tried to peel the seed coat and then surface-sterilise the seeds for 30 seconds with sodium hypochlorite (see • in Table 3.3). No bacteria were isolated from these seedlings, which confirmed our assumption that the source of bacterial contamination was the seed and not external sources. However, the roots of these seedlings were highly deformed due to the lack of seed coat, which protected the young tissue against sodium hypochlorite damage. In light of these results, we hypothesised that bacterial contamination of the seedlings occurred through the cracks in seeds after the radicle emergence during germination (Figure 3.4). Consequently, we tested a second sterilisation with sodium hypochlorite after seed germination (see ▲ in Table 3.3). However, the second application damaged the tissue as well. Thus, it was not possible to further pursue any of these methods as we wanted to measure the effect of AHL on root phenotype. Therefore, we optimised a 6% (v/v) sodium hypochlorite/10 minutes followed by an antibiotic treatment using Augmentin solution for six hours (see \* in Table 3.3). This protocol was the most effective in reducing the bacterial load to the minimum possible without damaging the delicate tissues of the seedling (see Chapter 5, Figure 5.13) and was subsequently used for all seed sterilisations.

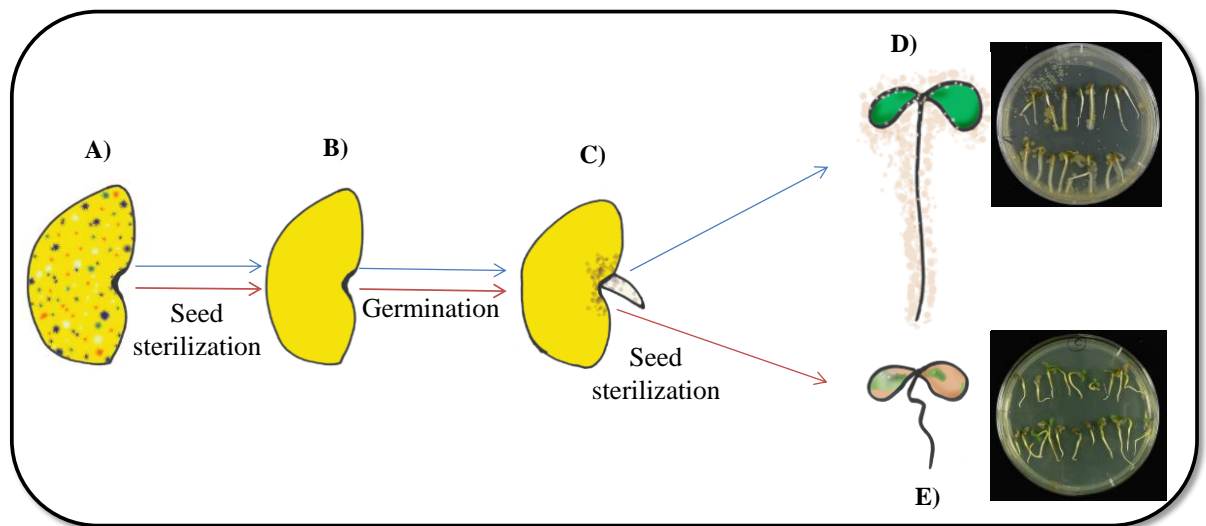




**Figure 3.3** Bacterial isolates from *M. truncatula* seedlings growing in magenta jars containing F media. Bacterial isolates were grown on Lysogeny broth (LB) agar plates at 28 °C for four days.

**Table 3.3** *M. truncatula* seed surface sterilisation methods used in this study. ● indicate seed coat peeling protocols. ▲ indicate a second sterilisation. \* indicate the protocol chosen for this study. Visible bacterial colonies were observed after growing *M. truncatula* seedlings on LB agar at 28°C for 72 hours.

Surface-seed sterilization Protocol	Visible bacterial colonies
6% (v/v) sodium hypochlorite/10 min	Yes
10% (v/v) sodium hypochlorite/20 min	Yes
70% (v/v)Ethanol/2 min+10% Sodium hypochlorite/5 min	Yes
70% (v/v)Ethanol/5 min+10% Sodium hypochlorite/20 min	Yes
70% (v/v)Ethanol/5 min+0.6% Sodium hypochlorite/5 min	Yes
80% Ethanol(v/v)/5 min+10% (v/v)Sodium hypochlorite/20 min	Yes
80% Ethanol(v/v)/60 min+3% (v/v)Hydrogen peroxide/3 min	Yes
95% (v/v)Ethanol/60 min+12.5% Sodium hypochlorite/10 min	Yes
95% (v/v)Ethanol/60 min+12.5% Sodium hypochlorite/10 min	Yes
95% (v/v)Ethanol + Tween 20/60 min+10% Sodium hypochlorite/20 min	Yes
100% ethanol/30 sec+8% Sodium hypochlorite/40 min+ water overnight	Yes
25% (v/v) Sodium hypochlorite + 1mL HCL/3 h	Yes
25% Sodium hypochlorite + Hydrogen chloride/3 h	Yes
70% ethanol/ 3 minutes/25% (v/v) sodium hypochlorite, 75% (v/v) H <sub>2</sub> O + Tween-20/5 min	Yes
80% Ethanol(v/v)/60 min+3% (v/v)Hydrogen peroxide/3 min/10% Sodium hypochlorite few sec.	Yes
80% Ethanol(v/v)/60 min+12.5% (v/v)Sodium hypochlorite/10 min	Yes
Sulfuric acid/3% Sodium hypochlorite/2 min	Yes
Sulfuric acid/3% Sodium hypochlorite/5 min	Yes
Sulfuric acid/6% Sodium hypochlorite/10 min	Yes
10% Sodium hypochlorite/20 min (v/v)/H <sub>2</sub> O overnight at 4°C 60/ 1% Sodium hypochlorite/30 sec.	Yes
3% (v/v)Hydrogen peroxide/30 sec/H <sub>2</sub> O overnight at 4°C 60/ 1% Sodium hypochlorite/30 sec.	Yes
80% Ethanol(v/v)/60 min+3% (v/v)Hydrogen peroxide/3 min	Yes
Peeled seed coat+3.75% sodium hypochlorite/30 sec ●	No
Peeled seed coat+3% sodium hypochlorite/30 sec ●	No
Peeled seed coat+2.5% sodium hypochlorite/30 sec ●	No
6% (v/v) sodium hypochlorite/10 min. Second sterilisation 3.75% sodium hypochlorite/30 sec ▲	No
6% (v/v) sodium hypochlorite/10 min. Second sterilisation 3% sodium hypochlorite/30 sec ▲	No
6% (v/v) sodium hypochlorite/10 min. Second sterilisation 2.5% sodium hypochlorite/30 sec ▲	No
6% (v/v)sodium hypochlorite/10 min followed by a solution of 200 mg/L of amoxicillin and clavulanic acid (Augmentin)/6 hours *	No

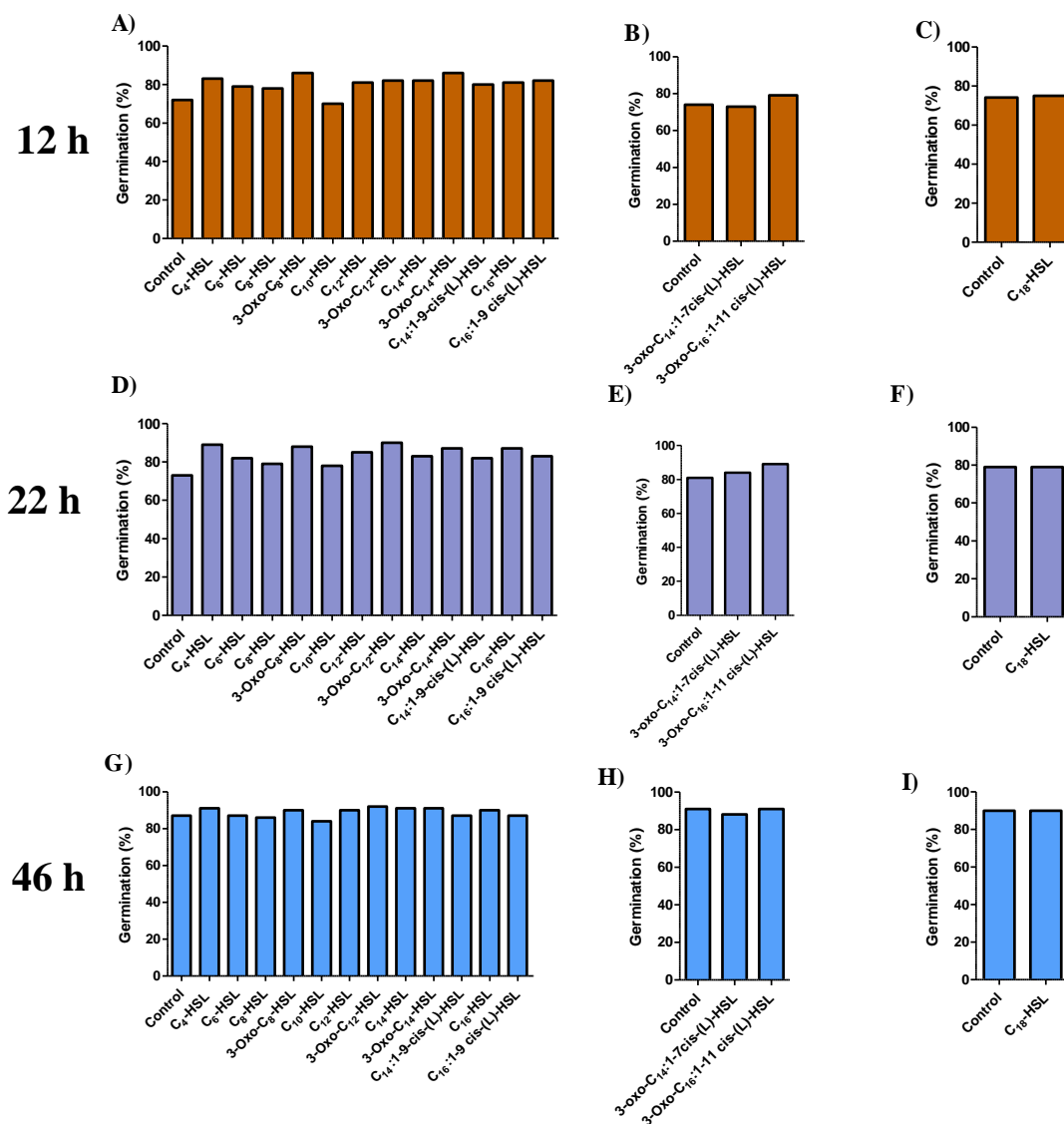


**Figure 3.4** Flow model of the rationale of *M. truncatula* surface seed sterilisation. Blue and red arrows: A) Seeds were surface sterilised. B) Bacteria on the seed coat were eliminated. C) When seeds germinated the cracks made by the radicle carried bacteria from inside the seed coat to the outside colonising the whole seedling (D). E) Red arrow only: In case of a second sterilisation or seed coat peeling sterilisation, the seedlings were uncontaminated but showed root growth defects. In D) and E) Photos show *Medicago* seedlings grown on Lysogeny broth (LB) media and incubated at 28°C for 72 hours.

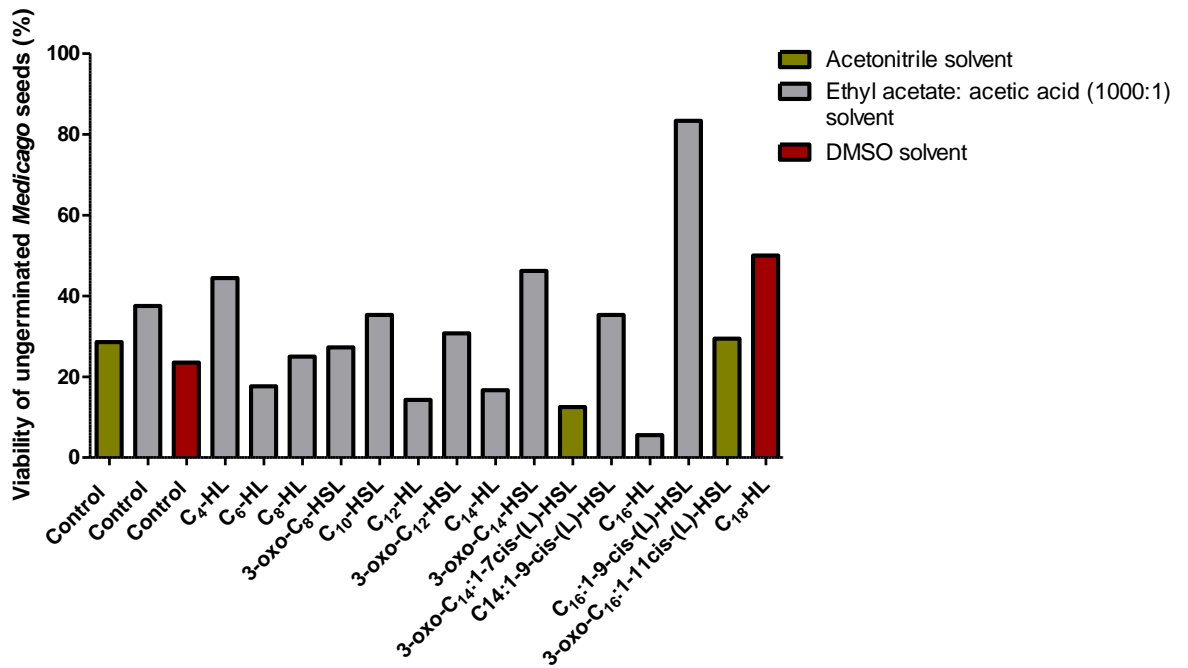
### 3.3.2.2 *Medicago truncatula* seed germination assay

In order to test whether AHLs could affect germination of *Medicago* seeds an assay was established. *Wt Medicago* seeds were scarified mechanically with sand paper, seed surface-sterilised and placed in a microtiter plate into a volume of 40  $\mu\text{L}$  of 10  $\mu\text{M}$  AHL solution or equivalent dilution of solvent. Then, seeds were stratified for 48 hours at 4 °C. Seeds were germinated individually in 96 well microtiter plates at 25°C in darkness. The percentage of germination was evaluated at different time points, 12, 22 and 46 h (Figure 3.5). Seeds with presence of bacteria were discarded. The experiment was repeated twice with similar results. No significant difference was found in seed germination at any time point between control and AHL treatments (Figure 3.5). Ungerminated seeds were soaked in 1% tetrazolium chloride solution (Gosling, 2004; Porter *et al.*, 1947) for two hours to test their viability. The viability of seeds was variable between treatments. Of these, ungerminated seeds treated with C<sub>16</sub>:1-9-cis-(L)-HSL showed the highest percentage of viable seeds (83%) (Figure 3.6). Ungerminated

seeds treated with C<sub>16</sub>-HSL showed the lowest seed viability. Perhaps, the ungerminated seeds did not germinate due to issues with the sterilisation protocol e.g. a poor scarification process, which was variable across treatments, or in response to the AHLs. This question was not further investigated.



**Figure 3.5** Effect of 10  $\mu$ M of different AHLs on seed germination at 12, 22 and 46 hours in *M. truncatula*. A, D, G) Control: Ethyl acetate: acetic acid (1000:1); B, E, H) Control: Acetonitrile; C, F, I) Control: Dimethyl sulfoxide (DMSO). No significant differences at  $p < 0.05$ ; Pearson's chi-square test ( $n = 94-96$ ).

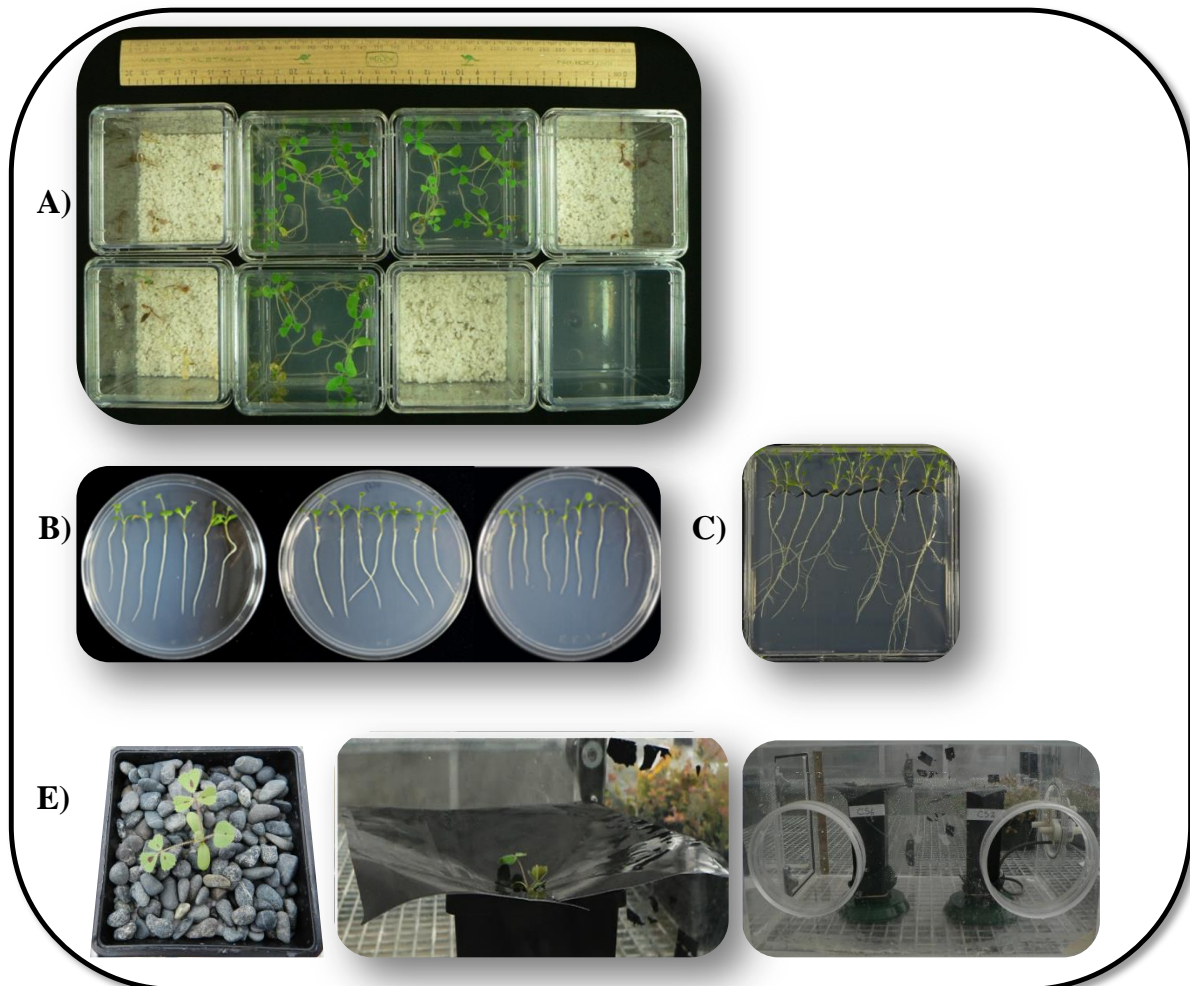


**Figure 3.6** Test of seed viability of *M. truncatula* ungerminated seeds using 1% tetrazolium chloride solution for two hours. Different colours indicate different solvents.

### 3.3.2.3 Magenta jars, pouches and plates to grow *Medicago truncatula* plants

We tested several systems to grow *Medicago* seedlings under sterile conditions. The first was magenta jars using fine perlite (Australian Perlite Pty Ltd, Australia) or F media agar (Fåhraeus, 1957) as a substrate (Figure. 3.7A). This is a closed system which ensures an axenic environment. However, as the jars are relatively small (77 mm×77 mm×97 mm) seedlings can only be grown for a maximum of one week. In addition, the root system is confined to a reduced space being affected by the size and shape of the container, which interferes with the root phenotype. We further tested paper pouches in plastic bags, which had more space for root growth but not the required axenic conditions. The last in vitro system that consisted in growing *Medicago* plants in plates, round (150mm × 15mm) (Figure 3.7B) and square petri dishes (245mm×245mm×18mm) (Figure 3.7C). The plates were sealed with an adhesive porous tape to allow gas exchange. This semi-closed system allowed plants to grow up to three weeks under controlled conditions. To ensure that AHLs dissolved in F media had an optimum pH to avoid degradation, the pH of all AHL-containing media was

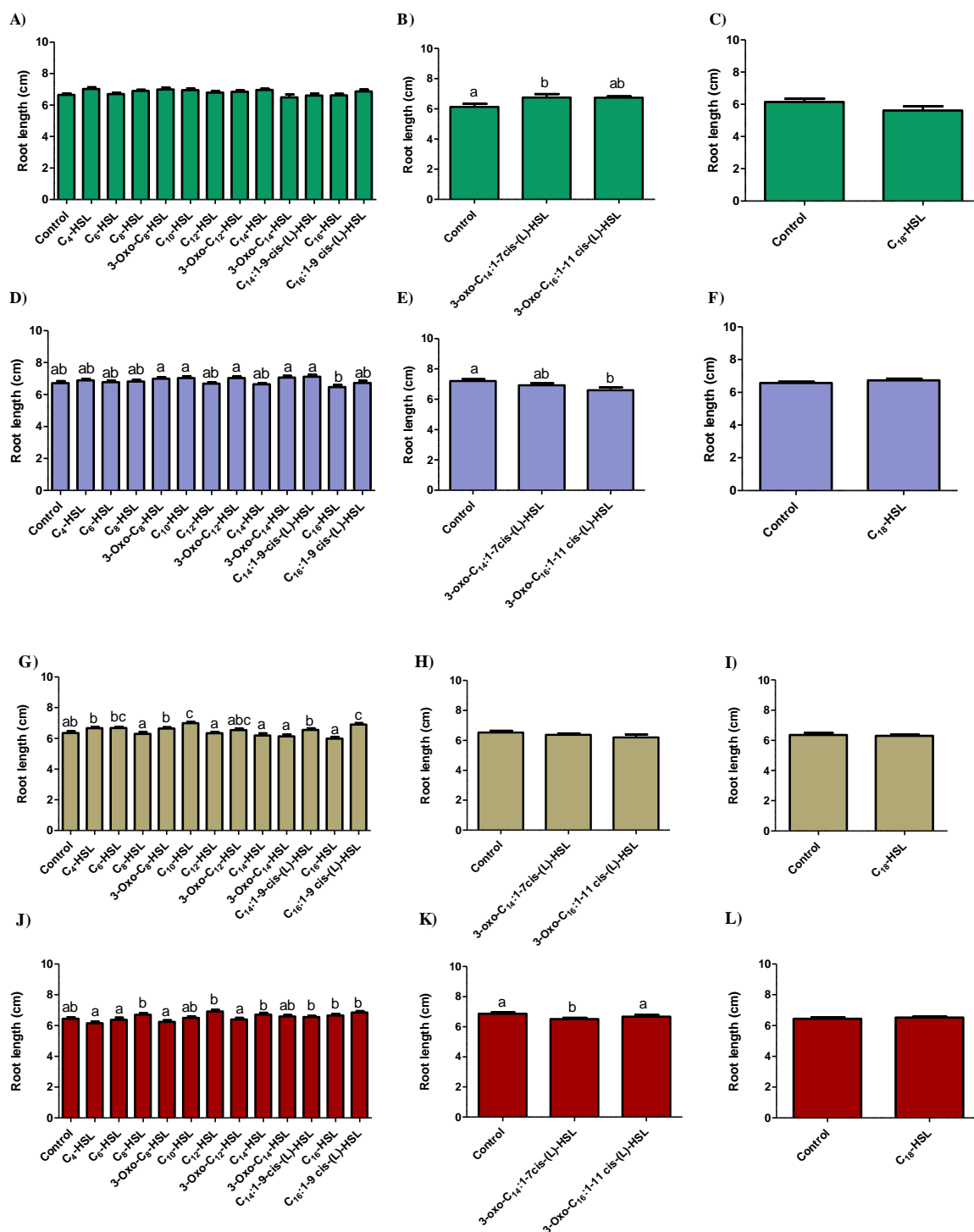
measured, and was found to be mildly acidic in all cases, which is important to prevent pH-dependent hydrolysis or degradation (Yates *et al.*, 2002).



**Figure 3.7** Different systems used to grow *M. truncatula* plants *in vitro* and in the glasshouse. A) Magenta jars containing perlite or F medium. B) Round plates with *Medicago* seedlings seven days after germination. C) Square plates with *Medicago* seedlings 25 days after germination. E) Left: Pots containing perlite and covered with pebbles. Middle: Plastic sheet covering soil for shoot AHL applications. Right: Perspex box for C<sub>4</sub>-HSL shoot applications.

### 3.3.3 *Medicago truncatula* phenotypic responses are AHL concentration-dependent.

First, after establishing an axenic growth system for *M. truncatula* seedlings, we started measuring the effect of a range of AHLs on root phenotypes. We assessed whether *Medicago* root phenotypes were AHL-concentration dependant at physiologically relevant concentrations. For these and subsequent experiments, *M. truncatula* seedlings were grown on Petri dishes containing Fåhraeus agar after surface-sterilisation followed by antibiotic treatment. Root length was differently affected by AHL concentrations although none of the changes were large. At 0.001  $\mu\text{M}$  concentration none of the changes in root length were significant, except after treatment with C<sub>14</sub>:1-9-cis-(L)-HSL, which slightly but significantly increased root length in comparison to the control ( $p < 0.05$ ; Figure 3.8B). However, this difference was not observed at 0.1  $\mu\text{M}$  or at 1  $\mu\text{M}$  concentration (Figure 3.8E, H). Root length was decreased in response to 10  $\mu\text{M}$  C<sub>14</sub>:1-9-cis-(L)-HSL ( $p < 0.05$ ; Figure 3.9K). Root length was not significantly affected by AHLs dissolved in ethyl acetate: acetic acid (1000:1) at 0.1  $\mu\text{M}$  or 10  $\mu\text{M}$  in comparison to the control, even though some of the AHLs caused significant differences between the different AHLs treatments (Figure 3.8D). However, at 1  $\mu\text{M}$  concentration, C<sub>10</sub>-HSL and C<sub>16</sub>:1-9 cis-(L)-HSL increased root length significantly in comparison to the control. C<sub>18</sub>-HSL did not have an effect on root length at any of the concentrations tested (Figure 3.8C, F, I, L). Different solvents in the three controls did not differ from each other. This experiment suggested that AHLs have structure and concentration-dependent effects on *M. truncatula* growth under the growth conditions chosen. Therefore, this system was used to further explore the effects of AHLs on *M. truncatula* nodulation and root architecture.



**Figure 3.8** Effect of AHL concentration on *M. truncatula* root length. Seeds were surface sterilised with antibiotics. Seedlings were grown on plates. A-C) 0.001  $\mu\text{M}$ ; D-F) 0.1  $\mu\text{M}$ ; G-I) 1  $\mu\text{M}$ ; J-L) 10  $\mu\text{M}$ . Control left: Ethyl acetate: acetic acid (1000:1), middle: acetonitrile; right: Dimethyl sulfoxide (DMSO). (A, B, D, E, G, H, J, K: Kruskal-Wallis test with Dunn’s post-test; C, F, I, L: Student t test). Data points indicate mean  $\pm$  SE ( $n = 24\text{-}32$ ). Treatments that do not share a common letter are significantly different at  $p < 0.05$ .



### 3.3.4 Effects of AHLs on nodulation and root architecture of *wild type Medicago truncatula*

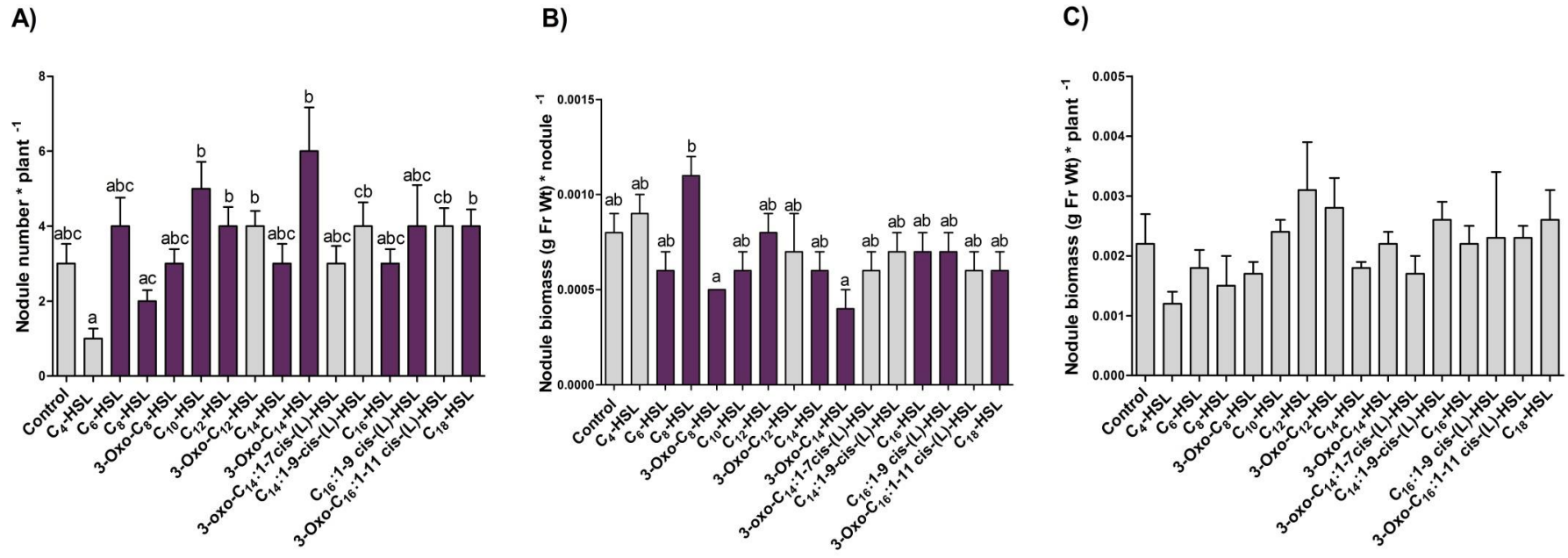
To determine whether AHLs modulate the interaction of *M. truncatula* roots with its symbiont, *Sinorhizobium meliloti*, we exposed surface-sterilised (Table 3.3 protocol marked with \*), germinated seedlings to 15 different AHLs (See Chapter 2, Table 2.5 and Figure 2.2). Seedlings were placed on Fåhraeus media agar with the addition of 1  $\mu\text{M}$  of each AHLs, or solvent as the control. We chose a concentration of 1  $\mu\text{M}$  because it was within the range of concentrations that were previously shown to elicit plant responses (Mathesius *et al.*, 2003; von Rad *et al.*, 2008; Ortíz-Castro *et al.*, 2008; Liu *et al.*, 2012; Palmer *et al.*, 2014) and AHL concentrations in the  $\mu\text{M}$  to  $\text{mM}$  range were previously measured in the tomato rhizosphere (Schuhegger *et al.*, 2006).

Seedlings were exposed to AHLs for three days before being inoculated with *S. meliloti* strain Rm1021. Previous experiments showed that major changes in protein accumulation occurred in *M. truncatula* within 24-48 h (Mathesius *et al.*, 2003), and gene expression changes were found within 4 h to 4 days after exposure to AHLs in *A. thaliana* (von Rad *et al.*, 2008), thus we hypothesised that an exposure of 3 days would ensure biological responses to occur in roots prior to inoculation with rhizobia. Nodule numbers were counted and nodule biomass determined three weeks post inoculation. At the same time, we counted the number of emerged lateral roots and determined the tap root length.

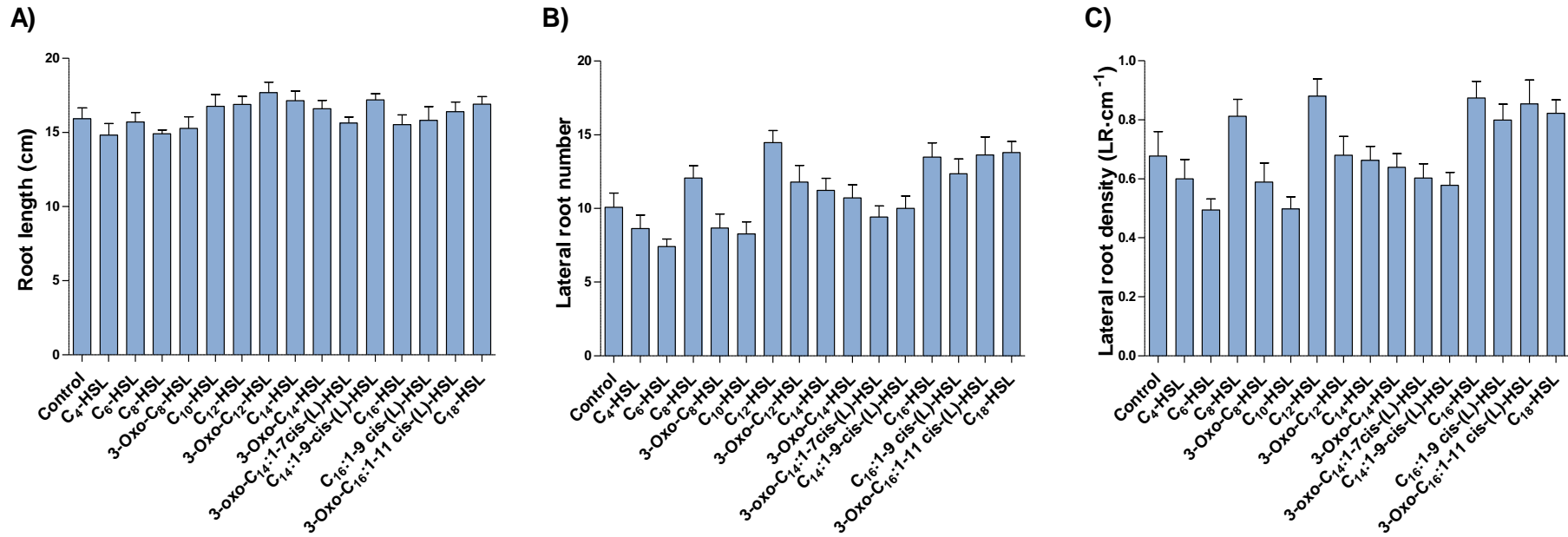
AHL exposure led to significant differences in the numbers of nodules between treatments (Figure 3.9A). However, there was no clear trend towards increased nodule numbers with AHLs synthesised by *S. meliloti* compared to AHLs from other bacteria. The *S. meliloti* AHL, 3-oxo-C<sub>14</sub>-HSL caused the highest increase in nodule numbers, with almost double the numbers of nodules per plant compared to the control. While this difference was not significantly different from the control in the ANOVA, it was repeated in other independent experiments where 3-oxo-C<sub>14</sub>-HSL significantly increased nodule numbers in comparison to the control (*cf.* Figure 4.15). We also determined nodule weight per plant and per nodule, which showed that the 3-oxo-C<sub>14</sub>-HSL treatment resulted in the lowest nodule biomass per nodule (Figure 3.9B), suggesting that nodule numbers in this treatment were increased at the expense of nodule biomass. Differences in the nodule biomass per plant (Figure 3.9C) showed the same trend as

nodule numbers per plant, although none of the treatment differences were statistically significant ( $p>0.05$ ).

To test whether the effects on nodule numbers were linked to other aspects of root development, we determined lateral root numbers and root length, two phenotypes that are modulated by AHLs in *A. thaliana* at concentrations from 1 to 100  $\mu\text{M}$  (e.g. Ortíz-Castro *et al.*, 2008; von Rad *et al.*, 2008; Liu *et al.*, 2012). However, in *M. truncatula* we found no significant changes in root length, lateral root number or lateral root density at 1 $\mu\text{M}$  treatments of the different AHLs (Figure 3.10), most likely because of the large variation in lateral root numbers between individual plants.



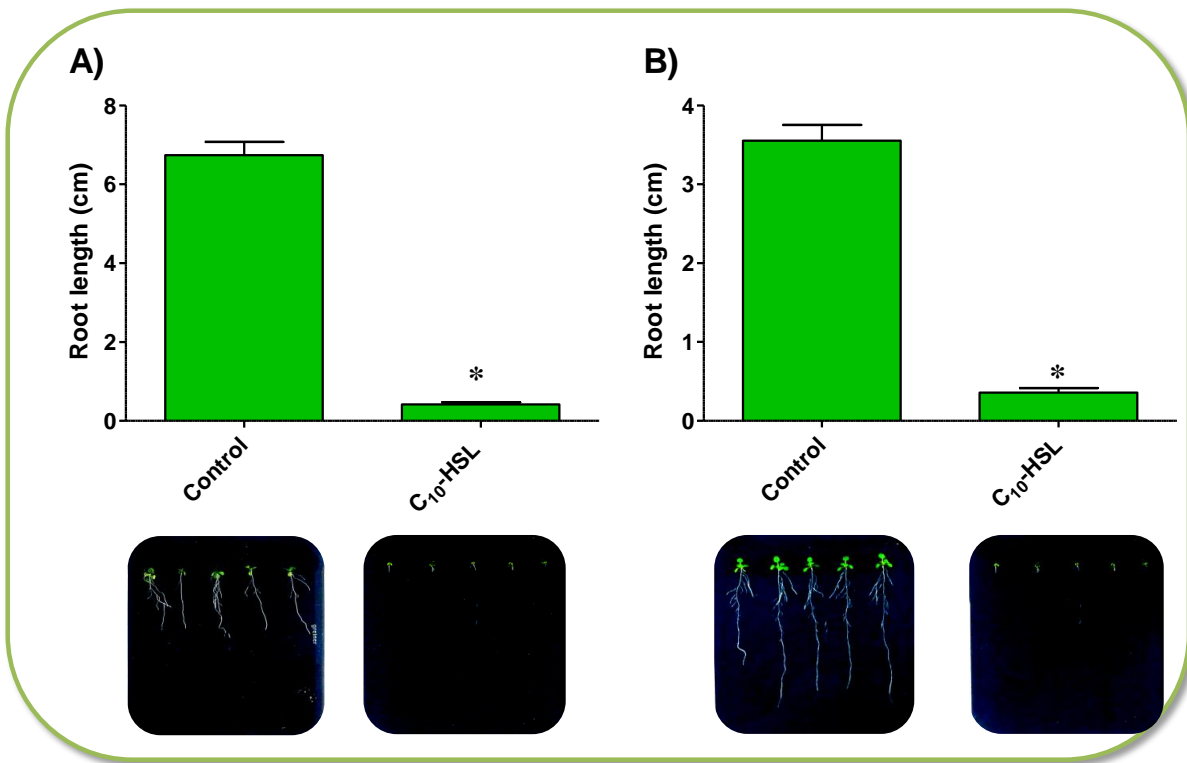
**Figure 3.9** Effect of 1  $\mu$ M AHLs on nodulation at 21 days after inoculation in *wild type M. truncatula*. A) Nodule numbers per plant; purple bars indicate AHLs synthesised by *S. meliloti*, grey bars indicate AHLs synthesised by other bacteria (cf. Table 2.5); B) Nodule biomass (in grams of fresh weight) per nodule; C) Nodule biomass (in grams of fresh weight per plant). Data points indicate mean  $\pm$  SE, ( $n = 25-27$ ). A: Kruskal-Wallis test with Dunn's post-test; B, C: One-way ANOVA with Tukey post-test at  $p < 0.05$ . Treatments in A) and B) that do not share a common letter are significantly different at  $p < 0.05$ . No statistically significant differences were found in C).



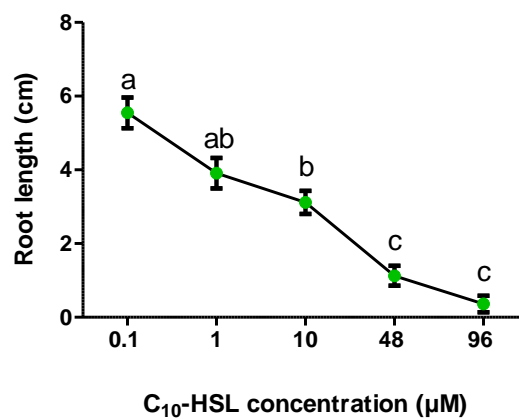
**Figure 3.10** Effect of AHLs on *M. truncatula* root architecture. Treatments correspond to 21 days after inoculation in wild type *M. truncatula* treated with 1  $\mu$ M AHL. A) Root length; B) Lateral root number per plant; and C) Lateral root density (number of lateral roots/ cm root length). No significant differences at  $p < 0.05$  (One-way ANOVA with Tukey post-test). Data points indicate mean  $\pm$  SE, ( $n = 25-30$ ).

### 3.3.5 AHL effects on root architecture are species-dependent.

Previous studies in the non-legume model plant *A. thaliana* clearly showed that AHLs affected root architecture (von Rad *et al.*, 2008; Ortíz -Castro *et al.*, 2008). For instance, C<sub>10</sub>-HSL significantly reduced root length in a study by Ortiz-Castro *et al.* (2008), long acyl side chain AHLs reduced root length while short acyl side chain AHLs increased it (Schenk *et al.*, 2012) and C<sub>4</sub>-HSL increased root length (von Rad *et al.*, 2008). As our results showed that AHLs only affected nodulation in *M. truncatula* we developed a biological assay using *Arabidopsis* to validate this lack of root growth in *Medicago*. The AHL C<sub>10</sub>-HSL was chosen since *Arabidopsis* plants treated with this compound had a consistent reduction in root length in comparison with other AHLs previously tested (Ortiz-Castro *et al.*, 2008; von Rad *et al.*, 2008). Plants were grown on 0.5 × MS media containing C<sub>10</sub>-HSL at a concentration of 48 μM or 96 μM, as was done previously by Ortiz-Castro *et al.*, (2008). We obtained significant reductions in main root length with both concentrations ( $p < 0.05$ ; Figure 3.11) as reported in previous studies by other laboratories. Both 48 μM and 96 μM are extremely high concentrations not likely reached over large areas of the root surface (Palmer *et al.*, 2014). We therefore also used 0.1 μM, 1 μM and 10 μM to test whether the effect of C<sub>10</sub>-HSL on root growth was concentration dependent and whether it is still relevant at lower AHL concentrations. We found that the root growth was reduced in an AHL concentration dependent manner ( $p < 0.001$ ; Figure 3.12). These results confirmed that under our growth conditions, the AHLs used within this thesis were biologically active on *A. thaliana*. This result suggests that the responses we observed in *M. truncatula*, i.e. relatively strong nodulation responses but weaker responses on root architecture are indeed due to species-specificity. This experiment, therefore, confirmed that AHLs may have taken on a specific role in legume nodulation, which will be further investigated in Chapter 4.



**Figure 3.11** Effect of  $C_{10}$ -HSL on root length of 10 days old *wt Arabidopsis* plants. A) 48  $\mu$ M B) 96  $\mu$ M. Data points indicate mean  $\pm$  SE ( $n = 17-23$ ). Significant differences  $p < 0.05$  denote with an asterisk (Student's t-test). Control treatments contained only solvent (Ethanol).



**Figure 3.12** Effect of  $C_{10}$ -HSL on root length on 10 days old *wt Arabidopsis* plants. Data points indicate mean  $\pm$  SE ( $n = 25$ ). Significant differences are denoted with different letters ( $p < 0.001$ , Dunn's test).

### 3.4 Discussion

Plants are surrounded by bacteria, which in turn can produce quorum sensing signals including AHLs. In order to evaluate the effect of AHLs on plant phenotype we needed to ensure that plants would be able to grow under axenic conditions, so that plant associated bacteria would not interfere with the synthetic AHLs applied. However, the *Medicago* seed batch, which was field-grown, contained culturable plant-associated bacteria, which were difficult to eliminate by surface seed sterilisation protocols. Several protocols were evaluated in order to reduce the presence of bacteria, which came from the seed, particularly under the seed coat. This is not a surprise as bacteria have been found in inner tissues of seed teguments (Compant *et al.*, 2011). We successfully reduced the bacterial contamination by optimising the method with the addition of an antibiotic treatment. Likewise, Caetano-Anollés *et al.*, (1990) tested different surface seed sterilisation techniques without complete success. The same authors stated that sterilisation-resistant bacteria, borne within the seed, proliferated on plant surfaces after germination. In our optimised surface seed-sterilisation we used Augmentin, which has a wider spectrum of action against bacteria, specially Gram negative, as it is a combination of amoxicillin and clavulanic acid, a penicillin antibiotic and  $\beta$ -lactamase inhibitor, respectively (Grayson *et al.*, 2010). In our protocol, Augmentin was removed by several washes after application, so in this way seedlings grew on the media without antibiotics. Many plant-associated bacteria living in the rhizosphere promote plant growth, suppress pathogens, increase nutrient availability, synthesise phytohormones and increase plant disease tolerance/resistance and may thus have co-evolved with plant hosts (Klein *et al.*, 2009). Seed bacterial endophyte communities are transmitted vertically via seeds. Plant associated bacteria and seed bacterial endophytes play a crucial role in plant survival and success (Hardoim *et al.*, 2015). Therefore, it is not surprising that plants select and keep bacteria in their reproductive organs to ensure that the next generation will not only survive, but also thrive. Interestingly, *Arabidopsis* seeds did not show visible bacterial colonies growing on the seedlings as *Medicago* did, suggesting that these plant-bacteria interactions are species-specific. Considering that *M. truncatula* can form a mutualistic symbiosis with *S. meliloti*, it is reasonable to speculate that *Medicago* has developed mechanisms to recognise and coexist with certain bacteria, and that these responses may be different to non-legumes. This could be tested further by examining a range of legume and non-legume plants for the presence of culturable endophytes.

Germination and early seedling developmental stages are critical for the establishment of a plant when they are the most vulnerable to biotic and abiotic stresses. After germination, plants sense their environment in order to initiate a complex transcriptional and transductional cascade (photomorphogenesis), which controls shoot and root growth. As observed here, the seed harbours bacteria, and these could potentially influence AHL-related responses during early seed development. Therefore we tested whether AHLs could influence germination in *M. truncatula* seeds as to the best of our knowledge no studies have been done testing the effect of AHL on seed germination. Under our conditions, no significant effect was found between different AHL treatments on germination, at least at the concentration used here.

We wanted to assess whether was feasible to evaluate AHLs in an open system having in mind potential future experiments under field conditions. We conducted a pilot study in order to evaluate the effect of AHLs on *M. truncatula* plants. However, the results were not consistent, probably due to a variety of biotic and abiotic factors, including the presence of microorganisms, which may influence the plant response towards to AHLs. In addition, the AHL spatial distribution present in the rhizosphere, under this open system, may have been heterogeneous creating the so called “hot spots” with higher AHL concentrations. Surprisingly, control treatments of shoot and root applications had significant effects on root and shoot biomass, and this was dependent on the age of the plant. Irrespective of low sample numbers and large variability between plants, treating shoots by spraying and wetting the leaves could have affected plant growth or led to (undetected) contamination. Overall, no significant effects of the AHLs on plant growth were observed. Because of the variability of this open system to grow *Medicago* we did not choose this method for further studies at this stage. We turned to the evaluation of different closed systems to study AHL responses on *Medicago* plants under controlled conditions. Plates were the best method due to the long term growth, sterile conditions with an appropriate gas exchange, easy and fast symbiont inoculation and AHL application which allowed a simultaneous screening of different AHLs. One of the abiotic factors that can cause hydrolysis of the AHL molecule is pH. Acidic conditions are required for the AHL molecule stability. In a study conducted by Yates *et al.*, (2002), the homoserine ring of C<sub>4</sub>-HSL was found to be largely intact below pH 6 and being completely opened at pH 8. In our experiments, plates containing media with AHLs were at or just below pH 6 in F media (growing *Medicago* plants) and in MS



media (growing *Arabidopsis* plants). Therefore, we used plates to test nodulation and other root phenotypic responses to AHLs in all experiments.

In order to find a suitable system to study AHLs on root phenotype, including nodulation, and to narrow down the number of AHL compounds to work with, we carried out a root architecture screening on *Medicago*. We found that most AHLs tested, whether synthesised by *S. meliloti* or other bacteria, had no significant effect on nodule numbers in *M. truncatula* at the tested concentration (1  $\mu\text{M}$ ). However, significant differences in nodule numbers were found for specific AHL compounds, with 3-oxo-C<sub>14</sub>-HSL application from *S. meliloti* resulting in the highest nodule numbers. This effect was specific to an increase in nodule numbers, while lateral root numbers and root length were not altered. Subsequently, four AHL compounds were chosen, which caused the strongest nodulation responses, including both short and long chain AHLs and AHLs from different bacterial origin (from symbiont and pathogen). These compounds were: C<sub>4</sub>-HSL, C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL. The subsequent Chapter investigates more detailed nodulation responses to these AHLs. Other root phenotypes were not repeatable or not significantly affected by any of the AHLs tested.

In order to validate *Medicago* root architecture responses under our growth conditions, we developed a biological assay with *Arabidopsis* plants seeking to reproduce previous results described in the literature (Ortíz-Castro *et al.*, 2008). We used C<sub>10</sub>-HSL as this compound has shown the most dramatic root length reduction in *Arabidopsis* plants (von Rad *et al.*, 2008; Ortíz-Castro *et al.*, 2008). We obtained similar root length reductions with 48  $\mu\text{M}$  and 96  $\mu\text{M}$  concentrations, suggesting that the observed lack of root length responses in *Medicago* is likely a species-specific response. There is evidence that AHLs systemically translocate throughout the plant (Götz *et al.*, 2007). Systemic transport rate in the plant is lower for lipophilic long-chain AHLs than for more water soluble short-chain AHLs (Sieper *et al.*, 2014). Perhaps the strong root reduction seen at high AHL concentrations might be explained by toxicity as longer AHL acyl-side chains like C<sub>10</sub>-HSL exhibit higher hydrophobicity than the short AHL acyl-side chains (e.g. C<sub>4</sub>-HSL), which presumably might cause an AHL over accumulation in root tissues affecting cell division in the meristem (von Rad *et al.*,

2008; Ortíz-Castro *et al.*, 2008). Therefore, the next step was to assess whether this effect was AHL concentration-dependent. Root length decreased inversely with the AHL concentration. Thus, low C<sub>10</sub>-HSL concentrations had less root length reduction than higher concentrations, confirming that the reduction in root growth in *Arabidopsis* plants is concentration-dependent and therefore suggesting that the root length reduction in this assay is caused by a specific AHL effect rather than a toxic effect.

## Chapter 4 The phenotypic effect of AHLs on legume nodulation

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### 4.1 Abstract

*N*-acyl homoserine lactones (AHLs) act as quorum sensing signals that regulate cell-density dependent behaviours in many gram-negative bacteria, in particular those important for plant-microbe interactions. AHLs can also be recognised by plants, and this may influence their interactions with bacteria. Here we tested whether the exposure to AHLs affects the nodule-forming symbiosis between legume hosts and rhizobia. We treated roots of the model legume, *Medicago truncatula*, with a range of AHLs either from its specific symbiont, *Sinorhizobium meliloti*, or from the potential pathogens, *Pseudomonas aeruginosa* and *Agrobacterium vitis*. We found increased numbers of nodules formed on root systems treated with the *S. meliloti*-specific AHL, 3-oxo-C<sub>14</sub>-homoserine lactone, at a concentration of 1 µM, while the other AHLs did not result in significant changes to nodule numbers. We did not find any evidence for altered nodule invasion by the rhizobia. 3-oxo-C<sub>14</sub>-HSL primed *Medicago* plants before inoculation with rhizobia, indicating that the increased in nodule numbers occurs at early stages and as a direct effect on the plant and not on the rhizobia. Quantification of flavonoids that could act as *nod* gene inducers in *S. meliloti* did not show any correlation with increased nodule numbers. Increased nodule numbers following 3-oxo-C<sub>14</sub>-HSL lactone treatment were independent of autoregulation of nodulation and were still observed in the autoregulation mutant, *sun4* (*super numeric nodules4*). However, increases in nodule numbers by 3-oxo-C<sub>14</sub>-HSL were not found in the ethylene-insensitive *sickle* mutant. Increase in nodule numbers were associated more strongly with higher AHL concentrations compared to lower AHL concentrations. A comparison between *M. truncatula* with *M. sativa* (alfalfa), *Trifolium repens* (white clover) and *Lotus japonicus* (Lotus) showed that the observed effects of AHLs on nodule numbers were specific to *M. truncatula*, despite *M. sativa* nodulating with the same symbiont. It was concluded that plant perception of the *S. meliloti*-specific 3-oxo-C<sub>14</sub>-homoserine lactone influences

nodule numbers in *M. truncatula*, most likely via an ethylene-dependent, but autoregulation-independent mechanism.

## 4.2 Introduction

Many species of the legume family interact with nitrogen-fixing bacteria collectively called rhizobia, leading to the formation of root nodules, in which the bacteria are housed. This provides a source of nitrogen to the plant, while the bacteria benefit from a carbon source from the plant host. Rhizobia, like most gram-negative bacteria, synthesise and perceive *N*-acyl-homoserine lactone (AHL) quorum sensing signals (González and Marketon, 2003; Sanchez-Contreras *et al.*, 2007). AHLs contain a homoserine lactone moiety with variable acyl chain length, and different bacterial species produce specific mixtures of AHLs. AHLs mediate a number of cell-to-cell signalling functions in bacteria, and are particularly important for bacteria that interact with plants. Among the traits regulated by AHLs in bacteria, bacterial movement, biofilm formation, production of virulence factors and degradative enzymes have been shown to be important for bacteria-plant interactions (e.g. Parsek and Greenberg, 2000; von Bodman *et al.*, 2003; De Angelis *et al.*, 2008). In rhizobia, AHLs mediate exopolysaccharide synthesis important for bacterial attachment and invasion, plasmid transfer, swarming behaviour, regulation of nitrogen-fixation genes and nodulation efficiency (e.g. Marketon *et al.*, 2002; Wisniewski-Dyé and Downie, 2002; González and Marketon, 2003; Sanchez-Contreras *et al.*, 2007; Cao *et al.*, 2009; Mueller and González, 2011; Gao *et al.*, 2012; Nievas *et al.*, 2012).

While AHLs regulate communication between bacterial cells, there is emerging evidence that AHLs could also regulate eukaryotic responses (Lowery *et al.*, 2008; Pacheco and Sperandio 2009). In response to synthetic AHLs, plants have been shown to specifically adjust the production levels of more than 150 proteins, including defence related proteins, metabolic enzymes, and enzymes of the flavonoid pathway (Mathesius *et al.*, 2003). This plant perception could give an advantage to the plant not only in perceiving the numbers of surrounding bacteria but also alter its behaviour in response to AHLs (Bauer and Mathesius, 2004; Hartmann *et al.*, 2014). In support of this hypothesis, a number of studies have demonstrated that AHLs trigger changes in plant development and plant defence (Hartmann *et al.*, 2014). For example, AHLs were

shown to alter root architecture in *Arabidopsis thaliana* and mung bean, in part by targeting hormone signalling (von Rad *et al.*, 2008; Ortíz-Castro *et al.*, 2008; Bai *et al.*, 2012; Liu *et al.*, 2012; Zuñiga *et al.*, 2013), and similar results were demonstrated in Chapter 3. In addition, AHLs mediate plant defence responses towards pathogens in tomato and *A. thaliana* (Schuhegger *et al.*, 2006; Schikora *et al.*, 2011; Schenk *et al.*, 2012; Zarkani *et al.*, 2013; Schenk *et al.*, 2014). So far, it is not known how plant responses to AHLs alter the interaction of legumes with their rhizobia symbionts.

During the symbiosis of legumes with rhizobia, the plant exudes signal molecules, in most cases flavonoids, into the rhizosphere to attract rhizobia and to induce the expression of nodulation (*nod*) genes in rhizobia (Firmin *et al.*, 1986; Peters *et al.*, 1986; Redmond *et al.*, 1986; Peck *et al.*, 2006). This leads to the synthesis of Nod factors that are necessary for the induction of cell divisions in the host root and the formation of infection threads, leading to the development of an infected nodule (Oldroyd and Downie, 2008). The absence of flavonoids in roots inhibits nodulation (Subramanian *et al.*, 2006; Wasson *et al.*, 2006; Zhang *et al.*, 2009), whereas the addition of external flavonoids acting as *nod* gene inducers has been shown to increase or decrease nodule numbers in legumes, depending on their concentration (Novák *et al.*, 2002). Host-exuded flavonoids have also been shown to increase the production of AHLs in rhizobia, possibly to coordinate the production of AHLs in the vicinity of the host in preparation for successful symbiosis (Pérez-Montaña *et al.*, 2001). There is also evidence that in *Medicago* plants stress and defense related genes are induced at early stages of rhizobia infection, implying that plants initially perceive their beneficial symbionts as a potential threat (Zamioudis and Pieterse, 2012; El Yahyaoui *et al.*, 2004)

Nodule numbers on the legume root system are under strict control from environmental factors, e.g. nitrogen availability, as well as an internal autoregulation system controlled through receptor-like kinases acting in the shoot (Reid *et al.*, 2011; Mortier *et al.*, 2012). When rhizobia first infect the root system, they induce the formation of regulatory plant peptides of the CLE family, which are transported to the shoot, interact with a receptor-like kinase and thereby generate an inhibitory signal that moves back to the root to limit further nodule initiation (Delves *et al.*, 1986; Okamoto *et al.*, 2013). The receptor-like kinase has been identified in several legumes, including the model legume *Medicago truncatula*, where it was named *SUNN* (*SUPER NUMERIC NODULES*) (Schnabel *et al.*, 2005). The *sun* mutant and other autoregulation mutants are characterised by the formation of excessive numbers of nodules, typically associated with a smaller shoot

and root system (Schnabel *et al.*, 2005; Penmetsa *et al.*, 2003). In addition, nodule numbers are controlled through ethylene signalling, and ethylene-insensitive mutants also show excessive nodule numbers, although this is root- and not shoot-determined. For example, the ethylene insensitive *sickle* mutant of *M. truncatula* hypernodulates, and this is likely due to reduced defence responses during root and nodule infection (Penmetsa *et al.*, 1997; 2003; 2008).

In Chapter 3 it was observed that certain AHLs specifically increased nodule numbers in the model legume *M. truncatula* (*cf.* Figure 3.9). This response was nodulation-specific while root length and lateral root density were not altered by the same treatment. This Chapter explored in more detail the possible mechanism behind the AHL effect on nodulation, focusing mainly on compounds that produced a strong nodule numbers response. In particular, it was tested whether AHLs from the rhizobial symbiont of *M. truncatula*, *Sinorhizobium meliloti*, would exert specific nodulation-related responses in its host. It was also tested whether the responses are restricted to *M. truncatula* or can be detected in other legumes.

## 4.3 Results

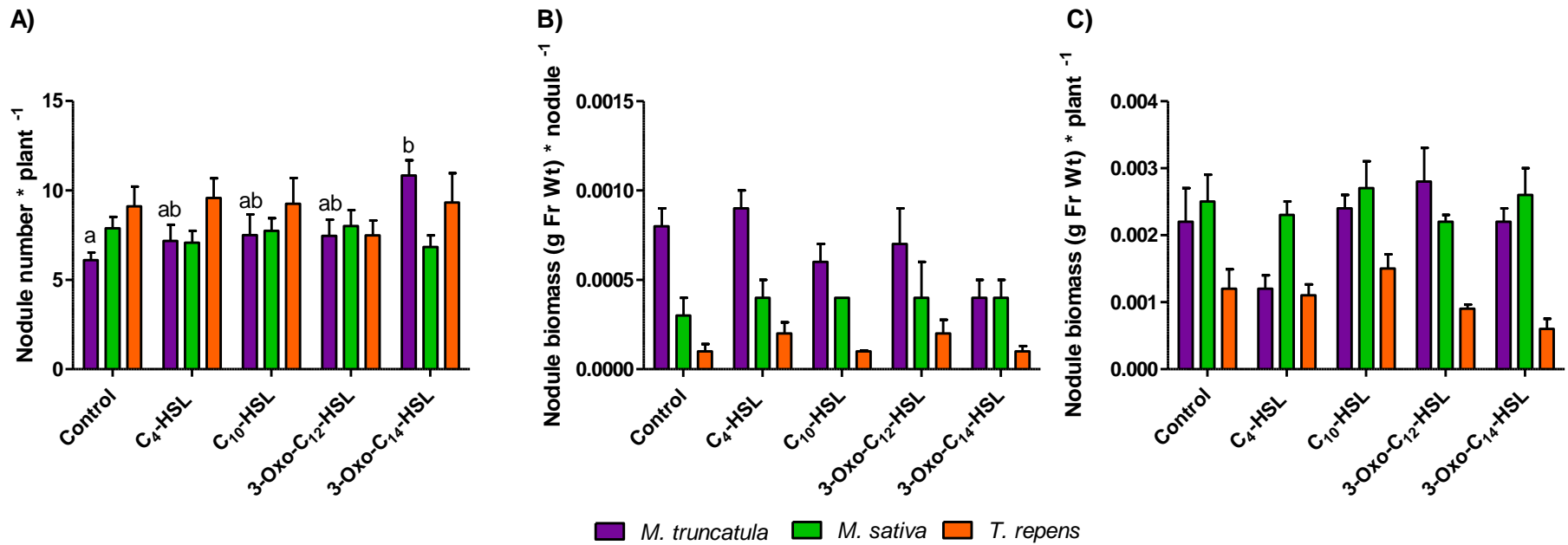
### 4.3.1 Effects of AHLs on nodulation and root architecture of different legumes

To test whether the observed responses on nodule numbers and root architecture in *M. truncatula* were conserved in other legumes, we conducted an experiment in which we compared *M. truncatula* (barrel medic), *M. sativa* (alfalfa), which also nodulates with *S. meliloti*, and *T. repens* (white clover), which nodulates with *Rhizobium leguminosarum*, *bv. trifolii*. This experiment was continued with only four AHLs that showed the most prominent changes in nodule numbers in the initial screening experiment (*cf.* Figure 3.9A). C<sub>10</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL from *S. meliloti* and C<sub>4</sub>-HSL and 3-oxo-C<sub>12</sub>-HSL from *P. aeruginosa* were selected. Interestingly, only *M. truncatula* showed significant differences in nodule numbers between AHL treatments (1 µM), with no significant effects in the other two legumes ( $p < 0.05$ ; Figure 4.1A). Only 3-oxo-C<sub>14</sub>-HSL caused a statistically significant increase in nodule numbers in *wild type* seedlings (Figure 4.1A). There were no significant differences in nodule biomass per nodule (Figure 4.1B), nodule biomass per plant (Figure 4.1C), or shoot and root dry biomass (Figure 4.2) in any of the three legumes. We noted that biomass per nodule was reduced with the 3-

oxo-C<sub>14</sub>-HSL treatment, similar to results shown in Chapter 3 (*cf.* Figure 3.9), but this decrease was not statistically significant in this experiment.

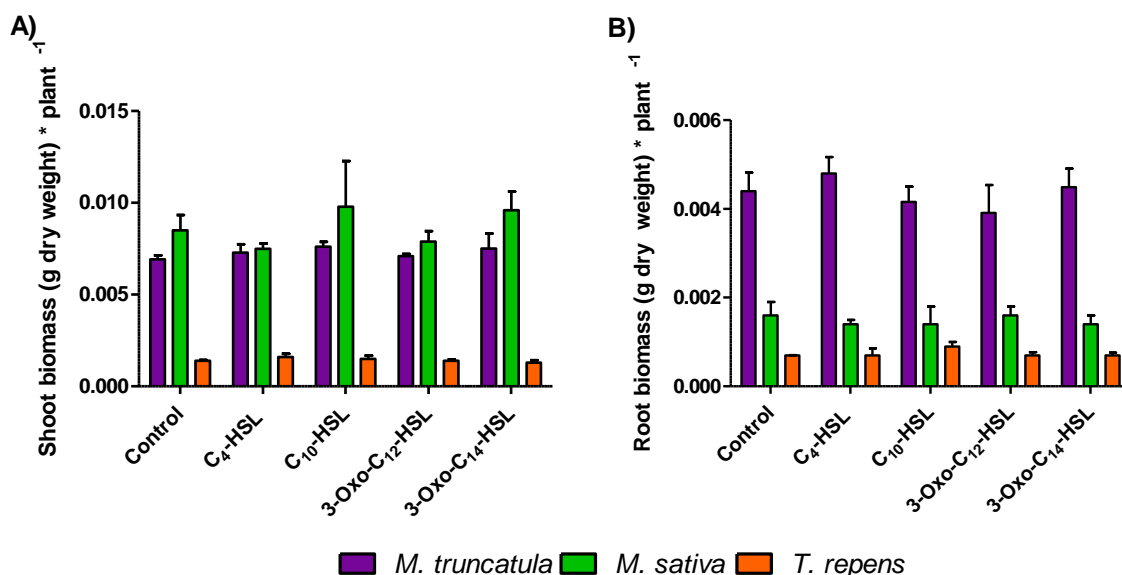
Root length was also unaffected by AHL treatment (Figure 4.3A), whereas lateral root density was significantly altered by AHLs in *T. repens*, but not *M. truncatula* or *M. sativa* ( $p < 0.05$ ; Figure 4.3B). While there were no significant differences between the control and any of the AHL treatments in *T. repens*, treatments with C<sub>10</sub>-HSL and 3-oxo-C<sub>12</sub>-HSL resulted in significantly different lateral root density.

Indeterminate and determinate nodules have differences in their developmental program (Terpolilli *et al.*, 2012). For this reason, we also wanted to evaluate the effect of AHLs on a model legume forming determinate nodules, *Lotus japonicus*. It has been reported that C<sub>12</sub>-HSL and C<sub>10</sub>-HSL are produced by *Mesorhizobium loti*, one of the *L. japonicus* symbionts (Yang *et al.*, 2009). However, no significant differences were found in nodule numbers of *L. japonicus* treated with different AHLs (Figure 4.4).

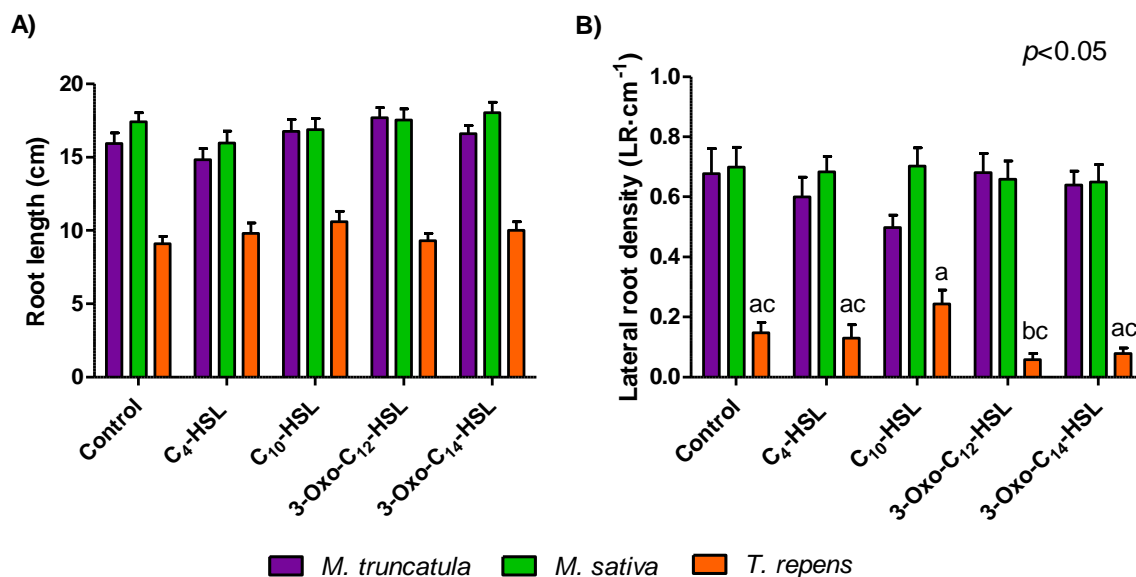


**Figure 4.1** Effect of AHLs on nodulation in three legume species 21 days after inoculation. *M. truncatula*, *M. sativa* and *T. repens* were treated with 1  $\mu\text{M}$  AHLs and inoculated with their respective nodulation rhizobia. A) Nodule number per plant. B) Nodule biomass (in grams of fresh weight) per plant. C) Nodule biomass (in grams of fresh weight) per nodule. Data points indicate mean  $\pm$  SE ( $n = 25-30$ ). In panel A), treatments of *M. truncatula* plants that do not share a common letter are significantly different at  $p < 0.05$  (one-way ANOVA with Tukey post-test). Differences in panels B) and C) were not significant at  $p < 0.05$ .

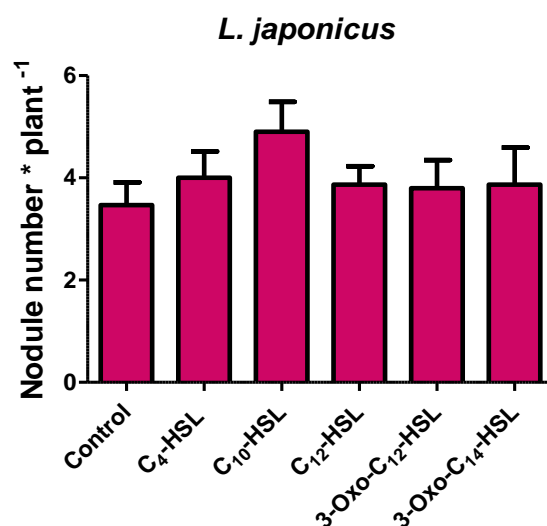




**Figure 4.2** Effect of AHLs on the shoot and root biomass of different legume species 21 days after inoculation. *M. truncatula*, *M. sativa* and *T. repens* were treated with 1  $\mu\text{M}$  AHLs and inoculated with *S. meliloti*. A) Shoot dry biomass (g) B) Root dry biomass (g). Data points indicate mean  $\pm$  SE ( $n = 25-30$ ). No significant differences at  $p < 0.05$  level (one-way ANOVA with Tukey post-test).



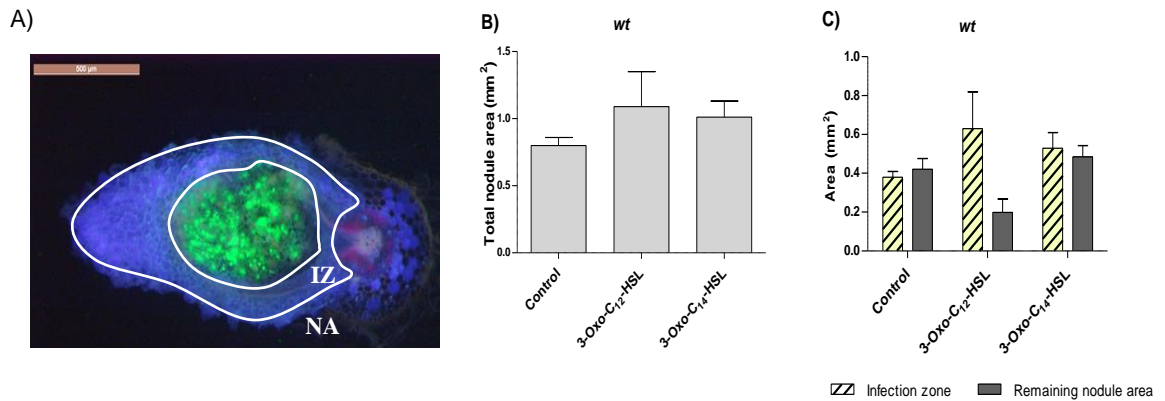
**Figure 4.3** Effect of AHLs on root architecture of different legume species 21 days after inoculation. *M. truncatula*, *M. sativa* and *T. repens* were treated with 1  $\mu\text{M}$  AHLs and inoculated with *S. meliloti*. A) Root length, B) Lateral root density. Data points indicate mean  $\pm$  SE ( $n = 25-30$ ). In panel B), treatments of *T. repens* plants that do not share a common letter are significantly different at  $p < 0.05$  (one-way ANOVA with Tukey post-test).



**Figure 4.4** Effect of AHLs on nodule numbers in *Lotus japonicus* 21 days after inoculation treated with 1  $\mu$ M AHLs and inoculated with *M. loti*. Data points indicate mean  $\pm$  SE ( $n = 10-17$ ). No significant differences were found at  $p < 0.05$  (Kruskal-Wallis with Dunn post-test).

#### 4.3.2 Effect of AHLs on nodule cross sectional area and nodule occupancy

AHLs affected nodule numbers exclusively in *M. truncatula* plants (*cf.* Figure 3.9A). Therefore, further nodulation experiments were done in the model legume *Medicago*. As nodule numbers were changed in response to AHLs, we evaluated other nodule phenotypes, including nodule cross sectional area and nodule occupancy. First, to test for changes in nodule occupancy by rhizobia, an experiment with *S. meliloti* Rm1021 strain expressing a constitutive GFP marker was carried out. Two AHLs, 3-oxo-C<sub>14</sub>-HSL from *S. meliloti*, which led to increased nodule numbers, and 3-oxo-C<sub>12</sub>-HSL from *P. aeruginosa*, which did not alter nodule numbers, were tested (*cf.* Figure 3.9A). Three week-old nodules were sectioned and the uninfected and infected nodule area in a section through the centre of each nodule were measured (Figure 4.5A). No statistically significant differences in total nodule area, remaining nodule area or infected nodule area between treatments were found (Figure 4.5B, C).



**Figure 4.5** Nodule area of *M. truncatula* treated with 1  $\mu\text{M}$  AHLs. Treatments correspond to 21 days after inoculation in *wild type M. truncatula* treated with 3-oxo-C<sub>12</sub>-HSL or 3-oxo-C<sub>14</sub>-HSL. A) Magnification of a nodule containing GFP labelled *S. meliloti* 1021 showing Nodule Area (NA) and Infection Zone (IZ) of 3 week-old *M. truncatula* seedlings. Magnification bar indicates 500  $\mu\text{m}$ . The image was taken after exposure to blue light ( $\sim 470$  nm, emission 515 nm). Blue colour is indicated by flavonoids and green colour by GFP labelled rhizobia; B) Total nodule area; C) Infection zone and remaining nodule area. No significant differences at  $p < 0.05$  (One-way ANOVA with Tukey post-test). Data points indicate mean  $\pm$  SE, ( $n = 5-8$ ).

### 4.3.3 Effect of AHLs on nitrogenase activity and nodulation of *M. truncatula*

As no significant changes in nodule area and nodule occupancy were found, we further investigated whether the effect of AHL on nodule numbers affected nitrogen fixation. In addition, previously it was observed that the increase in nodule numbers decreased nodule biomass per nodule (*cf.* Figure 3.9A, B). Therefore, we evaluated whether the increase in nodule numbers accompanied with a decrease in nodule biomass caused changes in overall nitrogen fixation. To determine this, we measured the nitrogenase activity *in vivo*, by an acetylene reduction assay (ARA), in plants exposed to 1  $\mu\text{M}$  of 3-oxo-C<sub>14</sub>-HSL. For this experiment 3-oxo-C<sub>14</sub>-HSL was used as it induced the highest nodule numbers in previous experiments (*cf.* Figure 3.9A). This experiment was performed at the University of Sydney in the Faculty of Agriculture and Environment.

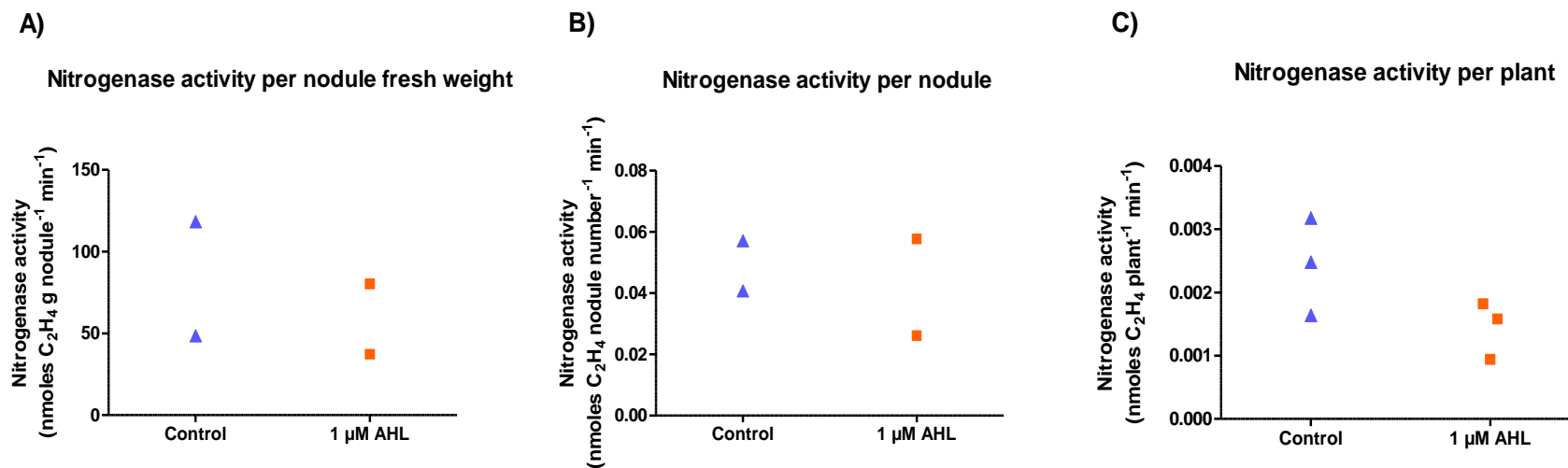
We could observe similar levels of nitrogenase activity in plants exposed to 3-oxo-C<sub>14</sub>-HSL compared to the control treatments (Figure 4.6). Unfortunately, we were not able to measure enough replicates to perform statistics for the nitrogenase activity per nodule and nitrogenase activity per nodule fresh weight (Figure 4.6A, B). The statistical

analysis for the nitrogenase activity per plant showed no significant differences between the AHL and control treatment (Figure 4.6C). The plants used in this assay were part of a larger experiment where we determined other plant phenotypes exposed to different concentrations of 3-oxo-C<sub>14</sub>-HSL (section 4.3.4). In this experiment plants exposed to 1 μM 3-oxo-C<sub>14</sub>-HSL did not form higher numbers of nodules than the control treatment (Figure 4.7C). Therefore, this experiment should be repeated before drawing firm conclusions.

#### **4.3.4 Effect of different concentrations of AHL on plant phenotype of *M. truncatula***

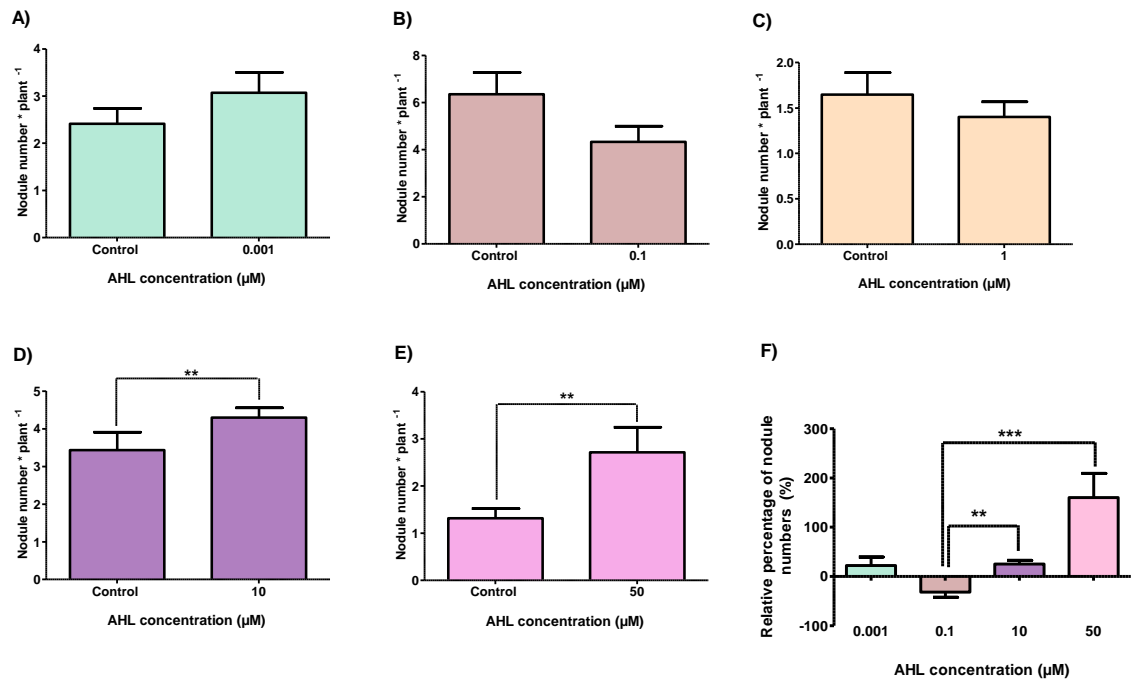
AHLs have been shown to have a concentration dependent effect on plant performance (e.g. Figure 3. 12) (von Rad *et al.*, 2008; Mathesius *et al.*, 2003; Gao *et al.*, 2012). Thus, we used different concentrations of 3-oxo-C<sub>14</sub>-HSL to investigate whether a range of AHL concentrations affected plant phenotype, including nodulation. A range of concentrations most likely to be found in natural conditions in the rhizosphere (from 0.001 μM to 10 μM) were used as well as a high concentration likely to be found in small dense patches of bacterial colonies or biofilms on root surfaces (50 μM) (hot spots) (Palmer *et al.*, 2014; Popat *et al.*, 2012).

The effect of 3-oxo-C<sub>14</sub>-HSL on nodule numbers was found to be concentration dependent. At low concentrations of AHLs such as 0.001 and 0.1 μM, nodulation was not significantly affected (Figure 4.7A, B). At higher concentrations (10 and 50 μM), nodule numbers increased significantly ( $p < 0.01$ ; Figure 4.7D, E). However, there was no a direct proportional relation between AHL concentration and nodule numbers. Plants exposed to 50 μM showed a significant increase in nodule numbers, up to ~ 2.7 times higher than the control ( $p < 0.01$ ; Figure 4.7E). Although no significant difference in nodule numbers was found at 1 μM in this specific experiment (Figure 4.7C), we used this AHL for further investigations as it showed to have the highest nodule numbers in a reproducible way along this project, and we wanted to avoid very high concentrations of AHLs that may not be physiologically relevant.

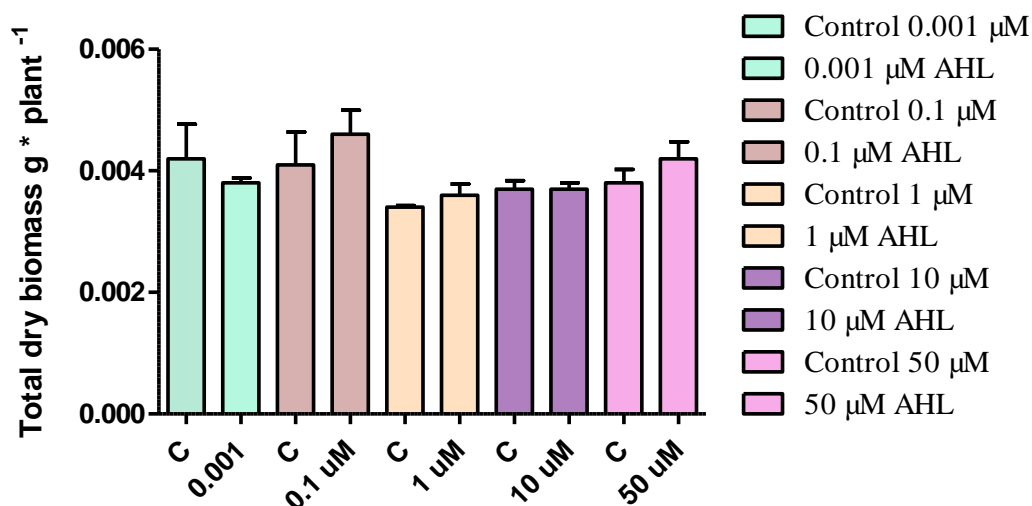


**Figure 4.6** The effect of the AHL 3-oxo-C<sub>14</sub>-HSL on nitrogenase activity (Nitrogen fixation rate) of *Medicago* plants 21 days after inoculation with *S. meliloti* after treatment with 1 μM and 10 μM. Controls were done for each of the AHL concentrations with the equivalent concentration of the solvent A) Nitrogenase activity per nodule fresh weight (g), B) Nitrogenase activity per nodule, C) Nitrogenase activity per plant. Data points indicate replicates per treatment. Due to insufficient number of replicates per treatment ( $n=2$ ), statistical analysis was not able to be performed in A) and B). However, a Student's t-test with no significant differences at  $p<0.05$  ( $n=3$ ) was performed between control and 1 μM AHL treatment for the nitrogenase activity per plant (C).

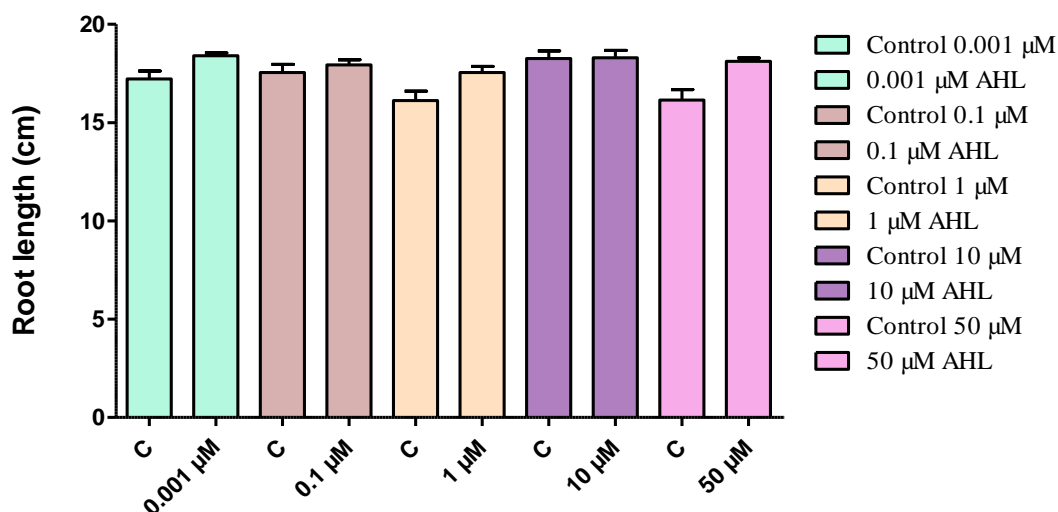
In Figure 4.7F, the relative percentage in nodule numbers in relation to their respective control is presented. The highest significant difference was found in plants treated with 50  $\mu\text{M}$  and 0.1  $\mu\text{M}$ . Plants treated with 0.1  $\mu\text{M}$  were significantly different from plants treated with 10  $\mu\text{M}$  ( $p < 0.01$ ; Figure 4.7F) but not from plants treated with 0.001  $\mu\text{M}$ . The number of nodules in the different control treatments varied considerably. Perhaps, the solvent had an effect on the plant physiology which can be seen at different concentrations for example; control treatments at 0.1  $\mu\text{M}$  and 50  $\mu\text{M}$  differ largely (Figure 4.7B, E). No significant differences were found in total dry biomass between *Medicago* 21 days after inoculating seedlings exposed to different concentrations of 3-oxo- $\text{C}_{14}$ -HSL (Figure 4.8). The highest total dry biomass was found in plants exposed to 0.1  $\mu\text{M}$  3-oxo- $\text{C}_{14}$ -HSL, even though this difference was not significant, and not in plants treated with either 10  $\mu\text{M}$  or 50  $\mu\text{M}$ , despite of significant increase in nodule numbers in plants with the same treatments (Figure 4.8). No significant differences were found in *Medicago* root length treated with different AHL concentrations when AHL treatments were compared to their respective controls (Figure 4.9). Likewise, no significant differences in lateral root density were found in any of the treatments in study (Figure 4.10). These results indicate that nodule numbers are modulated by 3-oxo- $\text{C}_{14}$ -HSL independent of other root architecture phenotypes and that this occurs at high but not low AHL concentration.



**Figure 4.7** The effect of a range of AHL concentrations on nodule numbers of *Medicago* plants 21 days after inoculation with 3-oxo-C<sub>14</sub>-HSL. A) 0.001 μM, B) 0.1 μM, C) 1 μM, D) 10 μM, E) 50 μM, F) Relative percentage of nodule numbers (%) normalized to their respective control. The different controls were adjusted to the appropriate dilution of solvent used for each AHL treatment. Different letters denote significant differences at  $p < 0.05$ . (Unpaired t-test with Welch's Test). Significant differences are indicated with asterisks. \*\*\* indicates significant differences at  $p < 0.001$  and \*\* at  $p < 0.01$ . (Kruskal-Wallis Test with Dunn's Multiple Comparisons Test). Data points indicate mean  $\pm$  SE ( $n = 28-30$ ).

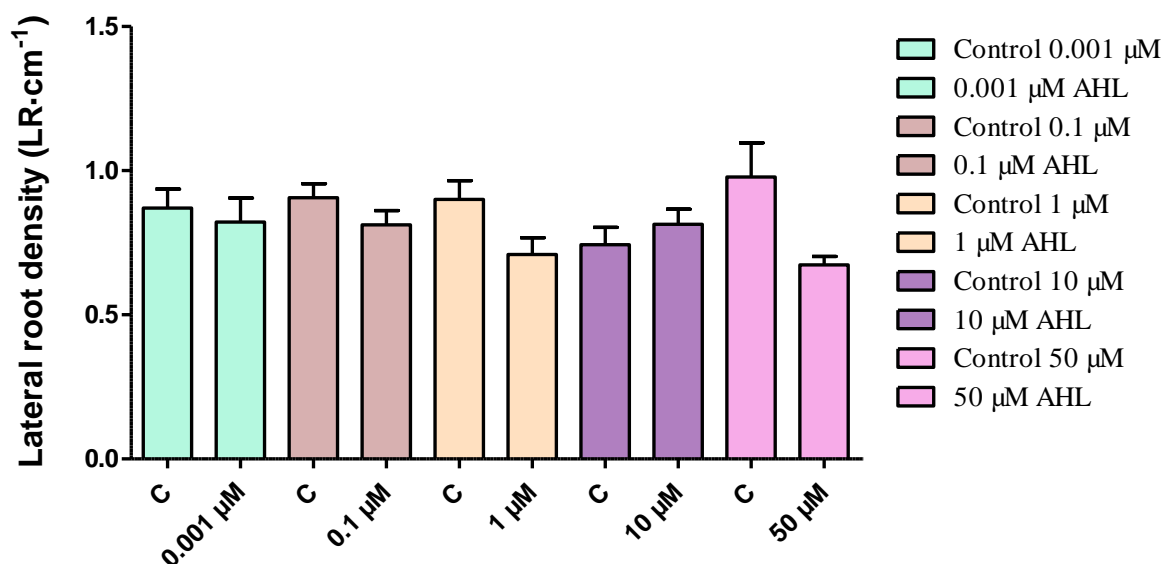


**Figure 4.8** The effect of 3-oxo-C<sub>14</sub>-HSL on total dry biomass per plant of *Medicago* plants 21 days after inoculation with 0.001 μM, 0.1 μM, 1 μM, 10 μM and 50 μM. The different controls were adjusted to the appropriate dilution of solvent used for each AHL treatment (Kruskal-Wallis Test with Dunn's Multiple Comparisons Test). Data points indicate mean ± SE ( $n = 3$ ). None of the means differed significantly with their respective control at  $p < 0.05$ .



**Figure 4.9** The effect of 3-oxo-C<sub>14</sub>-HSL on root length of *Medicago* plants 21 days after inoculation with 0.001 μM, 0.1 μM, 1 μM, 10 μM and 50 μM. The different controls were adjusted to the appropriate dilution of solvent used for each AHL treatment (Kruskal-Wallis Test with Dunn's Multiple Comparisons Test). Data points indicate mean ± SE ( $n = 28-30$ ). None of the means differed significantly with their respective control at  $p < 0.05$ .





**Figure 4.10** The effect of 3-oxo-C<sub>14</sub>-HSL on lateral root density of *Medicago* plants 21 days after inoculation with 0.001 μM, 0.1 μM, 1 μM, 10 μM and 50 μM. The different controls were adjusted to the appropriate dilution of solvent used for each AHL treatment (Kruskal-Wallis Test with Dunn's Multiple Comparisons Test). Data points indicate mean ± SE ( $n = 28-30$ ). None of the means differed significantly at  $p < 0.05$ .

#### 4.3.5 Effects of AHLs on flavonoid production by *M. truncatula*

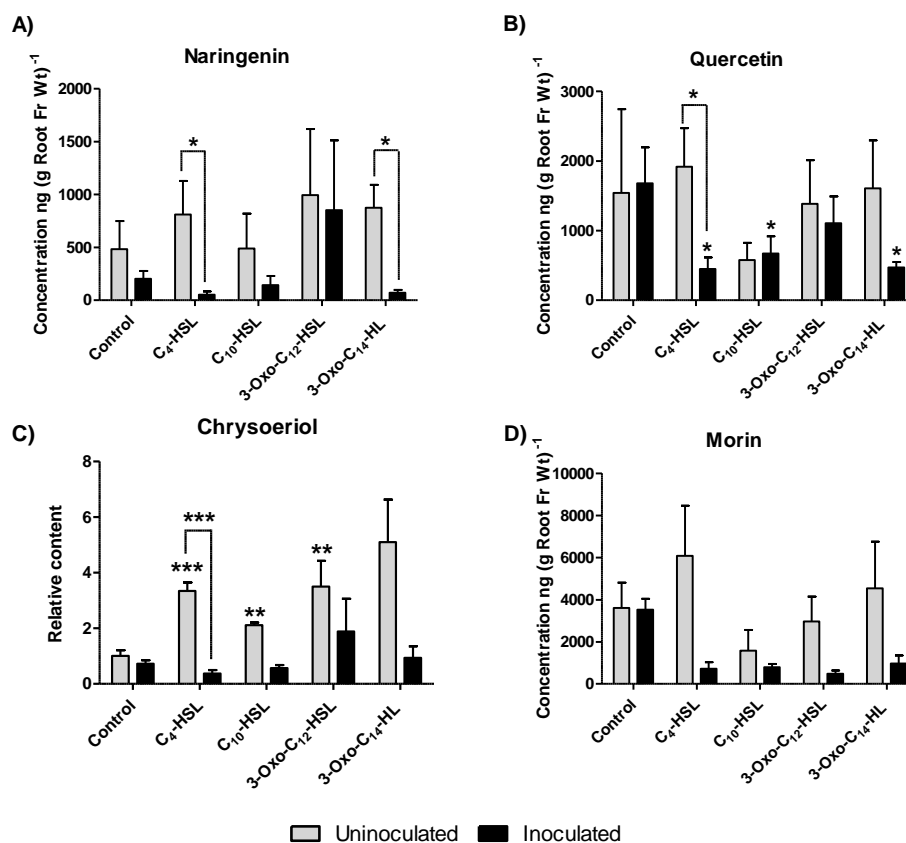
To investigate possible mechanisms for increased nodule numbers in 3-oxo-C<sub>14</sub>-HSL treated plants, we tested whether the AHL treatment could be associated with a different root flavonoid profile to increase *nod* gene inducing or *nod* gene inhibiting flavonoids as it has been seen in previous studies (e.g. Mathesius *et al.*, 2003; Zhang *et al.*, 2009; Ng *et al.*, 2015). We quantified the amounts of flavonoids extracted from roots of *M. truncatula* exposed to a subset of the previously tested AHLs (C<sub>4</sub>-HSL, C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL) using LC-ESI-QTOF MS/MS. Of these, only 3-oxo-C<sub>14</sub>-HSL had led to significant increase in nodule numbers (*cf.* Figure 3.9A, 4.1A). We hypothesized that an increase in nodule numbers is associated with an increase in the concentration of nod-gene inducing flavonoids, e.g. chrysoeriol (Hartwig *et al.*, 1990) and a reduction in nod-gene inhibiting flavonoids, e.g. medicarpin (Zuanazzi *et al.*, 1998), which can also act as a defence compound (Blount *et al.*, 1992; Guenoune *et al.*, 2001). In addition, increased concentrations of naringenin and quercetin have previously

been associated with successful nodulation and auxin transport control in *M. truncatula* (Ng *et al.*, 2015).

Naringenin, quercetin and morin content were quantified against an authentic internal standard, while the relative quantity of the rest of the flavonoids, for which standards were not available at the time, was estimated comparing the mass spectra of the flavonoid compounds with the mass bank database (Horai *et al.*, 2010) and the literature (Guo *et al.*, 2008; Li *et al.*, 2013).

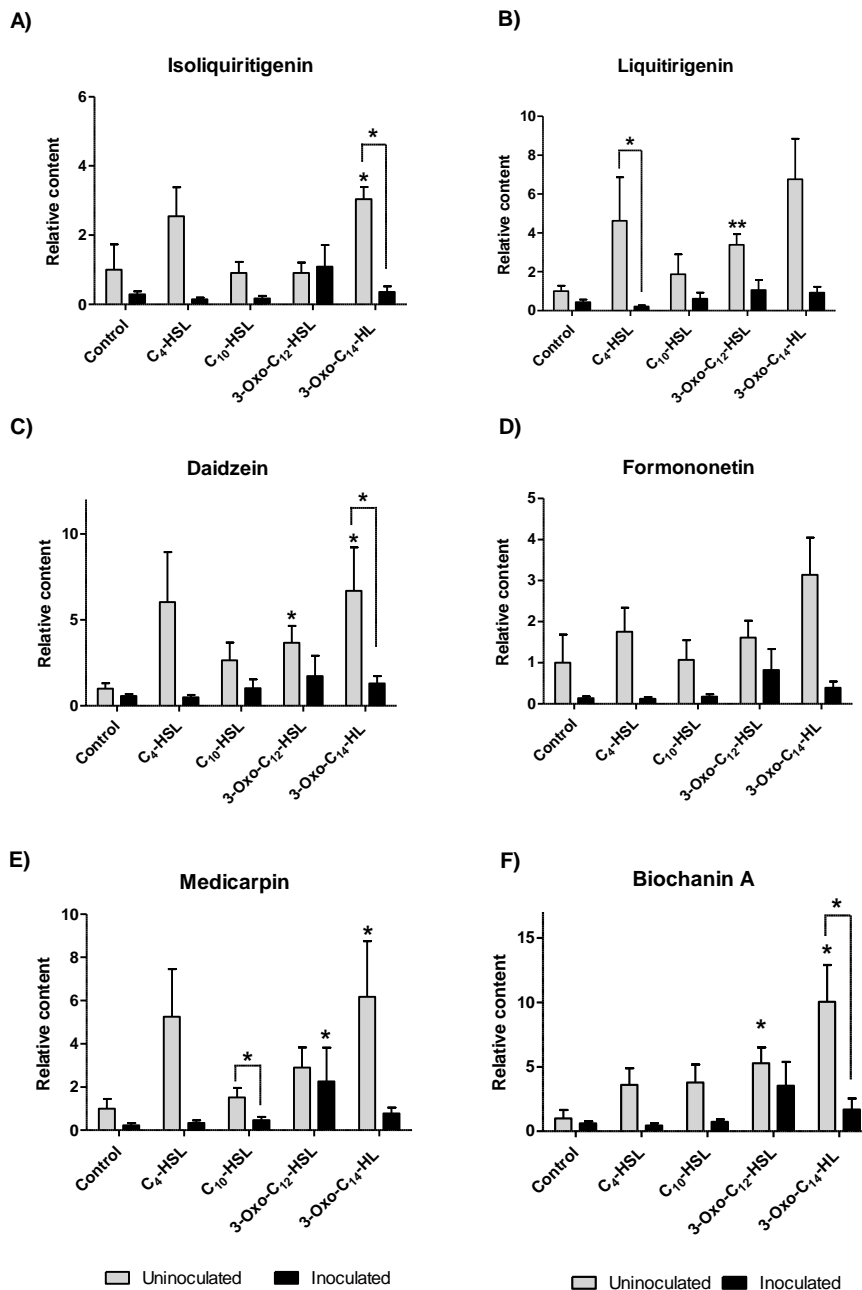
Roots were grown on AHL-containing Fåhræus agar for 24 h before inoculation or mock-inoculation with *S. meliloti* strain 1021 and harvested for analysis 24 h after inoculation. At this time point, flavonoid and auxin responses associated with nodule initiation have previously been detected in *M. truncatula* (Mathesius *et al.*, 2003; van Noorden *et al.*, 2007; Wasson *et al.*, 2006; Ng *et al.*, 2015). We found that flavonoids changed in relative abundance following the AHL application, with remarkable differences between *S. meliloti*-inoculated and mock-inoculated roots (Figures 4.11, 4.12). We detected the *nod* gene inducers chrysoeriol (Figure 4.11C) and isoliquiritigenin (Figure 4.12A) and the *nod* gene repressor medicarpin (Figure 4.12E) (Hartwig *et al.*, 1990; Zuannazzi *et al.*, 1998).

The concentrations of chrysoeriol and isoliquiritigenin did not increase in inoculated and AHL-treated roots, even though their concentrations did increase after AHL treatment alone. The concentration of medicarpin was significantly increased in 3-oxo-C<sub>12</sub>-treated roots compared to solvent control treated roots, but this did not correlate with a change in nodule numbers in the 3-oxo-C<sub>12</sub>-HSL treatment. The concentrations of the flavonol quercetin, which could act as an auxin transport inhibitor during nodulation (Zhang *et al.*, 2009; Ng *et al.*, 2015), was significantly reduced in inoculated roots treated with C<sub>4</sub>-HSL, C<sub>10</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL. However, only one of these treatments, 3-oxo-C<sub>14</sub>-HSL, increased nodule numbers. Overall, these results do not point to an increase in *nod* gene inducing flavonoids or a decrease in *nod* gene repressing flavonoids in *S. meliloti*-infected roots treated with AHLs that increased nodule numbers (3-oxo-C<sub>14</sub>-HSL) compared to those that did not. Similarly, no increase in flavonols that could act as auxin transport inhibitors during nodulation (Zhang *et al.*, 2009; Ng *et al.*, 2015) was found in treatments that increased nodule numbers.



**Figure 4.11** Effect of AHLs on flavanone, flavonol and flavone content in roots of *M. truncatula* four day-old seedlings treated with 1  $\mu$ M AHLs. A) naringenin (flavanone), B) quercetin (flavonol), C) chrysoeriol (flavone), and D) morin (flavonol). Significant differences between the treatments and the respective control are indicated with asterisks. \*\*\* indicates significant differences at  $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  (Student's t-test and Mann-Whitney-Wilcoxon test). Data points indicate mean  $\pm$  SE ( $n = 5$ ), i.e. five batches of roots with approx. 20 root segments per batch. g Root Fr Wt<sup>-1</sup> indicates gram per root fresh weight of the extracted root segments. Significant differences between inoculated and uninoculated plants are indicated with brackets.

Interestingly, AHL treatment in the absence of rhizobia led to the induction of several of the isoflavonoids and their precursors (liquiritigenin, daidzein, biochanin A and medicarpin; Figure 4.12) and the concentration of the flavone chrysoeriol (Figure 4.11C), while inoculation with *S. meliloti* generally attenuated the increases in flavonoid concentrations (Figure 4.12). In order to clarify these results we further evaluated some of the genes involved in the flavonoid pathway of *M. truncatula*.



**Figure 4.12** Effect of AHLs on isoflavonoid content of four day-old *M. truncatula*, seedlings treated with 1  $\mu$ M AHLs. A) isoliquiritigenin, B) liquiritigenin, C) daidzein, D) formononetin, E) medicarpin, F) biochanin A. Significant differences between the treatments and the respective control are indicated with asterisks. \*\* indicates significant differences at  $p < 0.01$  and \* indicates significant differences at  $p < 0.05$  level (Student's t-test and Mann-Whitney-Wilcoxon test). Data points indicate mean  $\pm$  SE ( $n = 5$ ), i.e. five batches of roots with approx. 20 root segments per batch. Significant differences between inoculated and uninoculated plants are indicated with brackets.

#### 4.3.6 Effects of AHLs on the regulation of flavonoid-related, defence-response and hormone-related gene expression in *M. truncatula*

There is a high level of interconnectivity between molecular pathways of primary and secondary metabolisms in order to ensure a successful nodulation. Plant hormones such as ethylene, auxins and cytokinins (Gage 2004; Desbrosses and Stougaard 2011) as well as flavonoids and accumulation of reactive species, among others, have been found to converge at different steps of the nodulation pathway (Amor *et al.*, 2003; Ferguson and Mathesius 2003). Auxin and cytokinin are two crucial hormones that regulate cell division, which are involved in nodule organogenesis (Kondorosi *et al.*, 2005; Mathesius, 2008). For example, there is an activation of cytokinin signalling in the cortical cells with an increase in cytokinins synthesis and concentration at the nodule initiation site leading to cortical cell division and nodulation gene expression (Cooper and Long, 1994; Mortier *et al.*, 2014; van Zeijl *et al.*, 2015). Furthermore, changes in auxin accumulation and auxin transport are needed for early nodule primordia and nodule initiation (Mathesius *et al.*, 1998; Mathesius, 2008) and these are under regulation of cytokinin signalling (Ng *et al.*, 2015). It has been reported that auxin is also involved in rhizobial root hair infection (Laplaze *et al.*, 2015). On the other hand, ethylene is involved in nodule number regulation, infection thread formation, nodule morphology and positioning (Oldroyd *et al.*, 2001; Guinel and Geil, 2002). Flavonoids, particularly flavones, exuded by legume roots can enhance nodulation by activating nodulation genes in rhizobia (Redmond *et al.*, 1986; Peck *et al.*, 2006). By contrast, some flavonoids, particularly isoflavonoids, inhibit nodulation gene expression (Zuanazzi *et al.*, 1998). Moreover, some flavonoids can modulate auxin transport (Santelia *et al.*, 2008; Ng *et al.*, 2015). In addition, there is evidence that during the early stages of nodulation, plant defence responses are induced and subsequently repressed (El Yahyaoui *et al.*, 2004; Zamioudis and Pieterse, 2012). Therefore, further experiments to determine changes in gene expression of flavonoid synthesis genes, plant defence response genes, nodulation genes and hormone responsive genes were conducted. *M. truncatula* seedlings were inoculated with *S. meliloti* or mock-inoculated and exposed to the same range of AHLs previously selected (C<sub>4</sub>-HSL, C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL, 3-oxo-C<sub>14</sub>-HSL). The expression of different defence-response genes and hormone-related genes were determined by qRT-PCR 24 hours after rhizobial inoculation. The genes tested are listed in Table 4.1.

**Table 4.1** Genes tested and biological processes influenced by their activity.

<b>Gene</b>	<b>Processes</b>	<b>Reference</b>
<b>Flavonoid synthesis</b>		
<i>Chalcone synthase (CHS)</i>	Initial step for flavonoid biosynthesis	Wasson <i>et al.</i> (2006) Zhang <i>et al.</i> (2009)
<i>Isoflavone synthase (IFS)</i>	Catalyses the synthesis of isoflavones	Jung <i>et al.</i> (2000)
<b>Plant-defence responses</b>		
<i>Peroxidase</i>	Catalyses the reduction of hydrogen peroxide, plant defence responses	Lambeth (2004)
<i>Glutathione S-transferase</i>	Plant defence responses, detoxification and metabolism	Edwards <i>et al.</i> (2000)
<i>Chitinase class III</i>	Hydrolysis of chitin, plant defence responses	Salzer <i>et al.</i> (2004)
<i>Pathogenesis related protein 5-1 (Prp)</i>	Plant defence responses	van Loon <i>et al.</i> (1994)
<b>Hormone-related</b>		
<i>Auxin responsive GH3 product</i>	Auxin-Regulated Transcription Auxin conjugation	Guilfoyle <i>et al.</i> (1993)
<i>Type A response regulator</i>	Cytokinin response	Kakimoto (2003)
<b>Nodulation</b>		
<i>LysM domain containing receptor like kinase 3</i>	Integral transmembrane receptor for carbohydrate-based signals	Riely <i>et al.</i> (2004)

Chalcone synthase (CHS) is the first enzyme in the flavonoid biosynthesis pathway (Tohge *et al.*, 2007). In inoculated plants, CHS expression was upregulated, with exception of 3-oxo-C<sub>14</sub>-HSL treatment, although this difference was not significant. Only the inoculated plants exposed to C<sub>10</sub>-HSL significantly increased CHS expression compared to the control and to the uninoculated plants ( $p < 0.001$ ; Figure 4.13A). Isoflavone synthase was significantly upregulated in uninoculated plants treated with 3-

oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL compared to the uninoculated control plants ( $p < 0.05$ ; Figure 4.13G). In addition, inoculated control plants exhibited a significant decrease in isoflavone synthase expression compared to uninoculated control plants. In contrast, the literature reports increased flavonoids after rhizobial inoculation (Zhang *et al.*, 2009; Harrison and Dixon, 1993, 1994; Ng *et al.*, 2015). Particularly, medicarpin was the only isoflavonoid induced in *Medicago* plants exposed to 3-oxo-C<sub>12</sub>-HSL after inoculation with *S. meliloti* (Figure 4.12E). Flavonoids can also be induced in response to pathogen infection (Makoi and Ndakidemi, 2007). It is also possible that the induction of IFS seen in uninoculated plants exposed to 3-oxo-C<sub>12</sub>-HSL and the induction of CHS in inoculated plants exposed to C<sub>10</sub>-HSL could be part of a defence response as well.

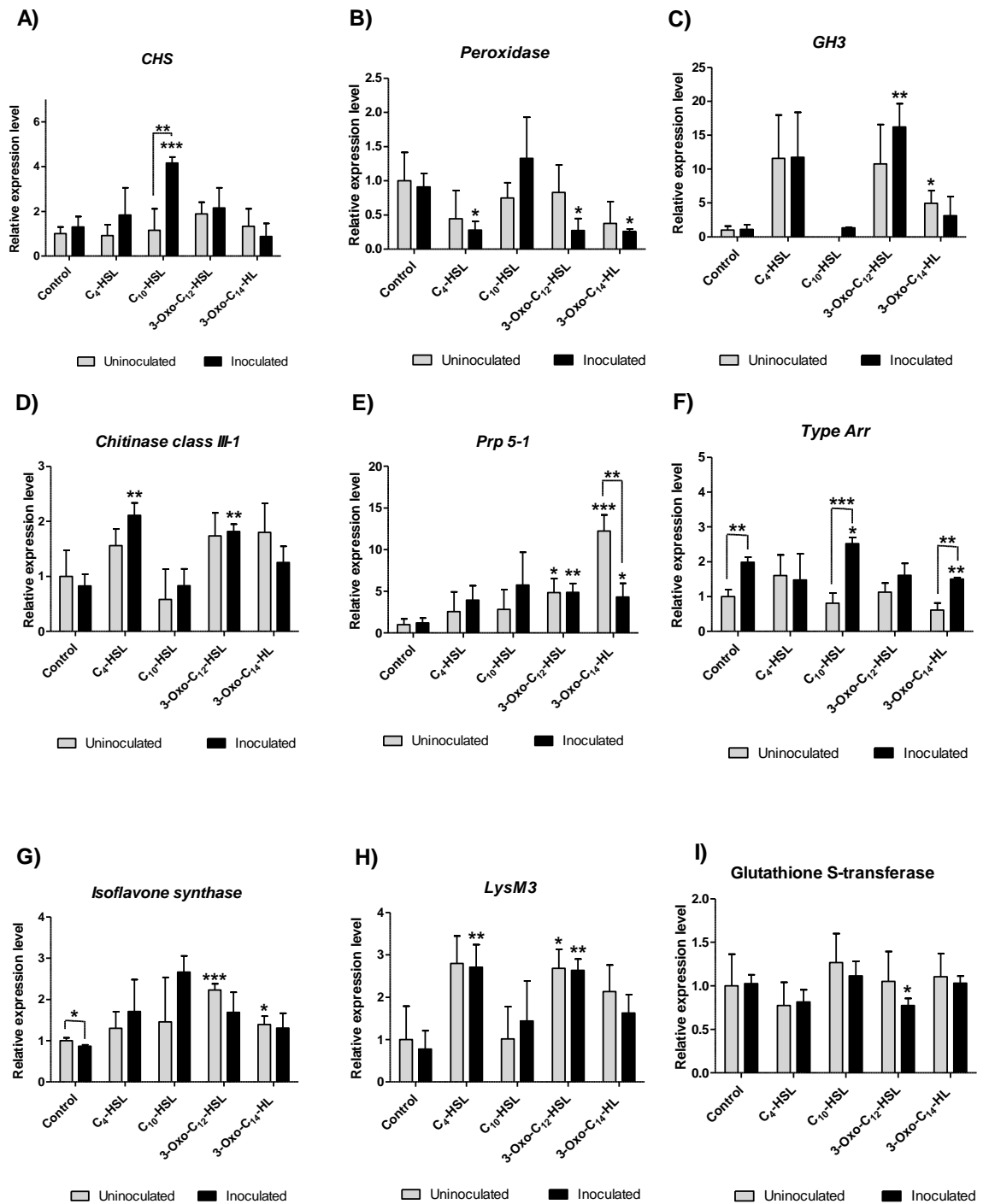
The auxin responsive *GH3* gene was significantly upregulated in inoculated plants exposed to 3-oxo-C<sub>12</sub>-HSL ( $p < 0.01$ ; Figure 4.13C), and in uninoculated plants treated with 3-oxo-C<sub>14</sub>-HSL (Figure 4.13C, F) indicating an induction of the action of auxin. However, contrary to our expectation (e.g. Ng *et al.*, 2015), *GH3* expression did not increase in response to rhizobia in any of the treatments. The cytokinin response gene *Type Arr* significantly increased its expression in inoculated plants exposed to 3-oxo-C<sub>14</sub>-HSL ( $p < 0.01$ ; Figure 4.13F). This was in support of our expectation, because a cytokinin response is part of a positive response during successful nodulation (e.g. van Zeijl *et al.*, 2015). However, this *ARR* gene was also induced by inoculation in the control and the C<sub>10</sub>-HSL-treated roots, so overall there was no clear relationship between its expression and the numbers of nodules induced.

The expression of plant stress and defence-related genes in inoculated plants treated with 3-oxo-C<sub>12</sub>-HSL was significantly upregulated with exception of glutathione-S-transferase and peroxidase, which were significantly downregulated ( $p < 0.05$ ; Figure 4.13B, D, E, I). Lohar *et al.*, (2006), reported that peroxidases did not show a strong pattern of regulation. However, eight peroxidase genes showed a marked induction at 2 and 24 hours post inoculation (hpi) in opposite to our results in which inoculated plants exposed to C<sub>4</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL significantly reduced peroxidase gene expression. Further, Lohar *et al.*, (2006), indicated that glutathione-S-transferase gene expression were mostly downregulated at 6 and 12 hpi although some were upregulated at 24 hpi. In our results no significant changes were observed in glutathione-S-transferase gene expression except in inoculated plants exposed to 3-oxo-C<sub>12</sub>-HSL, in which glutathione-S-transferase gene expression was significantly downregulated ( $p < 0.05$ ; Figure 4.13I). Lohar *et al.*, (2006), observed an overall

reduction of stress and defence genes during the first three days of the symbiosis (stage II, 6-12 hpi and stage IV, 72 hpi) in inoculated roots of *M. truncatula*. It has been reported by Salzer *et al.*, (2000) that the induction of Chitinase class III-1 in *M. truncatula* occurs in response to pathogens, and not to arbuscular mycorrhiza or *S. meliloti* inoculation. Interestingly, inoculated plants treated with C<sub>4</sub>-HSL and 3-oxo-C<sub>12</sub>-HSL, both derived from an opportunistic pathogen, significantly upregulated chitinase class III-1 gene expression in comparison with their control ( $p < 0.01$ ; Figure 4.13D). The rest of the treatments did not induce significant changes to chitinase class type III-1 expression.

LysM proteins constitute a recognition receptor of rhizobial Nod factors and they have been found crucial for the legume-rhizobia symbiosis (Gust *et al.*, 2012). In our study, LysM is a related lysine motif domain-containing receptor-like kinase3 (LysM-RLKs). Many of the LysM kinase family are induced in inoculated roots within hours in a Nod factor-dependent and ethylene regulated manner (Larrainzar *et al.*, 2015). However, 3-oxo-C<sub>14</sub>-HSL did not upregulate LysM significantly (Figure 4.13H). Overall, there was no consistent change in gene expression in any of the tested genes that could explain the increase in nodule numbers in 3-oxo-C<sub>14</sub>-HSL-treated roots compared to other treatments that did not increase nodule numbers. However, the selection of genes in this array was small and a broader range of genes could be measured in the future experiments.

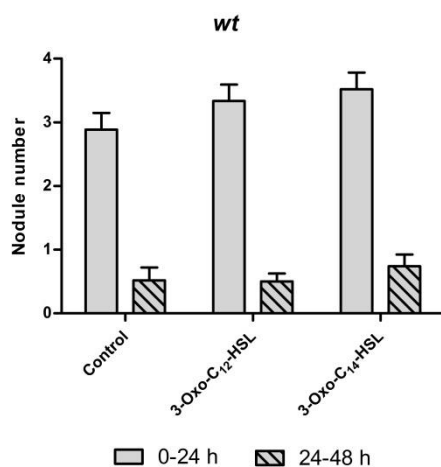




**Figure 4.13** Effect of AHLs on the relative level of gene expression of four days old *M. truncatula* seedlings, 24 h post inoculation, treated with 1  $\mu$ M AHLs. Significant differences between the AHL treatments and the respective control are indicated with asterisks. Significant differences between inoculated and uninoculated roots within each AHL treatment are indicated with asterisks on brackets. \*\*\* indicate significant differences at  $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ . Data points indicate mean  $\pm$  SE ( $n = 5$ ), i.e. five batches of roots with approx. 20 root segments per batch.

#### 4.3.7 Effects of AHLs on wild type and hypernodulation mutants of *M. truncatula*

Nodulation can be affected by two main biological components: the host and the rhizobia. In the case of the host, it is known that nodule numbers can be regulated at early stages of the nodulation pathway by formation of infection threads and control of defence responses that involve local ethylene signalling (Penmetsa and Cook, 1997), and at later stages by a molecular mechanism called autoregulation of nodulation (AON) (Krusell *et al.*, 2002; Penmetsa *et al.*, 2003; Reid *et al.*, 2011, Mortier *et al.*, 2012). The increase in nodule numbers by AHL could be influenced by the systemic autoregulation of nodulation (AON) mechanism. To test this, nodule numbers were counted on the root of wild type seedlings in the root segment corresponding to the nodulation zones at 0-24 h and 24-48 h after inoculation with *S. meliloti* (*cf.* Figure 2.1). Because of AON, nodules are initiated on roots of *M. truncatula* during first 24 h post inoculation, whereafter nodule numbers are reduced by systemic AON (van Noorden *et al.*, 2006). We found that treatment of roots with either 3-oxo-C<sub>12</sub>-HSL or with 3-oxo-C<sub>14</sub>-HSL led to the expected reduction of nodule numbers in the 24-48 h window, suggesting that the AHL treatment does not prevent AON (Figure 4.14).

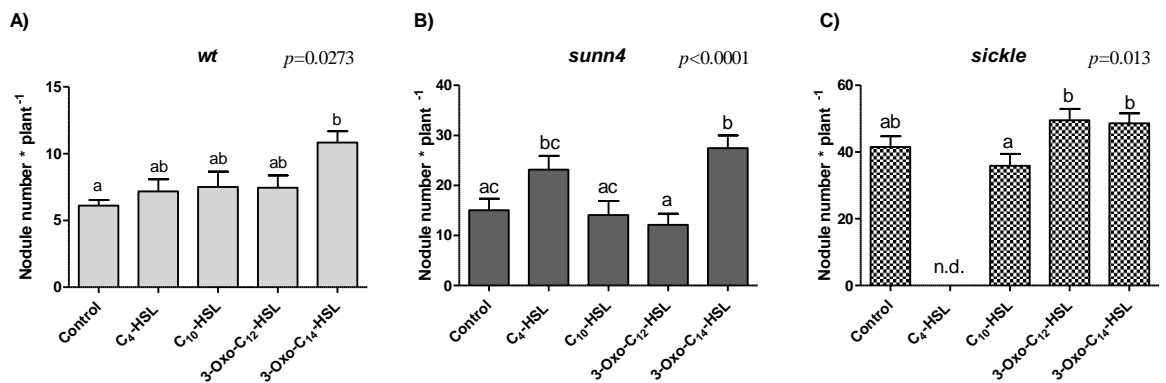


**Figure 4.14** Autoregulation of nodulation (AON) in *wild type M. truncatula* seedlings. Number of nodules formed after inoculation at two time points (0-24 h) and (24-48 h), for experimental setup see Figure 2.1. No significant differences at  $p < 0.05$  (Kruskal-Wallis test with Dunn's post-test). Data points indicate mean  $\pm$  SE, ( $n = 26-30$ ).

To further validate that AHL treatment does not increase nodule numbers by preventing AON, the effect of the selected subset of AHLs were tested on nodule numbers in the

systemic autoregulation mutant *sun4* (Penmetsa *et al.*, 2003). A significant increase in nodule numbers following treatment with 3-oxo-C<sub>14</sub>-HSL in the *sun4* mutant was found, similar to the *wild type* (Figure 4.15A, B), suggesting that this AHL increased nodule numbers independent of AON.

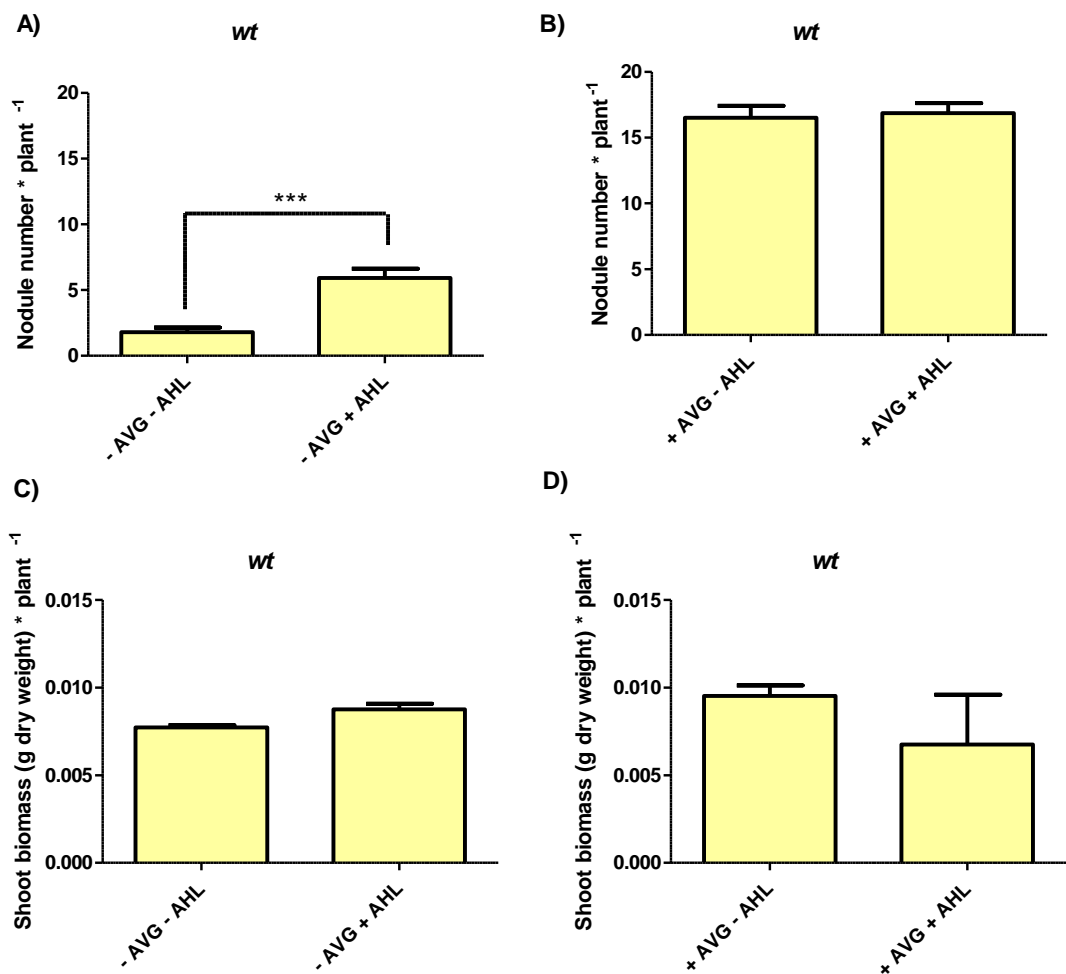
A further possibility to explain increased nodule numbers by AHLs is through reduced ethylene signalling. To test this, nodulation experiments in the ethylene-insensitive *skl* mutant were carried out (Penmetsa and Cook 1997). In this mutant, the significant increase in nodule numbers following 3-oxo-C<sub>14</sub>-HSL treatment was lost ( $p < 0.05$ ; Figure 4.15C). This suggests that ethylene signalling might be required for the increase in nodule numbers following 3-oxo-C<sub>14</sub>-HSL exposure. However, nodule numbers in both 3-oxo-C<sub>14</sub>-HSL- and control-treated *skl* mutants were a lot higher than in wild type or *sun4* mutants roots and may have reached a maximum for the root system.



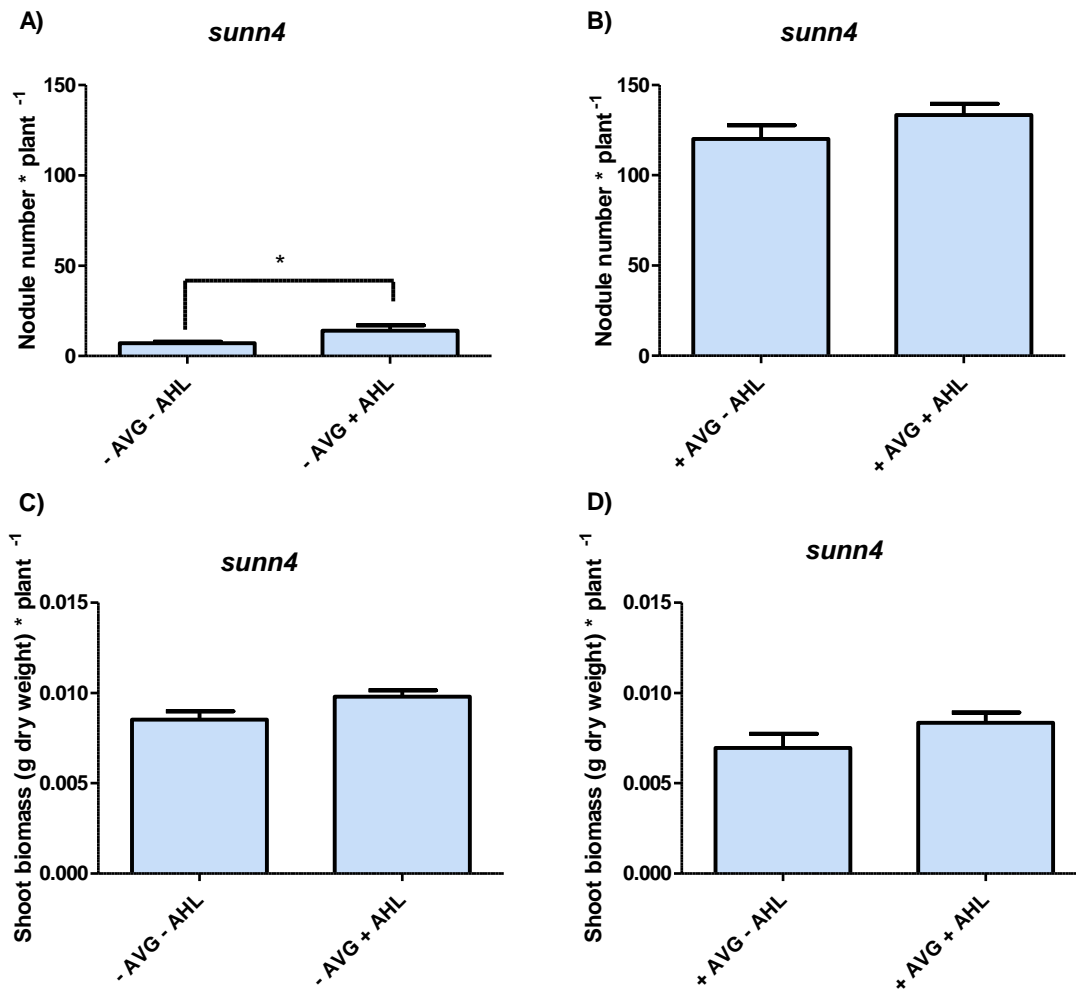
**Figure 4.15** Nodule numbers of supernodulating mutants at 21 days after inoculation. A) *wild type* (A17); B) *sun4* mutant; C) *sickle* mutant treated with 1  $\mu$ M of the indicated AHLs. Data points indicate mean  $\pm$  SE ( $n = 25-30$ ). Treatments that do not share a common letter are significantly different at  $p < 0.05$  (A and B: Kruskal-Wallis test with Dunn's post-test; C: One-way ANOVA with Tukey post-test). n.d.= no determined.

Moreover, it has been shown that inhibition of ethylene biosynthesis by Aminoethoxyvinyl-glycine (AVG) promotes nodulation in *wt M. truncatula* (Prayitno *et al.*, 2006a). Thus, in order to confirm the *skl* mutant results, *wt M. truncatula* plants

were treated with 0.1  $\mu\text{M}$  AVG, along with 3-oxo- $\text{C}_{14}$ -HSL. We chose 0.1  $\mu\text{M}$  since this concentration has been tested before on wild-type *M. truncatula* roots resulting in the optimum increase in nodulation Prayitno *et al.*, (2006a). 3-oxo- $\text{C}_{14}$ -HSL significantly increased nodule numbers in the treatments without AVG ( $p < 0.001$ ; Figure 4.16A). However, this significant increment of nodule numbers after AHL application did not occur in plants treated with AVG (4.16B). This suggests that ethylene might be involved in this nodule phenotypic response. Interestingly, shoot biomass was not significantly altered by AVG (Figure 4.16C, D). We also tested the response to AVG in the *sun4* mutant to test whether *sun4* would be able to form even more nodules. A significant increase in nodule numbers was found in the treatment without AVG exposed to 3-oxo- $\text{C}_{14}$ -HSL in comparison to its control (Figure 4.17A). Plants treated with AVG significantly increased their nodule numbers in comparison with plants without AVG ( $p < 0.05$ ; Figure 4.17B). The highest number of nodules reached by a plant treated with AVG plus AHL was up to 195 nodules with an average of 133 (Figure 4.18). This effect shows that the *sun4* mutant in the presence of AVG is capable of harbouring a larger number of nodules than the *skl* mutant. Interestingly, we observed nodules above the inoculation zone in AVG treated plants in the presence or in the absence of 3-oxo- $\text{C}_{14}$ -HSL. Notably, shoot biomass was not affected by the AHL treatment (Figure 4.17C, D).



**Figure 4.16** Effect of AHLs on nodule numbers and shoot biomass of *wt M. truncatula* plants 21 days after inoculation treated with 10  $\mu$ M of 3-oxo-C<sub>14</sub>-HSL and 0.1  $\mu$ M AVG. A, B) Nodule numbers per plant. C, D) Shoot biomass (g  $\times$  dry weight)  $\times$  plant<sup>-1</sup> of *wt Medicago*. Significant differences between the treatments and the respective control are indicated with asterisks. \*\*\* indicates significant differences at  $p < 0.0001$ , \* $p < 0.05$  (Student's t-test and Welch corrected t-test). Data points indicate mean  $\pm$  SE ( $n = 29-30$ ).



**Figure 4.17** Effect of AHLs on nodule numbers and shoot biomass of *sunn4* mutant *Medicago* plants 21 days after inoculation treated with 10  $\mu\text{M}$  of 3-oxo-C<sub>14</sub>-HSL and 0.1  $\mu\text{M}$  AVG. A, B) Nodule numbers per plant. C, D) Shoot biomass (g  $\times$  dry weight)  $\times$  plant<sup>-1</sup> of *sunn4* mutant. Significant differences between the treatments and the respective control are indicated with asterisks. \*\*\* indicates significant differences at  $p < 0.0001$ , \* $p < 0.05$  (Student's t-test and Welch corrected t-test). Data points indicate mean  $\pm$  SE ( $n = 29-30$ ).

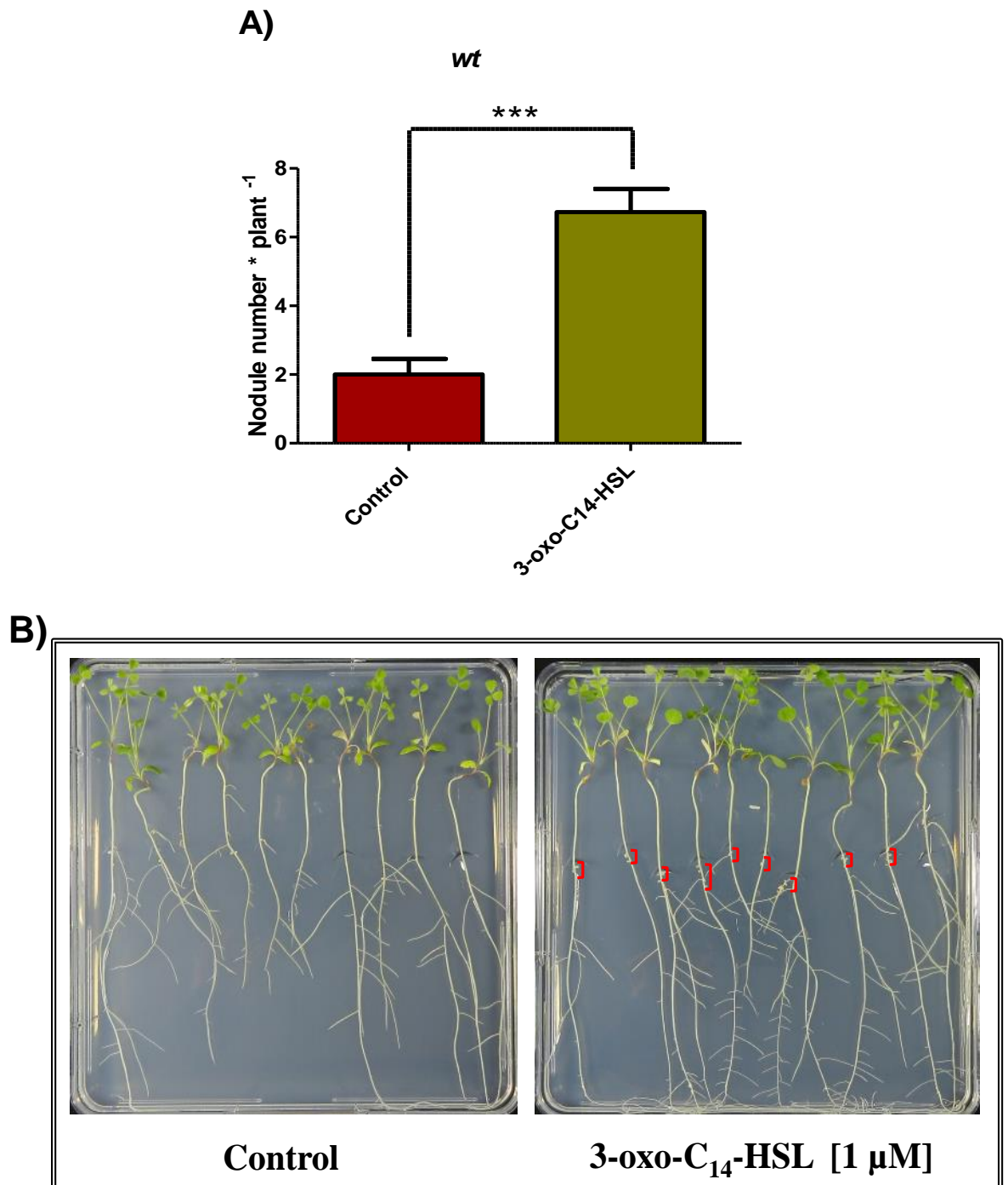


**Figure 4.18** Effect of AHLs on nodule numbers of *sunn4* mutant *Medicago* plants 21 days after inoculation treated with 10  $\mu$ M of 3-oxo-C<sub>14</sub>-HSL (AHL) and 0.1  $\mu$ M AVG. From left to right: *sunn4* mutant without AVG, without AHL; *sunn4* mutant without AVG, with AHL; *sunn4* mutant with AVG, without AHL and *sunn4* mutant with AVG, with AHL.

#### 4.3.8 Effects of AHLs on ‘priming’ of *Medicago truncatula*

Considering that ethylene has been found to control nodule numbers at early stages in the nodulation pathway (Penmetsa *et al.*, 2003; Guinel and Geil, 2002; Oldroyd *et al.* 2001), we proceeded to investigate whether the increase of nodule numbers observed by 3-oxo-C<sub>14</sub>-HSL was at early stages of the Nod factor signalling pathway. In our previous experiments, plants were grown on AHL-containing plates for the duration of the experiment and rhizobia inoculated onto the roots. Therefore, rhizobia as well as the plant were exposed to AHLs. As a consequence, it was tested whether the host or the rhizobia were responsible for the increase of nodule numbers by 3-oxo-C<sub>14</sub>-HSL via plant or bacterial perception of AHLs. In addition, it was also assessed whether the increase in nodule numbers by 3-oxo-C<sub>14</sub>-HSL occurred at early or later stages in the nodulation pathway. We ‘primed’ *wild type (wt) M. truncatula* plants by exposing seedlings to 1 µM 3-oxo-C<sub>14</sub>-HSL for four days and then we transferred the seedlings to a new plate without the compound. After seedling transfer we inoculated the plants with *S. meliloti*. We chose 3-oxo-C<sub>14</sub>-HSL since this compound produced the highest nodule numbers in *M. truncatula* (*cf.* Figure 3.9A). *Medicago* plants were primed with 3-oxo-C<sub>14</sub>-HSL at 1 µM. A highly significantly increased of nodule numbers in comparison with the non-primed or control plants was observed ( $p < 0.001$ ; Figure 4.19). This result suggests that the increase in nodule numbers occurs through direct plant perception and not through bacterial perception of this AHL. As observed previously (e.g. Figure 4.14), the nodules all formed in a small cluster at the inoculation site (Figure 4.19). This suggests that the increase in nodule numbers was likely due to increased numbers of successful infections early during the nodulation program, and this would be consistent with a temporary reduction of ethylene synthesis or signalling at the early stages of nodulation. We conducted further experiments in order to examine the molecular mechanism behind these observations.





**Figure 4.19** Priming experiment on *wt M. truncatula* plants 21 days after inoculation with 1 μM AHL. A) Treated plants were exposed for four days to 3-oxo-C<sub>14</sub>-HSL while mock-treated plants were exposed only to the solvent as controls. Data points indicate mean ± SE ( $n = 30$ ). Kruskal-Wallis Test with Dunn's Multiple Comparisons Test,  $p < 0.001$ ). B) Picture showing non-primed (control) and primed *M. truncatula* plants 21 days after inoculation. The brackets indicate the narrow zone where nodules were formed.

#### 4.3.9 Effects of AHLs on the regulation of nodulation and ethylene-related gene expression in *wt M. truncatula*

Taking into consideration that the increase in nodule numbers in plants treated with 3-oxo-C<sub>14</sub>-HSL is caused by plant and not rhizobial AHL perception, we decided to study the possible mechanisms that regulate nodulation in the host. In previous studies, exposure of plants to purified or synthetic AHLs led to the discovery that plants respond specifically to these bacterial signals (Mathesius *et al.*, 2003), altering plant defence and stress responses. In addition it has been reported that AHLs mediate plant defence responses towards pathogens in tomato and *A. thaliana* (Schuhegger *et al.*, 2006; Schikora *et al.*, 2011; Schenk *et al.*, 2012; Zarkani *et al.*, 2013; Schenk *et al.*, 2014). As determined in previous studies, nodule numbers are controlled through ethylene signalling, and ethylene-insensitive mutants also show excessive nodule numbers, although this is root- and not shoot-determined. For example, the ethylene insensitive *sickle* mutant of *M. truncatula* hypernodulates, and this is likely due to reduced defence responses during root and nodule infection (Penmetsa and Cook, 1997; 2003; 2008). Prayitno *et al.*, (2006) reported that in the *skl* mutant several proteins (ascorbate peroxidase (APX), 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO)) were induced after ACC treatment in the *wt* but not in *skl* mutants. Thus, ACO and APX are ethylene induced proteins (Table 4.2). In order to test this assumption and whether expression of the genes encoding these proteins is affected by AHL signalling, the relative expression of several ethylene inducible genes, including *ACO* and *APX*, were tested (Table 4.2). *Wt M. truncatula* seedlings were exposed to 1 µM 3-oxo-C<sub>14</sub>-HSL and inoculated with *S. meliloti* or mock-inoculated. The expression of different nodulation genes and ethylene inducible genes was determined by qRT-PCR 24 hours after rhizobial inoculation.

As expected, all nodulation genes (*ENOD11*, *ENOD40*, *NSP1* and *NSP2*) were upregulated in the inoculated treatments (Figure 4.20E-H) although induction of *ENOD40* was not quite statistically significant. 3-oxo-C<sub>14</sub>-HSL caused a strong increase in *NIN* expression in inoculated seedlings in comparison to the control ( $p=0.013$ ; Figure 4.20D). *NIN* is an early nodulin gene required for bacterial entry and autoactive CCaMK-induced nodule organogenesis (Marsh *et al.*, 2007). 3-oxo-C<sub>14</sub>-HSL from *S. meliloti* strain Rm1021 induced *NIN* expression in inoculated seedlings but not in controls and uninoculated plants. No significant changes in the expression of *ENOD11*,

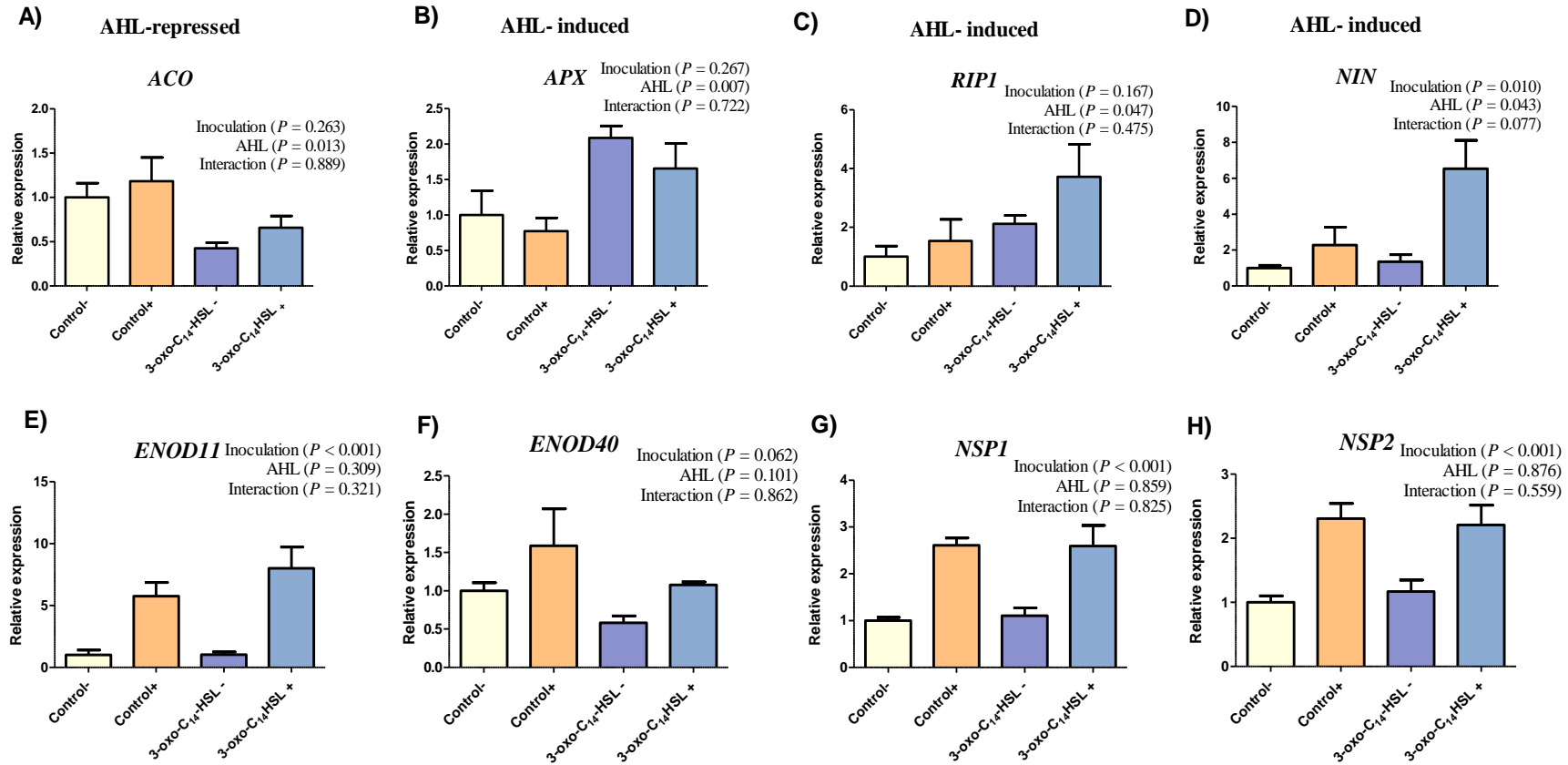
*ENOD40*, *NSP1* and *NSP2* were observed in response to AHL treatment ( $p>0.05$ ; Figure 4.20E-H).

Ramu *et al.*, (1999) indicated that the induction of a *Rhizobium-induced peroxidase I* (*RIP1*) is a result of an initial oxidative burst at very early stage of the Nod factor pathway, particularly infection. Our results showed an increase in *RIP1* expression levels in roots exposed to 3-oxo-C<sub>14</sub>-HSL compared to roots exposed to the solvent (controls), particularly inoculated roots ( $p=0.047$ ; Figure 4.20C). On the other hand, *ACO* was also induced in inoculated roots but was reduced in roots exposed to 3-oxo-C<sub>14</sub>-HSL ( $p=0.013$ ; Figure 4.20A). This suggests that 3-oxo-C<sub>14</sub>-HSL reduces ethylene synthesis as *ACO* catalyses the synthesis of ethylene from ACC substrate. *APX* is involved in ROS responses as it detoxifies H<sub>2</sub>O<sub>2</sub> (Caverzan *et al.*, 2012). Differential quantification of *APX* protein levels revealed no changes at 24 hpi between the *wt M. truncatula* and the *skl* mutant (Prayitno *et al.*, 2006). Roots treated with 3-oxo-C<sub>14</sub>-HSL presented an increased *APX* expression ( $p=0.007$ ; Figure 4.20B) suggesting that there is an increase of ROS in plants exposed to 3-oxo-C<sub>14</sub>-HSL.

**Table 4.2** Genes tested and biological processes influenced by their activity.

<b>Gene</b>	<b>Processes</b>	<b>Reference</b>
<b>Nodulation</b>		
<i>NSP1</i> ( <i>GRAS-type nodulation signalling pathway1</i> )	Symbiotically induced genes, responsive to cytokinin signalling	Smit <i>et al.</i> , (2005)
<i>NSP2</i> ( <i>GRAS-type nodulation signalling pathway2</i> )	Symbiotically induced genes, responsive to cytokinin signalling	Kaló <i>et al.</i> , (2005)
<i>NIN</i> ( <i>Nodule inception</i> )	Symbiotically induced genes, responsive to cytokinin signalling	Schauser <i>et al.</i> , (1999)
<i>ENOD11</i> ( <i>Early nodulin11</i> )	Symbiotically induced genes	Kouchi <i>et al.</i> , (1993)
<i>ENOD40</i> ( <i>Early nodulin40</i> )	Symbiotically induced genes	Asad <i>et al.</i> , (1994); Penmetsa <i>et al.</i> , (2003)
<b>Ethylene and defence responses</b>		
<i>ACO</i> ( <i>ACC oxidase (1-aminocyclopropane-1-carboxylate oxidase)</i> )	Ethylene biosynthetic process, Ethylene inducible gene	Yim <i>et al.</i> , (2014), Prayitno <i>et al.</i> , (2006)
<i>APX</i> ( <i>L-ascorbate peroxidase</i> )	Detoxification of ROS, ethylene inducible gene	Caverzan <i>et al.</i> , (2012), Prayitno <i>et al.</i> , (2006)
<i>RIP1</i> ( <i>Rhizobium-induced peroxidase1</i> )	Plant defence response, ethylene repressed gene, symbiosis	Cook <i>et al.</i> , (1995); Penmetsa <i>et al.</i> , (2003)

Abbreviations: ROS: reactive oxygen species.



**Figure 4.20** Effect of AHLs on the relative levels of gene expression in four days old *M. truncatula* seedlings treated with 1  $\mu\text{M}$  AHLs. REML test  $p < 0.05$  with Bonferroni's post-test. No post-hoc test when interaction is not significant. Data points indicate mean  $\pm$  SE ( $n = 5$ ). Inoculation: Rhizobia inoculated; AHL: with or without 3-oxo-C<sub>14</sub>-HSL; Interaction: Inoculation  $\times$  AHL. +: inoculated plants, -: uninoculated plants.

## 4.4 Discussion

This Chapter aimed at finding out whether exposure of legumes to AHLs from their symbionts, as opposed to those from non-symbionts, alters nodulation. This question arose from findings in Chapter 3 that *Medicago* plants exposed to AHLs specifically alter nodule numbers. Previous studies showed that gene and protein expression was altered by AHLs (e.g. Mathesius *et al.*, 2003; von Rad *et al.*, 2008), suggesting that plants interpret signals from surrounding bacteria that could alter the outcome of plant-microbe interactions (Hartmann *et al.*, 2014). For example, exposure of plants to AHLs has been shown to alter the outcome of plant-pathogen interactions (e.g. Schuhegger *et al.*, 2006; Schikora *et al.*, 2011; Schenk *et al.*, 2014).

3-oxo-C<sub>14</sub>-HSL specifically synthesised by its symbiont *S. meliloti*, repeatedly increased nodule numbers on *M. truncatula* roots. Interestingly, a comparison of nodulation phenotypes in four legume species showed that at the concentration of 1 µM used in these experiments, only *M. truncatula* showed significant changes in nodule numbers in response to AHLs, despite *M. sativa* nodulating with the same symbiont. It is possible that *M. sativa* responds more or less sensitively to AHLs, and future experiments could test a range of AHL concentrations in legumes to determine whether species show differences in the sensitivity to AHLs. The fact that *T. repens* responded with changes in lateral root numbers to 3-oxo-C<sub>12</sub>-HSL, while the other two legumes did not, suggests that different root phenotypes could respond to different thresholds and/or structures of AHLs. *A. thaliana* strongly responds to certain AHLs with changes in root growth and lateral root numbers (Ortíz-Castro *et al.*, 2008; Bai *et al.*, 2012; Liu *et al.*, 2012; Palmer *et al.*, 2014). Experiments in *M. truncatula* have shown that the AHL C<sub>10</sub>-HSL, which strongly reduces root growth in *A. thaliana*, does not inhibit root growth in *M. truncatula* at concentrations of 1 or 10 µM (See Chapter 3), suggesting that these responses are species-specific. Certain AHLs did affect root elongation in *M. truncatula* in a study by Palmer *et al.* (2014), and this, as well as other AHL responses, was highly concentration dependent. Our results from testing different 3-oxo-C<sub>14</sub>-HSL concentrations showed that high concentrations, i.e. 10 µM and 50 µM, increased nodule numbers significantly while low concentrations, i.e. 0.001 µM or 0.1 µM, did not or not consistently. Therefore, plant responses to AHLs are concentration-dependent. Lateral root density and root length were not affected by different 3-oxo-C<sub>14</sub>-HSL

concentrations, which confirmed our hypothesis that the effect of AHLs is nodulation-specific.

Even though the increase in nodule numbers by 3-oxo-C<sub>14</sub>-HSL was accompanied by a reduction in nodule biomass (*cf.* Figure 3.9), nodules appeared normal in their infection with rhizobia. At the time point measured (21 days post-inoculation), root and shoot biomass showed no significant changes, although it is possible that the increase of nodule numbers only results in an increase of biomass after a longer time interval. The nitrogenase activity *in vivo* measured in plants treated with 1 μM 3-oxo-C<sub>14</sub>-HSL was not changed compared to the control plants. This result is consistent with previous studies of hypernodulating mutants of *Medicago* (16 days post-inoculation) where the nitrogenase activity (pmol ethylene min<sup>-1</sup> root<sup>-1</sup>) of the hypernodulating mutants *sun4*, *skl* and a double mutant (*sun4/skl*) was similar to the *wild type* (Penmetsa and Cook, 1997; Penmetsa *et al.*, 2003). As nitrogenase activity was measured at early developmental stages of *Medicago* ontogeny it is also possible that at later stages this parameter might change. In consequence, the total biomass per plant in the treatments was not affected. As the experiments were carried out with limited numbers of plants, they would need to be repeated for confirmation of these results.

To further investigate the mechanism behind the increase of nodule numbers of *M. truncatula* in response to AHLs, particularly 3-oxo-C<sub>14</sub>-HSL, we determined whether altered flavonoid concentrations, which could act as Nod gene regulators in rhizobia, were associated with higher nodule numbers in plants exposed to 3-oxo-C<sub>14</sub>-HSL. Several of the isoflavonoids significantly increased after root exposure to AHLs, but not when roots were inoculated with rhizobia at the same time. This supports earlier data on the induction of isoflavone reductase by AHLs in *M. truncatula* using proteomics (Mathesius *et al.*, 2003). AHL-treated and *S. meliloti* inoculated roots did not show any increase or decrease in flavonoids in the roots that correlated with increased nodule numbers in 3-oxo-C<sub>14</sub>-HSL-treated roots. Therefore, currently there is no evidence that altered flavonoid profiles in the host roots could explain the alteration in nodule numbers in response to 3-oxo-C<sub>14</sub>-HSL.

To provide clarity whether the increase in nodule numbers was a direct effect of AHLs on *Medicago* plants or an indirect effect on rhizobia, we treated *wt Medicago* seedlings with 1 μM 3-oxo-C<sub>14</sub>-HSL for four days only (primed plants) or with solvent (non-primed plants). In this way we could investigate whether the increase in nodulation was

triggered at early stages of AHL exposure, before inoculation, or later stages, after inoculation with rhizobia. The results of this experiment revealed that *wt Medicago* plants that were ‘primed’ with 3-oxo-C<sub>14</sub>-HSL, significantly increased nodule numbers compared with non-primed plants. These findings indicate that 3-oxo-C<sub>14</sub>-HSL ‘primed’ plants before inoculation and that its direct effect on nodulation is on the plant and not on the symbiont. This is supported by previous studies that show evidence that AHLs also act as inter-kingdom signals (Hughes and Sperandio, 2008). Exposure of plants to purified or synthetic AHLs led to the discovery that plants respond specifically to these bacterial signals, and it has been speculated that this perception system may benefit the plant by sensing the presence and activity of nearby bacterial colonies and thus modifying its responses (Bauer and Mathesius, 2004; Teplitski *et al.*, 2010; Hartmann *et al.*, 2014).

We further used nodulation mutants of *M. truncatula* that are either defective in autoregulation of nodulation (AON), i.e. the autoregulation system reducing nodule numbers through systemic signalling (Schnabel *et al.*, 2005), or in ethylene signalling involved in regulation of nodule numbers through effects on defence responses (Penmetsa *et al.*, 1999; 2003; 2008) to investigate the possible mechanism behind the increase in nodule numbers by 3-oxo-C<sub>14</sub>-HSL. The AHL 3-oxo-C<sub>14</sub>-HSL still significantly increased nodule numbers in the AON mutant, *sun4*. This suggests that the increased numbers of nodules following 3-oxo-C<sub>14</sub>-HSL-treated roots are not a result of an inhibition of AON by this AHL. This agreed with a reduction of nodule numbers after 24 h post inoculation onward in 3-oxo-C<sub>14</sub>-HSL- as well as control-treated *wt* roots, the time window when the autoregulation signal is expected to travel from the shoot to the root to inhibit nodulation in *M. truncatula* (van Noorden *et al.*, 2006). However, 3-oxo-C<sub>14</sub>-HSL was not able to significantly increase nodule numbers in the ethylene-insensitive *skl* mutant, suggesting that the increase in nodule numbers, at least partly, involves ethylene signalling through EIN2. The lack of increase in nodule numbers in the *skl* mutant was not a result of the nodule forming capacity of the plant but an effect of ethylene. This result is supported by the fact that *sun4* plants treated with AVG were able to harbor ~ four times more nodule numbers than *skl* plants. Ethylene is a negative regulator of nodulation, so that it is most likely that 3-oxo-C<sub>14</sub>-HSL down-regulates ethylene synthesis and/or signalling to increase nodule numbers. Because of the already high number of nodules in the *skl* mutant, future experiments could test whether application of various concentrations of ethylene inhibitors would



result in a similar negation of AHL responses on nodulation. In *A. thaliana* the inhibition of root length by 3-oxo-C<sub>12</sub>-HSL and the breakdown product L-homoserine could be rescued by application of the ethylene synthesis inhibitor, AVG (aminoethoxyvinyl glycine), and similarly in the *M. truncatula skl* mutant, suggesting that ethylene mediates these root growth responses (Palmer *et al.*, 2014).

In an attempt to find out whether the increase in nodule numbers resulting from plant exposure to 3-oxo-C<sub>14</sub>-HSL caused changes in gene expression, we quantified the expression of nodulation, stress and defence response genes. As dramatic changes in hormone levels, especially in auxin and cytokinins, occur as a result of rhizobia infection (van Zeijl *et al.*, 2015; Mortier *et al.*, 2014; Ng *et al.*, 2015; Mathesius *et al.*, 1998, Mathesius, 2008). We explored whether altered expression of selected auxin-regulated and cytokinin-response genes (*GH3* and *Type Arr*, respectively) were associated with the increase in nodule numbers in response to 3-oxo-C<sub>14</sub>-HSL. Uninoculated plants exposed to 3-oxo-C<sub>14</sub>-HSL showed significantly upregulated *GH3* gene expression while inoculated plants exposed to this AHL showed upregulated expression of the *Type Arr* gene. This may suggest that 3-oxo-C<sub>14</sub>-HSL could increase auxin activity pre-rhizobial infection and this might have contributed to the higher nodule numbers observed with this AHL, as auxin has been shown to be essential for nodulation in *M. truncatula* and positively correlated with nodule numbers (van Noorden *et al.*, 2007). However, future studies would need to quantify auxin concentrations in response to AHLs during nodulation. On the other hand, the *Type Arr* gene was upregulated after inoculation of *S. meliloti* in plants exposed to C<sub>10</sub>-HSL, 3-oxo-C<sub>14</sub>-HSL and to the solvent (control). This result currently does not indicate a strong correlation between cytokinin response and nodule numbers as could have been expected (Crespi and Frugier, 2008). In the future, it would be interesting to test whether the effect of 3-oxo-C<sub>14</sub>-HSL on nodule numbers is reduced in mutants defective in auxin or cytokinin responses.

AHLs changed the expression of stress and defence response genes in *Medicago* plants. Plant defence responses such as those mediated by ethylene and ROS, are involved in nodulation and are reminiscent of pathogenic infections (Santos *et al.*, 2001). ROS may have important roles to play in legume-rhizobia nodulation. For example, in a semiaquatic legume, *Sesbania rostrata*, ROS production led to the formation of infection pockets and initiation of nodule primordia (D'Haeze *et al.*, 2003). In addition, overexpression of a catalase gene in *S. meliloti* affected infection thread formation

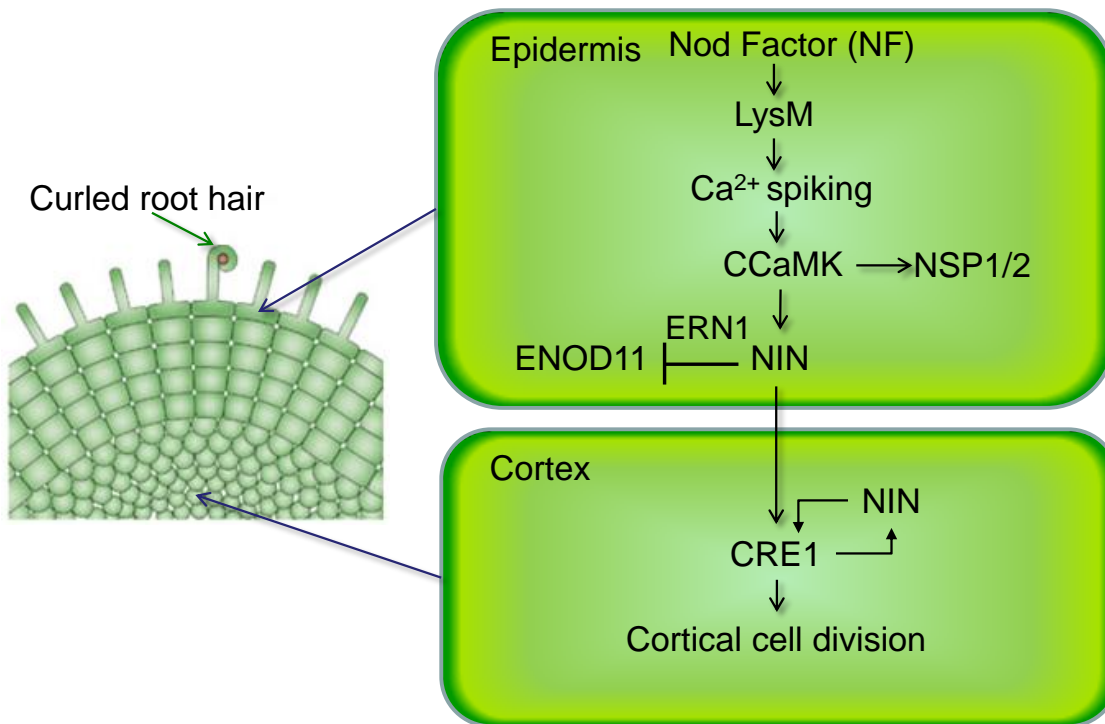
showing a delayed nodule phenotype in alfalfa (Jamet *et al.*, 2003). Thus, ROS could be important for infection thread formation. Downregulation of stress and defence response genes in four days old *Medicago* seedlings, at 24 hours post inoculation, has been reported to occur with simultaneous increase in accumulation of reactive oxygen species (ROS), which are necessary for symbiosis establishment (Hérouart *et al.*, 2002; Peleg-Grossman *et al.*, 2012). Our results showed that inoculated roots exposed to 3-oxo-C<sub>14</sub>-HSL increased *RIP1* expression levels compared to the controls while peroxidase gene expression was reduced at 24 h after inoculation. This may suggest that ROS accumulation in roots treated with this AHL is higher than the control treatments. This induction of *RIP1* expression correlates with the upregulation of APX in roots exposed to 3-oxo-C<sub>14</sub>-HSL, presumably to detoxify the increase of ROS. Thus, it would be interesting to examine in the future whether *Medicago* roots exposed to AHL results in increased ROS induction. Furthermore, ROS production mediates expression of *RIP1* nodulin gene in *M. truncatula* (Ramu *et al.*, 2002). *RIP1* is a peroxidase which could have multiple functions including the detoxification of ROS at the infection site (Glyan'ko, and Vasil'eva, 2010), cell wall modifications during infection thread formation and nodule organogenesis (Ramu *et al.*, 2002, Marino *et al.*, 2009) and regulating plant signalling through changes in protein activity (Ramu *et al.*, 2002). It would be interesting to further evaluate the effect of 3-oxo-C<sub>14</sub>-HSL on nodulation responses of *wt M. truncatula* exposed to ROS as well as the correlation with ethylene using the ethylene-insensitive *skl* mutant.

It has been reported that in *wt Medicago* plants, chitinases III-1 are upregulated in the presence of pathogenic fungi and arbuscular mycorrhiza but not in legume-rhizobia symbiosis (Salzer *et al.*, 2000). Interestingly, we found that a chitinase class III-1 was upregulated in inoculated plants with AHLs derived from *P. aeruginosa*. This may suggest that plants are able to distinguish AHLs as elicitors from non-symbiotic bacteria inducing the expression of chitinases class III-1. It would be interesting to examine whether the AHLs lead to other defence responses in *M. truncatula*.

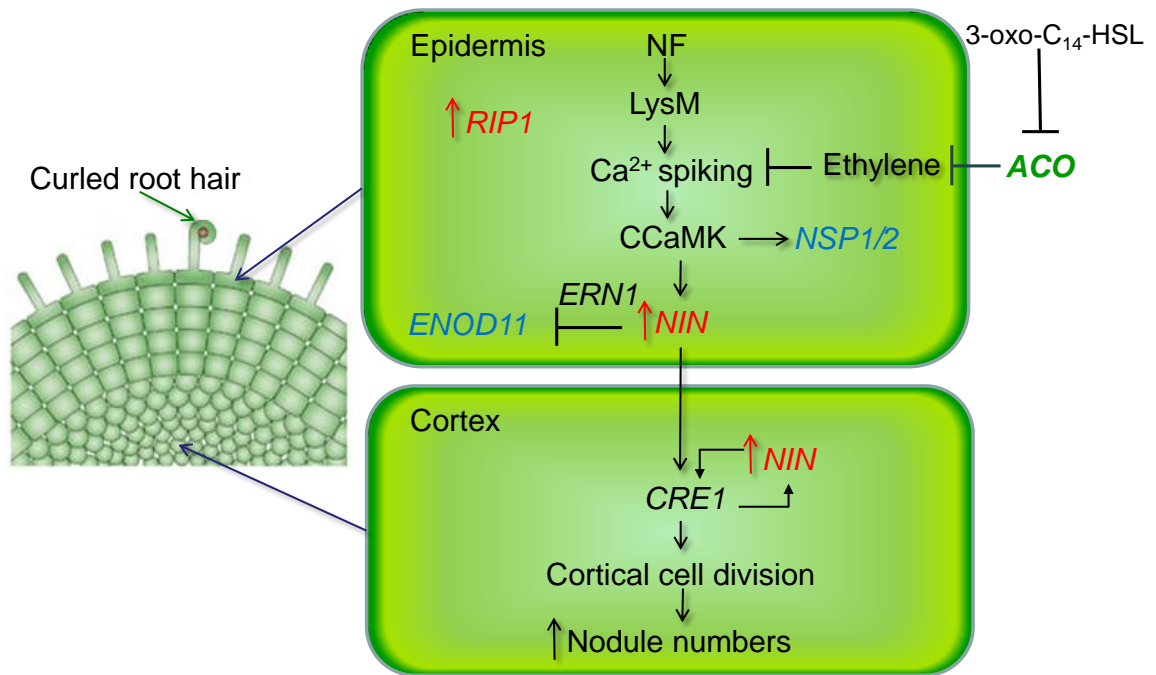
Nodulation gene expression is induced after inoculation with rhizobia (Lohar *et al.*, 2006; Larrainzar *et al.*, 2015). Vernié *et al.* (2015), in a recent model that examined several transcription factors in the nodulation pathway, proposed that the regulation of *NIN* (Nodule Inception) gene expression plays a major role in the Nod factor transduction signalling. When Nod factors are perceived by the LysM receptor transmembrane protein, located in the epidermal cells of *M. truncatula*, a Ca<sup>2+</sup> spiking

response is observed in root hairs, leading to a calcium and calmodulin-dependent protein kinase (CCaMK) induction, activating the expression of nodulation genes such as *NSP1*, *NSP2* and *NIN*. *NIN* represses *ENOD11* (*Early Nodulin11*) expression by competing with *ERN1* (*Ethylene Response Factor Required for Nodulation1*). *NIN* promotes the movement of a signal from the epidermis to the cortex initiating a cytokinin signalling via *CRE1* (*Cytokinin Response1*). In turn, *CRE1* promotes *NIN* expression in cortical cells creating a positive feedback loop increasing *CRE1* expression. As a result, cortical cell division is induced promoting nodule organogenesis (Figure 4.21). According to Vernié *et al.* (2015), an induction of *NIN* expression in the cortex leads to upregulation of *CRE1* expression, promoting a cytokinin signalling response and resulting in more cortical cell divisions and in more nodule numbers (Figure 4.21). Interestingly, our results showed that 3-oxo-C<sub>14</sub>-HSL from *S. meliloti* strain Rm1021 induced *NIN* expression in inoculated seedlings but not in either of the controls (uninoculated and inoculated plants) suggesting that the increase in nodule numbers might be partly mediated by upregulation of *NIN* expression (Figure 4.22). In the future, it would be interesting to evaluate *CRE1* and *ERN1* expression to confirm this hypothesis.

In addition, 3-oxo-C<sub>14</sub>-HSL down-regulated expression of *ACO*, encoding the enzyme that catalyses ethylene biosynthesis. This result suggests that 3-oxo-C<sub>14</sub>-HSL reduces ethylene synthesis in the root, resulting in an eventual increase in nodule numbers, as ethylene has been reported to down regulate Nod factor-triggered Ca<sup>2+</sup> spiking in root hairs of *M. truncatula*, and thus to negatively regulating nodulation (Oldroyd *et al.*, 2001; Larrainzar *et al.*, 2015) (Figure 4.22).



**Figure 4.21** Model of *NIN* action in the Nod factor transduction signalling pathway (Vernié *et al.*, 2015). Nod factors are perceived by the LysM receptor-like kinase which leads to a downstream activation of the nodulation pathway cascade, including the induction of the calcium (Ca<sup>2+</sup> spiking) and calmodulin-dependent protein kinase CCaMK activating the nodulin genes *NSP1* and *NSP2* and *NIN* gene expression. *NIN* competes with *ERN1* to repress *ENOD11*. In the cortex *NIN* induces the expression of *CRE1* which in turn induces the expression of *NIN* in the cortex creating a positive feedback loop. *CRE1* induces cortical cell division to finally nodule formation.



**Figure 4.22** Model showing the effect of 3-oxo-C<sub>14</sub>-HSL on nodulation gene expression and the possible mechanism behind the increase of nodule numbers by this AHL. Genes in blue: unaffected by 3-oxo-C<sub>14</sub>-HSL. Gene in red: upregulated by 3-oxo-C<sub>14</sub>-HSL. Gene in green: downregulated by 3-oxo-C<sub>14</sub>-HSL. Adapted from Vernié *et al.* (2015).

Interestingly, 3-oxo-C<sub>14</sub>-HSL from *S. meliloti* was shown to specifically enhance the resistance of *A. thaliana* towards the pathogens *Golovinomyces orontii* and *Pseudomonas syringae*, and resistance of *Hordeum vulgare* (barley) to *Blumeria graminis* (Schikora *et al.*, 2012; Schenk *et al.*, 2012; Zarkani *et al.*, 2014). Collectively these studies indicate a specific role for 3-oxo-C<sub>14</sub>-HSL in modulation of host defence responses that could alter the outcome of both pathogenic and symbiotic plant-microbe interactions. Further studies are necessary to investigate the mechanism of how this is achieved. It is likely that the AHLs that show effects on plants are processed before or after they are first perceived, e.g. by enzymatic degradation by the plant host (Palmer *et al.*, 2014). One of the breakdown products of AHLs, L-homoserine, has been shown to affect root length in *A. thaliana* (Palmer *et al.*, 2014), and it would be interesting to test its effect on nodulation in future studies. Future microscopic studies of infection threads using a *S. meliloti* GFP strain in the presence of AHLs could reveal whether AHLs, particularly 3-oxo-C<sub>14</sub>-HSL, have an effect on the hyper-nodulating phenotype if *NIN* (Marsh *et al.*, 2007).

## Chapter 5 Does the *Medicago truncatula* microbiome affect AHL phenotypic responses?

### 5.1 Abstract

Plant-associated bacteria play a key role in plant survival, health and productivity. As *Medicago* was shown to harbour culturable seed-borne bacteria (see Chapter 3), we evaluated whether *M. truncatula*-associated bacteria modulate plant responses to AHLs. Here, the microbiome is strictly referred to as the plant-associated bacteria whether endophytic or located on plant surfaces. The root microbiome significantly changed root phenotypes of *M. truncatula* in response to AHLs and these responses were AHL-concentration dependent. The most prominent phenotype affected by the interaction between AHLs and the microbiome was nodule numbers. The *M. truncatula* microbiome reduced nodule numbers in plants exposed to 3-oxo-C<sub>14</sub>-HSL. Gene expression and phenotypic analysis suggest that the increase in nodule numbers of *Medicago* plants exposed to 3-oxo-C<sub>14</sub>-HSL in the absence of their microbiome is ethylene-dependant.

The *M. truncatula* root microbiome (originated from the seed-borne microbiome) composition was determined via 454 pyrosequencing analysis, which revealed that plant-associated bacteria were mainly *Proteobacteria*. Interestingly, the AHL, 3-oxo-C<sub>14</sub>-HSL significantly reduced the relative abundance of *Pantoea* sp. specifically, showing that AHLs, in turn, may interfere with the plant-associated bacteria.

### 5.2 Introduction

Plants are colonised by a diverse range of microorganisms, particularly bacteria in either inside or outside of plant tissues. This plant microbiome is an extension of the plant itself as it closely and dynamically interacts with the plant in an ever changing environment. The root microbiome can have beneficial effects on the plant performance, e.g. enhancing agricultural productivity through different mechanisms including tolerance or resistance to diverse biotic and abiotic stresses (Berendsen *et al.*, 2012; Bulgarelli *et al.*, 2013; Philippot *et al.*, 2013). Thus, the root microbiome might constitute the “life vest” of plant survival and productivity, especially under stressful conditions. Moreover, plants and their bacterial communities are in continuous

communication through signals. There is evidence that quorum sensing signals, especially acyl homoserine lactones (AHLs) are involved in this cross-talk or inter-kingdom communication (Teplitski *et al.*, 2000; Gao *et al.*, 2003; Teplitski *et al.*, 2010).

Plants are able to shape their microbiome in a targeted selective process (Bodenhausen *et al.*, 2014). Interestingly, the soybean (*Glycine max* (L.) Merrill)-associated microbiome of non nodulating (Nod<sup>-</sup>), wild-type nodulating (Nod<sup>+</sup>), and hypernodulating (Nod<sup>++</sup>) mutants was found to be significantly different (Ikeda *et al.*, 2008). Moreover, plants can also inherit their bacterial consortium via seeds in a process called vertical transmission. Seed-borne bacterial community structure is able to disperse systemically throughout the plant, colonising roots and nodules (Johnston-Monje and Raizada, 2011; Hardoim *et al.*, 2012). Thus, the importance of the microbiome for plants is not only for their establishment but also for their entire life cycle.

From previous Chapters we had established that the batch of *Medicago* seeds used for our experiments harboured culturable bacteria, and these bacteria came from inside of the seed coat (Chapter 3). We also discovered that 3-oxo-C<sub>14</sub>-HSL significantly increased nodule numbers in a concentration-dependent and nodulation-specific manner, likely via ethylene signalling (Chapter 4). In the light of these observations we investigated whether the *M. truncatula* microbiome initially harboured by the seeds would affect plant nodulation responses towards AHLs, and whether this was ethylene-dependent. In order to investigate the effect of the root microbiome on phenotypic responses of *M. truncatula*, we evaluated the root microbiome of *Medicago* seedlings 21 days after inoculation, exposed to 3-oxo-C<sub>14</sub>-HSL in the presence and absence of the root microbiome. In addition, we also investigated the bacterial community structure of the *M. truncatula* root microbiome through pyrosequencing analysis. As the microbial communities vary at different developmental stages in *M. truncatula* (Mougel *et al.*, 2006) we evaluated the bacterial community structure at the same time point used to evaluate phenotypic nodulation assays in this study.

In this Chapter, the microbiome is strictly referred to as the plant-associated bacterial community and not to the whole consortium of microbes (e.g. fungi, archaea, protozoa, etc.).

## 5.3 Results

### 5.3.1 The effect of plant-associated bacteria on nodulation, other root parameters and plant biomass in inoculated *M. truncatula* plants

In order to test whether *Medicago* responses towards AHLs were affected by the microbiome, we conducted a series of experiments in which *Medicago* seeds were treated with and without antibiotics that were previously shown to eliminate culturable bacteria from the seedlings and then transferred to a sterile growth medium containing 1  $\mu\text{M}$  of the same 15 AHLs used in Chapter 3 (*cf.* Figure 3.9). The plant phenotypes were scored at 21 dai. Out of 15 AHLs tested, five showed significant differences, by increasing nodule numbers, at 1  $\mu\text{M}$  AHL concentration ( $p < 0.05$ ; Figure 5.1). The antibiotic treatment was significant in plants exposed to C<sub>4</sub>-HSL, C<sub>8</sub>-HSL, C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL, 3-oxo-C<sub>14</sub>-HSL, C<sub>16</sub>:1-9cis-(L)-HSL and 3-Oxo-C<sub>16</sub>:1-11cis-(L)-HSL ( $p < 0.05$ ; Figure 5.1). However, the AHL effect was significant in C<sub>6</sub>-HSL, C<sub>16</sub>:1-9cis-(L)-HSL and C<sub>18</sub>-HSL only ( $p < 0.05$ ; Figure 5.1B, M, O). Plant responses to AHLs in the presence of the microbiome were different than without the microbiome except for 3-oxo-C<sub>8</sub>-HSL, C<sub>12</sub>-HSL, C<sub>14</sub>-HSL, 3-oxo-C<sub>14</sub>:1-7-cis-(L)-HSL, C<sub>14</sub>:1-9-cis-(L)-HSL, C<sub>16</sub>-HSL, C<sub>16</sub>:1-9cis-(L)-HSL and C<sub>18</sub>-HSL. The interaction between the antibiotic treatment and the AHL exposure was significant for C<sub>4</sub>-HSL, C<sub>6</sub>-HSL, C<sub>8</sub>-HSL, C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL ( $p < 0.05$ ; Figure 5.1). This finding suggests that the effect of AHL treatment on nodulation is partly dependent on its microbiome. Moreover, plants treated with antibiotics increased nodule numbers in response to long acyl side chain AHLs but not to the exposure of short acyl side chain AHLs (Figure 5.1). This is not surprising in the light of that very long acyl side chain AHLs are specific to rhizobia (Cha *et al.* 1998; Marketon *et al.*, 2002; Llamas *et al.*, 2004). Thus, *Medicago* may recognise and respond to these compounds specifically. Interestingly, C<sub>4</sub>-HSL and C<sub>6</sub>-HSL showed a significant increase in nodule numbers with the presence of the microbiome but no effect in the absence of the microbiome ( $p < 0.05$ ; Figure 5.1A, B). By contrast, C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL significantly increased nodule numbers in the absence of the microbiome, but no in its presence ( $p < 0.05$ ; Figure 5.1E, G, I).

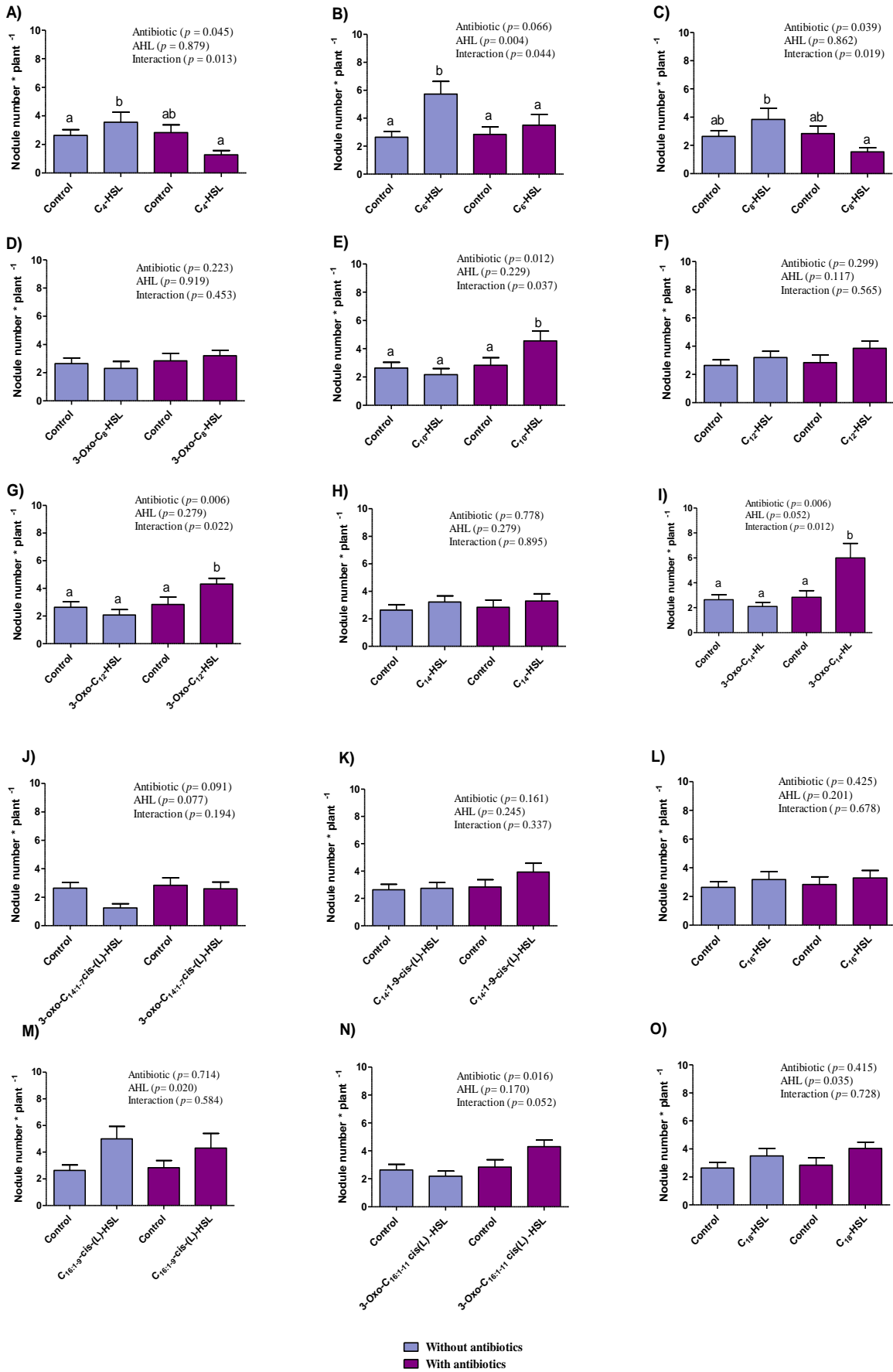
In the same experiment we further determined other root architecture parameters such as root length and lateral root density to investigate whether this effect was nodulation specific, as it was observed in previous experiments (see Chapter 4). In general, the root



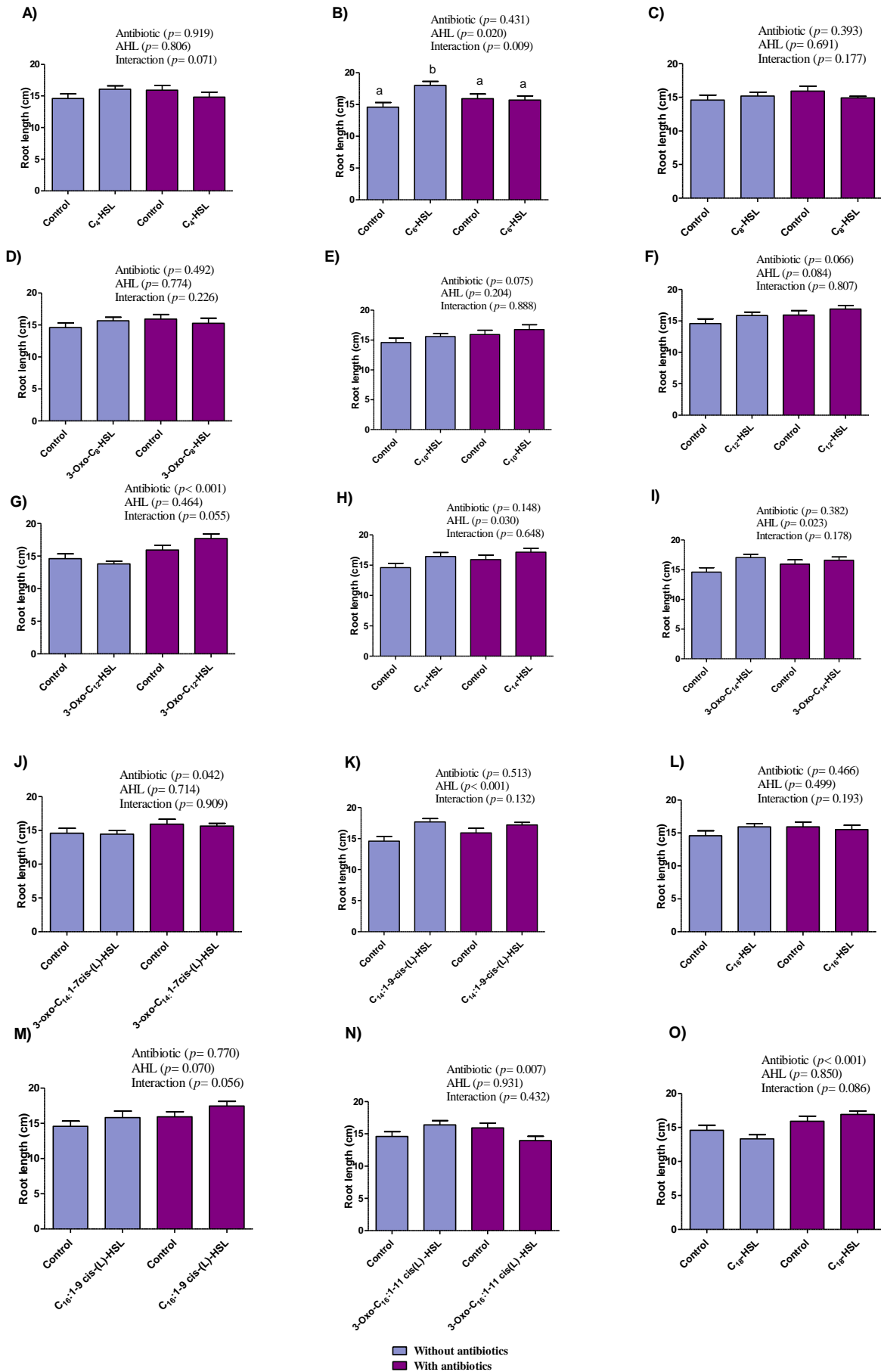
length and lateral root density were less affected than nodule numbers (Figures 5.2, 5.3). In terms of root length, the antibiotic effect was significant in plants exposed to 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL, 3-oxo-C<sub>14</sub>:1-7-cis-(L)-HSL and C<sub>18</sub>-HSL compounds (Figure 5.2G, I, J, O). The AHL effect was significant in plants exposed to C<sub>6</sub>-HSL, C<sub>14</sub>-HSL, 3-oxo-C<sub>14</sub>-HSL and C<sub>14</sub>:1-9-cis-(L)-HSL (Figure 5.2B, I, H, K). Lateral root density was affected significantly by the antibiotic treatment in plants exposed only to C<sub>12</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and C<sub>18</sub>-HSL (Figure 5.3F, G, O). In turn, the AHL treatment was only significant in plants exposed to C<sub>10</sub>-HSL and C<sub>16</sub>-HSL (Figure 5.3E, L). C<sub>6</sub>-HSL was the only compound that had significantly different effects on root length and lateral root density in the absence or presence of the microbiome. Lateral root density significantly decreased in the presence of C<sub>6</sub>-HSL with antibiotics but remained similar without antibiotics (Figure 5.3B).

In addition, we evaluated whether total dry biomass, which is the sum of root and shoot biomass was affected by the presence of the microbiome and the AHL application. However, there was no interaction between the antibiotic treatment and the AHL exposure (Figure 5.4). Only C<sub>4</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL, C<sub>14</sub>-HSL, 3-oxo-C<sub>14</sub>-HSL, 3-Oxo-C<sub>16</sub>:1-11cis-(L)-HSL and C<sub>18</sub>-HSL-treated plants showed a significant antibiotic effect (Figure 5.4A, G, I, H, N, O). 3-Oxo-C<sub>16</sub>:1-11cis-(L)-HSL was the only treatment with significant AHL effect (Figure 5.4N).

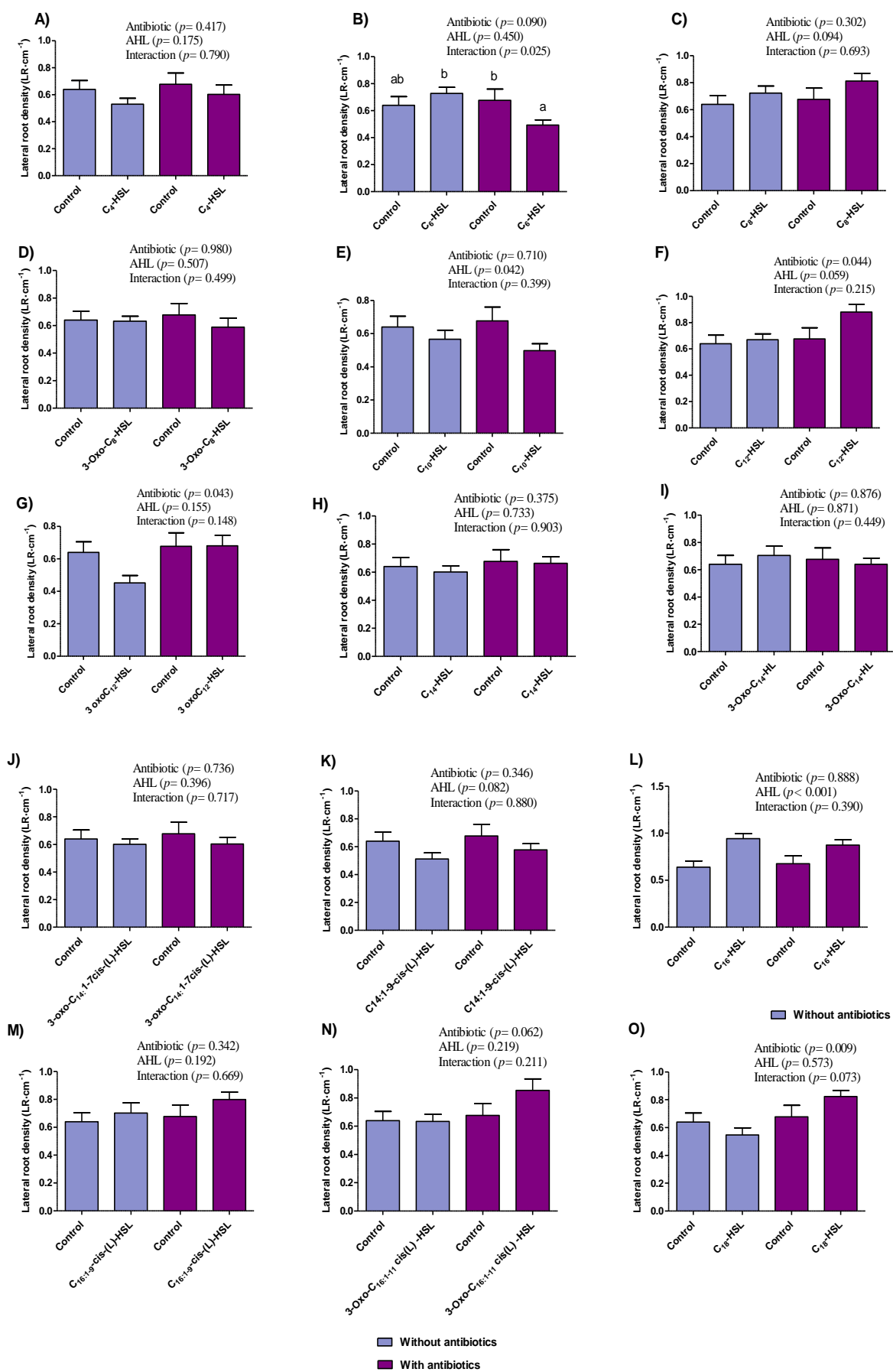
The effect of C<sub>4</sub>-HSL, C<sub>8</sub>-HSL, C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL on nodule numbers was specific for nodulation, and not for root length or lateral root density, and did not result in increased plant biomass (compare Figures 5.1-5.4).



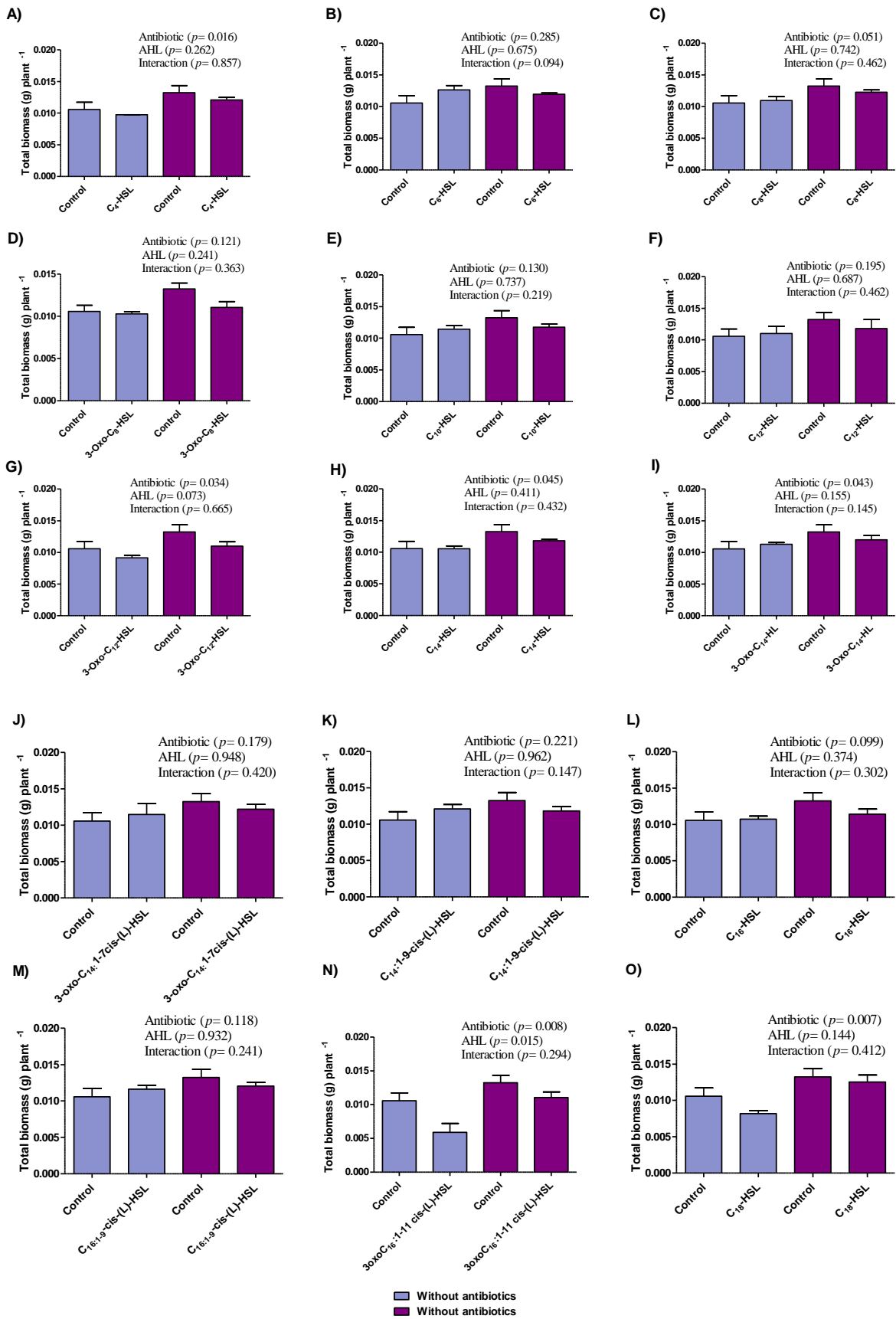
**Figure 5.1** Nodule numbers of *M. truncatula* plants 21 dai. exposed to 1  $\mu$ M AHLs inoculated with rhizobia. A) C<sub>4</sub>-HSL, B) C<sub>6</sub>-HL, C) C<sub>8</sub>-HSL, D) 3-Oxo-C<sub>8</sub>-HL, E) C<sub>10</sub>-HL, F) C<sub>12</sub>-HSL, G) 3-Oxo-C<sub>12</sub>-HSL, H) C<sub>14</sub>-HL, I) 3-Oxo-C<sub>14</sub>-HL, J) 3-oxo-C<sub>14</sub>:1-7cis-(L)-HSL, K), C<sub>14</sub>:1-9-cis-(L)-HSL, L) C<sub>16</sub>-HSL, M) C<sub>16:1-9</sub> cis-(L)-HSL, N) 3-Oxo-C<sub>16:1-11</sub> cis-(L)-HSL, O) C<sub>18</sub>-HSL. Different letters indicate significant differences between the treatments when the interaction is significant (REML test  $p < 0.05$ ). No post-hoc test when interaction is not significant. Data points indicate mean  $\pm$  SE, ( $n = 27-30$ ). Antibiotic: with or without antibiotic treatment; AHL: with or without 3-oxo-C<sub>14</sub>-HSL; interaction: Antibiotic treatment  $\times$  AHL treatment.



**Figure 5.2** Root length (cm) of *M. truncatula* plants 21 dai exposed to 1  $\mu$ M AHLs inoculated with rhizobia. A) C<sub>4</sub>-HSL, B) C<sub>6</sub>-HL, C) C<sub>8</sub>-HSL, D) 3-Oxo-C<sub>8</sub>-HL, E) C<sub>10</sub>-HL, F) C<sub>12</sub>-HSL, G) 3-Oxo-C<sub>12</sub>-HSL, H) C<sub>14</sub>-HL, I) 3-Oxo-C<sub>14</sub>-HL, J) 3-oxo-C<sub>14</sub>:1-7cis-(L)-HSL, K), C<sub>14</sub>:1-9-cis-(L)-HSL, L) C<sub>16</sub>-HSL, M) C<sub>16:1</sub>-9 cis-(L)-HSL, N) 3-Oxo-C<sub>16:1</sub>-11 cis-(L)-HSL, O) C<sub>18</sub>-HSL. Different letters indicate significant differences between the treatments when the interaction is significant (REML test  $p < 0.05$ ). Data points indicate mean  $\pm$  SE, ( $n = 20-30$ ). Antibiotic: with or without antibiotic treatment; AHL: with or without 3-oxo-C<sub>14</sub>-HSL; interaction: Antibiotic treatment  $\times$  AHL treatment.



**Figure 5.3** Lateral root density (LR cm<sup>-1</sup>root) of 21 days old *M. truncatula* plants exposed to 1 μM AHLs inoculated with rhizobia. A) C<sub>4</sub>-HSL, B) C<sub>6</sub>-HL, C) C<sub>8</sub>-HSL, D) 3-Oxo-C<sub>8</sub>-HL, E) C<sub>10</sub>-HL, F) C<sub>12</sub>-HSL, G) 3-Oxo-C<sub>12</sub>-HSL, H) C<sub>14</sub>-HL, I) 3-Oxo-C<sub>14</sub>-HL, J) 3-oxo-C<sub>14</sub>:1-7cis-(L)-HSL, K), C<sub>14</sub>:1-9-cis-(L)-HSL, L) C<sub>16</sub>-HSL, M) C<sub>16:1</sub>-9 cis-(L)-HSL, N) 3-Oxo-C<sub>16:1</sub>-11 cis-(L)-HSL, O) C<sub>18</sub>-HSL. Different letters indicate significant differences between the treatments when the interaction is significant (REML test  $p < 0.05$ ). No post-hoc test when interaction is not significant. Data points indicate mean  $\pm$  SE, ( $n = 25-30$ ). Antibiotic: with or without antibiotic treatment; AHL: with or without 3-oxo-C<sub>14</sub>-HSL; interaction: Antibiotic treatment  $\times$  AHL treatment.



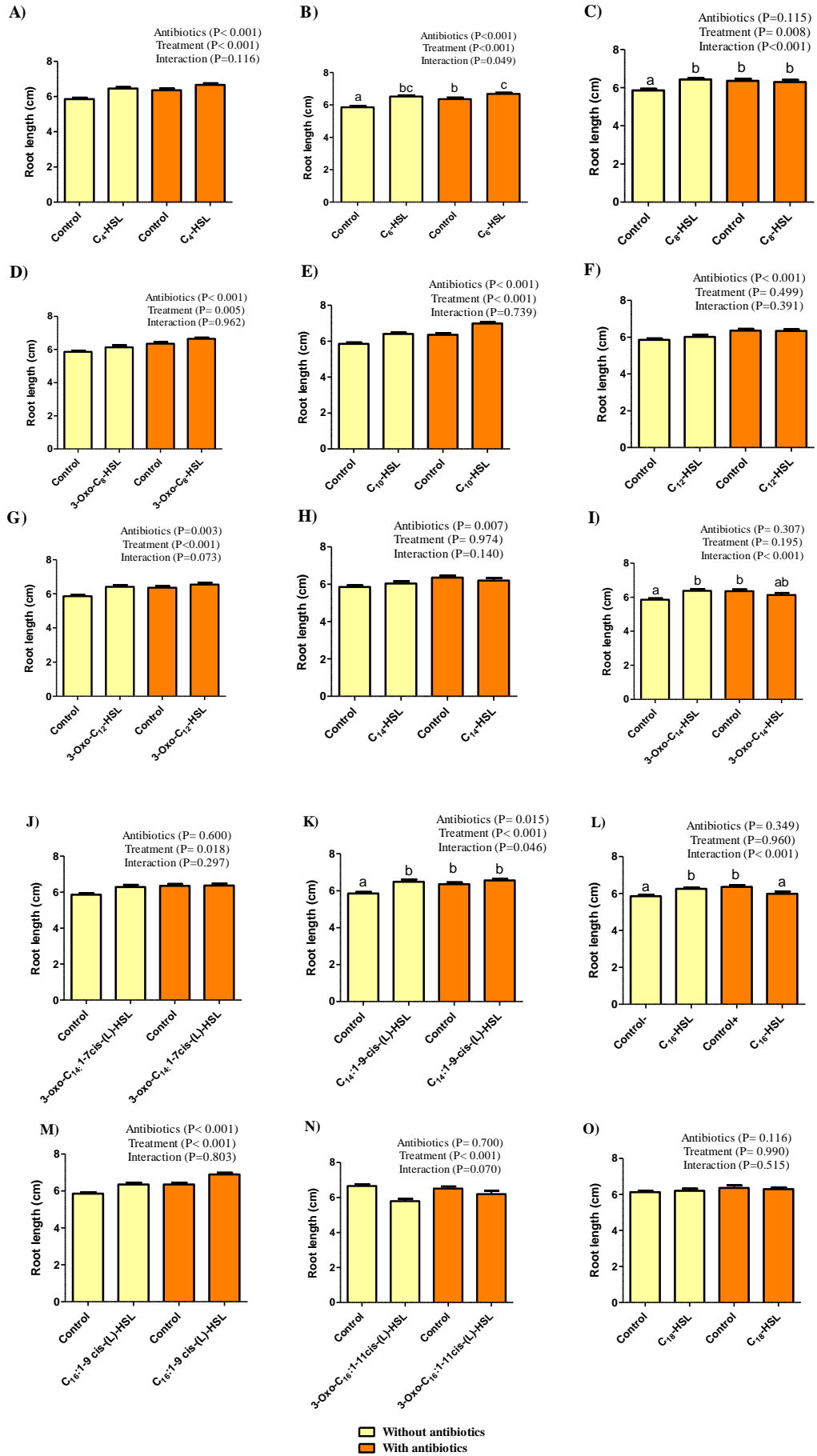


**Figure 5.4** Total dry biomass (root and shoot biomass) ( $\text{g plant}^{-1}$ ) of 21 days old *M. truncatula* plants exposed to 1  $\mu\text{M}$  AHLs inoculated with rhizobia. A) C<sub>4</sub>-HSL, B) C<sub>6</sub>-HL, C) C<sub>8</sub>-HSL, D) 3-Oxo-C<sub>8</sub>-HL, E) C<sub>10</sub>-HL, F) C<sub>12</sub>-HSL, G) 3-Oxo-C<sub>12</sub>-HSL, H) C<sub>14</sub>-HL, I) 3-Oxo-C<sub>14</sub>-HL, J) 3-oxo-C<sub>14</sub>:1-7cis-(L)-HSL, K), C<sub>14</sub>:1-9-cis-(L)-HSL, L) C<sub>16</sub>-HSL, M) C<sub>16</sub>:1-9 cis-(L)-HSL, N) 3-Oxo-C<sub>16</sub>:1-11 cis-(L)-HSL, O) C<sub>18</sub>-HSL. Different letters indicate significant differences between the treatments (REML test  $p < 0.05$ ). No post-hoc test when interaction is not significant. Data points indicate mean  $\pm$  SE, ( $n = 3$ ). Antibiotic: with or without antibiotic treatment; AHL: with or without 3-oxo-C<sub>14</sub>-HSL; interaction: Antibiotic treatment  $\times$  AHL treatment.

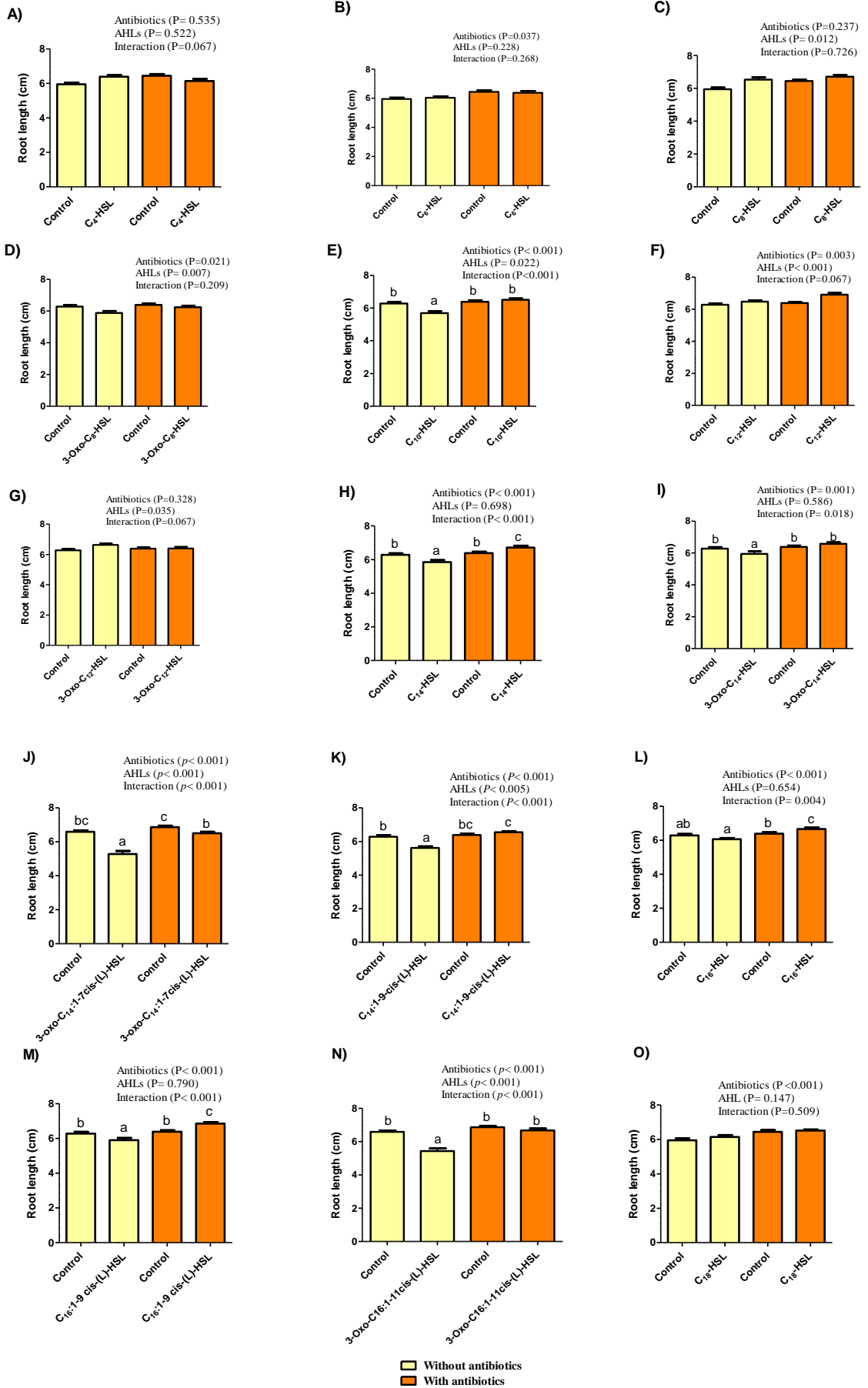
### 5.3.2 The effect of the microbiome and the AHL treatment on root length and total biomass of uninoculated *M. truncatula* plants

To evaluate whether the AHL effect on plant root architecture was dependent on the presence of rhizobia we further measured root length of *Medicago* plants without rhizobia exposed to 1 or 10  $\mu\text{M}$  AHL concentration. Root length of *Medicago* plants was affected by the presence of the microbiome. Different AHLs had significant effect on root length of *M. truncatula* either at 1  $\mu\text{M}$  or at 10  $\mu\text{M}$  concentrations. For example, C<sub>6</sub>-HSL significantly increased root length at 1  $\mu\text{M}$  but not at 10  $\mu\text{M}$  ( $p < 0.001$ ; Figure 5.5B; 5.6B), while C<sub>8</sub>-HSL significantly increased root length, in the absence of the microbiome, at 1  $\mu\text{M}$  but not at 10  $\mu\text{M}$  ( $p = 0.008$ ; Figure 5.5C, 5.6C). The root length of *Medicago* plants were more responsive towards AHLs at 10  $\mu\text{M}$  than at 1  $\mu\text{M}$  as eight out of 15 AHLs had significant effect on root length ( $p < 0.05$ ; Figure 5.6E, H-N) compared to five out of 15 AHLs at 1  $\mu\text{M}$  concentration ( $p < 0.05$ ; Figure 5.5B, C, I, K, L). Plants exposed to C<sub>6</sub>-HSL, C<sub>8</sub>-HSL, C<sub>14</sub>:1-9-cis-(L)-HSL and 3-oxo-C<sub>16</sub>:1-11cis-(L)-HSL at 1  $\mu\text{M}$  showed a significant interaction where AHL treatment contributed significantly ( $p \leq 0.008$ ; Figure 5.5B, C, N, K). On the other hand, plants exposed to C<sub>10</sub>-HSL, C<sub>14</sub>-HSL, 3-oxo-C<sub>14</sub>-HSL, 3-oxo-C<sub>14</sub>:1-7-cis-(L)-HSL, C<sub>14</sub>:1-9-cis-(L)-HSL, C<sub>16</sub>-HSL and 3-oxo-C<sub>16</sub>:1-11cis-(L)-HSL at 10  $\mu\text{M}$ , showed a significant interaction at 10  $\mu\text{M}$ , in which the AHL and the antibiotic treatment contributed significantly ( $p < 0.05$ ; Figure 5.6E, H-L, N). While 3-oxo-C<sub>14</sub>-HSL increased root length of *M. truncatula* at 1  $\mu\text{M}$  in the presence of the microbiome, at 10  $\mu\text{M}$  this increase disappeared when treated with antibiotics (Figure 5.5I; 5.6I). These results indicate that *Medicago* responses to AHLs in the presence or absence of its microbiome are AHL concentration-dependent.

Root length was significantly increased in plants exposed to C<sub>6</sub>-HSL, C<sub>8</sub>-HSL, 3-oxo-C<sub>14</sub>-HSL, C<sub>14</sub>:1-9-cis-(L)-HSL and C<sub>16</sub>-HSL at 1 μM concentration, only in the presence of the microbiome ( $p < 0.008$ ; Figure 5.5B, C, I, K, L). By contrast, 3-Oxo-C<sub>16</sub>:1-11cis-(L)-HSL significantly decreased root length at both AHL concentrations only in the presence of the microbiome ( $p < 0.001$ ; Figure 5.5N, 5.6N). Control treatment of plants without antibiotics had smaller root length than control treatments of plants treated with antibiotics in all AHL conditions except for 3-oxo-C<sub>16</sub>:1-11cis-(L)-HSL at 1 μM AHL concentration (Figure 5.5). AHL treatment was significant in all treatments except in plants exposed to 3-oxo-C<sub>14</sub>-HSL at 1 μM. 3-oxo-C<sub>14</sub>-HSL and 3-Oxo-C<sub>16</sub>:1-11cis-(L)-HSL showed significant changes in root length with and without the microbiome (Figure 5.5F, H; 5.6F, H). 3-Oxo-C<sub>16</sub>:1-11cis-(L)-HSL showed a significant reduction in root length in the presence of the microbiome ( $p < 0.001$ ; Figure 5.6N). However, this reduction disappeared when treated with antibiotics at both 1 μM and 10 μM AHL concentrations (Figure 5.5N, 5.6N). The AHL effect did not contribute to the plant responses to 3-oxo-C<sub>14</sub>-HSL either in the presence or absence of the microbiome (Figure 5.5I; 5.6I). No specific correlation of either short or long side chain was observed under both AHL concentrations studied.



**Figure 5.5** Root length (cm) of uninoculated *M. truncatula* plants 21 dai plants derived from seed treated with or without antibiotics exposed to 1  $\mu$ M AHLs. A) C<sub>4</sub>-HSL, B) C<sub>6</sub>-HL, C) C<sub>8</sub>-HSL, D) 3-Oxo-C<sub>8</sub>-HL, E) C<sub>10</sub>-HL, F) C<sub>12</sub>-HSL, G) 3-Oxo-C<sub>12</sub>-HSL, H) C<sub>14</sub>-HL, I) 3-Oxo-C<sub>14</sub>-HL, J) 3-oxo-C<sub>14</sub>:1-7cis-(L)-HSL, K), C<sub>14</sub>:1-9-cis-(L)-HSL, L) C<sub>16</sub>-HSL, M) C<sub>16:1-9</sub> cis-(L)-HSL, N) 3-Oxo-C<sub>16:1-11</sub> cis-(L)-HSL, O) C<sub>18</sub>-HSL. Different letters indicate significant differences between the factors (REML test  $p < 0.05$ ). No post-hoc test when interaction is not significant. Data points indicate mean  $\pm$  SE ( $n = 25-30$ ). Note: The results shown in Figures 5.5 and 5.6 of root length of seeds treated with and without antibiotics at 1  $\mu$ M and 10  $\mu$ M AHLs, were also presented in Figure 3.9 as they were carried out in the same experiment)



**Figure 5.6** Root length (cm) of uninoculated *M. truncatula* plants 21 dai plants derived from seed treated with or without antibiotics exposed to 10  $\mu$ M AHLs. A) C<sub>4</sub>-HSL, B) C<sub>6</sub>-HL, C) C<sub>8</sub>-HSL, D) 3-Oxo-C<sub>8</sub>-HL, E) C<sub>10</sub>-HL, F) C<sub>12</sub>-HSL, G) 3-Oxo-C<sub>12</sub>-HSL, H) C<sub>14</sub>-HL, I) 3-Oxo-C<sub>14</sub>-HL, J) 3-oxo-C<sub>14</sub>:1-7cis-(L)-HSL, K), C<sub>14</sub>:1-9-cis-(L)-HSL, L) C<sub>16</sub>-HSL, M) C<sub>16</sub>:1-9 cis-(L)-HSL, N) 3-Oxo-C<sub>16</sub>:1-11 cis-(L)-HSL, O) C<sub>18</sub>-HSL. Different letters indicate significant differences between the factors at  $p < 0.05$  (REML test with Bonferroni's post-test). No post-hoc test when interaction is not significant. Data points indicate mean  $\pm$  SE ( $n = 25-30$ ). Note: The results shown in Figures 5.5 and 5.6 of root length of seeds treated with and without antibiotics at 1  $\mu$ M and 10  $\mu$ M AHLs, were also presented in Figure 3.9 as they were carried out in the same experiment).

In summary, a survey to examine effects of a number of AHLs on *M. truncatula* was carried out in the absence and the presence of antibiotics that eliminated visible contamination of the seedlings. The antibiotic treatment modified some of the root and nodulation phenotypes, but this was specific for the AHL and the phenotype measured. There was no strong trend towards modulation of phenotypes by the antibiotic treatment.

Interestingly the positive effect of C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL on nodule numbers was eliminated in the absence of antibiotics (Figure 5.1I), i.e. in the presence of the *M. truncatula* microbiome. This suggests that the presence of some of the plant-associated bacterial species interfere with the effects of these AHLs on nodulation, either by destroying the signal compound, or by modifying the plant responses to this compound. To investigate this observation further, we examined the composition of the *M. truncatula* microbiome present in our seedlings.

### **5.3.3 The effect of *M. truncatula* microbiome on nodulation through ethylene signalling**

As previously suggested, the AHL effect on nodulation in *M. truncatula* is, at least partially, ethylene dependent (see Chapter 4). In order to assess whether the microbiome also changed *Medicago* nodulation responses to AHLs on a molecular level via ethylene signalling, we further analysed the gene expression of nodulation, plant defence and

ethylene inducible genes. The analysed genes are listed in Table 5.1. Our first hypothesis was that genes positively correlated with successful early nodulation, including *NIN*, *ENOD11*, *ERN1* and *RIP1* would correlate with increased nodule numbers after 3-oxo-C<sub>14</sub>-HSL application in the presence of antibiotics. Our second hypothesis was that 3-oxo-C<sub>14</sub>-HSL in the absence of the microbiome increases nodule numbers by decreasing ethylene synthesis or signalling, and that ethylene-repressed genes would therefore be upregulated. We selected 3-oxo-C<sub>14</sub>-HSL in these experiments as it was our previous focus in Chapter 4.

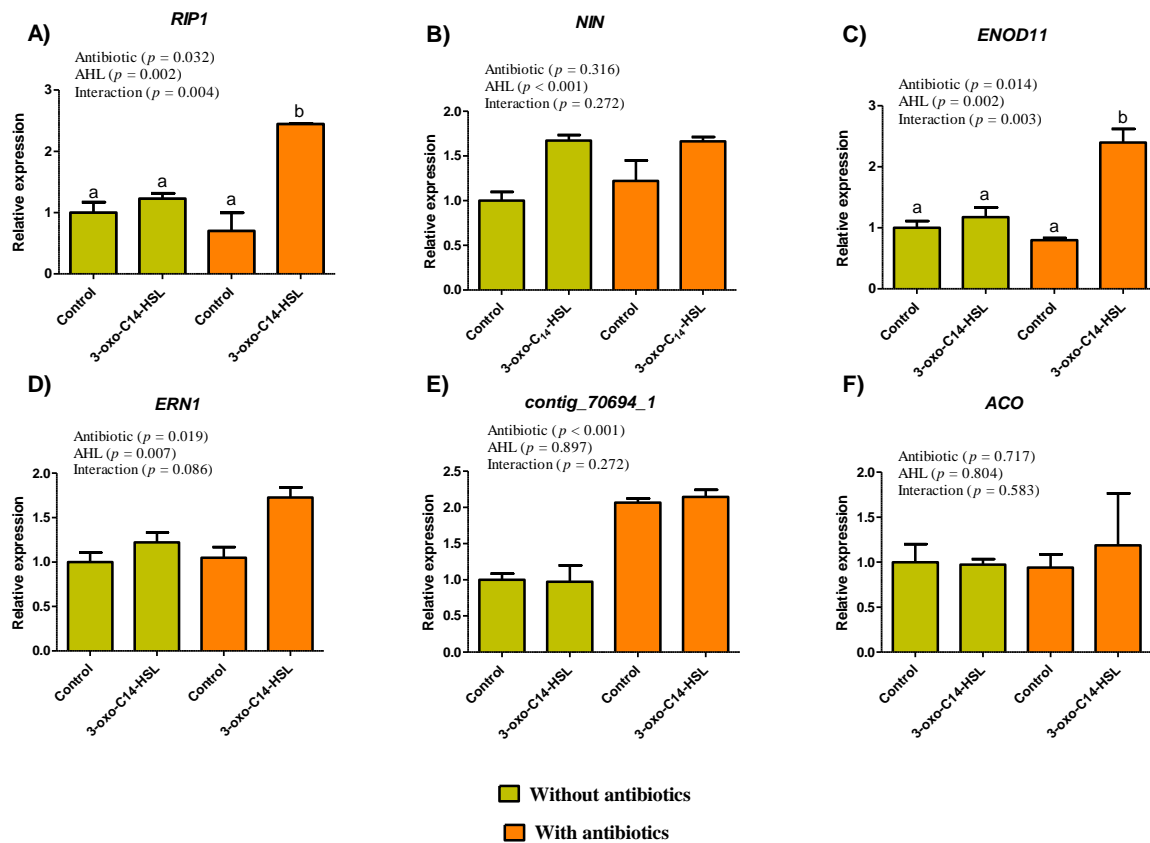
**Table 5.1** Genes tested and the biological processes they regulate.

<b>Gene</b>	<b>Ethylene regulation</b>	<b>Reference</b>
<i>NIN (Nodule inception)</i>	Ethylene-repressed gene Higher induction in <i>skl</i> mutant after inoculation	Schauser <i>et al.</i> , (1999)
<i>ENOD11 (Early nodulin11)</i>	Ethylene-repressed gene Higher induction in <i>skl</i> mutant after inoculation	Journet <i>et al.</i> , (2001)
<i>ERN1 (Ethylene response factor required for Nodulation1)</i>	Ethylene-repressed gene Higher induction in <i>skl</i> mutant after inoculation	Middleton, <i>et al.</i> , (2007) Larrainzar <i>et al.</i> , (2015) Vernié <i>et al.</i> , (2015)
<i>contig_70694_1 (ortholog of Ethylene response factor (AtERF1) in Arabidopsis )</i>	Ethylene-repressed gene Upregulation during early nodulation. <i>AtERF1</i> in ethylene signalling Involved in (a)biotic stress responses	Anderson <i>et al.</i> , (2010) Larrainzar <i>et al.</i> , (2015) Cheng <i>et al.</i> , (2013)
<i>RIP1(Rhizobium-induced peroxidase1)</i>	Ethylene-repressed gene Upregulated in <i>skl</i> mutant	Cook <i>et al.</i> , (1995); Penmetsa <i>et al.</i> , (2003)
<i>ACO (ACC oxidase (1-aminocyclopropane-1-carboxylate oxidase))</i>	Ethylene-induced gene ACO protein downregulated in <i>skl</i> mutant, ethylene induced	Yim <i>et al.</i> (2014), Prayitno <i>et al.</i> , (2006)



The presence or absence of the microbiome significantly modulated the gene expression of *RIP1*, *ENOD11*, *ERN1*, a putative ortholog of an *Arabidopsis* ethylene response factor and *ACO* ( $p < 0.05$ ; Figure 5.7). The 3-oxo-C<sub>14</sub>-HSL treatment significantly upregulated the expression of the early nodulation genes *RIP1*, *NIN*, *ENOD11* and *ERN1* confirming our first hypothesis (Figure 5.7A-E). *RIP1* and *ENOD11* gene expression were significantly increased only in *Medicago* seedlings exposed to 3-oxo-C<sub>14</sub>-HSL and treated with antibiotics ( $p < 0.004$ ; Figure 5.7A). Even though *ERN1* gene expression was also modulated by the antibiotic treatment and 3-oxo-C<sub>14</sub>-HSL exposure, there was not significant interaction between these treatments (Figure 5.7D).

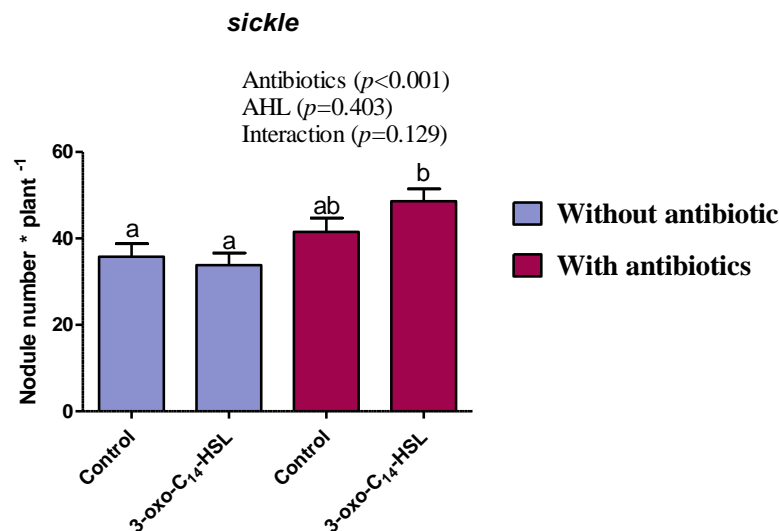
The literature has shown that early nodulation genes, including *RIP1*, *NIN*, *ENOD11* and *ERN1* had higher induction in the *skl* mutant after nodulation resulting in more nodule numbers (see Table 5.1 for references). Thus, these genes are induced under low levels of ethylene perception and, in turn, repressed at high levels of ethylene perception. Our results showed an upregulation of the expression of the ethylene-repressed genes *RIP1*, *NIN*, *ENOD11* and *ERN1* in the presence of the 3-oxo-C<sub>14</sub>-HSL treatment (Figure 5.7). These results suggest that *Medicago* plants perceive a reduced ethylene, resulting in an increase in nodule numbers which, in turn, supports our second hypothesis that ethylene-repressed genes would be upregulated indicating a reduction of ethylene synthesis or signaling. Interestingly, *ACO* gene expression showed a significant antibiotic treatment effect, but not an AHL effect or a significant interaction between treatments (Figure 5.7F). This may suggest that the microbiome represses ethylene synthesis in these *M. truncatula* seedlings.



**Figure 5.7** Effect of AHLs on the relative quantification of gene expression of four days old *M. truncatula* roots (24 h after rhizobia inoculation) treated with 1  $\mu$ M AHLs. A) *RIP1*, B) *NIN*, C) *ENOD11*, D) *ERN1*, E) *contig\_70694\_1*, F) *ACO*. REML test  $p < 0.05$  with Bonferroni's post-test. No post-hoc test when interaction is not significant. Data points indicate mean  $\pm$  SE ( $n = 3$ ). Note: the gene expression was measured in root segments not in the whole seedling.

Furthermore, we measured nodulation plant responses of the ethylene insensitive mutant *skl* to determine whether the microbiome changed plant nodulation responses to AHLs in an ethylene-dependent manner. As the hypernodulating mutant *skl* is unable to perceive ethylene, we expected that *skl* plants exposed to 3-oxo-C<sub>14</sub>-HSL and treated with antibiotics would not significantly increase nodule numbers compared to the control treatment as shown previously in Chapter 4. This was exactly what we observed in *skl* plants exposed to 3-oxo-C<sub>14</sub>-HSL in the absence of the microbiome (Figure 5.8). This suggests that the positive effect of 3-oxo-C<sub>14</sub>-HSL in the absence of the microbiome on nodule numbers is ethylene dependent, as proposed in Chapter 4.

Treatments with antibiotics resulted in more nodule numbers per plant than treatments without antibiotics even though only 3-oxo-C<sub>14</sub>-HSL significantly increased nodule numbers but only compared to the non-antibiotic treatments ( $p < 0.05$ ; Figure 5.8). This suggests that there is an ethylene-independent effect of the microbiome on nodule numbers.



**Figure 5.8** Nodule numbers of the ethylene insensitive mutant *sickle* (*skl*) 21 days after inoculation (dai) exposed to 3-oxo-C<sub>14</sub>-HSL at 1 μM. Different letters indicate significant differences between the treatments (REML test  $p < 0.05$ ). Data points indicate mean ± SE, ( $n = 27-29$ ).

### 5.3.4 Bacterial community of *Medicago* roots

We further investigated whether the differences observed in nodule numbers with and without antibiotics in plants exposed to 3-oxo-C<sub>14</sub>-HSL were due to bacterial composition and/or bacterial numbers. In order to provide insights into the root bacterial composition, including unculturable bacteria, we used a high-throughput sequencing approach, pyrosequencing as a tool to study the *Medicago* root microbiome. We also identified the most dominant viable bacteria present in *Medicago* seedlings through traditional methods of bacterial isolation.

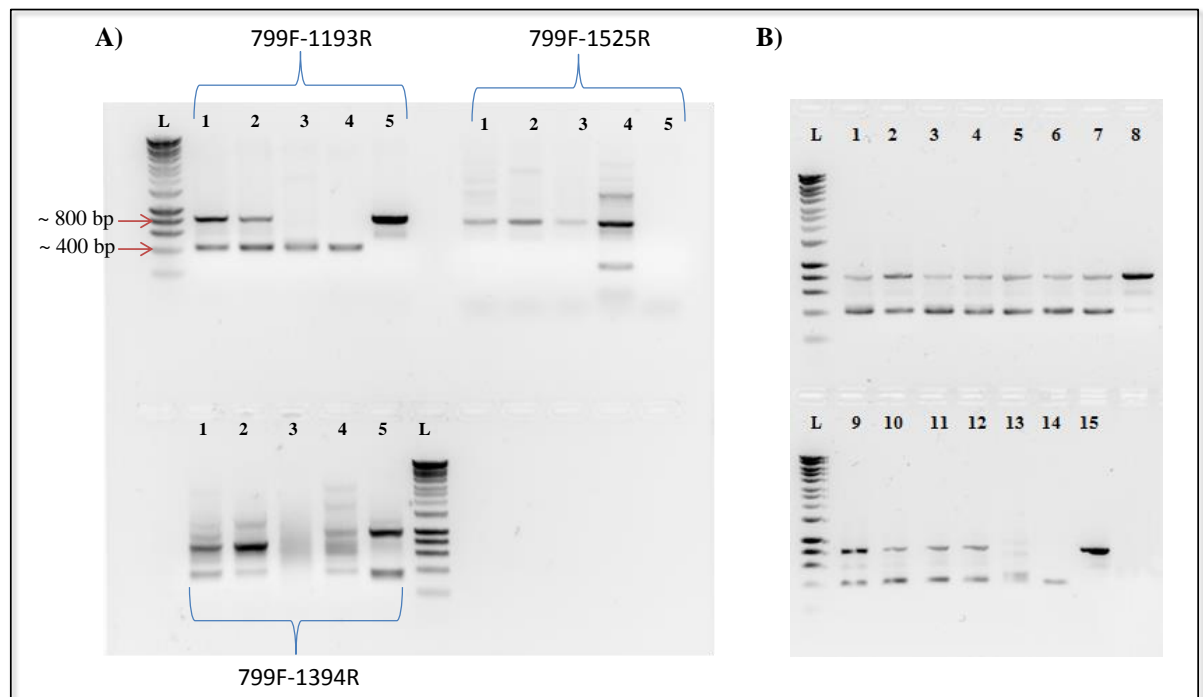
#### 5.3.4.1 Sample preparation

In order to identify the most dominant bacteria in three days old *Medicago truncatula* roots we isolated the most prevalent bacteria by culturing on LB media. After DNA extraction and 16S rRNA gene amplification, samples were sent for Sanger sequencing (AGRF, Australia). The sequences were BLAST searched against the NCBI data base of microorganisms. The most prominent isolates were identified as *Erwinia sp.*, *Pantoea sp.*, *Pseudomonas sp.* and *Enterobacter sp.* (Appendix A).

We further conducted an experiment to evaluate bacterial composition in *Medicago* roots. Four different treatments were established: Control plants with and without antibiotic treatment exposed to DMSO (control) and plants exposed to 3-oxo-C<sub>14</sub>-HSL with and without antibiotics. Three biological replicates per treatment, with five plants per replicate, were prepared. Plants from all treatments were grown on agar containing either solvent or AHL at 1  $\mu$ M final concentration. Four days after germination (dag) roots were collected for DNA extraction, PCR amplification of 16S rRNA genes and bacterial community analysis with pyrosequencing. We chose this time point in order to compare results with previous experiments. Thus, all plants were at the same developmental stage. This is particularly important as it has been shown that the rhizosphere microbiome can be affected by plant development (Chaparro *et al.*, 2014).

In order to amplify the bacterial 16S rRNA gene, we tested three different primer pair combinations on several DNA samples extracted from *Medicago* root tip, soil and *S. meliloti*. While soil and *S. meliloti* samples were used as controls to visualize the bacterial 16S rRNA amplicon, *Medicago* root tip was used as “clean plant tissue” to visualize plant 16S rRNA. *Medicago* root tips were taken from antibiotic-treated seedlings grown for only 48 h after germination, to minimize bacterial presence. From the three primer combination, 799F-1193R could successfully differentiate the plant 16S rRNA from the bacterial 16S rRNA gene (Figure 5.9A). Most of plant samples had two visible bands on the gel, except for the root tip (line 5 Figure 5.9A). One band corresponds to the plant plastid amplicon (~800 pb) (mitochondria and/or chloroplast) and the other correspond to the bacterial amplicon (~400 pb). These results are similar to previous studies using 799F-1193R in roots of *Arabidopsis thaliana* where the 400 bp amplicon corresponds to the hypervariable region V5-V7 of the bacterial 16S rRNA gene (Schlaeppli *et al.*, 2014). Subsequently, all samples were amplified with the 799F-1193R. The fragments were separated on 1.2% agarose gels (Figure 5.9). The DNA was

extracted from the 400 bp band and sent for 454 pyrosequencing to Molecular Research LP (Shallowater, Texas, USA).

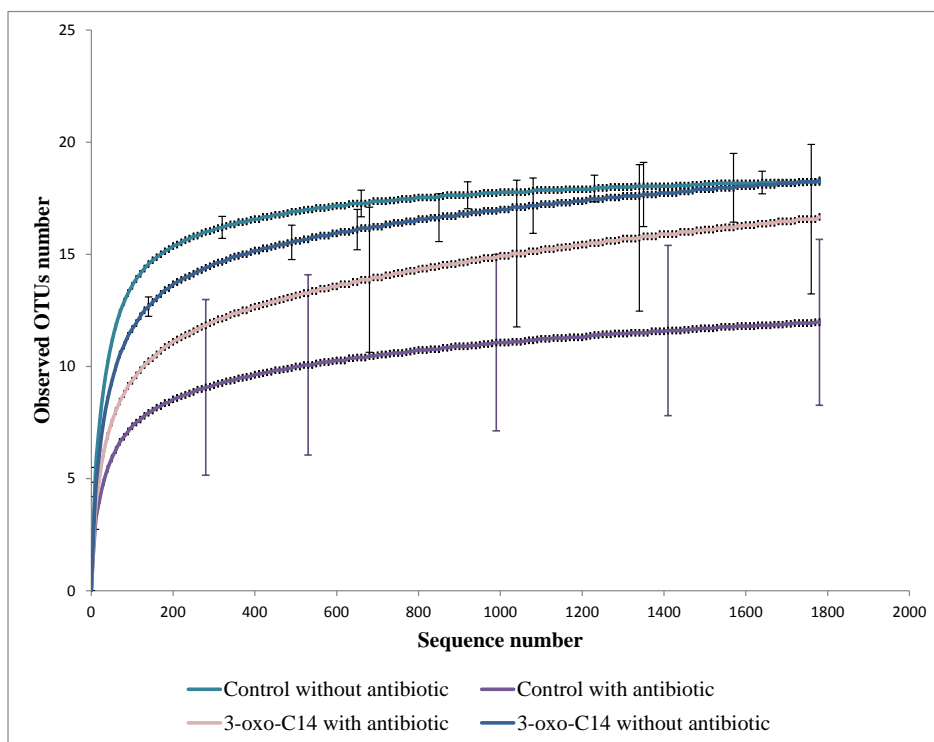


**Figure 5.9** 16S DNA PCR amplification on 1.2% agarose gels. **A)** Testing three primer pairs combination to differentiate plant from bacterial 16S rRNA gene; 799F-1193R, 799F-1525R, 799F-1394R. 1: DNA of *Medicago* seedlings derived from seeds treated with antibiotics, 2: DNA of *Medicago* seedlings derived from seeds treated without antibiotics, 3: DNA from soil sample, 4: DNA from pure culture of *Sinorhizobium meliloti* (Rm1021), 5: DNA of *Medicago* root tip. L: Hyper Ladder I. **B)** 16S rRNA amplicons for all treatments amplified with 799F-1193R primers. L: Hyper Ladder I. 1-3: Control without antibiotics, 4-6: Control with antibiotics, 7-9: 3-oxo-C<sub>14</sub>-HSL without antibiotics, 10-12: 3-oxo-C<sub>14</sub>-HSL with antibiotics, 13: soil sample, 14: *S. meliloti*, 15: *Medicago* root tip.

#### 5.3.4.2 Defining the root microbiome of *M. truncatula*

In order to verify the quality of our 454 pyrosequencing and also determine relative abundance we performed a rarefaction method. A rarefaction analysis is a measurement of the relative abundance or the diversity of a community based on sampling data (Gotelli and Colwell, 2001). In order to compare the bacterial diversity between the

treatments a rarefaction curve was produced. As it can be seen from the rarefaction curves, treatments with antibiotic have a decreased bacterial diversity compared to untreated samples, with the mean observed out of 18 OTUs at the sequencing depth of 1780 sequences (Figure 5.10). The rarefaction curve reaches the *plateau* at 1780 sequences.



**Figure 5.10** Rarefaction analysis of OTUs showing overall bacterial diversity of *Medicago* root. Bars indicate standard error ( $p < 0.05$ ;  $n=3$ ).

After the quality filtering, 2720-9514 valid sequences were obtained from each sample. According to Figure 5.10, all of these samples reached the plateau value of 1780 sequences, suggesting that pyrosequencing was deep enough to accurately represent the whole *Medicago* root bacterial community. These sequenced were classified into 88 operational taxonomic units (OTUs) according to the minimum of 97% similarity. Taxonomical assignment to the OTUs revealed that bacteria present in *wt Medicago* seedlings were mostly Gammaproteobacteria (99.78%). The antibiotic treatment changed the diversity and proportion of the plant *Medicago* root microbiome. Fourteen different genera were identified in treatments without antibiotics (Figure 5.11) but after the antibiotic treatment, mainly *Pseudomonas* and *Rhizobium* remained, with traces of

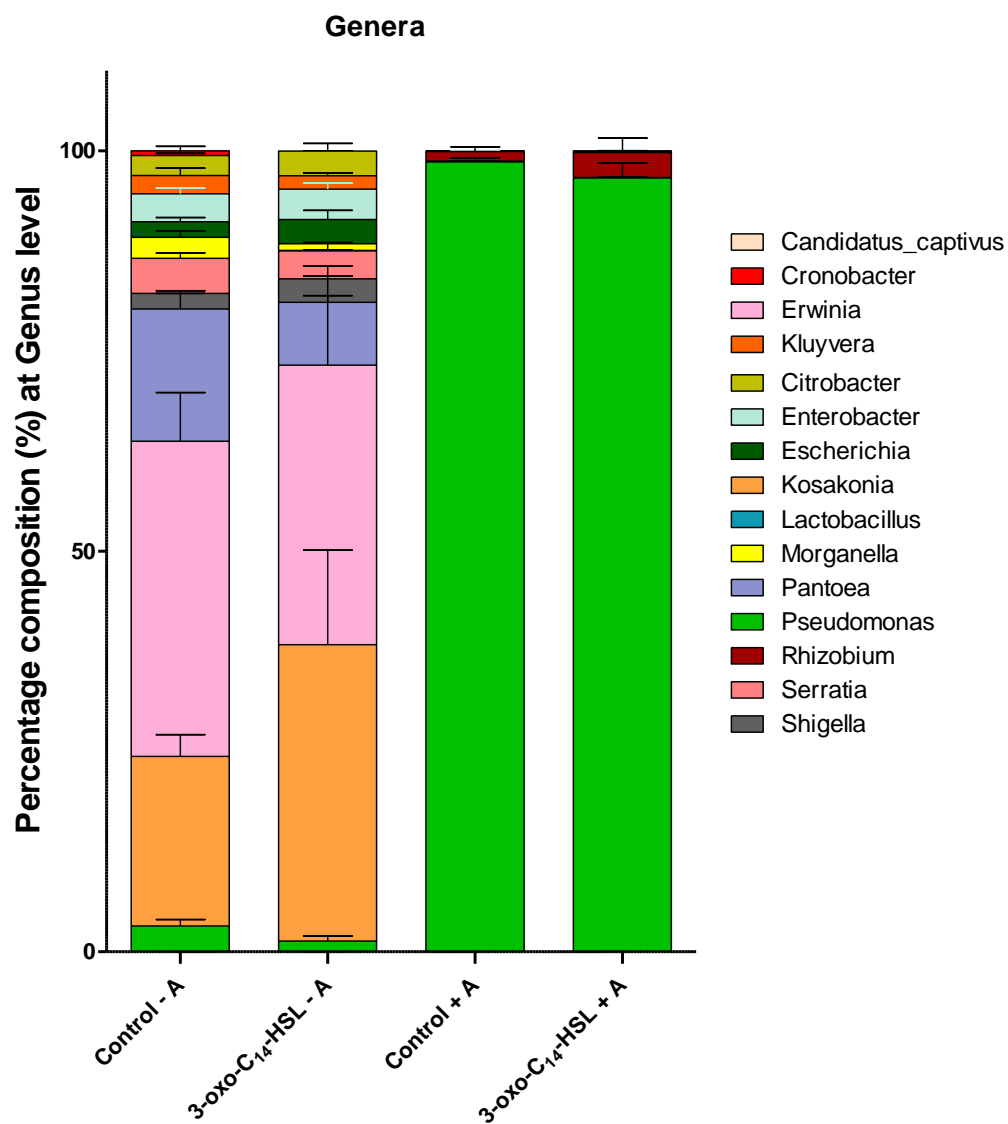
*Pantoea*, *Lactobacillus*, *Erwinia* and *Enterobacter* (Table 5.2). Out of 32 species identified in treatments without antibiotics only 21 and 16 species persisted in control and 3-oxo-C<sub>14</sub>-HSL after the antibiotic treatment, respectively (Figure 5.12). However, as the results are expressed in relative abundance it is not possible to know whether the antibiotics reduced the actual bacterial numbers. Thus, to confirm that antibiotics decreased bacterial populations we counted colony forming units (cfu) on LB agar from serial dilutions of seedlings treated with and without antibiotic. The antibiotic reduced significantly the cfu per root biomass (fresh weight) ( $p < 0.05$ ; Figure 5.13). In this way, the results observed in figures 5.10 and 5.11 can be complemented with figure 5.13, where antibiotic treated roots indicate that bacteria in and on roots are actually in low numbers. This result was confirmed on a molecular level via amplification of the bacterial 16S of *Medicago* roots treated and untreated with antibiotics (Figure 5.14). The advantage of this approach is that we are able to detect uncultivable bacteria present in *Medicago* roots via 16S RNA gene amplification. The relative abundance was calculated by ImageJ through quantifying band density. The ratio of the band densities was plotted as a proxy to determine the effect of the antibiotic treatment on the *Medicago* microbiome. The antibiotic treatment significantly decreased the bacterial relative abundance of *M. truncatula* roots from 1 to 0.20 ( $p = 0.0025$ ; Figure 5.14).

In order to assess the biological importance of all treatments in this study, an ANOVA test at genus level was performed. The results indicated that the relative abundance of 11 genera was significantly affected by the treatments ( $p < 0.05$ ; Table 5.4). Among the 11 genera, *Pseudomonas* and *Pantoea* had the lowest  $p$  values with the highest effect size (Table 5.2). The effect size shows how big the significant differences between treatments are. In this way, the ANOVA removes features with small effect sizes leaving only the active or significant features. In our study, out of the 11 active features only five, *Pseudomonas*, *Pantoea*, *Shigella*, *Enterobacter* and *Serratia*, dramatically changed their relative abundance showing effect sizes from 0.832 to 0.999 (Table 5.2). As the antibiotic treatment changed the composition of the root microbiome dramatically, positioning *Pseudomonas* as the dominant bacterial genus, we further compared the relative mean abundance of treatments untreated with antibiotics. 3-oxo-C<sub>14</sub>-HSL significantly decreased the relative abundance of *Pantoea* genus in *Medicago* roots ( $p = 0.015$ ; Figure 5.17A). This effect was specific to *Pantoea* as none of the other members of the *Medicago* microbiome were affected by the 3-oxo-C<sub>14</sub>-HSL treatment.

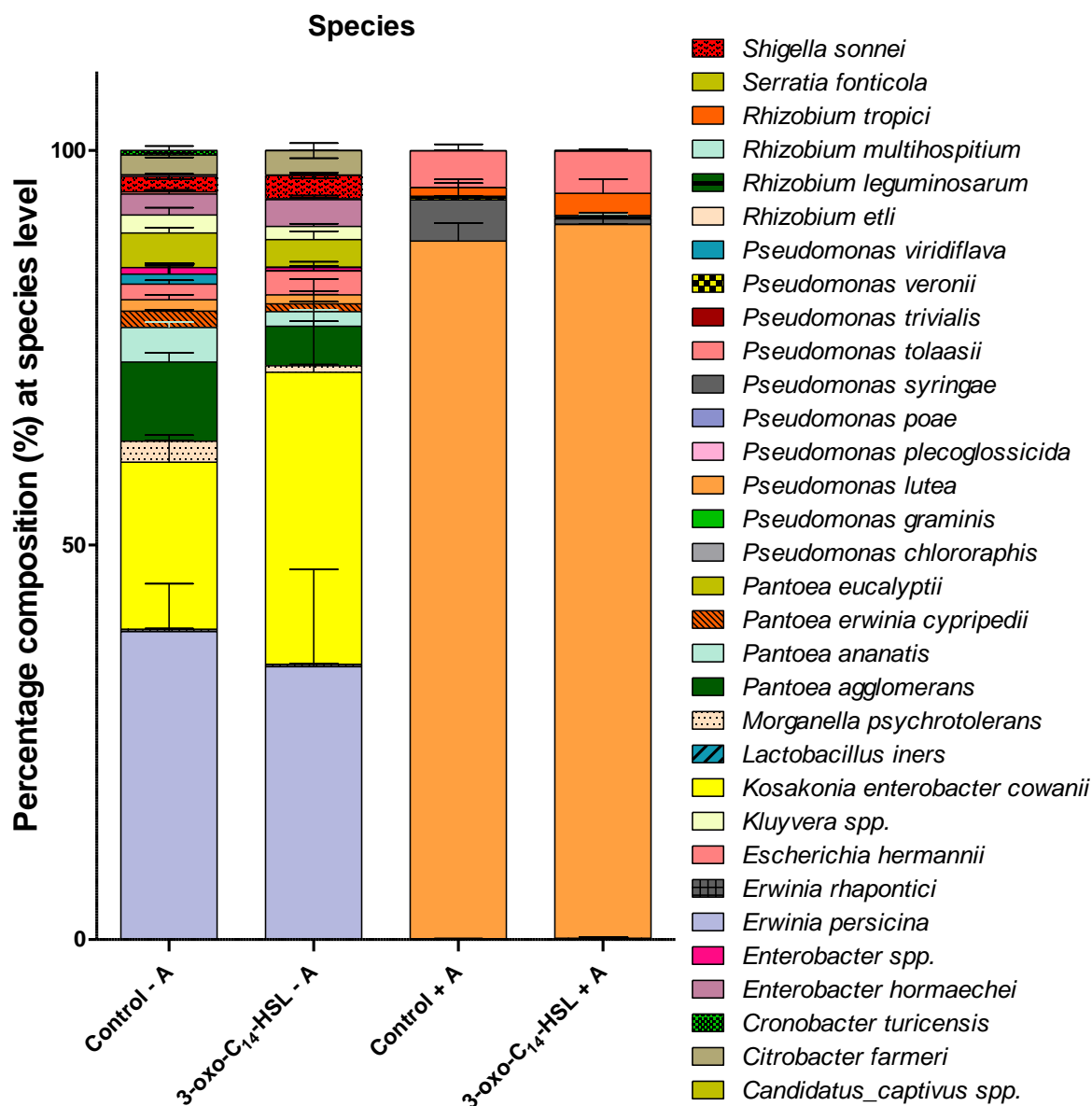
This result correlates with the effect observed by 3-oxo-C<sub>14</sub>-HSL on *Pantoea* which is similar to the samples without antibiotic treatment (Figure 5.16).

A principal component analysis (PCA) from each sample was used to determine how the antibiotic application was correlated with the exposure to 3-oxo-C<sub>14</sub>-HSL. The first two components explained 61.59% of the total variation. The PCA plot showed that the addition of antibiotic treatment can be explained by the first axis (50.18%), while on the second axis (11.41%) explained the AHL exposure (Figure 5.15). A further analysis comparing the PCA scores within either the antibiotic or without antibiotic treatment was done to eliminate the antibiotic effect. Subsequently, we investigated whether the AHL treatment had an effect on the composition of the *Medicago* root associated bacteria. However, no significant effect of the AHL 3-oxo-C<sub>14</sub>-HSL was found perhaps due to the low number of replicates used ( $n=3$ ). The Figure 5.16 shows that roots without antibiotic treatment showed higher bacterial diversity than roots treated with antibiotics. In addition, the relative abundance of different bacterial genera in plant roots depends on the presence or absence of the microbiome. *Kosakonia*, *Erwinia* and *Pantoea* were dominant genera in roots treated without antibiotics, while *Pseudomonas* genus was the dominant genus in roots treated with antibiotics. It was also interesting to observe that *Medicago* root-associated bacteria included several species of rhizobia that do not nodulate *Medicago* (Figure 5.12). It would be interesting to further study the bacterial root composition with more replicates per treatments to confirm these results.

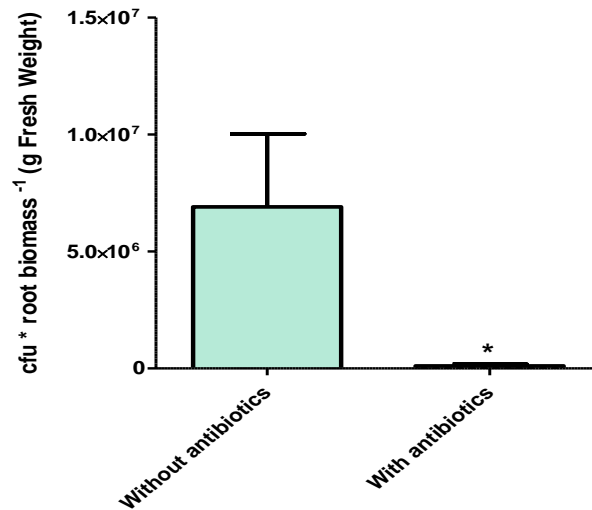




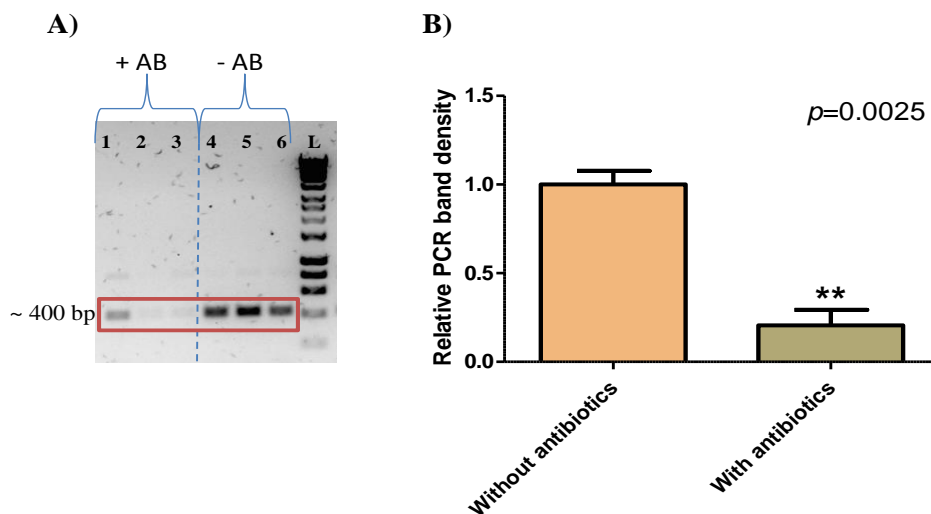
**Figure 5.11** Relative abundance of bacterial genera associated with *M. truncatula* roots treated with and without 3-oxo-C<sub>14</sub>-HSL in the presence or absence of antibiotics (-A or + A) revealed by pyrosequencing. 16S RNA gene amplified with the 799F and 1193R primer set.



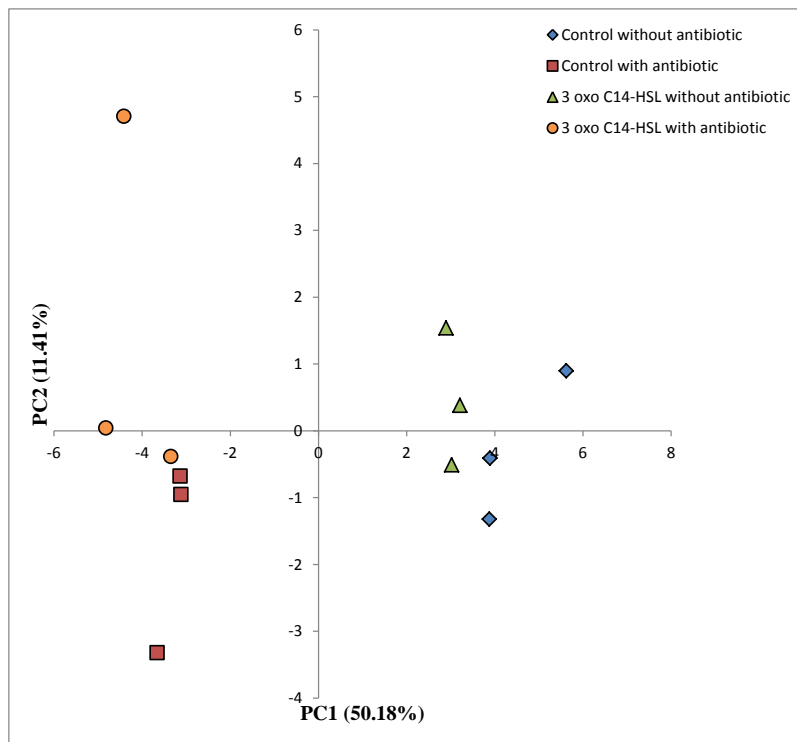
**Figure 5.12** Relative abundance of bacterial species of *M. truncatula* root seedlings treated with and without 3-oxo-C<sub>14</sub>-HSL in the presence or absence of antibiotics (-A or + A) revealed by pyrosequencing. 16S RNA gene amplified with 799F and 1193R primer set.



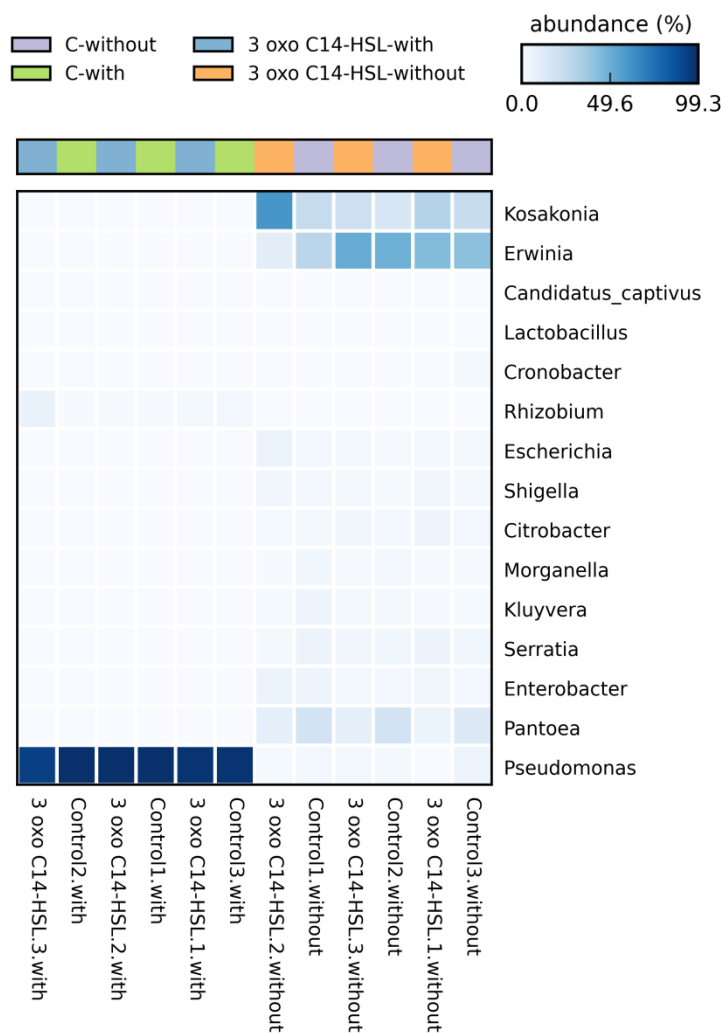
**Figure 5.13** Effect of antibiotics on *M. truncatula* associated bacteria present in roots four days after germination. Bacterial abundance was estimated from culturable bacterial colonies. Significant differences between the treatments and the respective control are indicated with an asterisk  $p < 0.05$  (Student's t-test). Data points indicate mean  $\pm$  SE ( $n = 5$ ). Abbreviations: cfu: colony forming unit.



**Figure 5.14** Relative abundance of bacterial 16S of *Medicago truncatula* roots treated and untreated with antibiotics. A) Agarose gel showing bacterial 16S amplicon (red rectangle) of *Medicago* roots treated with antibiotics (+AB) and *Medicago* roots untreated with antibiotics (-AB). B) Relative PCR band density ratio calculated by ImageJ. Significant differences between the treatments and the respective control are indicated with an asterisk  $p < 0.05$  (Student's t-test). Data points indicate mean  $\pm$  SE ( $n = 3$ ). Abbreviations: +AB: With antibiotics, - AB: Without antibiotics.



**Figure 5.15** Principal Component Analysis (PCA) of the root associated bacteria composition of four days old *Medicago* roots according to pyrosequencing data. PCA shows that 50.18% of the variation is explained by the antibiotic application.

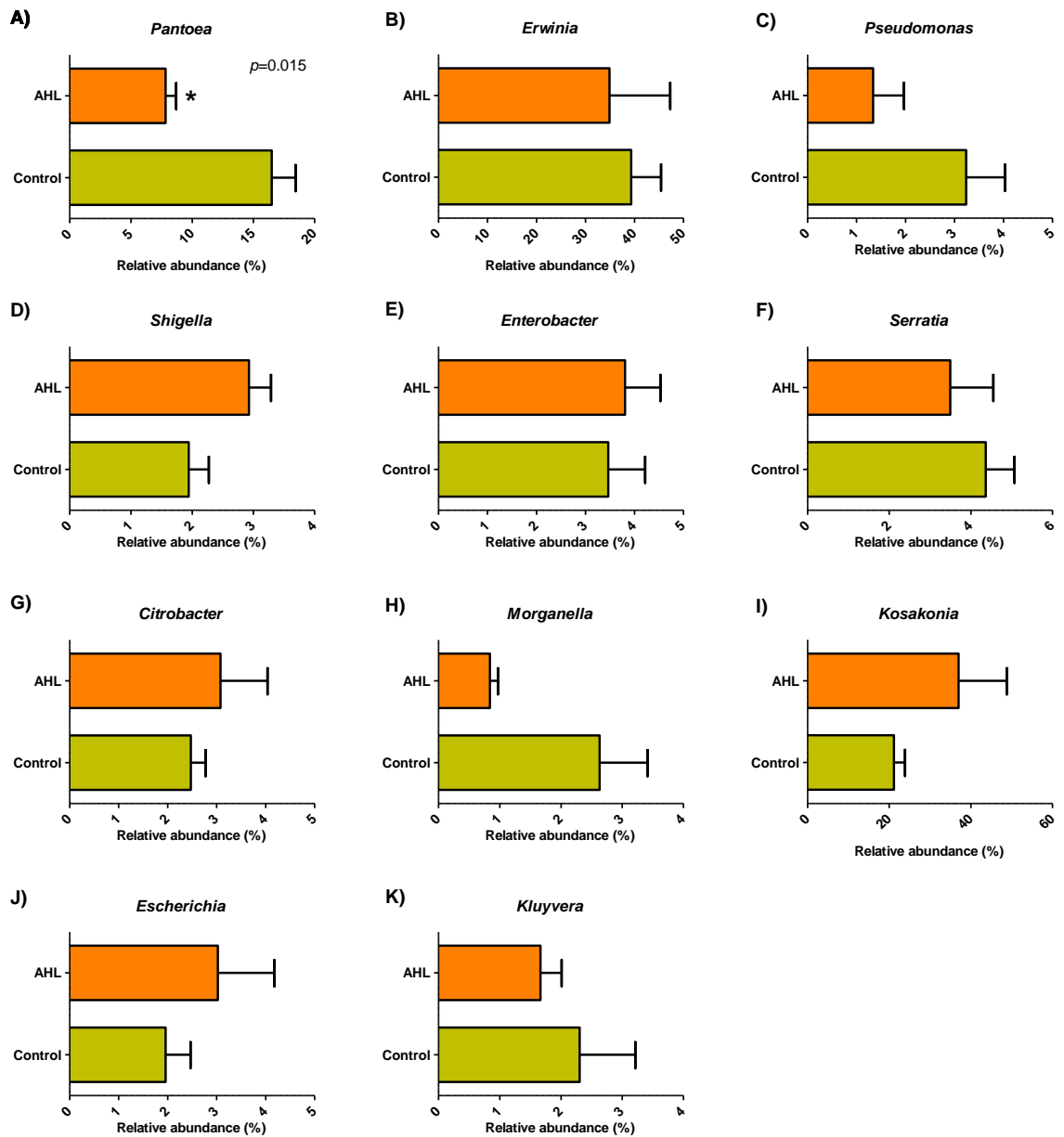


**Figure 5.16** Heat map showing bacterial relative abundance at Genus level of all treatments in study. The heat map analysis was conducted with STAMP software (Donovan Parks and Robert Beiko 2.1.3). Abbreviations: C-without: Control without antibiotic treatment, C-with: Control with antibiotic treatment, 3-oxo-C<sub>14</sub>-HSL-with: 3-oxo-C<sub>14</sub>-HSL with antibiotic treatment, 3-oxo-C<sub>14</sub>-HSL-without: 3-oxo-C<sub>14</sub>-HSL without antibiotic treatment. Data are shown for each biological replicate.

**Table 5.2** Relative abundance means of the treatments in study analysed by ANOVA using multiple test correction (Benjamini-Hochberg FDR) and a post-hoc test (Tukey-Kramer,  $p < 0.05$ ), an effect size (Eta-Squared) and a q-value ( $< 0.05$ ). 11 active features are shown for bacterial Genus. ( $p < 0.05$ ).

Genus level	Mean abundance (%)				<i>p</i> -values	Effect size
	Control without antibiotics	Control with antibiotics	AHL without antibiotics	AHL with antibiotics		
<i>Pseudomonas</i>	3.2376	98.6210	1.3393	96.6307	4.42E-11	0.999
<i>Pantoea</i>	16.5093	0.0181	7.8434	0.0234	8.08E-05	0.954
<i>Shigella</i>	1.9441	0.0061	2.9277	0.0000	0.0003	0.930
<i>Enterobacter</i>	3.4646	0.0717	3.8067	0.0806	0.004	0.853
<i>Serratia</i>	4.3679	0.0000	3.4994	0.0000	0.006	0.832
<i>Citrobacter</i>	2.4740	0.0000	3.0780	0.0000	0.010	0.795
<i>Morganella</i>	2.6329	0.0000	0.8379	0.0000	0.010	0.785
<i>Erwinia</i>	39.3512	0.0520	34.9049	0.0234	0.009	0.784
<i>Kosakonia</i>	21.1644	0.0107	36.9994	0.0000	0.011	0.767
<i>Kluyvera</i>	2.3054	0.0045	1.6652	0.0000	0.031	0.686
<i>Escherichia</i>	1.9588	0.0000	3.027603	0.0000	0.030	0.682

AHL: 3-oxo-C<sub>14</sub>-HSL



**Figure 5.17** The effect of 3-oxo-C<sub>14</sub>-HSL on the relative abundance of bacterial genera in *M. truncatula* roots untreated with antibiotics. Significant differences between the AHL treatment and the respective control are indicated with an asterisk,  $p < 0.05$  (Student's t-test). Data points indicate mean  $\pm$  SE ( $n=3$ ).

## 5.4 Discussion

### 5.4.1 The effect of the *M. truncatula* root microbiome on root phenotype and ethylene-regulated gene expression

We aimed to (i) investigate the effect of the root microbiome on phenotypic responses of *M. truncatula* exposed to 3-oxo-C<sub>14</sub>-HSL and (ii) determine the expression of nodulation and ethylene-regulated genes in *M. truncatula* exposed to 3-oxo-C<sub>14</sub>-HSL in the presence/absence of the root microbiome. The *M. truncatula* microbiome did affect plant responses toward several AHLs. The long acyl AHL increased nodule number while short acyl-side AHL did not. Conversely, C<sub>10</sub>-HSL, 3-oxo-C<sub>12</sub>-HSL and 3-oxo-C<sub>14</sub>-HSL showed a significant increase on nodule numbers in the absence of the microbiome, but this effect was lost in the presence of the microbiome. Thus, 3-oxo-C<sub>14</sub>-HSL was not the only compound that significantly increased nodule numbers as seen in previous Chapters (Chapters 3 and 4). Considering that very long acyl-side chain AHLs are specifically produced by rhizobia is not surprising that *Medicago* is able to distinguish these AHLs and respond specifically as *Medicago* has been shown to perceive and distinguish different AHLs from its symbiont and from an opportunistic pathogen (Mathesius *et al.*, 2003). On the other hand, short acyl-side chain AHLs, C<sub>4</sub>-HSL and C<sub>6</sub>-HSL, showed a significant increase in nodule numbers with the presence of the microbiome but no effect in the absence of the microbiome. Short acyl-side chain AHLs are less specific to bacteria synthesising them, and this might explain the lesser response of *M. truncatula* to these AHLs. For example, C<sub>4</sub>-HSL is synthesised by *P. aeruginosa* (Pearson *et al.*, 1995), C<sub>6</sub>-HSL by *S. meliloti* AK631 (Teplitski *et al.*, 2003), *Pantoea* sp. (Morohoshi *et al.*, 2007) and *Yersinia pseudotuberculosis* (Atkinson *et al.*, 1999) among others. 3-oxo-C<sub>6</sub>-HSL is produced by many bacteria, including *Erwinia carotovora*, *Pantoea* sp., *Vibrio fischeri* and *Yersinia enterocolitica* (Atkinson *et al.*, 2006; Fray *et al.*, 1999; Dong *et al.*, 2000). Thus, it might be possible that the *Medicago* root microbiome modulates nodule numbers by altering these compounds (i.e. enzymatic degradation of AHLs) which might, in turn, influence the root microbiome. The *M. truncatula* root architecture and also the plant biomass were affected by the microbiome and/or the AHL treatment. However, nodule numbers was the most affected phenotype of all.



Root phenotypic responses in non-nodulated *Medicago* seedlings were specifically altered in response to the type of AHL and the phenotype measured. Root length at 1  $\mu\text{M}$  and 10  $\mu\text{M}$  showed significant changes, which depend on the AHL concentration. For example, C<sub>10</sub>-HSL significantly decreased root length in the presence of the microbiome at 10  $\mu\text{M}$  but not at 1  $\mu\text{M}$ . However, none of these effects were quantitatively large.

The increment of nodule numbers observed in *M. truncatula* roots exposed to 3-oxo-C<sub>14</sub>-HSL only occurred in the absence of the microbiome and after the AHL treatment. Taking into consideration that ethylene might be involved in *Medicago* responses towards AHLs, particularly 3-oxo-C<sub>14</sub>-HSL, it is also possible that reductions in bacterial numbers (due to the antibiotic treatment) decreases ethylene due to a downregulation of plant defence signalling including ethylene induced stress genes. This hypothesis is supported by the findings of Hontzeas *et al.*, (2004), who showed that the plant growth promoting rhizobacteria *Enterobacter cloacae* UW4 (ACC deaminase-containing bacteria) decreased the expression of ethylene-induced stress genes. On the other hand, Iniguez *et al.*, (2005) showed that ethylene was involved in the decrease of bacterial endophytic colonisation in monocotyledons and dicotyledons. This premise was demonstrated by experiments done with the ethylene-insensitive mutant of *M. truncatula*, *sickle* (*skl*), which harboured more endophytes than the *wild type* (Iniguez *et al.*, 2005). In addition applications of ethylene early during seedling development resulting in the lowest endophytic colonisation of roots and hypocotyls of *wt M. truncatula*, suggesting that this response occurs at early stages of the plant-bacterial colonisation. Furthermore, ethylene has been found to participate in plant innate immunity processes such as induced systemic resistance (ISR) in the plant defence pathway at very early stages (Mersmann *et al.*, 2010). In the present study, the nodule numbers of the *skl* mutant with and without antibiotic treatment changed significantly. The highest nodule number per plant was achieved by the *skl* seedlings exposed to 3-oxo-C<sub>14</sub>-HSL in the absence of the microbiome but not in seedlings exposed to 3-oxo-C<sub>14</sub>-HSL with the microbiome. It is also possible that the *skl* mutant may not have the same bacterial population composition of the *wt M. truncatula* (A17) as it has been reported that microbial communities are shaped by different genotypes even within the same specie (Berendsen *et al.*, 2012).

Additionally to the ethylene mediated phenotypic responses, we investigated whether they were also detectable at a molecular level. We assessed the expression levels of

different nodulation and ethylene-regulated genes. All nodulation and ethylene-repressed genes were significantly modulated by the AHL treatment. Interestingly, *RIP1* and *ENOD11* gene expression were significantly regulated by the presence of the microbiome, AHL exposure and their interaction. Furthermore, the expression of the early nodulins and ethylene-repressed genes *RIP1* and *ENOD11* was significantly upregulated in *Medicago* plants only exposed to 3-oxo-C<sub>14</sub>-HSL in the absence of the microbiome indicating that the plants perceive less ethylene. The significant upregulation of *RIP1* and *ENOD11* genes correlates with the significant increase in nodule numbers observed in plants exposed to 3-oxo-C<sub>14</sub>-HSL in the absence of the microbiome. As these genes are ethylene-repressed and nodulation inducible, these results indicate that, *Medicago* nodulation responses to 3-oxo-C<sub>14</sub>-HSL and the microbiome are ethylene-dependent. *RIP1* and *ENODs* are involved in the modulation of the root hair curling and infection thread formation (Ramu *et al.* 2002; Peleg-Grossman *et al.* 2007). Thus, 3-oxo-C<sub>14</sub>-HSL application in the absence of the microbiome might regulate nodule numbers through increased infection thread formation. The upregulation of *NIN*, *ERN1*, *ENOD11* and *RIP1* indicates that initially there is a positive regulation of the Nod factor signalling pathway and reduced ethylene-perception, which in turn, could correlate with more nodule numbers. *NIN* gene expression was regulated in an AHL-dependent and antibiotic-independent manner. Thus, the modulation of *NIN* gene expression is controlled specifically by the AHL exposure. *NIN* has been proposed to be involved in the activation of cortical cell division, which leads to nodule formation (Vernié *et al.* 2015). Therefore, the upregulation of *NIN* gene expression in response to 3-oxo-C<sub>14</sub>-HSL might enhance cortical cell division, resulting in more nodule numbers. *ERN1* gene expression was significantly modulated by 3-oxo-C<sub>14</sub>-HSL and the antibiotic treatment. However, the interaction between AHL and antibiotic treatment was not significant. *skl* plants significantly increased nodule numbers by 3-oxo-C<sub>14</sub>-HSL with antibiotics but only compared to the non-antibiotic treatments. The *skl* response and the upregulation of *ERN1* and *NIN* in the presence of the 3-oxo-C<sub>14</sub>-HSL independently of the presence of the microbiome suggest that the microbiome might not interfere with the ethylene pathway. This suggests that there is an ethylene-independent effect of the microbiome on nodule numbers. On the other hand, *ACO* gene expression showed a significant antibiotic treatment effect, but not an AHL effect or a significant interaction between treatments suggesting that the microbiome represses ethylene synthesis. Therefore, we cannot strongly conclude that the microbiome mediates ethylene signalling in *M.*

*truncatula*. Further studies incorporating more genes will be needed to establish definitive conclusions about how the root microbiome affects nodule number responses to 3-oxo-C<sub>14</sub>-HSL in *M. truncatula*. It would also be interesting to evaluate *Medicago* nodulation responses to AHLs, in particular whether 3-oxo-C<sub>14</sub>-HSL application increases the number of infection threads and cortical cell divisions will be needed to investigate the mechanism behind this nodulation response. In addition, it would be interesting to measure ethylene concentration inside the infected roots to test whether AHL application directly affects ethylene concentration during the early stages of nodulation.

#### **5.4.2 The *M. truncatula* root microbiome composition**

Further, we aimed to determine the bacterial community structure of the *M. truncatula* root microbiome through 454 pyrosequencing analysis. The amplification of bacterial 16S ribosomal RNA (rRNA), which contains nine hypervariable regions (V1-V9), has been used to determine bacterial species in diagnostic assays and in bacterial community structure (Chakravorty *et al.*, 2007). Next generation DNA sequencing methods such as pyrosequencing 454 platform has contributed novel and valuable knowledge to explore microbial diversity (Margulies *et al.*, 2005; Fabrice and Didier, 2009). We used 454 pyrosequencing to determine the bacterial community composition of *M. truncatula* plants used in this study. The antibiotic application shifted the bacterial dominance and community composition of *Medicago* roots four days after germination regardless of the presence of 3-oxo-C<sub>14</sub>-HSL. In our study, bacteria present in *Medicago* roots were mostly proteobacteria. Many bacteria belonging to this phylum use AHLs to control the expression of a diversity of specific genes (Christensen *et al.*, 2013). The proteobacteria present in this study belong to the Enterobacteriaceae family. Some species from this family are commonly known to cause human disease (Mahon *et al.*, 1997; Tyler and Triplett, 2008). Different enteric bacteria such as *Salmonella enterica* serotype *Typhimurium* and *Klebsiella pneumoniae* that have been found in alfalfa sprouts (*Medicago sativa*) and *M. truncatula* had originated from seeds (Mahon *et al.*, 1997; Dong *et al.*, 2003). Several seed surface sterile methods have been tested in order to clean legume seeds, including *Medicago sativa* and *M. truncatula* (Hong *et al.*, 2016; Choi *et al.*, 2015). Exogenous applications of ethylene as well as 3-oxo-C<sub>14</sub>-HSL have reduced the occurrence of human pathogens in plants, probably due to triggering plant defence responses (Iniguez *et al.*, 2005; Hernández-Reyes *et al.*, 2014). Therefore, it is not surprising that in this study *M. truncatula* seedlings harbour such a variety of

bacteria as both organisms have co-adapted to develop specific mechanisms and strategies to orchestrate intimate interactions. It is also possible that another batch of *Medicago* seeds harbors a different bacterial population composition as it has been shown that the environmental effect (e.g. soil and air) can also contribute to the plant microbiome (Hardoim *et al.*, 2012).

The most relative abundant genera of the *M. truncatula* microbiome, *Kosakonia*, *Erwinia* and *Pantoea*, appeared quite consistently in seedlings without antibiotic treatment. As it was shown in Chapter 3, the bacterial community present in *Medicago* seedlings originated from the seeds. The consistent pattern of bacterial genera in all samples of this study suggests that *M. truncatula* has a strong interaction with these genera, selecting the type of bacteria in a targeted process. This selection may play a critical role in plant survival as this bacterial community will be vertically transmitted to the next generation via a seed-borne microbiome (Johnston-Monje and Raizada, 2011; Hardoim *et al.*, 2012). This microbial consortium can help the plant against abiotic and biotic factors e.g. while the seeds are on the soil waiting for the right conditions to germinate or when they have begun the germination process (Kaga *et al.*, 2009). In consequence, the seed-borne microbiome can help to secure the establishment of the young seedling becoming the foundation of the bacterial community composition of the new seedling (Kaga *et al.*, 2009; Johnston-Monje and Raizada, 2011). Plant colonisation by microorganisms has been shown to be specific (Bulgarelli *et al.*, 2012; Cardinale *et al.*, 2015). This is in line with the thought that plants invest energy and resources to select and nurture their microbiota as they play a crucial role in plant growth and health, particularly at the early stages of plant development. One reason for why the presence of the microbiome negatively affected nodule numbers of *M. truncatula* could be due to the lack of other environmental components that modulate the plant microbiome, like soil (Berendsen *et al.*, 2012). Over the time, the seed-borne microbiome interacts with the soil microbiome where the seedling grows. Possibly, this interaction provides the adequate balance for the plant to grow and develop. Our experiments missed out this interaction as the plants were growing under controlled conditions on agar plates. Perhaps, this also influenced the suitable and delicate balance of *Medicago* associated bacteria causing the decrease in nodule numbers observed in the presence of the microbiome.

Interestingly, 3-oxo-C<sub>14</sub>-HSL addition to non-antibiotic exposed plants significantly decreased the relative abundance of *Pantoea* spp., and this effect was specific to this

bacterial genus. *Pantoea* has been found to produce C<sub>6</sub>-HSL, 3-oxo-C<sub>6</sub>-HSL and 3-oxo-C<sub>8</sub>-HSL (Morohoshi *et al.*, 2007; Jiang *et al.*, 2015) and also it has been isolated from different crops like rice and pea (Kaga *et al.*, 2009; Elvira-Recuenco and Van Vuurde, 2000). QS controls exopolysaccharide production and biofilm formation in *Pantoea* (Morohoshi *et al.*, 2007). In addition, QS controls the release of extracellular hydrolytic enzyme in *P. ananatis* (Jatt *et al.*, 2015). As *Pantoea* spp. can be affected by 3-oxo-C<sub>14</sub>-HSL, it is possible that it can also perceive and respond to 3-oxo-C<sub>14</sub>-HSL, producing enzymes such as acylases or lactonases which would degrade 3-oxo-C<sub>14</sub>-HSL, which may explain why 3-oxo-C<sub>14</sub>-HSL did not increase nodule numbers in the presence of the antibiotic treatment. As reported for other rhizobacteria such as *Salmonella* (Subramoni and Venturi, 2009), *Enterobacter* and *Erwinia* (Sabag-Daigle and Ahmer, 2012), *Pantoea* possess a solo LuxR ortholog of Sdi system which, in turn, allows to 'listen' to an unusually large variety of AHLs synthesised by other organisms (Venturi and Ahmer, 2015). Thus, it would be possible for *Pantoea* to 'listen to' other AHLs. Another member of the *M. truncatula* microbiome identified in this study, *Kosakonia* sp., has been found to interfere with quorum sensing. For example, isolates of *Kosakonia* sp. from citrus leaves interfered with quorum signalling in *Xanthomonas citri* subsp. *citri*, the agent of citrus canker (Caicedo *et al.*, 2015). *Pseudomonas* spp. also has been found to interfere with quorum sensing of *Xanthomonas campestris* pv. *campestris* (Newman *et al.*, 2008).

It is also possible that the *M. truncatula* root microbiome modulates plant metabolism by interfering with ethylene signalling as it has been shown previously (Nonaka and, Ezura, 2014; Ribaudó *et al.*, 2006). PGPRs can decrease ethylene concentration in plants by synthesising 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which reduces the ethylene precursor ACC into  $\alpha$ -ketobutyrate and ammonia, resulting in decreased ethylene production (Nonaka and, Ezura, 2014). For instance, it has been reported that *Pseudomonas putida* GR12-2 can promote root elongation in canola seedlings most likely due to reduced endogenous ethylene levels (Glick *et al.*, 1994). However, the PGPR *Azospirillum brasilense* FT326, unable to produce ACC deaminase, promoted root hair and shoot development in tomato plants via increased ethylene levels (Ribaudó *et al.*, 2006). Thus, it is difficult to draw firm conclusions about how the *M. truncatula* root microbiome or some members, might modulate nodulation responses to AHLs. It would be interesting to further investigate the mechanism by which the microbiome influences nodule numbers in *M. truncatula* by (i)

quantifying the AHL concentration in the plant by mass spectrometry, (ii) detecting plant and bacterial enzymes that might degrade AHLs using a bioassay and (iii) measuring ethylene levels in the plant.

Seed-borne bacteria (rhizobial and non-rhizobial species) will eventually colonise other parts of the plant like roots and nodules, usually resulting in a plant growth-promoting effect (Dudeja *et al.*, 2012). It has been proposed that different bacterial strains have contradictory effects on nodulation either to promote or to inhibit nodulation (Oehrle *et al.* 2000). Previous works have demonstrated that single strain co-inoculation of members of the bacterial nodule microbiome has had a positive effect on plant nodulation (Stajković *et al.*, 2009; Ibáñez *et al.*, 2009). For instance, co-inoculation of *Bacillus* and *Rhizobium* strains increased nodule numbers of pigeon pea (*Cajanus cajan*) (Rajendran *et al.*, 2008). Conversely, Oehrle *et al.* (2000), reported that multiple species of seed-borne bacterial isolates reduced the attachment and infection of *Bradyrhizobium japonicum* in soybean (*Glycine max*). In the same study, germinating seeds were treated with antibiotic with no effects on plant development and the attachment of *B. japonicum* increased 200-325% after the antibiotic treatment. This might be another reason why the seed-borne microbiome of *M. truncatula* prevented an increase nodule numbers by 3-oxo-C<sub>14</sub>-HSL in our study as they might also inhibit the attachment of rhizobia. Thus, during plant growth, the community of plant associated bacteria can change, e.g. strains that inhibit nodulation overcome strains that enhance nodulation reducing the available sites for infection in *Medicago* roots.

The *Rhizobium* genus was found present in *M. truncatula* roots treated either with or without antibiotic treatment. None of the four different *Rhizobium* species identified were specific to nodulate *M. truncatula*. The genus *Rhizobium* has been able to colonise a range of plants promoting their growth through different mechanisms specially biocontrol (Mahdy *et al.*, 2001; Klock *et al.*, 2015). For instance, *R. etli* has been found to induce resistance towards root knot nematodes as well as to assist mycorrhizal colonisation in tomato as a helper bacterium (Reimann *et al.*, 2008). It has been suggested that *Rhizobium* isolates from nodules that are incapable to nodulate legumes act as helper bacteria (Dudeja *et al.*, 2012). *R. leguminosarum* bv. *phaseoli*, isolated from red clover nodules, promoted plant growth (Sturz and Christie, 1995). Thus, *Rhizobium* species found in our work could act as helpers.

*Pseudomonas* was prevalent in roots treated with the antibiotic and became the most dominant bacterial genus in this study. This is presumably because *Pseudomonas sp.* can develop antibiotic resistance to a broad range of antibiotics. For instance, *P. aeruginosa* isolates from clinical samples were 100% resistant to augmentin, imipenem, and erythromycin (Breidenstein *et al.*, 2011; Bibi *et al.*, 2015).

## 6 General Discussion

Previous studies established that plants are able to recognise AHLs, and differentiating AHL signals from pathogenic and symbiotic bacteria (Mathesius *et al.*, 2003; Gao *et al.*, 2003). Moreover, plants can also respond to AHLs by altering plant growth and development (Mathesius *et al.*, 2003; Joseph and Phillips, 2003; von Rad *et al.* 2008; Klein *et al.* 2009). For example, in response to physiologically relevant concentrations of AHLs, the model legume *Medicago truncatula* have been shown to adjust the production levels of more than 150 proteins, including defense related proteins, metabolic enzymes, and enzymes of the flavonoid pathway (Mathesius *et al.*, 2003). Legumes have co-adapted to interact with rhizobia in a fine-tuned process to allow rhizobial root colonisation, and because AHLs are necessary to regulate several bacterial behaviours linked to successful nodulation (González *et al.*, 1996; Marketon and González, 2002; Hoang *et al.*, 2008), it was interesting to examine if plant perception of AHLs affects nodulation. However, the effect of AHLs on the legume-rhizobia symbiosis, particularly nodulation has so far remained unexplored. The hypothesis of this work was that quorum sensing signals affect nodulation in *M. truncatula*.

This thesis explored the following questions:

- i. Do quorum sensing signals affect plant nodulation and how specific is this effect?
- ii. What are the mechanisms involved in the plant response towards quorum sensing signals during nodulation?
- iii. Do plant associated bacteria influence plant responses to quorum sensing signals?

### 6.1 Summary of the main results

#### 6.1.1 Developing a growth system to test AHLs on plant performance

AHL molecules can be degraded by enzymes produced by other bacteria (Dong *et al.*, 2001, Lin *et al.*, 2003, Helman and Chernin, 2015). Therefore, we evaluated different methods to surface sterilise *M. truncatula* seeds. Through serendipity we discovered that the batch of seeds we used, collected from *Medicago* plants growing under field conditions, harbored a culturable bacterial consortium coming from underneath the seed



coat. This discovery led us later on to evaluate whether these seed-borne associated bacteria present in the root of *M. truncatula* seedlings affect plant responses to AHLs (Chapter 5). We optimised the seed surface sterilisation protocol by treating the seeds with antibiotics. The antibiotic treatment significantly reduced the presence of bacteria in *Medicago* seedlings (*cf.* Figure 5.13, 5.14). As AHLs can be degraded by enzymes produced by other bacteria we developed a growth system suitable to test AHLs on plant development. Different open and closed systems were evaluated. However, growing *Medicago* plants on plates proved to be the most satisfactory. Furthermore, we investigated the possible effect of AHLs on different *Medicago* phenotypes including root architecture, seed germination, leaf area and biomass. Interestingly, nodulation, specifically nodule numbers, showed the biggest significant differences between the treatments, indicating that the effect of certain AHLs on *Medicago* is nodulation-specific. As previous studies showed that AHLs affect root phenotype in *Arabidopsis thaliana* (Ortíz-Castro *et al.*, 2008; von Rad *et al.*, 2008), we further confirmed whether our AHL-testing conditions were appropriate for evaluating AHLs on *Medicago*. We set a biological assay to reproduce the significant reduction in root length of *A. thaliana* by the AHL C<sub>10</sub>-HSL (Ortíz-Castro *et al.*, 2008). The application of C<sub>10</sub>-HSL significantly reduced root length in *A. thaliana* in our conditions validating our method to test AHLs on *Medicago*.

### **6.1.2 The effect of AHLs on *M. truncatula* is nodulation-specific**

AHLs, specifically 3-oxo-C<sub>14</sub>-HSL, significantly increased nodule numbers in *M. truncatula* and this effect was nodulation-specific and not a result of increased root growth or plant biomass. Even though some AHLs occasionally changed other root parameters, these were quantitatively small and not always consistent (*cf.* Figure 3.9; 5.2; 5.3).

### **6.1.3 The effect of AHLs on root architecture is concentration dependent**

*A. thaliana* and *M. truncatula* plants responded to AHLs in a concentration-dependent manner. While the root length of *Arabidopsis* was inversely related to AHL concentration (*cf.* Figure 3.12), root length of *Medicago* was differentially affected at several physiologically relevant concentrations (*cf.* Figure 3.9). Nodule numbers of *Medicago* were significantly changed at different AHL concentrations (*cf.* Figure 4.7) with higher concentrations showing stronger responses.

#### 6.1.4 The effect of AHLs on nodulation is species-specific

We examined the effect of AHL compounds on nodule numbers in several species including temperate and tropical legumes. Nodule numbers were significantly increased only in *M. truncatula* seedlings in response to 3-oxo-C<sub>14</sub>-HSL (*cf.* Figure 4.1).

#### 6.1.5 The effect of AHLs is structure-specific

The effect of AHLs on nodule numbers was dependent on the chemical structure of AHLs. For example, short acyl side chain AHLs tended to decrease nodule numbers in *M. truncatula* while long side chain increased them (*cf.* Figure 3.9; 5.1). This might be due to *Medicago* recognising long AHLs produced by its symbiont as it is known that rhizobia produce very long acyl side chain AHLs (Marketon *et al.*, 2002; Llamas *et al.*, 2004). It might be possible that short acyl side chain AHLs might alter plant defence responses as previous studies have reported that short-chain AHL C<sub>6</sub>-HSL, produced by *Serratia liquefaciens*, upregulated defence genes in tomato plants (Schuhegger *et al.*, 2006). In addition, 3-oxo-C<sub>6</sub>-HSL produced by *Serratia plymuthica*, triggered plant immunity responses in cucumber (Pang *et al.*, 2009). Thus, this possible modulation of plant defences by short-acyl AHLs might result in increased rhizobial infection.

#### 6.1.6 The effect of 3-oxo-C<sub>14</sub>-HSL on nodule numbers in *M. truncatula* is ethylene-dependent

We explored the possible mechanism behind the effect of the AHL, particularly 3-oxo-C<sub>14</sub>-HSL, on the increase in nodule numbers of *M. truncatula* through the evaluation of nodulation and ethylene inducible gene expression and the ethylene insensitive *sickle* (*skl*) mutant. The significant increase of nodule numbers observed in the *wt Medicago* in response to 3-oxo-C<sub>14</sub>-HSL disappeared in the hypernodulating *skl* mutant (*cf.* Figure 4.15) suggesting that ethylene mediates this response in *Medicago*. We further investigated whether nodulation and ethylene-regulated genes were differentially expressed in response to 3-oxo-C<sub>14</sub>-HSL. An up regulation of nodulation gene expression was observed in *Medicago* plants inoculated with rhizobia compared to uninoculated plants. The expression of ethylene-repressed genes *NIN*, *ENOD11*, *ERN1* and *RIP1* was positively modulated in *Medicago* plants exposed to 3-oxo-C<sub>14</sub>-HSL (*cf.* Figure 5.7).

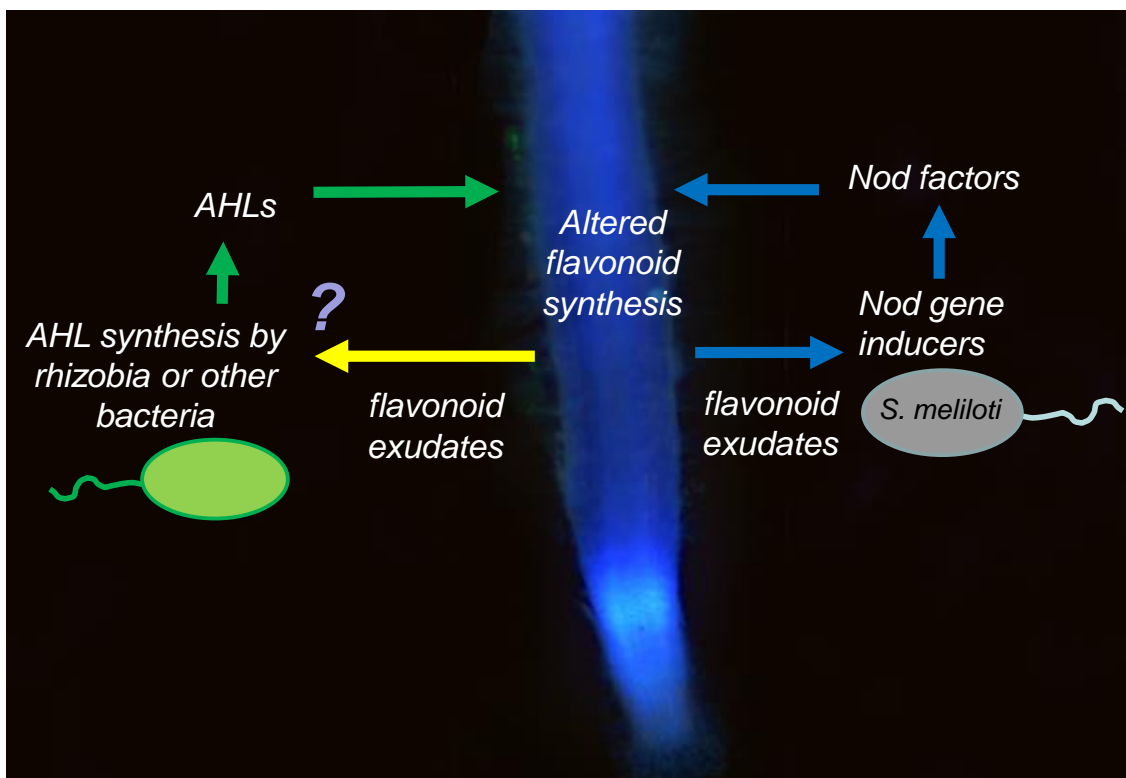
### **6.1.7 The effect of 3-oxo-C<sub>14</sub>-HSL on nodule numbers is dependent on the presence of the *Medicago* root microbiome**

In order to investigate whether the plant associated bacterial community of *M. truncatula* changed the nodulation responses to 3-oxo-C<sub>14</sub>-HSL, we treated *Medicago* seeds with and without antibiotics. The antibiotic treatment significantly reduced and changed the composition of *Medicago* root microbiome (cf. Figure 5.11; 5.12). The increase of nodule numbers of plants exposed to 3-oxo-C<sub>14</sub>-HSL was due to the interaction of the microbiome and the AHL treatment. The root microbiome of *Medicago* changed the effect of 3-oxo-C<sub>14</sub>-HSL on other root parameters to a lesser extent than nodulation. Interestingly, the relative abundance of *Pantoea* was significantly reduced in plants exposed to 3-oxo-C<sub>14</sub>-HSL in the presence of the microbiome. Thus, the microbiome affected AHL responses, but AHLs in turn, affected plant-microbial composition to some extent.

## **6.2 Ethylene: A possible mechanism behind the increase in nodule numbers of *M. truncatula* by 3-oxo-C<sub>14</sub>-HSL**

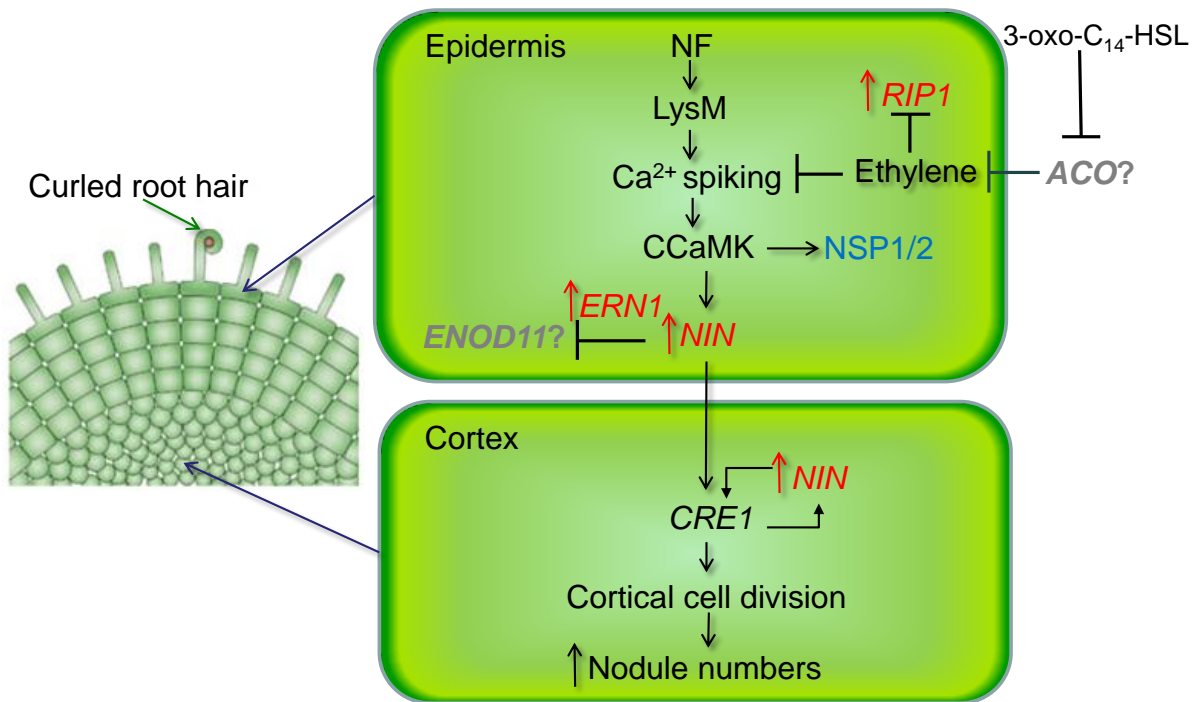
As we found a significant upregulation of nodule numbers by AHLs we further investigated the possible mechanism behind this response. As flavonoids can induce Nod gene expression in rhizobia, which in turn induce Nod factors synthesis enhancing the Nod factor signalling transduction, we quantified flavonoids via mass spectrometry. However, no correlation between flavonoid content and the increase in nodule numbers was found. Flavonoids can act as quorum mimic compounds interfering with quorum sensing (Vandeputte *et al.*, 2010; 2011). Even though we did not find any correlation between the increased nodule numbers and flavonoid content, overall flavonoids changed in response to AHLs. It could be possible that *Medicago* perceive AHLs and respond, altering the flavonoid synthesis, which in turn could affect the AHL synthesis of rhizobia or other rhizobacteria (Figure 6.1). Thus, it would be interesting to further investigate these plant responses to specific bacteria in the rhizosphere (i.e. through collection of root exudates). We further examined whether the autoregulation of nodulation (AON), mechanism that reduces nodule numbers through systemic signalling, was involved in the increase of nodules by 3-oxo-C<sub>14</sub>-HSL. This AHL still significantly increased nodule numbers in the AON mutant, *sun4* (Penmetsa *et al.*, 2003). Therefore, the inhibition of AON was not involved in this response.

An increase in nodule numbers of *M. truncatula* requires an increase in successful infection events. Infection events are regulated by nodulation gene expression and ethylene (Oldroyd *et al.*, 2001; Guinel and Geil, 2002). Therefore, we explored whether the increase in nodule numbers was primed by 3-oxo-C<sub>14</sub>-HSL at early stages of the Nod factor signalling pathway. *Medicago* seedlings four days after germination were ‘primed’ with 3-oxo-C<sub>14</sub>-HSL to highly significantly increase nodule numbers. As ethylene can negatively regulate nodule numbers in *M. truncatula*, we tested nodulation responses of the ethylene-insensitive *sickle* (*skl*) mutant (Penmetsa and Cook, 1997) towards 3-oxo-C<sub>14</sub>-HSL. The significant increase in nodule numbers following 3-oxo-C<sub>14</sub>-HSL treatment was lost in *skl*. This suggested that the nodulation response is mediated by ethylene signalling.



**Figure 6.1** Possible flavonoid-AHL interactions in *M. truncatula*-rhizobia symbiosis. *M. truncatula* exudates flavonoids into the rhizosphere, which up-regulate the expression of Nod genes in *S. meliloti*. This, in turn, induces the synthesis of Nod factors which are perceived by the host and altering the flavonoid synthesis. Flavonoid exudates can also alter AHL synthesis by rhizobia or other bacteria Photo courtesy of Ulrike Mathesius.

To further confirm these results we assessed the expression of some nodulation and ethylene inducible genes in response to 3-oxo-C<sub>14</sub>-HSL. The nodulation genes *RIP1*, *NIN* and *ERN1* were significantly upregulated by the AHL treatment only in the absence of the microbiome except for *NIN*, which was the only gene that was upregulated independently of the microbiome. *ACO* and *ENOD11* gene expression patterns were not completely conclusive as they showed inconsistent results when repeated (*cf.* Figure 4.20, 5.7). This might be due to different sets of experiments. It would be necessary to repeat these experiments to draw a firm conclusion. The spatial and temporary induction of *RIP1* expression has been correlated with the transient root hair susceptibility to rhizobial infection (Cook *et al.*, 2005). In addition, ROS production mediates expression of *RIP1* nodulin gene in *M. truncatula* (Ramu *et al.*, 2002). Thus, the upregulation of *RIP1* may suggest that ROS accumulation in roots treated with this AHL is higher than the control, allowing more rhizobial infections. This hypothesis is supported by an AHL-priming study in which 3-oxo-C<sub>14</sub>-HSL seemed to prime barley, wheat and tomato plants by inducing accumulation of ROS to induce resistance against pathogenic fungus (Hernández-Reyes *et al.*, 2014). *NIN* has been proposed to promote the movement of a signal from the epidermis to the cortex inducing *CRE1* gene expression, which in turn promotes cortical cell division and an upregulation of *NIN* in the cortex creating a positive feedback loop resulting in more nodule numbers (Figure 6.2) (Vernié *et al.* 2015). *NIN* gene expression was significantly upregulated in *Medicago* plants exposed to 3-oxo-C<sub>14</sub>-HSL independently of the presence of the microbiome showing that *NIN* expression responds specifically to the AHL treatment.



**Figure 6.2** Model showing the effect of 3-oxo-C<sub>14</sub>-HSL on nodulation gene expression and the possible mechanism behind the increase of nodule numbers by AHL. Genes in blue: unaffected by AHL. Genes in red: upregulated by 3-oxo-C<sub>14</sub>-HSL. Genes in grey: did not show consistent results. All gene expression measurements were made at 24 hours post inoculation in root segments comprising the nodulation zone.

### 6.3 Ecological role of AHL-plant microbiome interactions on plant nodulation

The role of the plant microbiome in plant responses to different stimuli has been largely ignored or given an insignificant consideration. However, it is now becoming increasingly evident that the plant microbiome is a key player not only of plant health and productivity but also in global biogeochemical cycles (Berendsen *et al.*, 2012; Philippot *et al.*, 2009). The microbiome can interfere with growth, organ development, immune responses and hormonal signalling in the plant (Chaparro *et al.*, 2014; Ortíz-Castro *et al.*, 2009). A new concept of plant as a ‘holobiont’ has been proposed, where the ‘holobiont’ is the plant plus its intimately associated microbes (Zilber-Rosenberg and Rosenberg, 2008; Vandenkoornhuyse *et al.*, 2015).

The root microbiome is extremely dynamic and can be influenced by the soil and the plant (Berendsen *et al.*, 2012; Bulgarelli *et al.*, 2012; Lebeis *et al.*, 2014). Plants shape their microbiome by selecting, feeding and influencing the composition and activity of the root microbiome (Mendes *et al.*, 2011). The composition of either beneficial or detrimental microorganisms in the rhizosphere (for plants and humans) will depend on their relative abundance. Considering that the presence and the relative abundance of bacteria is higher in the rhizosphere than in the bulk soil, it is not surprising that AHLs are also richer in the rhizosphere (DeAngelis *et al.*, 2008; Berendsen *et al.*, 2012). In our study, the plant associated bacteria (referred to as the microbiome) modulated the nodulation responses of *M. truncatula* to 3-oxo-C<sub>14</sub>-HSL, ie. the increase in nodule numbers by 3-oxo-C<sub>14</sub>-HSL was only observed in plants treated with the antibiotics. In the light of these results we conclude that the increase in nodule numbers of *M. truncatula* by 3-oxo-C<sub>14</sub>-HSL was due to the interaction between the AHL treatment and ethylene-signalling in the absence of the microbiome.

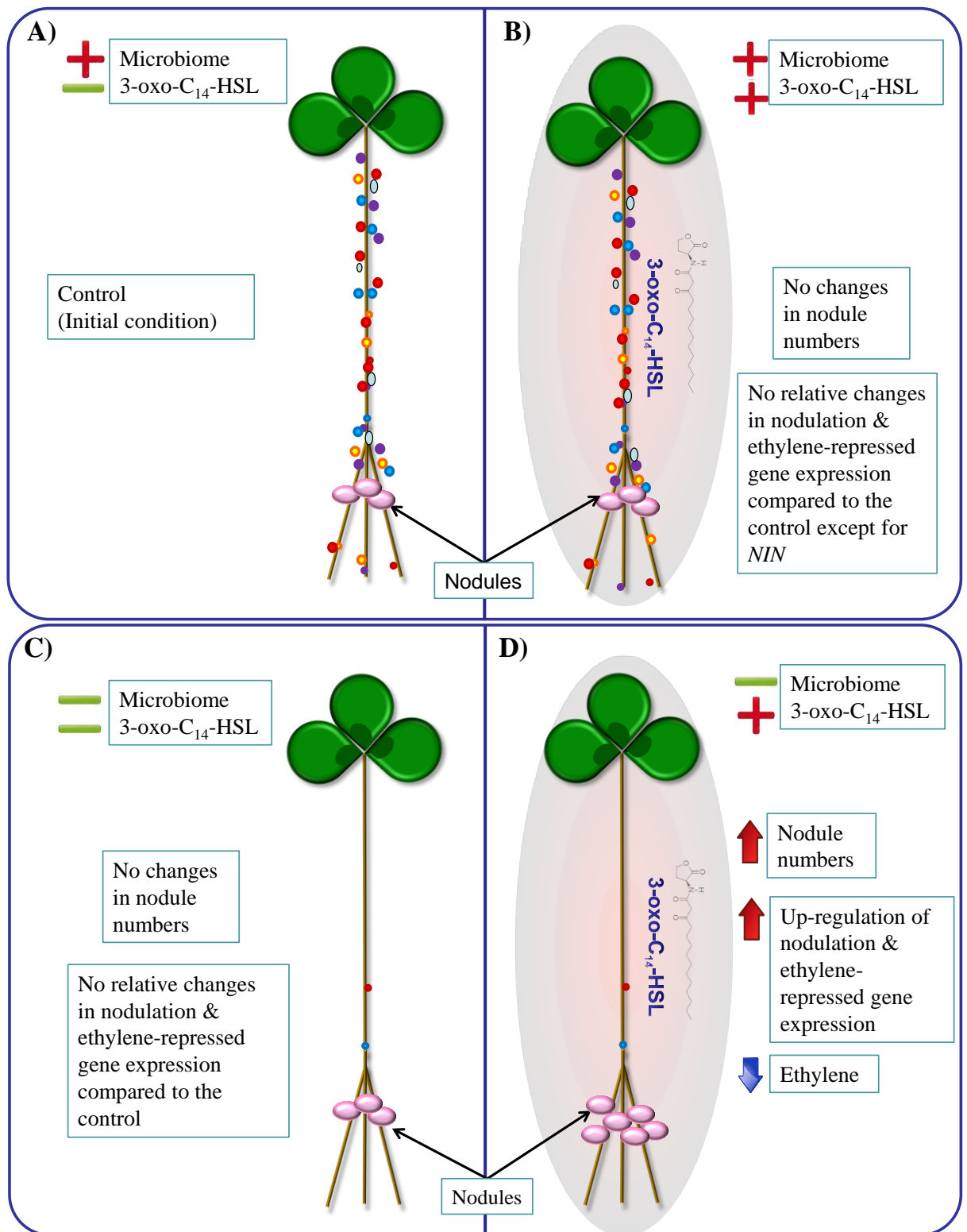
To further evaluate the composition of the *Medicago* root microbiome (originated from the seed of our seed batch), we determined relative abundance at genus and species level by 454 pyrosequencing. The highest relative abundance of members of *Medicago* microbiome corresponded to *Pantoea*, *Erwinia* and *Kosakonia* species belonging to the Enterobacteriaceae family. Interestingly, only the relative abundance of *Pantoea* was significantly reduced in response to 3-oxo-C<sub>14</sub>-HSL treatment. As *Pantoea* was affected by this AHL might be possible that it can feed back to 3-oxo-C<sub>14</sub>-HSL (e.g. producing enzymes to degrade 3-oxo-C<sub>14</sub>-HSL). On the other hand, after the antibiotic treatment *Pseudomonas* became the most dominant genus. Even though the bacterial numbers after the antibiotic treatment were extremely low it is also possible that they might have an effect on nodule numbers of *M. truncatula* as it has been reported that approximately 30% of *M. truncatula* gene expression was regulated by extremely low doses (an average of two bacteria per plant) of *Salmonella enterica* and *Escherichia coli* (Jayaraman *et al.*, 2014).

The consistent pattern of bacterial genera in all samples without the antibiotic treatment suggests that *Medicago* actively selected these genera in a targeted process. This seed-borne microbiome is subsequently vertically transmitted (from seed to the next generation) most likely to provide protection against biotic and abiotic factors (Kaga *et al.*, 2009). In our study, *Medicago* plants were grown under controlled conditions, which did not provide the natural interaction of plants grown in soil. Consequently, the

seed-borne microbiome was not able to balance its members (in type and numbers) with the bacterial populations from the soil. This might have had an impact on the lack of increase of nodule numbers in *M. truncatula* when treated with 3-oxo-C<sub>14</sub>-HSL. Another reason why the presence of the root microbiome presented an increase of nodule numbers in *Medicago* in response to AHLs in this work could be that the root microbiome interferes with the root hair attachment and infection of *S. meliloti*. This hypothesis has been reported previously in soybean (*Glycine max*) where the microbiome reduced attachment and infection of *Bradyrhizobium japonicum* resulting in decreased nodule numbers (Oehrle *et al.*, 2000).

Figure 6.3 summarizes our findings and our proposed model. *M. truncatula* plants in the presence of the microbiome (treatment without antibiotics) unexposed to 3-oxo-C<sub>14</sub>-HSL constitute the control treatment (initial condition) (Figure 6.3A). When *Medicago* plants were exposed to 3-oxo-C<sub>14</sub>-HSL in the presence of the microbiome, nodule numbers were not affected by this AHL compared to the control treatment (*cf.* Figure 5.1I, Figure 6.3B). In addition, there were no relative changes in nodulation and ethylene-regulated gene expression, except for the ethylene-repressed gene *NIN*, which was shown to be induced by 3-oxo-C<sub>14</sub>-HSL (*cf.* Figure 5.7B). When *Medicago* plants were treated with antibiotics, eliminating its microbiome, no significant changes in nodule numbers were observed as a result of the antibiotic treatment (Figure 6.3C). However, when 3-oxo-C<sub>14</sub>-HSL was added to *Medicago* plants treated with antibiotics (in the presence of its microbiome), a significant increase in nodule numbers was observed (*cf.* Figure 5.1I) as well as an induction of nodulation and ethylene-repressed genes including *RIP1*, *NIN*, *ENOD11*, *ERN1* (Figure 6.3D). This might be due to decreased ethylene signalling in the plant. Therefore, we postulate that 3-oxo-C<sub>14</sub>-HSL reduces ethylene synthesis or signalling in the plant, resulting in increased nodule numbers of *M. truncatula*.





**Figure 6.3** Diagram of the proposed model for the increase of nodule numbers of *M. truncatula* in response to 3-oxo-C<sub>14</sub>-HSL. A) *Medicago* plants unexposed to 3-oxo-C<sub>14</sub>-HSL and in the presence of the microbiome constitute the control treatment (initial condition). B) When *Medicago* plants were exposed to 3-oxo-C<sub>14</sub>-HSL no changes in nodule numbers or gene expression of nodulation and ethylene were shown. C) *Medicago* plants unexposed to 3-oxo-C<sub>14</sub>-HSL in the presence of the microbiome

(plants treated with antibiotics), neither change nodule numbers nor gene expression. D) By contrast, in the absence of the microbiome, *Medicago* plants exposed to 3-oxo-C<sub>14</sub>-HSL increased nodule numbers and nodulation and ethylene-repressed gene expression, possibly due to a reduction of ethylene synthesis or signalling in the plant. -: without, +: with. The coloured spots represent different bacterial species ('microbiome'), and while indicated on the surface of the plant, most of the bacteria are likely to colonise the inside of the plant tissue.

## 6.4 Concluding remarks and future outlook

For the first time has been shown that AHL perception by the plant host affects nodulation in the legume-rhizobia symbiosis. Moreover, the AHL 3-oxo-C<sub>14</sub>-HSL from the *M. truncatula* symbiont, *Sinorhizobium meliloti*, significantly increased nodule numbers in a nodulation, structure, concentration and species-specific manner. We also discovered that this response occurred at early stages of the Nod factor signalling pathway and is ethylene-dependent. We further investigated the possible effect of *M. truncatula* microbiome (plant associated bacteria) on the plant responses to AHLs. We determined that the root microbiome negated the increase of nodule numbers by 3-oxo-C<sub>14</sub>-HSL treatment.

Future investigations exploring ROS production and accumulation as well as infection thread formation could sharpen our knowledge about the mechanism behind of the increased nodule numbers in *M. truncatula* by 3-oxo-C<sub>14</sub>-HSL.

Most of the studies done with plant-associated bacteria are limited to single or co-inoculations. In this study, we investigated the effect of the microbiome as a whole on plant nodulation. It is important to take into consideration the plant microbiome as it can strongly influence the plant responses to a certain stimulus, and this response can feed back to modulate the behaviour of the microbiome. It would be interesting to test in the future whether the addition of the microbiome back onto the plants treated with antibiotics changes nodulation responses to AHLs. However, this could be hampered by the inability to culture these bacteria, and to deliver them to the appropriate cell or tissue type harbouring the individual bacteria.

Understanding the process that modulates the root microbiome in terms of composition and function is essential for food productivity. Further studies in selecting rhizosphere

signalling molecules such as AHLs could be used to manipulate the root microbiome to enrich beneficial members, thereby increasing plant growth, health and yield in a sustainable manner. Moreover, these strategies could be extrapolated in the prevention of outbreaks due to plant-originated infections with human pathogens.

It has to be taken into consideration that when trying to influence nodulation or infection of plants by application of AHLs, the presence of bacteria present in and on plant surfaces and in the rhizosphere will likely influence the outcomes of AHLs responses by plants. Thus, positive effects of AHLs on nodulation as seen here under sterile and controlled conditions, may not be observable in the field. Future attempts at defining plant responses to AHLs will clearly rely on the identification of the AHL receptors in plants, which have not been reported to date.

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**Appendix A** Blast results of bacterial isolates from *M. truncatula* roots.

<b>Isolates</b>	<b>Description</b>	<b>Max score</b>	<b>Total score</b>	<b>Query cover</b>	<b>E value</b>	<b>Identity</b>	<b>NCBI Accession</b>
<b>1</b>	<i>Enterobacter sp.</i> A529 16S ribosomal RNA gene	1899	1899	100%	0	99%	KU312048.1
<b>2</b>	<i>Enterobacter sp.</i> HT-Z60 16S ribosomal RNA gene	1561	1561	100%	0	99%	KJ526920.1
<b>3</b>	<i>Pseudomonas sp.</i> FBF56 partial 16S rRNA gene	1862	1862	100%	0	99%	HG805730.1
<b>4</b>	<i>Pantoea vagans</i> strain Hb-11 16S ribosomal RNA gene	1465	1465	100%	0	96%	KC139414.1
<b>5</b>	<i>Pantoea agglomerans</i> strain DsppB7 16S ribosomal RNA gene	145	145	100%	1.00E-31	98%	KT958491.1
<b>6</b>	<i>Pseudomonas gessardii</i> strain MM1-4 16S ribosomal RNA gene	1868	1868	100%	0	99%	JX144945.1
<b>7</b>	<i>Erwinia rhapontici</i> strain A534 16S ribosomal RNA gene	1275	1275	100%	0	99%	KU312051.1
<b>8</b>	<i>Pantoea agglomerans</i> strain TSD 16S ribosomal RNA gene	1127	1127	99%	0	99%	KT075207.1